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Cytokine Intervention: A Double Edged Sword in the Nkg2d System Regulation

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Abstract

The natural killer group 2 members D (NKG2D) is an activating receptor which plays a major role in immune surveillance, and the detection and elimination of malignant tumors and infected cells. NKG2D acts over both arms of the vertebrate immune response, and is expressed in some human and mouse myelopoietic, γδ T, NKT and CD4+ cells, but is present in all NK and CD8+ T cells in humans and activated mouse CD8+ T cells. In humans, eight ligands which selectively bind to the NKG2D receptor have been identified. These ligands are not systemically expressed, but are triggered in response to stress and expressed only under specific pathological states. Several research results point to the importance of cytokines for increasing expression of NKG2D to restore the functionality of NK cells as well as their ligands in the target cells. However, the NKG2D system itself in an activated state, also release pro and anti-inflammatory cytokine transcripts to establish communication with other cells or for self-regulation. Additionally, type I antiviral interferon is largely produced. Such cytokine interactions could be regarded as a double edged sword. This behavior is emphasized by a discrepancy regarding the functionality of cytokines which interact with, or on the NKG2D system. Indeed, they seem to protect the host and rather can induce ligand expression, cell proliferation or dissemination of malignant tumors, generating complicated cytokine-mediated messenger loops which are far from being fully understood. Whatever the case, cytokines related to the NKG2D system could be an attractive and useful target for immunotherapeutic approaches. Thus, here we briefly review recent findings on the main aspects involved in the regulation of this system and, particularly, attempt to clarify the role played by cytokines in the activating or inhibitory function they exert over the NKG2D system in different contexts.

Keywords: Cytokines; Immunotherapy; MICA/B; NKG2D system; Stress; Tumor; ULBP

Abbreviations: APC: Antigen Presenting Cell; CD: Cluster of Differentiation; CIK: Cytokine-induced Killer Cell; DC: Dendritic Cell; DAP: Disulphide Adaptor Molecule; 5-FU: Fluorouracil; γc: Gamma chain; CSF2: Granulocyte-Macrophage Colony-Stimulating Factor; Grb2: Growth Factor Receptor-Bound Protein 2; H60: Histocompatibility Antigen 60; IBD: Inflammatory Bowel Disease; IEL : Intraepithelial Lymphocyte; IFN: Interferon; IL: Interleukin; JNK: Jun N-terminal Kinase; KIR2DL1: Killer Cell Immunoglobulin-Like Receptor, Two Domains, Long Cytoplasmic Tail 1; LTA: Lymphotoxin Alpha; MHC: Major Histocompatibility Complex; MICA/B: MHC class I Chain–Related Proteins A/B; miRNA: micro RNA; MyD88: Myeloid Differentiation Primary Response 88; NK: Natural Killer; NKG2D: Natural Killer Group 2 Member D; NKRs: Natural Killer Receptors; NKT: Natural Killer T Cell; FOLFOX4: Oxaliplatin-Folinic Acid-Fluorouracil; PBMCs: Peripheral Blood Mononuclear Cells; PI3K: Phosphatidyl Inositol 3 Kinase; Src: Proto-oncogene Tyrosine-protein Kinase; RAET-1: Retinoic Acid Early Transcript; RAE-1: Ribonucleic Acid Export 1; Syk: Regulatory T Cells (Treg) Spleen Tyrosine Kinase; TGFB-1: Transforming Growth Factor Beta 1; TNF: Tumor Necrosis Factor; ULBP: UL16-Binding Proteins; YINM: Tyrosine-Isoleucine-Asparagine-Methionine Motif; ZAP70: Zeta-Chain-Associated Protein Kinase 70

Introduction

Cells communicate with one another through extracellular signalling proteins know as cytokines. Each cytokine is produced immediately in response to many different stressors and binds to the extracelular domain of either one or two matching membrane-bound receptors denoted as α-, β- or γ-chain [1]. Due this matching specificity, cytokines enables the rapid propagation of immune signaling and have long been regarded as the regulators of host responses to infection, immune responses, inflammation, and trauma [2]. Nevertheless, the immune system's primary task telling friend from foe is not one that can be easily solved solely through the indiscriminate production of cytokines to pass on instructions to cells. Faced with a serious threat such as infection, wound or tumors, the system needs to mount a defense response immediately. However, it also needs to know when not to interfere with innocuous visitors. To deal with dangerous outsiders, vertebrates have developed two lines of defense. These are termed learned immunity, which is acquired by exposure to a pathogen, either from the environment or through a vaccine, and innate immunity, which is the immediate hard-wired reaction to outside invaders [3]. A wide set of immune competent cells actively interact between the two arms of the immune system to mount an effective inflammatory defense against a particular threat. However, inflammatory processes intend to mediate against invaders, if not properly controlled, could irreversibly damage host tissues [4]. At cellular level, the key ingredients in linking the innate and adaptive responses are the innate-like cells. The best example of this particular cell type is the natural killer (NK) cell, a subset of lymphocytes involved in early defenses triggered through receptors that respond to infected,

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transformed and/or stresed cells [5,6]. Among the different receptors expressed by NK cells, the most studied is the NKG2D, which, upon stimulation, binds to any of the eight different ligands (MICA, MICB and ULBP 1 to 6) of the NKG2D system [7]. Upon detection of some pathological alterations in autologous cells, stimulation of the receptor can lead to the enhancement of innate immune functions, mediated by NK cells and myeloid cells, and the enhancement of adaptive immunity mediated by CD8+ and γδ T cells [8]. In human NK cells, at least, crosslinking with multivalent soluble ligands of NKG2D stimulates the production of several cytokines, including interferon gamma (IFN-γ), tumor-necrosis-factor alpha (TNF-α), lymphotoxin and the colony stimulating factor 2 (granulocyte-macrophage) (CSF2), as well as chemokines such as CCL4 and CCL1 [9-11]. However, the cytokines produced by NKG2D positive cells seldom play a single role. This notion is supported by several recent investigations which have established that, upon activation [12,13] or inhibition [14,15] of the NKG2D system, the same set of cytokines might be acting in both responses. Several research groups have reported functional aspects of cytokines in which IL-2 [16,17], IL-15 [18,19] or the combination of IL-15 and TNF-α [20], have participated as key molecules, and observed a marked increased expression of the NKG2D receptor. In other studies, the dual functional role of cytokines has been demonstrated using IL-4 [21] or IL-21[22], two well known inducers of NK cell maturation [23]. This last study suggested that both cytokines, the IL-4 and the IL-21, act as negative regulators of the NKG2D system in both NK and CD8+ T cells. Additionally, the modulation of NKG2D ligands has also been observed by IFN-γ, which downregulates the expression of MICA and ULBP2 [24,25], and IFN-α that upregulates the same [26,27]. Nevertheless, in a regular setting, the activation of the NKG2D system by any of its ligands, triggers cytokine production to activate innate or adaptive immune responses following a canonical pathway. Therefore, to define the exact role that a certain cytokine may play in the NKG2D system is far from straightforward since, as mentioned above, evidence suggests their involvement in both directions depends on the context of the study (Table 1). In this brief review, we intend to summarize and discuss recent advances made in our understanding of the double edged nature of cytokines in the regulation of the NKG2D

Name	Cytokine	Producer cell	Cell target	Organ target	Condition	Organism	Main function	Ref
Interleukin 1, alpha	Il1a	Macrophages	Tumor cell	Skin	Merkel Cell Carcinoma	M	Down-regulation of RAE-1, tumor-cell resistance to NK-mediated control of virus-induced tumors	[86]
Interleukin 1, beta	Il1b							
Interleukin 2	IL2	T cells	NK	-	Viral infection / tumor	H	Up-regulation of NKG2D-DAP10 surface expression	[69], [68]
Interleukin 4	Il4	Th cells	CD8+ T cells	-	Th2 pathology	M	Down-regulation of NKG2D and increasing of the activation threshold of memory CD8+ T cells	[21]
Interleukin 6	IL6	dNK	dFibroblast	Placenta	HCMV infection	H	Control and spreading suppression of HCMV infection by NKG2D of dNKs	[72]
Interleukin 7	IL7	-	NK	-	Viral infection / tumor	H	Up-regulation of NKG2D-DAP10 surface expression	[69]
Interleukin 8	IL8	Myoblasts	NK	Muscle	Inflammatory myopathies	H	Up-regulation of NKG2D and NK cell-mediated lysis of muscle cells	[123]
Interleukin 9	IL9	CD8+ T cells	in vitro	-	Viral infection / tumor	H	Decreased expression of antiinflammatory cytokines through NKG2D expression	[89]
Interleukin 10	IL10	DCs	DCs	-	HIV infection	H	Immune dysfunction due to NK cell-mediated elimimatiom of DCs	[124]
Interleukin 12	IL12	PBMNc	NK	-	HCMV infection	H	Down-regulation of NKG2D to control NK cell reactivity against normal cells expressing NKG2D ligands	[125]
		NK	-	-	Infection/tumor	H	Up-regulate the expression of NKG2D in NK cells	[9]
Interleukin 13	IL13	CD8+ T cells	-	-	Viral infection / tumor	H	Decreased expression of antiinflammatory cytokines through NKG2D expression	[89]
Interleukin 15	IL15	in vitro	NK	-		H	Up-regulation of NKG2D-DAP10 surface expression	[69]
			IELs	Intestine	Celiac disease	H	Induction of MICA surface expression	[128], [66]
		DCs	NK	-	Hepatitis C	H	Up-regulation of MICA/B in DCs and activation of NK cells	[25]
Interleukin 17	IL17	CD4+ T cells	IELs	Intestine	Crohn's Disease	H	Th17 immune response	[126]
Interleukin 18	IL18	Macrophages, DCs, Keratinocytes	Leukemia cells	-	Leukemia	H	Up-regulation of ULBP2	[85]
		in vitro	NK	-	Tumor	H	Down-modulation avoidance of NKG2D by TGF- β1 (together with IL-2)	[68]
Interleukin 21	IL21	Ovaric cancer cell line	NK	Ovary	Ovarian cancer	H	Up-regulation of NKG2D-MICA expression and the cytokines IFN-γ and TNF-α	[70]
		Th cells	NK/CD8+ T cells	-	Autoimmunity	H	Down-regulation of NKG2D/DAP10 in autoimmune diseases (immunotherapy)	[22]
Interleukin 22	IL22	CD4+ T cells	IELs	Intestine	Crohn's Disease	H	Potentiate Th17 immune response	[126]

Interleukin 33	Il33	Macrophages	Tumor cell	Skin	Merkel Cell Carcinoma	M	Down-regulation of RAE-1 for tumor-cell resistance to NK-mediated control of virus-induced tumors	[86]
Tumor necrosis factor	Tnf/TNF	in vitro	ECs	Endothelium	Activated endothelium	H	Inhibition of NK cytotoxic activity by soluble MICA	[90]
Interferon alpha 1	IFNA1	DCs	NK	-	Hepatitis C	H	Up-regulation of MICA/B in DCs and activation of NK cells	[25]
		in vitro	Tumor cell	-	Infection/tumor	H	Promotion of MICA expression in tumor cells, enhancing their sensitivity to NK lysis	[26]
Interferon beta 1	IFNB1	PBMNc	NK	-	HCMV infection	H	Down-regulation of NKG2D to control NK cell reactivity against normal cells expressing NKG2D ligands	[125]
Interferon gamma	ifng/IFNG	CD4+ T cells	IELs	Intestine	Crohn's Disease	H	Up-regulation of MICA in IELs during Crohn's disease	[127]
		chNKG2D T cells	Tumor cells	-	Lymphoma/ Murin ovarian cancer	M	Stimulation of DCs, NK and T cells with the subsequent antitumor effect	[89], [88]
		in vitro	ECs	Endothelium	Activated endothelium	H	Inhibition of NK cytotoxic activity by soluble MICA	[90]
Colony stimulating factor 2(granulocyte-macrophage)	Csf2/CSF2	Ovaric cancer cell line	NK	Ovary	Ovarian cancer	M	Up-regulation of NKG2D-MICA expression and the cytokines IFN-γ and TNF-α	[70]
		chNKG2D T cells	Tumor cells	Lymph node/ Ovary	Lymphoma/ Murin ovarian cancer	M	Stimulation of DCs, NK and T cells with the subsequent antitumor effect	[89], [88]
		NK	in vitro	-	Infection/tumor	H	ULBPs induce NK cells to produce this chemokine to recruit and activate NK and other immune cells	[9]
Transforming growth factor beta 1	TGFB1	in vitro	NK	-	Viral infection / tumor	H	Supression of NKG2D-DAP10 surface expression and MICA	[87], [67], [69]
Chemokine (C-C motif) ligand 4	CCL4	NK	in vitro	-	Infection/tumor	H	ULBPs induce NK cells to produce these chemokines to recruit and activate NK and other immune cells	[9]
Chemokine (C-C motif) ligand 1	CCL1					H		
Chemokine (C-C motif) ligand 2	Ccl2/CCL2	Tumor cells	NK	Liver	Carcinoma	M	Elimination of senescent tumors by NK cells due to p53 and NKG2D ligand expression in tumor cells	[71]
Chemokine (C-X-C motif) ligand 1	CXCL1	dNK	dFibroblast	Placenta	HCMV infection	H H	Crucial role of NKG2D in dNK cells in controlling HCMV infection and spreading	[72]

Table 1: Major cytokines and chemokines related to the NKG2D system Abreviations: CCL: Chemokine c-c motif Ligand; CXCL: Chemokine c-x-c motif Ligand; chNKG2D: chimeric NKG2D; DCs: Dendritic Cells; dFibroblast: Decidual Fibroblast; dNK: Decidual Natural killer; ECs: Epithelial Cells; H: Human; HCMV: Human Citomegalovirus; HIV: Human Immunodeficiency Virus; IELs: Intestinal Epithelial Cells; IFN: Interferon; IL: Interleukin; M: mouse; NK: Natural Killer; PBMCs: Peripheral Blood Mononuclear Cells; TGF: Transforming Growth Factor; Th: T helper; TNF: Tumor Necrosis Factor; (-) not specified.

system, highlighting the importance of the full co-operation between both components to achieve a succesful immune response.

Natural Killer (NK) Cells: the Link between Innate and Adaptive Immunity

Herberman [28] and Kiessling [29] described NK cells for the first time in 1975, but not until the last decade a true and unique identity of this cellular group were firmly established. NK cells are a subset of lymphocytes that lack antigen-specific cell surface receptors [30] which provide innate effector mechanisms against viruses and tumor cells through direct cytotoxic effects and the release of cytokines [5]. NK cells detect microbial insults by means of innate receptors like the Toll-like receptors, or in response to pro-inflammatory cytokines produced by dendritic cells (DCs) [31-33] macrophages and neutrophils [34]. To achieve their basic effector functions, fulfil their intrinsic development, and to survive or proliferate, all NK cells are dependent on cytokines of the common gamma chain (γc) family (IL-2, IL-7, IL-15, and IL-21) [35-37]. Of particular interest is interleukin-2, a key cytokine which enhances NK cell proliferation both in vitro and in vivo [38] potentiates and mediates NK-cell functions, orchestrates the interaction with T-lymphocytes and DCs [39], while contributing to achieve the homeostasis of mature NK cells [36]. In a different setting, during a viral infection, type I interferons (IFN-I), especially IFN-α, and type II IFN-γ are potent cytokines that trigger NK cell activation, turning them into surveillance entities that limit viral replication by binding to and sequestering specific virus-encoded proteins [40,41]. This viral dampening is a notable feature of NK cells, although recent studies have demonstrated that, in certain conditions, activated NK cells also have the ability to produce immunosuppressive cytokines such as TGF-β1 and IL-10, which may induce tolerance in local NK cells [42,43] and contribute to virus-mediated T-cell exhaustion [44,45]. Thus, this behavior suggests that, in addition to their positive role in combating viral treats when activated by IFNs, NK cells also have a negative regulatory role, releasing anti-inflammatory cytokines during acute and a chronic virus infection [41]. In addition, a different NK feature promotes their rapid extravasation from blood vessels or specific tissue to enhance the recruitment of immune cells at infection sites or tumor foci. These is achieved through the secretion of a wide set of chemokines, which include CCL2 (MCP-1), CCL3 (MIP1-a), CCL4 (MIP1-b), CCL5 (RANTES), XCL1 (lymphotactin) and CXCL8 (IL-8) [31]. This secretion of chemokines by NKs is an additional key feature that promotes their co-localization with other hematopoietic cells in areas where inflammatory processes are taking place or in tumor immunosurveillance sites [46]. But, despite this ability of NK cells to mediate the transcription of cytokines, they need to be primed

in advance by exogenous cytokines such as IL-15 [47,48], IL-12 [49] or IL-18 [50] to complete their full effector potential.

Hallmarks of NK Cells Receptors

NK cells possess a particular set of receptors that can sense microbial and non-microbial signals emitted by target cells through a variety of lectin-type receptor families. Of particular interest are the type II transmembrane proteins, which include the natural killer receptor (NKR) family such as FcgRIIIA activator [51], or the mammalian inhibitory CD94/NKG2 receptors [6]. CD94 forms heterodimers with various NKG2 receptors but not with NKG2D, which is a homodimer. In addition to CD94/NKG2 receptors, in some rodent species the inhibitory Ly49 family of lectin-type NKRs is also present. Additionally, cytokine receptors coupled to the common (γc) IL-15R, IL-2R and IL-21R are also involved in NK cell development and their effector functions. Among these receptors, IL-15R is essential for the maturation and survival of all NK cells, while IL-1R in humans [52] and IL-18R in mice [50], which are linked to the adapter protein MyD88, have a particular role in the NK cell maturation process. Thus, based on the above evidence, it can be concluded that, despite the considerable intra-and interspecific variation in gene numbers and complexity among mammalian Ig-type and lectin-type NKRs, they are expressed on NK cells, bind to MHC I or proteins that share structural similarities with MHC I, and can inhibit or activate target cell killing and/or cytokine release through competing signaling pathways [6].

Cytokine Induced Killer Cells (CIK): Antitumor Inducers

Cytokine-induced killer cells (CIK) cells are a heterogeneous subset of ex-vivo expanded T lymphocytes with a mixed T-NK phenotype [53]. Among the several advantages of conducting research with CIKs, the most interesting are their rapid proliferation in vitro, the strong antitumor activity and the broader target tumor spectrum they possess, compared with other antitumor effector cells, have been investigated so far [54]. Additionally, CIK cells have the capacity to provide a non-cross-resistant mechanism of antitumor activity that can be incorporated in surgery, radiation or chemotherapy treatments [55]. Thus, in the last years application of CIK cells in combination with chemotherapy has raised a powerful tool to treat cancer patients. In a classical approach, these cells are derived from peripheral blood mononuclear cells (PBMCs), but can also be generated from bone marrow or umbilical cord precursors [56]. The ex vivo expansion of CIK cells takes 3-4 weeks and is driven by the addition of IFN-γ, Ab-anti CD3 and IL-2 [57] to the cell culture. When the expansion is finished, the predominant subsets of CD3+ T lymphocytes are: CD3+CD56- and CD3+CD56+, formerly known as cytotoxic NK-like T cells. The antitumor activity of CIK cells relies solely on cell–cell contact [17]. Briefly, following an elegant research approach using antibodies against CD54, CD11c and NKG2D, Verneris and colleagues succeeded to attenuate the cytotoxic effect by blocking the cell-cell interaction, thus demonstrating that such effect is based on MHC-unrestricted mechanisms which rely on the interaction of the NKG2D receptor present in CIK cells and the ligands expressed in tumor cells. Therefore NKG2D-ligand interaction triggers the last step of tumor-killing, which is mediated by perforin and granzyme. These enzymes play a fundamental role in generating a pore in the cell membrane, which finally causes apoptosis of the target cell. Thus, capacity of CIKs as effector cells has been observed to be active in solid and hematological malignancies, which has been tested in vitro and confirmed in vivo with murine models of human tumor xenograft transplants. In a study of the cellular capacity of IL-2 primed CIK cells, resulted in the downregulation of the IL-18 levels which were mediated by IFN-γ [58,59]. Liu and colleagues, used oxaliplatin-folinic acid-fluorouracil (FOLFOX4) in combination with CIK cells as adjuvant to treat patients with gastric cancer [60]. The process described in their study identified the need for exogenous cytokines to prime CIKs. Briefly, the CIKs obtained from PBMCs and stimulated with INF-γ, IL-1α, IL-2 and anti-CD3 MAb for use as adjuvants resulted in increased NK cells activity and higher CD3+ and CD4+ T total cell counts, whereas CD8+ T cells decreased in number. In a parallel study, Shi and colleagues used fluorouracil (5-FU) with CIKs as adjuvant to successfully treat advanced gastric cancer [54]. The CIK cells used were activated by IFN-γ and IL-2 from the patients' PBMCs. The mix of 5-FU and activated CIK cells prolonged the disease-free state and significantly improved overall survival in patients with intestinal-type tumors. Thus, these studies conducted by different groups demonstrate that CIK cell priming by cytokines is essential for their activation and positive activity over malignant tumors. Interestingly, to determine the clinical value of autologous immunocyte therapy as a standard treatment against cancer, patients with colorectum, lung, breast, kidney, or stomach cancer received the DC vaccination once a week for six weeks and a CIK cell injection six times within four days. A positive cell-mediated cytotoxicity response was recorded, and improvements in physical strength, appetite and sleeping status were observed. Thus, they concluded that the therapy was safe since no serious adverse side-effects similar to those caused by chemotherapy and radiotherapy were observed [61]. Additionally, in recent publications it was suggested that CIK cells treated with IL-15 [62], or in combination with IL-2 [63] improve their cytotoxicity against leukemic cells or lung cancer cells, respectively. In both cases, the percentage of CD3+CD56+ cells was significantly increased in IL-15 stimulated CIK cells and their proliferative rate was higher. Taken together, these results realize that cytokine-activated cells may have a beneficial effect in the near future on the treatment of patients with cancer [61].

Cells Respond to Stress in Different Ways

Broadly defined, stress is the state in which cells deviate from the status quo in response to sudden environmental changes or frequent fluctuations in environmental factors [64]. Such changes can damage existing molecules, including proteins, mRNAs, DNA and lipids, and, if the damage is not dealt with, a metabolic imbalance may result in a redox alteration [65]. Several mechanisms are immediately triggered to overcome the stress responses; for example, damaged macromolecules are promptly cleared [66], molecular chaperones are induced [67,68] or growth arrest and "emergency" gene transcription kicks in [69]. When cells can no longer cope with excessive damage, straightforward cell death may occur through necroptosis. In contrast, stressors like heat shock, oxidative stress, viral infection or DNA damage may induce the expression of particular extracellular ligands [70]. Tumor cells can be stressed by multiple intrinsic or extrinsic stimuli, both of which may promote membrane expression or the release of 'eat-me', 'danger', or 'killing signals' that will facilitate immune recognition and the final eradication of stressed tumor cells [71]. Molecules from the MHC-I are ligands, which inhibit or activate receptors expressed on NK cells and T cells. The expression of MHC-I is frequently impaired in virus-infected or tumor cells, which results in lack of engagement on the inhibitory receptors and hence the activation of NKs. Hence, class I serves as a positive indicator for the integrity of cells, protecting against NK cell attack [72]. In contrast, NKG2D ligands, MICA/B and members of the ULBP/RAET1 may signal cellular distress and evoke immune responses. Although NK cells can eliminate tumor cells with

the loss or aberrant expression of class I, the interaction of MICA with NKG2D may promote antitumor responses in the presence of class I, depending on the balance of multiple inhibitory and activating signals, the relative amounts of receptors and their ligands, and the state of NK cell activation. However, this balance should be achieved through the differential expression of NKG2D-ligands, the modulation of the receptor and the cytotoxic activity of NKL cells after cell-cell contact with the tumor cells [73]. Thus, the interaction of NKG2D with MICA/B or ULBP/RAET1 may enhance diverse antitumor innate NK cell and antigen-specific T-cell responses. This recognition, in which self-encoded ligands are induced in stressed cells, is known as "induced self-recognition" [74].

The NKG2D System

Properties of the system in infection and tumor immunity

NKG2D is a cell-activating receptor that mediates non-MHC restricted and TCR-independent lysis. Cells expressing NKG2D are modulated by cytokines. Nevertheless, the NKG2D system has effector functions in which high levels of IFN-γ and TNF-α, among other cytokines with specific cytotoxic properties are produced [75]. Due this high plasticity, NKG2D is deeply involved in tumor immunosurveillance, which plays an important role in the cytotoxic activity of NK and CD8+ T cells. NKG2D is a C-type, lectin-like, type II transmembrane glycoprotein [76,77] which functions as an activating receptor through an interaction with the adaptor signalling molecules disulphide adaptor molecule (DAP)10 and/or (DAP)12 [78,79]. When the receptor is ligated, DAP10 provides signals that recruit the p85 subunit of phosphatidylinositol 3-kinase (PI3K) and a complex of GRB2 and VAV1 to complete the activation [80], whereas DAP12 activates protein tyrosine kinase Syk and ZAP70 [81], but this activation is not through the cytoplasmic signalling motif Tyrosine-Isoleucine-Asparagine-Methionine Motif (YINM) [82]. Therefore, the engagement of NKG2D with ligands in NK cells result in the induction of degranulation and cytokine production. The NKG2D receptor, is not only expressed by all NK cells [83] but also by NKT, subsets of γδ T cells [8], CD8+ T cells [71], activated mouse macrophages and a small subset of CD4+ T cells in humans. Thus, to trigger an effective immune response mediated by NK cells, the only prerequisite is engagement of the receptor with one of its ligands by means of a stimulus. However, in mouse T cells, co-stimulation by a subset of γδ T cells resident in the skin may be needed [84,85] . This co-stimulatory function is also present in αβ CD8+ T cells, but is much more noticeable when the cells lack CD28 marker expression, which is recognized as the normal co-stimulatory receptor for T cells [86]. Interestingly, the stimulation of CD8+ T cells with IL-15 together with CD3 generates a potent activation that leads to the engagement of the ligand with NKG2D. Such NKG2D priming driven by IL-15, triggers NK and T cell cytotoxicity, which is a key negative regulator in certain T cell–mediated pathologies such as in celiac disease [87]. Therefore, NKG2D is able to generate activating signals, which in some cases may co-activate cellular killing and produce undesired cytokines by NK cells and certain subsets of T cells. In the case of TGF-β1, it has been reported to decrease DAP10 levels and, as a consequence, NKG2D protein level [88]. Thus, to avoid the-induced NKG2D downregulation by TGF-β1 in NK cells, a previous activator engagement is required by the combination of IL-2/IL-18 with the receptor [89]. However, (γc) cytokines (IL-2, IL-7, IL-15) [89,90] and IL-18 [89] promote the induction of DAP10 and, consequently, the surface expression of the receptor. In ovarian cancer mice model, IL-21 has been also reported to play an activating role for the NKG2D expression [91] as well as innate tumor rejection activity, in tumors that can elicit an NKG2D-mediated immune response [92]. Therefore, two cytokines of the gamma-chain, the IL-7 and IL-15 seems to be key mediators in the upregulation of the NKG2D-DAP10 axis expression by NK cells. For instance, while IL-15 plays a common signaling role, also primes and regulates the NKG2D expression through the phosphorylation and further upregulation of the adaptor molecule DAP10 [93].

The role of cytokines on the NKG2D system is double edged

Several examples may illustrate this cytokine double edged behaviour. In one case, the enhanced expression of IL-15 increases the impaired expression of the NKG2D ligand MICA in monocyte/macrophages and induces the abnormal expansion of NKG2D+CD4+ T cells (NK cell-like CD4+ T cells) [75], like in a rare blood vessel disease, the granulomatosis with polyangiitis (Wegener's) [94]. Interferons are rapidly induced when NKG2D is activated, particularly IFN-α may upregulate NKG2D expression but can also dampen the expression of inhibitory receptors like NKG2A or KIR2DL1. The IFN-γ, which plays an inhibitory role in the expression of NKG2D, promotes the expression of NKG2A [27].In the case of chemokines, CCL1, CCL2, CCL4 and CXCL1 are known for their capacity to recruit and activate NK cells to the target cells [9,95,96]. Taken together, these results point to the difficulties involved in in vivo prediction on how the NKG2D system might behave under different scenarios. Thus, from above description is clear that a selection of cytokines may be specifically required for activation or inhibition of the NKG2D receptor or their associated ligands. However, the ways in which these cytokines may behave do not always correlate with their primary associated functional role. Further studies are required to throw light on how these proteins are integrated in the signaling pathways of NK cell activation and how the engagement of different activating receptors controls their activity.

NKG2D Ligands Modulation by Cytokines

NKG2D has multiple ligands including MHC class I chain-related-A (MICA), -B (MICB), and several UL-16 binding proteins (ULBP), which are preferentially expressed after cellular stress, infection, or DNA damage [78,97]. In humans, the MICA, MICB and the ULBPs, also known as retinoic acid early transcript RAET1 proteins, have been seen to be upregulated in cancer and infected cells [8,78,81,98]. These ligands are recognized by the immune activating receptor NKG2D. Upon engagement, allows the recognition and further elimination of infected and malignant cells. In mice, there are no orthologs for the MICA and MICB genes, but a family of genes orthologous to the human ULBP/RAET1 family is present. These genes are highly polymorphic and encode proteins that fall into three subgroups of NKG2D ligands, including five different isoforms of retinoic acid early inducible-1 (RAE-1) proteins (Rae-1α, Rae-1β, Rae-1γ, Rae-1δ and Rae-1ε), one murine UL16-binding protein-like transcript 1 (MULT1) protein, and three different isoforms of H60 proteins (H60a, H60b, H60c) [99]. In fact, to make the signalling pathways even more complex, there are over 60 MICA and 20 different MICB alleles. Human RAET1 genes are also polymorphic, as are Rae-1 and the histocompatibility antigen (H60) genes in mouse [100]. The effect of such polymorphism is that all the ligands engage with the NKG2D receptor with different degrees of affinity, which may affect the threshold of NK and T cell activation [7]. Moreover, some of the NKG2D ligands may be excreted to the extracellular environment, stay attached to the cell surface, remain at the transcription level biogenesis, be stabilized on the RNA or stabilized and cleaved from the cell membrane. Lastly, the effect of cytokines is no less confusing. As example, IL-18 increases the susceptibility of

target cells by inducing the surface expression of ULBP2 in leukemia cells [101]. However the pro-inflammatory cytokines IL-1α, IL-1β, IL-33, and TNF-α down regulate RAE-1 expression and susceptibility to NK cell-mediated cytotoxicity, leading to the avoidance of NK cell-mediated control of virus-induced tumors in mice [102]. In a similar way, TGF- β1 inhibits the transcription of the ligand MICA in humans [103]. In turn, CSF2 upregulates NKG2D-MICA expression [91] and stimulates antigen-presenting cells (APC) to initiate an antitumor response [104,105]. This last effect is shared with IFN-γ. Furthermore, IFN-γ upregulates MICA in intestinal epithelial lymphocytes (IELs) in Crohn's disease. However, it can also have a negative effect, leading to the inhibition of NK cytotoxicity by soluble MICA. This effect has been observed to be shared with TNF-α [106].

NKG2D Ligands Modulation by Alternative Players

Among the different cellular regulatory systems, it has recently been demonstrated that the translocation of mRNAs encoding the MICA/B in naive cells is inhibited by microRNAs (miRNA) [107]. However, upon stress, the transcription of MICA/B mRNAs is substantially upregulated and significant protein levels are detected. This observation suggests that the mRNA levels, when they exceed the amount of controlling cellular miRNAs, result in the overexpression of MICA/B proteins [70]. Until now, p53 has never been regarded as being essential for the expression of NKG2D ligands in cells suffering DNA damage. Nevertheless, co-operation between p53 induced-tumor cell senescence and the innate immune system has recently been highlighted [108]. The restoration of p53 function in established carcinomas leads to tumor regression, but only in mice with an intact immune system. Also, the inflammatory cytokines IL-15 and CSF2, and the chemokines CCL2 and CXCL1 were upregulated in tumors following p53 reactivation, correlating with the recruitment of neutrophils, macrophages and NK cells into tumors, where they are responsible in tumor shrinkage [108]. Thus, it seems that input from p53 to the NKG2D system is crucial at some stages of the signaling cascade, directly modulating the transcription of cytokines by tumor cells. Other unexpected players are the epigenetic changes, manifested by inhibitors of histone deacetylase, which can also induce the surface expression of NKG2D ligands on tumor cells [109]. From an evolutive perspective, these ligands are fairly conserved among vertebrates, and thus are not exclusively expressed in human and mice, but they are widely distributed among mammals [110]. From the information presented above, it is clear that a large variety of NKG2D ligands exist. However, so far the explanation for this variability, and the intriguingly diversity in which they are regulated, remains a matter of speculation.

The Double Edged Activity Exerted by Cytokines on Cancer Cells

During cell transformation and tumorigenesis numerous stress pathways are activated in affected cells [81]. During malignancies, NKG2D ligands are transcriptionaly induced and highly expressed on the surface of tumor cells. The ligands expressed depend on the type of cancer, which expresses MICA/B and at least one member of the ULBP family [111]. The receptor NKG2D participates in immune surveillance and has the capacity of eliminate NKG2D ligand-positive tumor cells in an early developmental stage [7]. A major feature of the antitumor response is mediated through type 1 cytokines, like TNF-α, or the IFN-γ. Indeed, increased amounts of IFN-γ and TNF-α were measured after treatment in the serum of patients [112]. Additionally, other cytokines, LTA, IL-13, IL-10 and CSF2, are produced upon contact with susceptible tumor target cells [113]. These cytokines can exert differential effects on the regulation of NKG2D ligands [7]. For example, melanoma cells exposed to IFN-γ downregulate MICA and ULBP2, and IFN-γ also reduces the expression of mouse H60 in sarcomas [24,114]. This reduced expression of NKG2D ligands has a negative effect, diminishing the susceptibility of tumors to NK cytolysis. In the supernatant of cultured HeLa and K562 cells, soluble MICA (sMICA) was observed to be upregulated by IFN-γ, demonstrating that IFN-γ modulates MICA expression not only at the transcriptional level, but also at the post-translational level by promoting proteolytic cleavage [27]. In contrast, IFN-α increases the expression of MICA in tumor cells and thereby enhances their sensitivity to NK lysis [27]. However, chronic exposure to tumor cells expressing NKG2D ligand alters NKG2D signalling and may facilitate the evasion of tumor cells from NK cell reactions [115]. One of the mechanisms that malignant cells are known to use for this purpose is the shedding of NKG2D ligands into the sera of cancer patients [116], where they weaken the immune response by downmodulating the receptor on effector cells and producing the consequent impaired immune response [114]. TGF-β1 has also been shown to decrease the transcription of MICA, ULBP2 and ULBP4 in human glioma [117], and downregulate NKG2D receptor expression on effector cells [118], while blocking TGF-β1 can lead to increased NKG2D expression [88,119,120]. This suggests that TGF-β1 secreted by tumors is a major mechanism that tumor cells employ to evade the NKG2D-DAP10-mediated cytotoxicity. Moreover, Clayton et al. [121] reported that tumor-derived exosomes also express NKG2D ligands and directly interact with NK and CD8+ cells. This response was demonstrated to be highly dependent on exosomal TGF-β1 and to induce the reduction in surface NKG2D expression. Using a mouse model, it was demonstrated that MICA did not mediate the downregulation of the receptor NKG2D [122]. The observed downregulation was explained through the sustained stimulation with tumor cell–bound ligand that disassociates the NKG2D receptor from the intracellular calcium mobilization and the exertion of NK cell–mediated cytolysis, while it induces the continuous production of IFN-γ. These functional changes are associated with a low abundance of the NKG2D signalling adaptors DAP-10 and DAP-12 [115]. So it was concluded that the low expression of MICA and MICB on resistant tumor cells may be another mechanism that allows tumor cells to escape from CIK cell-mediated cytolysis [17]. Although cytokine stimulation of cells may overcome receptor inhibition mediated by soluble ligands. To illustrate this mechanism, Song and colleagues [89] demonstrated that the combination of IL-2 with IL-18 can protect the TGF-β1-induced NKG2D down-modulation in NK cells via the JNK pathway. In addition, NKG2D contributes to the anti-tumor responses elicited by IL-2 and IL-12 cytokine therapy. As regarded the chemokine CCL2, it has been reported to be an active player in the elimination of tumor cells through the induction of NK cell recruitment into the tumor driven by p53 [95]. This mechanism has been demonstrated to be NKG2D-dependent and mediated by the recognition of ribonucleic acid export 1 (RAE-1) proteins in mice. Moreover, by pharmacological reactivation, p53 in specific cell lines has been reported to stimulate the expression of ULBP2 [123,124]. Perhaps, the use of ectopic NKG2D–DAP10 expression triggers the tumor-promoting capacity through ligand-mediated NKG2D self-stimulation [125]. An overview of these regulation mechanisms is provided in Figure 1.

A Key Player in Immunotherapy: the Cytokines

Cytokines directly stimulate immune effector and stromal cells at the tumor site and enhance recognition by cytotoxic mediators. Numerous animal model studies have demonstrated that cytokines

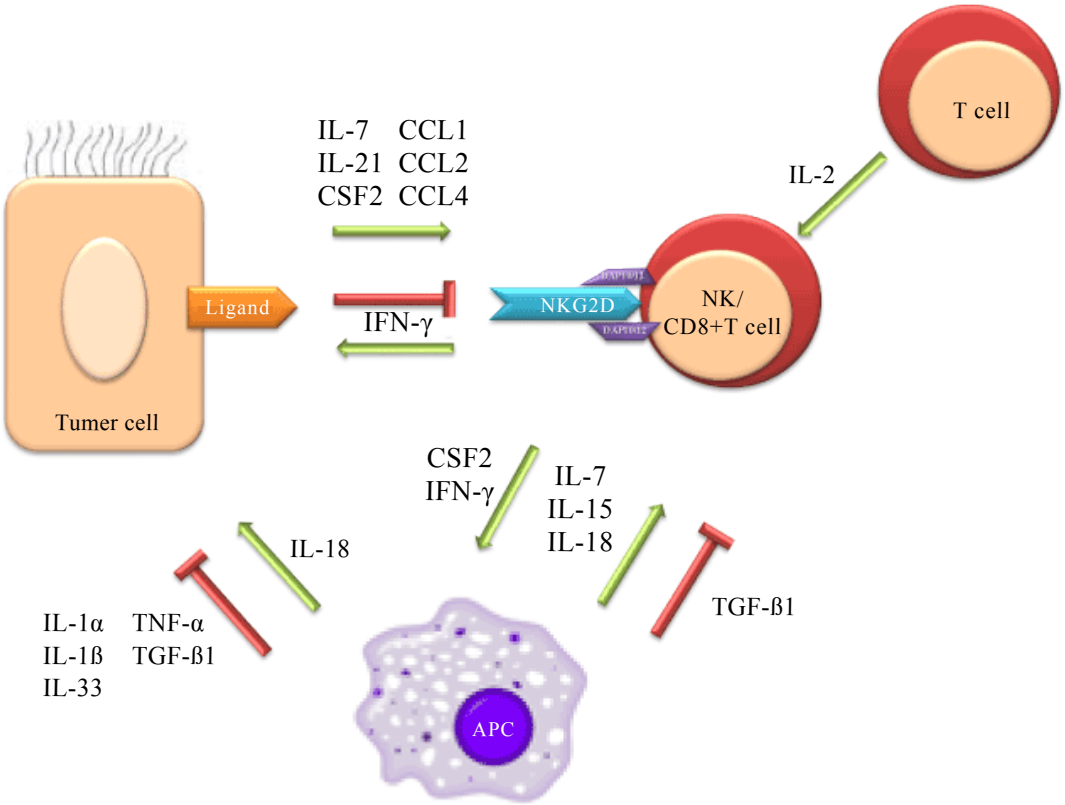

Figure 1: Within the tumor microenvironment, cytokines play a significant role in the regulation of the cytotoxic and cytolytic activity against tumor cells. Antigen-presenting cells (APC), dendritic cells (DCs) and macrophages, can up- or down-regulate the expression of NKG2D and NKG2D ligands on the surface of epithelial cells, leading to tumor elimination or tumor-cell resistance, respectively. The different output effects depend on the developmental stage of the tumor. Cytokines produced by NKG2D+ cells modulate ligand expression on the target cell and stimulate APC to exert antitumor effects. Schreiner [132] alter [133] Muntasell [134] pariente [135] allez [72]hüe [136].

have broad anti-tumor activity and this has been translated into a number of cytokine-based approaches for human therapy [126]. Additionally, the notable success of the targeted inhibition of several cytokines in patients with rheumatoid arthritis, psoriasis and many other diseases has fundamentally revised the treatment of inflammatory diseases. Together, these findings suggest that different conditions may share a common pathophysiology and may benefit from disruption of the cytokine network [127]. In cancer therapies, cytokines are critical for tumor immunosurveillance. Single-agent or the combination of cytokines with classical immune antibodies or TLR agonists resulted in potent CD8+ T cell-mediated antitumor effects [128,129]. The mix of CD40 antibody and IL-2 has been observed to have a synergistic antitumor effect [130,131], and a similar effect was observed when mice were treated with CpG motifs and IL-15 [129]. In both cases, the antitumor effects were dependent on the production of CD8+ T cells, IFN-γ, IL-12 and Fas ligand expression [129,130]. Additionally, it has been recognized that effector and memory CD8+ T cells express elevated levels of IL-12R and IL-18R, and secrete IFN-γ in response to stimulation with both cytokines [132]. In this setting, it is clear that the stimulation of CD8+ cells with cytokines results in antigen-nonspecific expansion, which is useful for immunotherapy in the treatment of advanced tumor models and represents a primary effector mechanism [131]. Interestingly, regulatory T (Treg) cells can inhibit NK cell cytolytic function and IFN-γ secretion, and have been shown to downregulate NKG2D in human and mouse NK cells through membrane-bound TGF-β1 [133]. However, not all the interactions between CD8+ cells and cytokines have been reproducible in NKG2D+CD4+ T cells. Even though , these cells proliferate and increase in number relative to other T cell populations, thereby causing imbalances in the lymphocyte pool and imposing an immunosuppressive cytokine milieu. In advanced cancer patients, however, cytokine mediated tumor expression and shedding, mainly of soluble MICA and B, can lead to a substantial proliferative expansions of the NKG2D+CD4+ T cells [125]. Thus, above presented evidences clearly point out that various clinical trials of immunotherapy for hematologic malignancies can induce regression to their pathophysiological state.

Conclusions

Based on the information reviewed above, it is clear that the cytokines involved on the regulation of the NKG2D system are an attractive target for therapy. However, many of the molecular processes associated in this system are not well understood and establishing their functional activity on particular scenarios remains a challenge. More knowledge is needed to understand the influences of these molecules on every single regulatory activity, ranging from target effector cells to the ligands they produce. A better molecular understanding on how cytokines regulate the effector response may provide important insights into the way in which the NKG2D system overcomes infection and combat tumors. We anticipate that answers to these questions will yield clinically useful information because the NKG2D system

clearly has an essential role in removing harmful cellular components. Moreover, impairment or disabling of the system has been linked to many human pathologies including cancer, autoimmune responses like inflammatory bowel disease [134], Celiac disease [135] or several types of gastritis. With this in mind, this review will hopefully contribute to stimulate much interest into the search for new answers in this intriguing research field.

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