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Seminars in Cancer Biology 16 (2006) 412–418

seminars in
CANCER BIOLOGY

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Review

Novel approaches using natural killer cells in cancer therapy

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Abstract

Strategies are emerging to apply natural killer (NK) cells as therapeutic agents against a broad range of malignancies. Novel clinical approaches aim to overcome limitations of original therapies, which have utilized lymphokine activated killer cells or systemic cytokine treatments. Remarkable results, including survival improvements and amelioration of graft versus host disease, were obtained with alloreactive NK cells in some cases. Other approaches in clinical evaluation include targeting heat-shock protein (Hsp) 70 expressing tumors with pre-stimulated autologous NK cells or the application of an NK cell line, NK-92, with enhanced cytolytic activity. Further mechanistic insights into NK cell cytotoxicity are a prelude to improved clinical cancer therapies.

Keywords: Natural killer cells; Adoptive immunotherapy; Alloreactivity; Hsp70; NK-92

Contents

1. General aspects of NK cells and cancer cell therapy

Natural killer (NK) cells are part of the innate immune system and important players in the first line of defense against diseases, including malignancies. In contrast to cells of the adaptive immune system, e.g., T-cells, immunization is not required to trigger NK cell cytotoxicity. NK cells therefore provide an immediate natural response. Their rapid cytolytic action and broad target range suggest that NK cells may be promising candidates for cancer cell therapy with the potential to target a wide range of malignancies. The clinical application of *ex vivo*-manipulated cells, including NK cells, is referred to as adoptive immunotherapy (AIT). The first clinical AIT trial exploited autologous *ex vivo* expanded and interleukin 2 (IL-2)-stimulated lymphokine-activated killer (LAK) cells and was published in the 1980's by Rosenberg et al. from the National Cancer Institute (NCI) [1,2]. However, no major benefit was reported from the original LAK protocols. As an alternative, infusion of high dose IL-2 to activate the endogenous NK cells of cancer patients did not increase therapeutic efficiency and caused severe toxic side effects. Outcomes and types of LAK cell and cytokine therapies targeting a variety of malignancies, including melanoma, renal carcinoma, lung carcinoma, ovarian cancer, brain tumor, have been reviewed in detail [3]. The low success rate of these therapies is likely due, at least in part, to insufficient cytolytic activity of NK cells from patients affected by severe malignancies [4]. Furthermore, NK cells represent only about 10% of the LAK graft population compared to T-cells, which constitute the major cell type [3]. Purification by specific selection and enrichment of NK cells on a clinical-scale before *ex vivo* expansion and stimulation can improve therapeutic outcomes [5]. Alternatively, stimulation of LAK cells

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1044-579X/$ – see front matter © 2006 Published by Elsevier Ltd.
doi:10.1016/j.semcancer.2006.07.006

with IL-15 [6] or IL-21 [7] instead of IL-2 may also increase efficacy.

Novel approaches aim to overcome the limitations of traditional AIT, and are enabled by an improved mechanistic understanding of NK cell cytotoxicity. NK cell mechanisms of action are complex and orchestrated by an array of receptors in a manner, which is unique for this subtype of immune cells. Soon after the discovery of NK cells [8,9], Klas Karre proposed a model, 'the missing self hypothesis', which remains the paradigm for NK cell function [10]. According to this model, NK cells eliminate those cells that lack, or 'miss', major histocompatibility (MHC) class I protein, molecules that define 'self'. Such a distinction between 'self' and 'non-self' by NK cells is enabled by the killer cell immunoglobulin (Ig)-like receptors (KIRs), that recognize and interact with the MHC class I human leukocyte antigens (HLA). Engagement of matching HLA ligands expressed by autologous cells with KIRs inhibits NK cell cytolytic activation pathways, thereby protecting autologous cells from being eliminated. Consequently, cells that downregulate HLA molecules, e.g. to escape a T-cell response, including malignant cells, become NK cell targets [11,12]. Co-engagement of NK cell activation receptors with target cell ligands activates NK cell cytotoxicity to destroy such aberrant 'non-self' cells. The field of NK cell receptor biology is rapidly developing and novel receptors, with inhibitory or activating function, continue to be discovered, e.g., natural killer cell receptors [13]. Further insight into NK cell receptor biology is a prelude for the development of novel, more promising NK cell therapies. This review will focus on recent progress that has been made in the field.

2. Exploiting NK cell alloreactivity

One novel AIT approach, with remarkable results reported in some cases, exploits the particular NK cell cytotoxic mechanism involving KIR receptors [14,15]. As described above, cytolytic pathways triggered by activation receptors are no longer inhibited when KIR receptors fail to recognize their respective HLA ligands. The therapy exploits the principle of KIR receptor mismatch initiating alloreactivity of NK cells. However, in the NK cell population of healthy individuals, each NK cell co-expresses at least one inhibitory KIR receptor for self-recognition, thereby preventing NK attack against autologous cells [16]. Nevertheless, KIR receptors and MHC class I molecules do not co-evolve. Both are highly polymorphic, allelelic molecule families, which are inherited independently. This raises the question as to how the crucial match between these groups is achieved, since failed ligand recognition by KIRs would activate NK cell cytotoxicity against autologous cells, a fatal scenario. Experimental evidence comes from studies with mice transgenic for the structurally different, but functionally related LY49 receptor [17,18] or more recently from an elegant model, using mouse strains generated to express only a single MHC class I molecule or combinations of three [19]. According to these data, developing NK cells are educated by MHC class I molecules in a quantitative and qualitative manner. MHC class I alleles expressed in a given individual determine the type and frequency of inhibitory KIR receptors expressed in the NK cell population [19,20], thereby preventing reactivity against self. However, alloreactive allogeneic NK cells can be exploited as tumor cell aggressors. *In vitro*, cytolysis of tumor targets by alloreactive NK cells has been demonstrated for chronic myelogenous leukemia (CML), non-Hodgkin's lymphoma, and multiple myeloma [16].

Andrea Velardi's group initially demonstrated a significant long-term benefit and prevention of leukemia relapse for haploidentical mismatched transplant recipients suffering from acute myeloid leukemia (AML) [21,22]. T-cell depleted donor grafts containing alloreactive NK cells improved the overall survival of patients in remission by mediating an enhanced graft versus leukemia effect (GvL) and significantly reducing graft versus host disease (GVHD) [23,24]. The decrease in GVHD was explained by donor NK cell alloreactivity against host antigen presenting cells (dendritic cells) that prevents presentation of host antigens to donor graft T-cells. However, in common phenotype acute lymhoblastoid leukemia (ALL) no major therapeutic effect could be demonstrated. Several similar clinical studies have been published since the first trial and extensively reviewed [15,16,23,25]. The overall outcome was contradictory, with success in some cases, but not in others. Generally, studies involving related (haploidentical) donors reported better efficacy compared with studies of unrelated donors [26]. Nevertheless, those trials differed in several parameters, which allowed defining crucial factors with potentially significant impact. For example, the extent of T-cell depletion varied, with more T-cells present in unrelated donor grafts versus haploidentical related grafts. However, T-cell depletion of the donor graft seems to be of major importance, since alloreactive donor T-cells can cause adverse clinical outcomes [26]. Furthermore, the overall transplanted effective stem cell number differed, with lower numbers in unrelated donor studies. It has been concluded that the extent of donor graft T-cell depletion, the number of transplanted stem cells and the degree of post transplant immunosuppression, which could negatively affect development of engrafted NK cells, are crucial factors influencing therapeutic outcome [26]. Moreover, engrafted donor NK cells become subject to host MHC class I education, acquiring a 'host-matched' receptor pattern, eventually annulling their alloreactivity [19]. Future AIT approaches exploiting NK cell alloreactivity will benefit from a better understanding of the complex scenario of NK receptor biology.

3. Targeting Hsp70 positive tumors

It has been found that tumor cells, different from normal healthy cells, selectively express stress-inducible plasma membrane-bound heat-shock protein (Hsp) 70 and an immunostimulatory function has been suggested for this protein [27]. Gabriele Multhoff's group was the first to identify that NK cells recognize such unusually on the surface expressed Hsp70 molecules [28,29]. Binding studies demonstrated direct interaction between NK cells and Hsp70 [30] and a quantitative correlation between Hsp70 surface expression and NK cell cytolysis was established. An extensive screen of different tumor biopsies (including colon carcinoma, liver metastases, pancreas carcinoma, lung carcinoma, glioblastoma, astrocytoma, leukemia

blasts and mammary carcinoma) revealed that, with the exception of mammary carcinoma, all tumor types tested expressed membrane-bound Hsp70 [31]. In contrast, membrane-bound Hsp70 was undetectable on bone marrow and normal tissues. These findings indicate that Hsp70 is widely expressed on the surface of tumor cells as a selective marker. Recently, Hsp70 surface-positive exosomes have been described that are released by tumor cells, with NK cell stimulatory function [32]. However, the responsive NK cell receptor, which binds to membrane-bound Hsp70, is not known and the mechanism of target cell lysis is not yet fully understood. Experimental evidence suggests a role for CD94 as the putative NK cell receptor for Hsp70 [30], thereby assigning a new function to this receptor, which is otherwise known to form heterodimers with NKG2 receptors. CD94-NKG2 heterodimers bind to non-classical MHC class 1 antigens (e.g. HLA-E), however, an involvement of MHC antigens in the NK cell recognition of Hsp70 could be excluded [30]. The C-terminal Hsp70 substrate-binding domain has been defined as the NK-cell interactive region. A derived 14-mer peptide (aa 450–463, TKDNNLLGRFELG, TKD) includes the recognition structure for NK cell activation. The finding that membrane-bound Hsp70 is a selective tumor cell marker triggering NK cell cytotoxicity and migration toward membrane-bound Hsp70-bearing tumor cells has recently been translated into a clinical phase I trial [33]. Autologous NK cells from peripheral blood were *ex vivo* stimulated for 4 days with the TDK Hsp70-peptide and low-dose IL-2, to improve the capability of the cells to recognize Hsp70 expressing tumors, before reinfusion into the patient. The leukapheresis product contained 8–20% activated NK cells of total lymphocytes. NK cell cytolytic activity was enhanced and cell surface expression of CD94 increased, further strengthening the previous findings, which implicated CD94 as a putative Hsp70 NK cell receptor. Also, NK cells demonstrated Hsp70 reactivity without further stimulation when re-isolated from patients after one and four reinfusion cycles. Eleven patients with metastatic colorectal cancer and one patient with non-small cell lung cancer were studied. All patients had failed standard therapies and presented with progressive, metastatic disease. The treatment was overall tolerated well; no serious side effects were noticed, demonstrating the safety of the procedure. Although all patients had high levels of tumor cell burden at the start of treatment, at least one patient showed stable disease during therapy, indicating a potential therapeutic effect. Future trials of patients with less tumor burden may better determine the feasibility and success of Hsp70-NK cell based therapies.

4. AIT with permanent NK cell lines

A novel approach different from cell therapy with endogenous NK cells of host (autologous) or donor (allogeneic) origin involves the permanent IL-2 dependent NK cell line, NK-92. A major advantage of permanent NK cell lines such as NK-92 is the ease of their maintenance and expansion in culture. Leukapheresis is unnecessary, which avoids potential adverse effects. Furthermore, the product can be made available in large quantities at high purity with reproducible characteristics. The only other cell line being used in clinical applications is the T cell line TALL-104 [34–36]. NK-92 was established by Hans Klingemann's group from a patient at diagnosis with non-Hodgkin's lymphoma (NHL) with large granular lymphocytes (LGL) [37]. NK-92 was shown to exhibit superior cytotoxicity compared with LAK cells or the T cell line TALL-104 [38]. The efficacy of NK-92 was demonstrated *in vitro* against several leukemia cell lines and primary leukemia blasts from relapse patients (AML, T-cell adult lymphocytic leukemia (T-ALL), B-lineage ALL, and CML) [39]. More than 50% of tumor samples were sensitive to NK recognition. However, tumor cell resistance to NK-92 lysis was also reported for B-precursor ALL [40].

The high cytolytic activity of NK-92 is thought to be the result of the lack of KIR receptors; only the unusual KIR with activating and inhibitory functions (KIR 2DL4) has been detected. The main inhibitory KIRs, expressed by most individuals, are KIR 2DL1, KIR 2DL2, KIR 2DL3, and KIR 3DL1, which recognize the two major MHC class I groups, HLA-C group 1 and 2, and HLABw4. These KIRs have been found to be represented on NK cells of 97% (KIR 2DL1), 100% (KIR 2DL2/3), and 94% (KIR 3DL1) of 162 individuals tested [16]. KIR 2DL1 binds to HLA-C group 2 alleles, KIR 2DL2/3 bind to HLA-C group 1 alleles, and KIR 3DL1 recognizes the HLA-Bw4 allele. Accordingly, application of allogeneic NK-92 cells would create a KIR mismatch situation. In our study, we detected 3 KIRs by PCR for NK-92, KIR 2DL5 (inhibitory function), KIR 3DL1 (inhibitory function), and KIR3DL3 (unknown function), potentially a result of the different culture conditions chosen, which in fact rendered the cell line less cytotoxic [41]. However, KIR expression by NK-92 has not yet been tested in a direct comparison under different culture conditions.

Feasibility for *in vivo* application of NK-92 was tested in SCID mouse models, xenografted with patient-derived leukemia (T-ALL, AML) [39] or melanoma cell lines [42]. Tumor burden was reduced or undetectable and survival of mice significantly improved [38,39,42]. Studies were conducted to define optimal culture conditions for NK-92 for large-scale expansions under good manufacturing practice conditions [43]. The optimal γ-irradiation dose was determined (10 Gray) that prevents engraftment of the tumorigenic NK-92 cells into the host but retains their cytotoxic capacity [44]. NK-92 has been suggested as an *ex vivo* purging agent for peripheral blood stem cell grafts [45], although the clinical benefit of this maneuver is questionable. The US Food and Drug Administration (FDA) and Institutional Review Board (IRB) have approved use of NK-92 cells from an established master cell bank (product ZRx101, ZelleRx, Chicago, US) which has tested pathogen free. Working cell banks derived from the master cell bank are individually re-tested. Phase I/II clinical trials to evaluate NK-92 feasibility and safety for clinical applications have been conducted in Frankfurt/Germany, by Ehrhard Seifried's group [38] for the treatment of children and young adults with advanced cancer (leukemia and sarcoma), initiated in Chicago and transferred to Pittsburgh/US by Hans-Georg Klingemann's group [46] for the treatment of advanced renal cell carcinoma and malignant melanoma, and, recently, in Toronto by Armand Keating's group for the treatment of leukemia and lymphoma in relapse, after autotransplant (A. Keating, personal communication).

The NK-92 trial conducted in Germany showed that infusion of 3×10^9 cells/m^2 body surface is safe (two transfusions, 48 h apart). Minor side effects, including mild fever and lower back pain, were documented with doses up to 5×10^9 cells/m^2 body surface although no serious adverse reactions were found. It is conceivable that NK-92 cells (due to the lack of KIRs) eliminate dendritic cells and prevent a T-cell response. Efficacy of treatment cannot be evaluated in the phase I trial and positive responses would be unlikely given the advanced and resistant disease in the patients enrolled [38].

In the US study, three infusions, 48 h apart, were given, with the highest dose of 3×10^9 cells/m^2. Toxic side effects, starting at a dose of 1×10^9 cells/m^2, included fever, but were transient and eventually resolved. It was concluded that NK-92 administration for renal cell cancer and melanoma is also safe [46]. Reports from the recently initiated Canada trial are awaited with great anticipation, especially because in this study some patients are likely to have relatively low tumor burden. Future studies will elucidate the potential of this novel, highly promising NK cell therapy.

Another NK cell line that may have potential as a novel candidate for AIT is KHYG-1 [41]. KHYG-1 was isolated from a patient with aggressive NK cell leukemia [47]. KHYG-1 is a highly cytotoxic cell line that exceeds the cytotoxicity of NK-92 against the standard leukemia target cell line K562 *in vitro* [41]. KHYG-1 cytolytic activity remains stable under different culture conditions, likely due to its unusually primed state [41,48]. KHYG-1 was highly cytotoxic against several leukemia and lymphoma cell lines, but showed no reactivity against peripheral blood from healthy individuals (Suck, Branch and Keating, unpublished observations). KHYG-1 retained its enhanced cytotoxicity after γ-irradiation with proliferation inhibiting doses [49]. It is reasonable to assume that KHYG-1 will be a potent anti-tumor agent *in vivo*. *In vivo* evaluation of KHYG-1 will further elucidate its potential in clinical applications.

4.1. NK-92 modifications and targeting approaches

Several NK-92 variants have been established that are independent of exogenous IL-2 [50–52]. Although NK-92 retains cytotoxicity for some time after IL-2 withdrawal, cytokine independence could allow prolonged cytolytic activity *in vivo*. The TR-IL-2-NK-92 variant with autocrine IL-2 secretion was shown to significantly exceed parental NK-92 cytotoxicity against several tumor targets and a survival benefit was demonstrated *in vivo* in tumor bearing mice [50]. However, the effect of irradiation on TR-IL-2-NK-92 activity has not been studied.

Other variants include NK-92CI and NK-92MI, which secrete IL-2 at low or moderate levels, respectively [51]. Despite similar cytolytic activity of those derivatives compared to parental cells, as shown *in vitro* against different tumor targets [51] and *in vivo* in xenografted SCID mice [42], the underlying cytotoxic mechanism seems to differ at least for NK-92CI [53].

The NK-92MI variant has been further evaluated as an adjuvant to photodynamic therapy (PDT) [54]. In NOD-SCID mice xenografted with human cervical squamous cell carcinoma, the addition of NK-92MI to PDT significantly improved therapeutic outcome, compared to PDT treatment alone. Similarly, in a second NOD-SCID mouse model, xenografted with human colorectal adenocarcinoma, addition of NK-92MI, but not of control NK-92 cells, improved the outcome. Moreover, in immunocompetent mice (syngeneic BALB/c), a beneficial effect of NK-92MI was noted, indicating that cells were not rejected as the result of a host immune response [54]. However, both variants, NK-92CI and NK-92MI are more sensitive to irradiation and responded with diminished cytotoxicity compared to parental NK-92 cells. Therefore, there is no obvious advantage for the use of irradiated NK-92CI and NK-92MI in AIT. However, the introduction of a suicide gene into those variants could potentially improve NK-92 based therapies. Indeed, this approach may allow a means to control the growth of these cells *in vivo*. This would be particularly important for the recently described stem cell factor expressing NK-92 variant, NK-92-SCF, which shows increased proliferation and cytotoxic potential after stimulation with IL-2 or IL-15 [55].

Another potential approach, which completely circumvents IL-2 secretion is provided by a novel NK-92 variant that expresses and retains IL-2 in the endoplasmic reticulum (ER) [52]. This autocrine IL-2 secretion, which does not leak into the extracellular compartment, is sufficient for survival and growth of NK-92 cells and retention of their cytotoxic potential. The insertion of a suicide gene (such as thymidine kinase) for potential clinical approaches is discussed [52].

A novel approach to design a NK-92 variant bearing a suicide gene is NK-92-CD20. NK-92 has been transformed with full-length CD20 antigen, which after engagement with anti-CD20 antibody (for example Rituximab), would trigger apoptosis in the cells thereby controlling their expansion *in vivo* [56].

It has been shown that NK cells expressing a chimeric ζ-receptor can be directed and kill otherwise resistant tumors [57,58]. This approach has been exploited to genetically modify NK-92 in several ways. One variant targets particularly epithelial tumors that express the tumor-associated antigen Her2/neu (ErbB2) [59]. A crucial role for Her2/neu as a therapeutic target has been shown previously [60–62]. The NK-92 anti-Her2/neu variant expresses an antibody fragment (scFv (FRP5) specific to Her2/neu, which is linked through a flexible hinge region to the signal transducing CD3-ζ chain (of the T-cell antigen receptor complex). Engagement of the Her2/neu specific antibody fragment by the tumor cell can directly trigger NK-92 cytotoxicity. Significant cytolytic activity of this variant could be demonstrated against several Her2/neu expressing primary and established tumor cells (including breast carcinoma, ovarian carcinoma, and squamous cell carcinoma), which were otherwise resistant to parental NK-92 cells. Furthermore, treatment of CD1-nude mice xenografted with tumorigenic fibroblasts with the NK-92 variant inhibited tumor growth for several days, however NK-92 alone did not have this effect [59]. Recently, the NK-92 anti-Her2/neu variant and the parental cell line, labeled with iron-oxide contrast agents, were tracked *in vivo* in mice xenografted with Her2/neu-expressing tumor cells [63]. This method could enable tracking of NK cells after therapy in patients to identify NK cell distribution. Strikingly, the NK-92 anti- Her2/neu variant cells, but not the parental NK-92 cells,

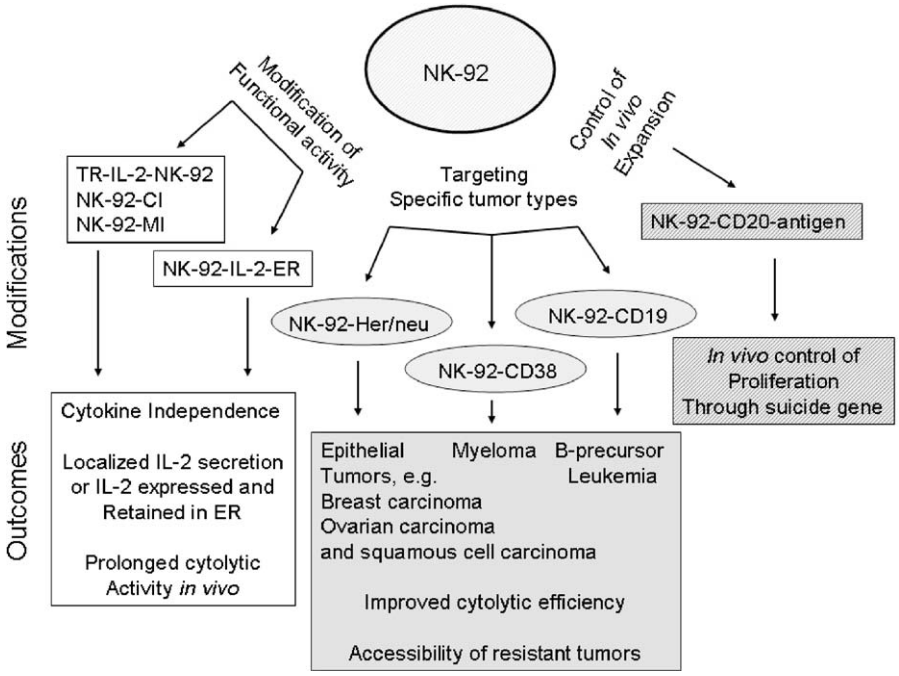

Fig. 1. NK-92 modifications and their potential effects. Endoplasmic reticulum (ER).

were found to accumulate in tumor tissue. Clinical studies with the NK-92 anti- Her2/neu variant are warranted to further test its suitability.

Other recently modified NK-92 variants are directed against multiple myeloma B cell neoplasm [64] or human B-precursor leukemia [65]. The same principle as for the NK-92 anti-Her2/neu variant, expressing a chimeric receptor, is exploited. In the case of myeloma, CD38 is targeted, a molecule highly expressed by plasma cells. In the case of human B-precursor leukemia, since ALL shows variable sensitivity to NK-92 mediated lysis, CD19 is targeted to overcome B-precursor ALL resistance [65]. CD19 is a universal marker for the B lineage and a therapeutic effect of anti-CD19 has been recently demonstrated in mice [66]. Resistance to B precursor ALL is a common phenomenon in NK cell cytotoxicity for which the mechanism is not known. A CD19 expressing NK-92 cell line could be of particular relevance in a clinical context [65].

Targeting malignant cells with modified NK cell lines, summarized in Fig. 1, holds promise as a powerful tool for cancer therapy. Furthermore, this approach is applicable to other potential NK cell lines such as KHYG-1. Further clinical studies are warranted and are likely to determine the clinical utility of NK cell lines.

5. Conclusions and outlook

In recent years a variety of novel approaches have been developed to exploit NK cells in cancer therapy. An improved understanding of NK cell cytotoxicity should lead to progress in clinical applications of this approach. The cytolytic action of NK cells is characterized by the differential action of an array of surface receptors with inhibitory or activating function. In addition to the predominant role of inhibitory KIRs predicted by the 'missing self hypothesis', a crucial function for activation receptors becomes increasingly more obvious. The role for activation receptors, rather than inhibitory receptors, may explain resistance of B-lineage acute lymphoblastic leukemia (B-lineage ALL) [40]. Nevertheless, hematological malignancies are likely to be the best targets for NK cell mediated AIT, in part because potential problems associated with inefficient trafficking are less likely [67]. The most promising clinical application involving NK cells exploits NK cell alloreactivity against leukemia in mismatched transplants. However, the therapeutic benefits, although remarkable in some cases, were negligible in others. Since several factors influencing outcome have now been identified, improvements in the near future are to be expected. Other novel concepts discussed in this review, including targeting Hsp70-expressing tumors and the application of the NK-92 cell line, require further assessment. However, these approaches appear to be safe and show promise. Since membrane-bound Hsp70 seems to be widely expressed on tumor cells, but not on normal cells, Hsp70 induced immunity may also be useful. Moreover, NK resistant tumors may be accessible to cell lines expressing chimeric receptor variants. Other NK cell lines, such as KHYG-1, may further extend the utility of this approach, which may be further expanded by targeting strategies. Additional studies of mechanisms of action, in particular the precise interplay of the 'receptor array', are likely to lead to new insights and the potential to enhance AIT.

Acknowledgements

This work was supported by an institutional grant to the Division of Biomedical Sciences, Johns Hopkins in Singapore from the Agency for Science, Technology and Research, A*STAR. I would like to thank Armand Keating, Donald

R. Branch and Victor Levitsky for critical reading of the manuscript.

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