UNIVERSIDADE FEDERAL DO RIO GRANDE DO SUL
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Natural Killer Cell Therapy: from Bench to Bedside

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“Knowing is not enough; we must apply. Willing is not enough; we must do.”

Johann Wolfgang von Goethe

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Resumo

A terapia celular é uma alternativa promissora para o tratamento do câncer. Células Natural Killer (NK) são linfócitos que se desenvolvem na medula óssea a partir de células progenitoras hematopoéticas de linhagem linfóide, e que podem ser encontradas no sangue periférico. Importantes efetoras do sistema imune inato, as células NK tem função citotóxica e papel imunoregulador, sendo consideradas leucócitos especializados no combate a neoplasias e células infectadas por vírus. Quando ativadas, as células NK tem um grande potencial terapêutico para o tratamento do câncer, podendo melhorar os benefícios de transplante de células-tronco hematopoéticas. Células NK também estão relacionadas com o desejado efeito do enxerto *versus* leucemia, sem indução de doença do enxerto contra o hospedeiro. Com base nisso, as células NK parecem ser candidatas naturais a serem utilizadas na imunoterapia adoptiva (IA). A segurança e a eficácia da IA utilizando células NK tem sido direcionada principalmente para o tratamento de leucemia mielóide aguda. Entretanto, a utilização de células NK para o tratamento de outras doenças hematológicas, tais como linfoma e mieloma múltiplo, também tem sido relatada. Há ainda alguns desafios a serem superados para tornar a terapia com células NK efetiva, segura e economicamente acessível, sendo primordial a necessidade de um número expressivo de células ativas. Nesta revisão são discutidos protocolos para isolamento, expansão e produção *in vitro* de grandes quantidades de células NK funcionais e que atendem aos critérios para aplicações clínicas. Dentre os métodos estudados estão: o uso de biorreatores para aumentar a produção, e expansão das células NK em presença de interleucinas e *feeder cells*. A revisão também traz novas metodologias visando otimizar a geração de produtos de grau clínico para IA.

PALAVRAS-CHAVE

Células Natural Killer, imunoterapia adotiva, câncer, produção *in vitro*.

Este artigo de revisão foi elaborado segundo as normas do Jornal *Frontiers in Immunology|NK Cell Biology*.

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NK – Natural Killer

AI – Adoptive immunotherapy

HSCT – Hematopoietic stem cell transplant

GVL – Graft versus leukemia

GVHD – Graft versus host disease

FBS – Fetal bovine serum

PB – Peripheral blood

TNF-α – Tumor necrosis factor-α

IFN-γ – Interferon-γ

KIR – Killer immunoglobulin-like receptor

AML – Acute myeloid leukemia

MM – Multiple myeloma

UCB – Umbilical cord blood

IL – Interleukin

aAPC – Artificial Antigen Presenting Cells

CAR – Chimeric Antigen Receptors

Summary

Natural Killer Cell Therapy: from Bench to Bedside

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Abstract

Cell therapy is a promising alternative for the treatment of cancer. Natural Killer (NK) cells are lymphocytes that develop in the bone marrow from lymphoid lineage hematopoietic progenitor cells, and can be found circulating in peripheral blood. Important effectors of the innate immune system, NK cells have cytotoxic function and immunoregulatory role, being considered specialized lymphocytes that fight cancer and virally infected cells.

Activated NK cells have a great therapeutic potential for cancer treatment and can improve the benefits of hematopoietic stem cell transplant. NK cells are also related to the desirable graft versus leukemia effect, without inducing graft versus host disease. Based on that, NK cells appear to be natural candidates to be used in adoptive immunotherapy (AI). The safety and efficacy of AI using NK cells has been primarily directed for treating acute myeloid leukemia. In the meantime, the use of NK cells to other hematological diseases like lymphoma and multiple myeloma has been also reported.

There are still some challenges to overcome to make NK cell therapy cost-effective, being the most important the need of an expressive number of active cells. In this review we discuss protocols for isolation, expansion and *in vitro* production of large quantities of functional NK cells that meet the criteria for clinical applications. Among the methods studied are: the use of bioreactors for scaling up production, and expansion of NK cells in

presence of interleukines and feeder cells. The review also brings novel methodologies aiming to optimize the generation of clinical grade products for AI.

Keywords: Natural Killer cells, adoptive immunotherapy, *ex vivo* expansion, hematopoietic stem cell transplant, graft versus host disease, graft versus leukemia, acute myeloid leukemia.

1. Introduction

Cell therapy is a promising alternative for the treatment of proliferative diseases like cancer, especially in the context of hematopoietic stem cell transplant (HSCT) for the treatment of malignancies. It is known today that the factor that effectively gives HSCT its curative potential is the effect of graft versus malignancy or graft versus leukemia (GVL) (1) exerted by the donor´s normal immune system. Despite all the advances in both diagnostic and therapeutic technology, allogeneic HSCT remains a procedure with high morbidity, associated to a mortality of 20 to 50 %, depending on the type of HSCT (related or unrelated) (2). The main cause of mortality related to HSCT are recurrence of primary disease and graft versus host disease (GVHD), acute or chronic (3).

The currently unquestionable existence of the GVL effect, without GVHD, has been exploited widely in the past few years (4–9). Knowing how to differentiate GVL and GVHD allows us to choose a lymphocyte type, and, without transplant, to infuse *in vitro* expanded activated lymphocytes active against malignant cells. The so called Adoptive Immunotherapy (AI). Lymphocytes of various subgroups have been explored for this purpose (10). Natural Killer (NK) cells appear to be natural candidates since it is a population of specialized lymphocytes with activity against cancer and virally infected cells (4), with little or no anti-HLA activity (4,9,11). However, *in vitro* expansion of NK cells may be accompanied by joint expansion of undesirable T lymphocytes. Therefore, purification techniques for NK cells cultures have been explored (8,12–14).

The *ex vivo* cultivation of immune cells for use in AI usually requires addition of supplements, some from animal origin like fetal bovine serum (FBS), with obvious risks of xenoreaction. The World Health Organization (15), the European Medicines Agency (16), and several researchers (17–20) have already manifested their concern on the subject, since laboratory cultured cells are being increasingly used in clinical trials (21,22) (registered at www.clinicaltrial.gov as clinical trials: NCT00625729, NCT01795378, NCT01787474).

There are still some challenges to overcome in order to make NK cell therapy safe and cost-effective, being the most important of it the need of an expressive number of active cells. In this study we review methodologies to obtain and expand NK cells. Possible clinical applications of AI are reviewed.

1.2. Natural Killer cells

NK cells develop during fetal life, and also after birth, from lymphoid lineage $CD34^+$ hematopoietic progenitor cells (23,24). NK cells constitute from 1 to 32.6 % of peripheral blood (PB) lymphocytes from normal individuals (5), and can be found in the lymph nodes, spleen, bone marrow, lung, liver, bowel, omentum, and placenta (25). Differentiation occurs in the periphery, in lymph nodes, where they acquire cytotoxic activity (1). Resting, or not activated, NK cells circulate in the blood, but following activation by cytokines, they are capable of extravasation and infiltrate into almost all tissues that contain pathogen-infected or malignant cells (26–29).

Human NK cells were firstly described by Trinchieri as nonadherent and nonphagocytic cells (30), being morphologically recognized as large lymphocytes containing azurophil granules (Large Granular Lymphocytes) (30,31), and depending on their activation status, some NK cells can also display normal small lymphocyte morphology (32).

Besides their size and morphology, human NK cells are immunophenotypically characterized by the expression of CD56, with or without CD16, and lack of expression of CD3 (13,33). Based on their CD56 expression, NK cells can be separated into two subsets: $CD56^{dim}$ and $CD56^{bright}$ (34). The first subset is defined by a low-density expression of CD56, and corresponds to the majority of the human NK cells, while the second corresponds to around 10 % of the human NK cells (34). Studies relate these subsets to functional properties of the NK cells, being the $CD56^{dim}$ subset associated to more cytotoxic cells, while $CD56^{bright}$ subset shows the ability to produce more cytokines than the first (34–36).

NK cell activation results from the balance between positive and negative signals provided by activating and inhibitory types of receptors (37), and the density of ligands of the interacting cells for these receptors dictates whether or not NK cells will be activated or have their cytotoxicity and/or cytokine secretion increased (38). NKG2D, NKp46, NKp30, NKp44 are the activating form known as KIR-S and CD16 (37), and the most studied inhibitory receptors are KIR2DL1, KIR2DL2/3, KIR3DL1, and immunoreceptor tyrosine-based inhibition intracellular motifs (38). Some ligands of the activating receptors remain unknown,

while ligands of the inhibitory receptors are well characterized as large families of major histocompatibility complex class I (38).

NK cells play an important role in the elimination of virally infected and tumor cells. When activated, NK cells have the capacity to lyse tumors with aberrant expression of the major histocompatibility complex class I, and to produce cytokines and chemokines for their self-regulation, or for other immune effectors regulation (7). NK cells, upon activation, lead to target cell apoptosis through contact-dependent cytotoxicity, primarily mediated by perforin and granzyme B (24,33,39), and can secrete large quantities of pro-inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interferon-γ (IFN-γ) (38,40–43).

1.2. Mode of action of NK as cell therapy agents

NK cells are important effectors of the innate immune system, and as previously mentioned, these cells have cytotoxic function and immunoregulatory role (13,24,41). They work significantly in the immune surveillance, especially in the initial phase of defense against infections caused by a variety of microorganisms, or in the control of malignant tumors (4,44,45). NK cells have been studied to be used in AI, in an autologous or allogeneic setting, alone or after allotransplantation (38).

NK cells play a significant role in the GVL reaction without inducing GVHD, through reacting against receptor´s dendritic cells (13), and this is associated with a better prognosis (4,45). In allogeneic HSCT, better grafting and lower relapse rate are observed when inhibitory receptors of donor NK cells are not compatible with HLA class I molecules of the receptor, therefore these receptors are not activated (45). Aloreactive potential of the NK cells is mainly due to the incompatibility of the killer immunoglobulin-like receptor (KIR) ligand (7), what has been exploited as a tool for cell immunotherapy (9), and can be used to eradicate residual disease after allogeneic HSCT in patients refractory to chemotherapy (46).

1.3. NK cells in the treatment of AML

Acute myeloid leukemia (AML) is a hematological malignancy, representing a heterogeneous group of myeloid neoplasms - some of it high-grade - mostly affecting elderly (mean age of 66 years). The annual incidence of new cases, is 4.4 per 100,000 in United States (47). In southern Brazil, the prevalence described so far is of 1.1 cases per 100,000 inhabitants per year (48).

Allogeneic HSCT is an effective and potentially curative treatment for AML (49–52). However, HSCT is associated with high morbidity and mortality rates, and GVHD is the

foremost serious complication of this procedure (53,54). It was reported that activated NK cells have a great therapeutic potential for the treatment of cancer and improve the benefits of HSCT (55). Ruggeri and colleagues demonstrated that patients with AML who received haploidentical transplantation with aloreactive NK cells showed decreased risk of refractory leukemia, no GVHD and better event-free survival (56).

NK cells may also exert anti-tumor activity in non-transplanted patients. In a study by Miller *et al.*, 5 out of 19 patients with poor prognosis AML achieved complete remission after treatment with haploidentical NK cells, and a significantly higher rate of complete remission was obtained when KIR mismatched donor cells were used (57).

1.4. Other clinical applications for NK cells

The safety and efficacy of AI using NK has been primarily directed for the treatment of AML. In the meantime, the use of NK cells to other hematological diseases has been also reported. In a pilot study published by Bachanova *et al.*, six patients with advanced B cell non-Hodgkin lymphoma received infusion of haploidentical NK cells, and after two months, four patients showed an objective clinical response. This finding suggests that allogeneic NK cell therapy can be safe, feasible and effective in patients with lymphoma (58). NK cells are endowed with a natural function to make antibody mediated cellular killing (59), and are known to be the principal cellular component to determine cancer cells destructions, mainly upon treatment with tumor antigen-associated specific antibody – such as anti-CD20 monoclonal antibody – which utilization changed the lymphoproliferative treatment scenario (60).

NK cells have been also studied to treat multiple myeloma (MM) (59). There are evidences suggesting that NK cells have anti-MM activity (61,62), contributing to the graft-versus-myeloma reactions along with T lymphocytes (59,63–65). In a study by Shi *et al.*, haploidentical KIR-ligand mismatched NK cells were infused into patients with relapsed/refractory MM. The results of this study indicate that the use of NK cells is safe, does not diminish engraftment, neither causes GVHD. However, the real contribution of the AI with NK is difficult to be confirmed due to the fact that patients, who also received autologous peripheral blood stem cell transplantation, were heavily pre-treated, so the authors point out suggestions to enhance the efficacy of future protocols (66). A more recent study also reports the safety of using NK cells for poor prognosis MM patients, and the results provide further support for the hypothesis that NK cell therapy can be optimized and become effective to the point of being incorporated as treatment for MM (67).

Although NK cells are cytotoxic and can infiltrate into solid tumors (68), these are complex and NK cells can have their activity prevented by the tumor microenvironment (69,70). However, due to their ability of precisely kill antibody coated cells, cancer cells, and genotoxically altered cells, while maintaining tolerance to healthy cells, NK cells are very attractive effectors to be tested, either alone or with standard therapy, against all forms of cancer, including disseminated solid cancer (71).

Patients with advanced non-small cell lung cancer were treated with a combination of chemotherapy and NK cells in a Phase I clinical trial. In this study, patients with adenocarcinoma and squamous cell carcinoma were enrolled and treatment was evaluated as safe and potentially effective (72). Krause *et al.* tested activated NK cells in patients with metastatic colorectal cancer and non-small cell lung cancer, in order to evaluate the tolerability, feasibility, and safety of these cells in a Phase I clinical trial. Overall, the results indicated that the use of the activated NK cells is safe (73). Regarding the clinical response, these findings may be somewhat limited by the fact that the patients were in an advanced disease state when they entered the study. Future clinical studies on patients with lower tumor burden can provide more information about the clinical value of the immunotherapy with NK cells (72,73).

Geller *et al.* used allogeneic NK cells in recurrent ovarian and breast cancer in a Phase II study, and an important finding was that *in vivo* sustainment of NK expansion may be limited by host rejection, competition with host lymphocytes or suppression by recipient T_{reg} or myeloid-derived suppressor cells. It is suggested by the authors that, in order to evaluate the clinical benefit of NK cells against solid tumors, more effective strategies to augment *in vivo* NK cell persistence and expansion are required (74). Or a more intense immunosuppression of the host like Rosenberg suggested (75,76).

A more recent study shows that NK cells can be effective against brain tumors like medulloblastoma. *In vitro* and *in vivo* experiments using animal model were performed, and the results indicate that NK cells can reach the tumor through the brain, being the tumor sensitive to NK cell lysis. These pre-clinical data provided the foundation for a Phase I clinical trial to be approved by the United States Food and Drug Administration, in which NK cells will be used in patients who have undergone re-resection of infratentorial tumors (77).

1.5. Defining the protocol and obtaining the cells

The efficacy of NK cells depends on their maturation and activation status, thus protocols for isolation, expansion and *in vitro* production following Current Good

Manufacturing Practices guidelines of large quantities of functional NK cells for clinical applications have been explored (38). *Ex vivo* expansion of NK cells can be performed in flasks, culture bags, gas-permeable static cell culture flasks (G-Rex), and in bioreactors (8,12,78–80), depending on the starting number of cells and their intended application.

Clinical trials usually require a large number of cells, being those cells preferably of the same product. The most commonly reported number of cells for infusion ranges from 5×10^6 to 5×10^7 NK cells per kilogram (57,58). Infusion of a number of cells as high as 1×10^8 NK cells per kilogram has been also reported (67).

Many studies, including clinical trials, use NK cells derived from apheresis (8,81,82) (registered at www.clinicaltrial.gov as clinical trials: NCT02123836, NCT01287104, NCT00846833), a technique that enables to harvest large numbers of PB lymphocytes (13). To obtain NK cells from apheresis, the protocol usually consists in removing white blood cells from PB, depleting the $CD3^+$ T cells, enriching the $CD56^+$ cells, and using the resulting product as treatment (8). Although apheresis can be a rapid approach for obtaining NK cells, the number of cells is limited, providing less than 2×10^7 NK cells per kg for a single infusion, which is considered insufficient (8). The apheresis product can also be relatively impure prior manipulation, containing less than 30 % NK cells (74). Clinical trials with NK cells demand high NK cell doses and often several infusions, and one apheresis product may not contain sufficient numbers of these cells (14). Besides, apheresis is considered an invasive and expensive technique (8).

Donor-to-donor differences can have an impact in the absolute number of NK cells obtained (66), and products with high NK cell numbers tend to be derived from donors who present high NK cell count in PB (81). Interestingly, in a study by Al-Ali *et al.*, it was reported that donor´s older age had an impact on NK cell count encountered in leukapheresis grafts. Significantly more NK cells were found in harvests from donors ≥ 60 years when compared with younger donors (82).

Although, PB has been the main source to obtaining NK cells in most of the studies up to date (83), NK cells can also be generated from, umbilical cord blood (UCB), bone marrow, human embryonic stem cells or induced pluripotent stem cells (84,85).

Bone marrow is the microenvironment where NK cells develop *in vivo*, being considered a rich source of $CD34^+$ stem cells to generate NK cells with mature properties. However, there are important drawbacks of using NK cells from bone marrow for cell therapy: harvesting procedure and the cell number that can be obtained (37). UCB can also be an alternative font to isolate NK cells (83,37,86–88). In a study by Shah *et al.*, expanded NK

cells were obtained from UCB, and both fresh and cryopreserved units were used. According to the authors, it was possible to obtain a log-scale expansion of pure NK cells (> 95 % $CD56^+/CD3^-$, < 1 % $CD3^+$ cells), and the cells were significantly active against MM in vivo (xenogeneic mouse model) (83). These results support the idea of UCB also being an important source of NK cells. It is important to bear in mind though, that the low initial numbers of NK cells in UCB can be an important limitation of this approach (89).

Apart from all the previously cited sources, another way of obtaining NK cells could be the buffy coat (9,13). Instead of blood banks discarding the white blood cells from the blood donations processing, they could be used to generate NK cells, and probably other immune cells. This strategy could bring some advantages: it is promptly available and the samples are previously screened for a range of infectious diseases and hematological conditions.

1.5.1. Use of activating cytokines

NK cells depend on cytokines for their development, survival, and function (37,90). The clinical use of cytokines is of great interest due to their role in sustaining and/or activating NK cell antitumor potential. Cytokines including interleukin (IL)-2, IL-12, IL-15, IL-18 and IL-21 have influence on NK cell (see table 1) (37,90).

IL-2 and IL-15 are the best studied cytokine activators of NK cells (91–95). Both can enhance antitumor response (90), having their combined efficacy also tested *in vitro*, showing additive response in NK cell stimulation (96) . When IL-2 is combined with IL-12, they synergy to stimulate NK cell cytotoxicity *in vitro* (97). A combined pre-activation with IL-12, IL-15, and IL-18 generate cytokine-induced memory-like NK cells, long-lived NK cells that exhibited enhanced functionality when re-stimulated, producing significantly more IFN-γ (98,99).

IL-2 was one of the first cytokines used clinically to induce antitumor immunity (90,100–102). IL-2 plays an immunoregulatory role on lymphocytes, and despite having no direct impact on cancer cells, it has the ability to mediate immune reactions directed against cancer antigens (102). IL-2 activated NK cells can lyse tumor targets which are not normally susceptible to their action (NK cells resistant targets) (103). However, this cytokine alone is not able to sustain the proliferation, and the association of it with feeder cells or artificial Antigen Presenting Cells (aAPC) seems to be more efficient for NK cell *in vitro* expansion (13,44,89,104,105).

Once NK cells are infused, IL-2 is administered systemically to support NK cell survival *in vivo* (66,101). Treatment with low dose of IL-2 may not be very effective (106), while using high doses of this cytokine may cause toxicity symptoms in patients (66). When incubated with IL-2 for longer periods, NK cell products contained significant amounts of pro-inflammatory cytokines IFN- γ and TNF-α, which were associated to symptoms like hypotension and chills presented by patients in a study reported by Shi and colleagues. The authors suggest that incubating NK cells with IL-2 only during the period of processing instead of overnight allows the administration of larger number of subcutaneous IL-2 doses to the patients to enhance NK cells survival in the body (66).

1.5.2. Feeder cells and artificial Antigen Presenting Cells

NK cells do not normally undergo sustained proliferation (79), so clinically applicable methods for *ex vivo* production of functional cells on a large scale are needed. In many studies, the proliferation of NK cells in response to cytokines, with or without co-culture with different types of feeder cells - used in culture systems to support NK cells *ex vivo* expansion (4), has been limited in number of cells and duration of proliferative response (107–109). To overcome this limitation, aAPC derived from K562 cells were genetically modified to express specific markers (13).

Being initially designed to generate tumor-specific T cells for allogeneic cell therapy of B-cell malignancies, the first aAPC used to propagate clinical-grade NK cells for AI human trials was the clone 4 (79), K562 cells genetically modified to co-express CD19, CD64, CD86, 4-1BBL, and surface membrane–bound IL-15 (110–112). However, *ex vivo* proliferation of NK cells mediated by aAPC clone 4 was limited by telomere shortening (13). Singh and coworkers developed a aAPC clone 9, genetically modified to co-express CD19, CD64, CD86, CD137L, as did the clone 4, and a mutein of interleukin 21 (IL-21) bound to the membrane (K562-CL9-mIL21) (113). Denman and colleagues compared the *ex vivo* proliferation of human NK cells using different aAPC, including the clones 4 and 9. According to the study, aAPC clone 9 promoted better proliferation of activated human NK cells, which supports the clinical use in the expansion of NK cells for AI (13). Another advantage of NK cell production mediated by aAPC is the reported reduction of T lymphocyte concurrent expansion (83,114).

In order to be used to produce NK cells for clinical protocols, aAPC are irradiated before culture (13,44,79,83,115), being lysed by the expanding NK cells (79,115). Although the risk of infusing viable aAPC is negligible, it is recommended to incorporate safety

measures to guarantee that the NK cell product can be released (79). At the end of the culture, NK cell product must be tested for the presence of viable aAPC (79) (as well as for other parameters further described) by flow cytometry. Cultures of irradiated aAPC can be prepared and have their growth and DNA synthesis rate monitored (79).

A particle-based feeder-free approach has been recently reported by Oyer *et al*. Their methodology consisted in ex vivo expansion of PB derived unsorted NK cells using plasma membrane particles derived from the aAPC K562-mbIL15-41BBL. The current study found that the extent of NK cell expansion and cell content depended on the concentration of the plasma membrane particles. Phenotype analysis and cytotoxicity assays demonstrated that these NK cells present cytotoxic phenotype and activity. The authors suggested that this novel and promising methodology is effective to expand active NK cells, and should be translatable into clinical setting (116).

1.5.3. Supplements used in NK cell culture

FBS still is a widely used supplement in cell culture media. In 1997, the World Health Organization issued a memo advising that, whenever possible, bovine derived inputs should not be used in the pharmaceutical practice, nor should be any product that is administered to patients for the risk of xenoreaction (15).

Besides the risk of xenoreaction, other factors corroborate the importance of finding alternatives to the use of FBS in cell culture for clinical application, and we can relate, for example its indefinite composition, the risk of contamination, concerns with animal welfare during its collection and production, problems due to limited availability, and cost (19,117). In Brazil, the *Agência Nacional de Vigilância Sanitária* has published a resolution referred as RDC 09/2011, which regulates cell therapy and use of animal products. It determines that the use of animal products should be avoided, and if used, the absence of infectious agents and contaminants must be certified (118).

Most studies that relate using culture medium supplemented with products of human origin instead of animal´s aim to mesenchymal stem cell culture, being the platelet lysate reported as an alternative (17,19,20,22,119–121). In 1985, Brown and co-workers have developed a culture medium free of bovine supplement, which was shown to be advantageous for the production of NK cells, since both proliferation and activation were better for cells grown in animal free medium, than those from cells grown in medium supplemented with FBS (122).

There is a growing number of studies reporting successful use of human serum or human serum albumin for expanding NK cells (6,8,58,66,123,124). Serum free medium have also been used (88,125,126), as well as supplemented with chemokines and monoclonal antibodies (125). Therefore, replacing animal origin supplements by optimized serum-free medium or human serum for *ex vivo* expansion of NK cells can be a promising approach for clinical immunotherapeutic application.

1.5.4. Purification of NK cells and release criteria

Minimizing $CD3^+$ T lymphocyte populations is a necessary step so that the expanded NK cells can be clinically applicable to adoptive allogeneic therapy (14). The presence of T cells in NK cells cultures intended to clinical use is undesirable due to the risk of GVHD, particularly when the donor is haploidentical (13). Therefore, release criteria must be established for the residual content of $CD3^+$ in the final product, regardless the manufacturing methodology of the NK cells. The purity criteria of NK cells products varies from study to study as a consequence of the manufacturing process (127). A study by Shah and coworkers has shown that aAPC-mediated expansion of NK cells from UCB can yield a product containing less than 1 % $CD3^+$ cells after CD3 depletion (83). In another study, an infusion target with less than 1.0×10^5/kg $CD3^+$ $CD56^-$ T cells was settled (66).

Depletion of $CD3^+$ T cells can be performed at any stage of NK cell expansion. Denman *et al* reported no difference in NK cell proliferation when depletion is performed prior to expansion, or by the third stimulation (13). Establishing the best time during the expansion of the NK culture in which T cell depletion is more efficient must take into account the starting number of cells, the expected yield of purified NK cells, and donor´s NK cell repertoire. Depletion of $CD3^+$ T cells on a large scale, like on the final product of manufactured NK cells for clinical use, requires higher total number of starting cells (78).

In addition to CD3 depletion, isolation of clinical grade NK cells for AI can also be accomplished by combining an enrichment step of NK cells, utilizing strategies such as CD56 enrichment (8,123,128–130), when $CD56^+$ cells are isolated (127), which enables to obtain a higher purity than using CD3 depletion alone. However, cell recovery is lower when CD56 enrichment is associated to CD3 depletion (106). Besides isolation protocols being costly and time consuming, in order to obtain elevated NK cell purity and extensive T cell depletion there is still a considerable loss of NK cells during the process (8).

If one compares the use of cells for therapy with medicines (mainly intravenous), the need to validate quality control methodologies is even more obvious. To guarantee sterility,

Gram stain, endotoxin, and *Mycoplasma* are among the tests that need to be done (67,79,123). To release the final NK cell product, it is essential to perform immunophenotypic analysis of the cells, contemplating NK cell antigens (CD56, CD16), and T cell antigens (CD3, CD4, CD8) (8,13,44,66). It is also recommended phenotyping NK cells during expansion protocol, identifying those which are $CD3^-$ and $CD16^+$ or $CD56^+$ (44). Other NK cell markers like HLA-E, NKG2A, NKG2D, NCR (NKp30, NKp44, NKp46), and KIR can also be evaluated (8,66,131). Levels of cytokines related to the functional state of NK cells - IFN-γ, TNF-α, and interleukins - can be determined using a flow cytometric bead array (5,66).

For a clinically efficacious NK cell product, not only the NK cell number must be taken into account, but cell purity and function are also key factors (132). Prior to infusion, products containing more than 95 % viable NK cells are desirable (66). However, lower viabilities have been also reported (106). Cell viability can be measured by flow cytometry using NK cells markers, as described above, in combination with propidium iodide or 7AAD staining, or by trypan blue exclusion (6,12,89). The ability of the NK cells to kill sensitive targets must also be tested. For evaluating NK cell activity, the ^{51}Cr release assay is considered the "gold standard" (133). The calcein release assay, a fluorimetric assay related to the chromium release assay (134), is also used in determining NK cell cytotoxicity (13,44). Flow cytometry-based NK cell cytotoxicity assays have been developed, and when compared to the traditional chromium release assay, they can be a viable alternative presenting interesting advantages (133,135–137).

Once infused, the successful *in vivo* expansion of the allogeneic NK cells product in the receiving patient can be evaluated by measuring donor chimerism using a standard short-tandem repeat assay on unsorted mononuclear cells (74).

1.6. Genetically engineered NK cells

T cells can be genetically modified to express specific antigens receptors via transduction with viral vector encoding Chimeric Antigen Receptors (CAR) (138–140). Similar approaches have been studied using NK cell lines, as well as NK cells (141), and the genetic modification can be applied either to induce the proliferation and survival of NK cells (142,143) or to specifically direct them to malignant targets (144), like cancer or infected cells/tissues.

Genetically engineered NK cells with CARs directed against CD20 have been proposed as treatment for B cells malignancies (145), and CD19 transfection restored the

capacity of NK-92 cells to kill previously resistant leukemic cells (146). In another study, NK cells had their cytotoxicity importantly enhanced against neuroblastoma after being transduced with a disialoganglioside GD(2)-recognizing CAR (147). These findings indicate that genetically engineered NK cells can have their therapeutical potential enhanced, and even be able to overcome inhibitory signals (114,145).

Overall, data obtained from experimental models indicate that this might be an interesting approach in immunotherapy with NK cells (106). A Phase I study, sponsored by St. Jude Children's Research Hospital, to determine the maximum tolerated dose of genetically modified NK cells in relapsed/refractory B-lineage acute lymphoblastic leukemia has been completed, but no study results have been reported so far (registered at www.clinicaltrial.gov as trial NCT00995137) (148). Further research should be carried out to establish the safety and feasibility of genetically modified NK cells in the clinical setting.

1.7. NK cell line

The use of cell lines can be an alternative in NK cell antitumor immunotherapy (84,106), in allogeneic setting (84). Amongst several, NK-92 is the most studied NK cell line (141), in both pre-clinical and clinical scenario (84,141,149–152). When considering CD56 and CD3 expression, NK-92 cells show a typical NK profile, being positive for CD56 and negative for CD3 (150). They express activating receptors such as NKp30 and NKp46, express few inhibitory receptors, lacking most of the KIR receptors, and also express high levels of molecules involved in the cytolytic/cytotoxic activity (TNF family factors and perforin-granzyme) (153).

The advantages of cell lines like NK-92 are that they can be utilized “off the shelf” (141), be grown under GMP conditions (106), and they provide a more homogeneous population when compared to NK cells isolated from PB (141). When infused to patients, the results of clinical trials indicate that NK-92 cells can be safe and potentially beneficial (126,149,154). The Food and Drug Administration approved NK-92 cell for testing in patients with advanced malignant melanoma and renal cell carcinoma, being currently the only NK cell line that has entered clinical trials (155). On the other hand, NK-92 is a tumor cell line derived from non-Hodgkin's lymphoma (141,150), with the need to be irradiated prior to infusion for safety reasons (24,141), but the irradiation can limit the efficacy of NK-92 cell *in vivo* (24). Further studies are still required to assess the long-term effects and safety of these cells in clinical trials.

2. Final considerations and concluding remarks

Anticancer therapy has progressed significantly over the last few years. Important example of this is the development of monoclonal antibodies, which are already available in the clinical setting, and are used to treat many diseases, including cancer (156). In the meantime, standard anticancer therapy like chemotherapy drugs still fails in a considerable number of patients (157), and even when successful, can cause serious adverse effects. Immune cells have the potential to bypass cellular mechanisms of drug resistance provided by tumor environment and spare normal tissues (38,45). Therefore, treating cancer with cell therapy has become a very attractive approach.

One of the principal challenges in cell immunotherapy as a whole is its cost. Producing cells in a GMP environment is very expensive, mainly when multiple purification and stimulation steps are required. In NK cell immunotherapy, the lack of a consolidated large scale clinical grade expansion method is the major barrier to be overcome, considering that there can be a substantial variability in the process, pointing to its need of optimization. Not to mention the variability of the NK cell content that can be obtained from each individual donor.

The efficacy of NK cells depend on their maturation and activation status, thus protocols for isolation, and *in vitro* expansion of large quantities of functional of NK cells that meet the criteria for clinical applications have been explored. The production of cells, traditionally grown in culture flasks, needed to be scaled up to bags and bioreactors. Manufacturing protocols combining different approaches like feeder cells mediated expansion of NK cells also stimulated with chemokines such as IL-2 have been exploited. Gene modification technologies, *e.g.* CAR (84,141,146), have been also applied, and novel methodologies started to be explored for NK cells, aiming to optimize the generation of clinical grade products for AI.

Regarding the clinical protocol for AI with NK cells, there is still no consensus on some parameters, such as the ideal dose, the possibility of dose dependent response or the maximum number of residual T cells per kg that can be infused. Defining what is the top priority during *ex vivo* NK cell expansion, if high yield or high purity, may raise debate. Release criteria and a "gold standard" methodology of obtainment and expansion of NK cells for clinical application might be difficult to be stated yet, because standardization of a procedure that is prone to so many variables is a great challenge.

When using feeder or aAPC cells in the manufacturing process, it is important to bear in mind that the required amount of these cells for mediating NK cell expansion meant to

clinical protocols is also significantly high. So proper banking of produced and tested cells under Current Good Manufacturing Practices needs to be maintained. For large scale manufacturing cell process, having master and working banks is recommended (79,158).

Issues that have not been previously addressed, but also need to be taken into consideration when NK cells are manufactured for clinical use are: cryopreservation and optimization/standardization of shipping conditions if the cells are used at distant sites. This is critical, because not just must the final product meet all the release criteria, it should also get to the patient in the same condition it was approved during quality control.

Despite all the previously mentioned difficulties, NK cell-based therapy has been continuously explored over the years due to the fact that NK cells have numerous properties that make them appealing for clinical applications. Besides all the issues involving the manufacturing process, there is still much to be learnt regarding NK cells. Studies addressing *ex vivo* kinetics expansion, *in vivo* biodistribution, and cell behavior after genetic modification are essential for further understanding of NK cells, and for helping improve their generation approaches, taking strategies so far mostly applied on bench to bedside.

3. Conflict of Interest Statement

The authors declare no conflict of interests.

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Table 1

Key interleukins and their role on Natural Killer cell.

Interleukine	Role on NK cell
IL-2	Supports *in vivo* activation and expansion (90) and *in vitro* survival (159), induces activation, triggers antitumor response, and upregulates IL-12 (106)
IL-4	Supports survival (159) and proliferation (108,159)
IL-7	Augments cell proliferation (108)
IL-10	Increases *in vitro* cytotoxicity and the expression of cell migration-related genes (160)
IL-12	Augments cell proliferation (108), induces activation (106), differentiation, and IFN-γ production (159), increases cytotoxic activity (90)
IL-15	Promotes *in vitro* proliferation and survival (159,127), supports maturation (159), and induce activation (106)
IL-18	May be involved in the functional final maturation to IFN-γ production (159)
IL-21	Enhances proliferative response (127), and cytotoxic activity (90,127), increasing expression of IFN-γ, perforin, and granzyme B (90)

Note: not all the studies were performed *in vivo* or utilized human derived cells.

5. References

1. Barrett AJ. Understanding and harnessing the graft-versus-leukaemia effect. Br J Haematol (2008) **142**:6. doi: 10.1111/j.1365-2141.2008.07260.x

2. Jagasia M, Arora M, Flowers MED, Chao NJ, McCarthy PL, Cutler CS, et al. Risk factors for acute GVHD and survival after hematopoietic cell transplantation. Blood (2012) **119**:1. doi: 10.1182/blood-2011-06-364265

3. Holtan SG, Pasquini M, Weisdorf DJ. Acute graft-versus-host disease: a bench-to-bedside update. Blood (2014) **124**:3. doi 10.1182/blood-2014-01-514786

4. Eissens DN, Meer A van der, Joosten I. "Licensed to Kill: Towards Natural Killer Cell Immunotherapy". In: Demirer T, editor. New Advances in Stem Cell Transplantation (2012) ISBN: 978-953-51-0013-3, InTech, doi: 10.5772/27482

5. Pittari G, Fregni G, Roguet L, Garcia A, Vataire A-L, Wittnebel S, et al. Early evaluation of natural killer activity in post-transplant acute myeloid leukemia patients. Bone Marrow Transplant (2010) **45**:5. doi: 10.1038/bmt.2009.265

6. Ahn Y-O, Kim S, Kim TM, Song EY, Park MH, Heo DS. Irradiated and activated autologous PBMCs induce expansion of highly cytotoxic human NK cells in vitro. J Immunother (2013) **36**:7. doi: 10.1097/CJI.0b013e3182a3430f

7. Miller JS. Therapeutic applications: natural killer cells in the clinic. Hematology Am Soc Hematol Educ Program (2013) **2013**:1. doi: 10.1182/asheducation-2013.1.247

8. Koehl U, Brehm C, Huenecke S, Zimmermann S-Y, Kloess S, Bremm M, et al. Clinical grade purification and expansion of NK cell products for an optimized manufacturing protocol. Front Oncol (2013) **3**:118. doi: 10.3389/fonc.2013.00118

9. Eissens DN, Michelo CM, Preijers FWMB, van Cranenbroek B, van Houwelingen K, van der Meer A, et al. Selective expansion of human natural killer cells leads to enhanced alloreactivity. Cell Mol Immunol (2014) **11**:2. doi: 10.1038/cmi.2013.56

10. Darcy PK, Neeson P, Yong CSM, Kershaw MH. Manipulating immune cells for adoptive immunotherapy of cancer. Curr Opin Immunol (2014) **27**. doi: 10.1016/j.coi.2014.01.008

11. Parham P. MHC class I molecules and KIRs in human history, health and survival. Nat Rev Immunol (2005) **5**:3. doi: 10.1038/nri1570

12. Berg M, Lundqvist A, McCoy P, Samsel L, Fan Y, Tawab A, et al. Clinical-grade ex vivo-expanded human natural killer cells up-regulate activating receptors and death receptor ligands and have enhanced cytolytic activity against tumor cells. Cytotherapy (2009) **11**:3. doi: 10.1080/14653240902807034

13. Denman CJ, Senyukov V, Somanchi SS, Phatarpekar P V, Kopp LM, Johnson JL, et al. Membrane-bound IL-21 promotes sustained Ex Vivo proliferation of human natural killer cells. PLoS One (2012) **7**:1. doi: 10.1371/journal.pone.0030264

14. Lapteva N, Szmania SM, van Rhee F, Rooney CM. Clinical grade purification and expansion of natural killer cells. Crit Rev Oncog (2014) **19**:121-132.

15. World Health Organization. Medicinal and other products and human and animal transmissible spongiform encephalopathies: memorandum from a WHO meeting. (1997). Bulletin of the World Health Organization, 75(6), 505–513.

16. European Commission. Note for guidance on minimising the risk of transmitting animal spongiform encephalopathy agents via human and veterinary medicinal products (EMA/410/01 rev.3). Off J Eur Union (2011) C:1–18.

17. Witzeneder K, Lindenmair A, Gabriel C, Höller K, Theiß D, Redl H, et al. Human-derived alternatives to fetal bovine serum in cell culture. Transfus Med Hemotherapy (2013) **40**:6. doi: 1.1159/000356236

18. Martin MJ, Muotri A, Gage F, Varki A. Human embryonic stem cells express an immunogenic nonhuman sialic acid. Nat Med (2005) **11**:2. doi: 10.1038/nm1181

19. Tekkatte C, Gunasingh GP, Cherian KM, Sankaranarayanan K. "Humanized" stem cell culture techniques: the animal serum controversy. Stem Cells Int (2011) 504723. doi: 10.4061/2011/504723

20. Bieback K, Hecker A, Kocaömer A, Lannert H, Schallmoser K, Strunk D, et al. Human alternatives to fetal bovine serum for the expansion of mesenchymal stromal cells from bone marrow. Stem Cells (2009) **27**:9. doi: 10.1002/stem.139

21. Killig M, Friedrichs B, Meisig J, Gentilini C, Blüthgen N, Loddenkemper C, et al. Tracking in vivo dynamics of NK cells transferred in patients undergoing stem cell transplantation. Eur J Immunol (2014) **44**:9. doi: 10.1002/eji.201444586

22. Silla L, Valim V, Amorin B, Alegretti AP, Dos Santos de Oliveira F, Lima da Silva MA, et al. A safety and feasibility study with platelet lysate expanded bone marrow mesenchymal stromal cells for the treatment of acute graft-versus-host disease in Brazil. Leuk Lymphoma (2014) **55**:5. doi: 10.3109/10428194.2013.823495

23. Luetke-Eversloh M, Killig M, Romagnani C. Signatures of human NK cell development and terminal differentiation. Front Immunol (2013) **4**:499. doi: 10.3389/fimmu.2013.00499

24. Cho D, Campana D. Expansion and activation of natural killer cells for cancer immunotherapy. Korean J Lab Med (2009) **29**:2. doi: 10.3343/kjlm.2009.29.2.89

25. Vivier E. What is natural in natural killer cells? Immunol Lett (2006) **107**:1. doi: 10.1016/j.imlet.2006.07.004

26. Morris MA, Ley K. Trafficking of natural killer cells. Curr Mol Med (2004) **4**:4. doi: 10.2174/1566524043360609

27. Fogler WE, Volker K, McCormick KL, Watanabe M, Ortaldo JR, Wiltrout RH. NK cell infiltration into lung, liver, and subcutaneous B16 melanoma is mediated by VCAM-1/VLA-4 interaction. J Immunol (1996) **156**:4707-14.

28. Biron CA. Activation and function of natural killer cell responses during viral infections. Curr Opin Immunol (1997) **9**:1. doi: 10.1016/S0952-7915(97)80155-0

29. Glas R, Franksson L, Une C, Eloranta ML, Ohlén C, Orn A, et al. Recruitment and activation of natural killer (NK) cells in vivo determined by the target cell phenotype. An adaptive component of NK cell-mediated responses. J Exp Med (2000) **191**:129-138.

30. Trinchieri G. Biology of natural killer cells. Adv Immunol (1989) **47**:187–376.

31. Herberman RB. Natural killer cells. Annu Rev Med (1986) **37**:347–52.

32. Dvorak AM, Galli SJ, Marcum JA, Nabel G, der Simonian H, Goldin J, et al. Cloned mouse cells with natural killer function and cloned suppressor T cells express ultrastructural and biochemical features not shared by cloned inducer T cells. J Exp Med (1983) **157**:843–61.

33. Campbell KS, Hasegawa J. Natural killer cell biology: An update and future directions. J Allergy Clin Immunol (2013) **132**:3. doi: 10.1016/j.jaci.2013.07.006

34. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol (1986) **136**:4480–6.

35. Cooper MA, Fehniger TA, Caligiuri MA. The biology of human natural killer-cell subsets. Trends Immunol (2001) **22**:633–40.

36. Anfossi N, André P, Guia S, Falk CS, Roetynck S, Stewart CA, et al. Human NK cell education by inhibitory receptors for MHC class I. Immunity (2006) **25**:2. doi: 10.1016/j.immuni.2006.06.013

37. Yoon SR, Kim TD, Choi I. Understanding of molecular mechanisms in natural killer cell therapy. Exp Mol Med (2015) **13**:47. doi: 10.1038/emm.2014.114

38. Romagné F, Vivier E. Natural killer cell-based therapies. F1000 Med Rep (2011) **3**:9. doi: 10.3410/M3-9

39. Srivastava S, Lundqvist A, Childs RW. Natural killer cell immunotherapy for cancer: a new hope. Cytotherapy (2008) **10**:8. doi: 10.1080/14653240802648181

40. Caligiuri MA. Human natural killer cells. Blood (2008) **112**:3. doi: 10.1182/blood-2007-09-077438

41. Moretta A, Locatelli F, Moretta L. Human NK cells: from HLA class I-specific killer Ig-like receptors to the therapy of acute leukemias. Immunol Rev (2008) **224**:1. doi: 10.1111/j.1600-065X.2008.00651.x

42. Vivier E, Ugolini S, Blaise D, Chabannon C, Brossay L. Targeting natural killer cells and natural killer T cells in cancer. Nat Rev Immunol (2012) **12**:4. doi: 10.1038/nri3174

43. Vivier E, Ugolini S. Natural killer cells: from basic research to treatments. Front Immunol (2011) **2**:18. doi: 10.3389/fimmu.2011.00018

44. Somanchi SS, Senyukov V V, Denman CJ, Lee D a. Expansion, purification, and functional assessment of human peripheral blood NK cells. J Vis Exp (2011) **48**:2540. doi: 10.3791/2540

45. Baier C, Fino A, Sanchez C, Farnault L, Rihet P, Kahn-Perlès B, et al. Natural killer cells modulation in hematological malignancies. Front Immunol (2013) **4**:459. doi: 10.3389/fimmu.2013.00459

46. Grzywacz B, Miller JS, Verneris MR. Use of natural killer cells as immunotherapy for leukaemia. Best Pract Res Clin Haematol (2008) **21**:3. doi: 10.1016/j.beha.2008.07.008

47. Kanate AS, Pasquini MC, Hari PN, Hamadani M. Allogeneic hematopoietic cell transplant for acute myeloid leukemia: Current state in 2013 and future directions. World J Stem Cells (2014) **6**:2. doi: 10.4252/wjsc.v6.i2.69

48. Capra M, Vilella L, Pereira WV, Coser VM, Fernandes MS, Schilling MA, et al. Estimated number of cases, regional distribution and survival of patients diagnosed with acute myeloid leukemia between 1996 and 2000 in Rio Grande do Sul, Brazil. Leuk Lymphoma (2007) **48**:12. doi: 10.1080/10428190701713622

49. Appelbaum FR, Clift RA, Buckner CD, Stewart P, Storb R, Sullivan KM, et al. Allogeneic marrow transplantation for acute nonlymphoblastic leukemia after first relapse. Blood (1983) **61**:949–53.

50. Appelbaum FR. Bone marrow transplantation or chemotherapy after remission induction for adults with acute nonlymphoblastic leukemia. Ann Intern Med (1984) **101**:5. doi: 10.7326/0003-4819-101-5-581

51. Champlin RE. Treatment of Acute Myelogenous Leukemia. Ann Intern Med (1985) **102**:3. doi: 10.7326/0003-4819-102-3-285.

52. Cassileth PA, Harrington DP, Appelbaum FR, Lazarus HM, Rowe JM, Paietta E, et al. Chemotherapy Compared with Autologous or Allogeneic Bone Marrow Transplantation in the Management of Acute Myeloid Leukemia in First Remission. N Engl J Med (1998) **339**:23. doi: 10.1056/NEJM199812033392301

53. Baron F, Baker JE, Storb R, Gooley TA, Sandmaier BM, Maris MB, et al. Kinetics of engraftment in patients with hematologic malignancies given allogeneic hematopoietic cell transplantation after nonmyeloablative conditioning. Blood (2004) **104**:8. doi: 10.1182/blood-2004-04-1506

54. Tabbara IA, Zimmerman K, Morgan C, Nahleh Z. Allogeneic hematopoietic stem cell transplantation. Arch Intern Med (2002) **162**:14. doi: 10.1001/archinte.162.14.1558

55. Ruggeri L, Capanni M, Urbani E, Perruccio K, Shlomchik WD, Tosti A, et al. Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants. Science (2002) **295**:5562. doi: 10.1126/science.1068440

56. Ruggeri L, Mancusi A, Capanni M, Urbani E, Carotti A, Aloisi T, et al. Donor natural killer cell allorecognition of missing self in haploidentical hematopoietic transplantation for acute myeloid leukemia: challenging its predictive value. Blood (2007) **110**:1. doi: 10.1182/blood-2006-07-038687

57. Miller JS, Soignier Y, Panoskaltsis-Mortari A, McNearney SA, Yun GH, Fautsch SK, et al. Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer. Blood (2005) **105**:8. doi: 10.1182/blood-2004-07-2974

58. Bachanova V, Burns LJ, McKenna DH, Curtsinger J, Panoskaltsis-Mortari A, Lindgren BR, et al. Allogeneic natural killer cells for refractory lymphoma. Cancer Immunol Immunother (2010) **59**:11 doi: 10.1007/s00262-010-0896-z

59. James AM, Cohen AD, Campbell KS. Combination immune therapies to enhance anti-tumor responses by NK cells. Front Immunol (2013) **4**:481. doi: 10.3389/fimmu.2013.00481

60. Seidel UJE, Schlegel P, Lang P. Natural killer cell mediated antibody-dependent cellular cytotoxicity in tumor immunotherapy with therapeutic antibodies. Front Immunol (2013) **4**:76. doi: 10.3389/fimmu.2013.00076

61. Frohn C, Hoppner M, Schlenke P, Kirchner H, Koritke P, Luhm J. Anti-myeloma activity of natural killer lymphocytes. Br J Haematol (2002) **119**:3. doi: 10.1046/j.1365-2141.2002.03879.x

62. Hsi ED, Steinle R, Balasa B, Szmania S, Draksharapu A, Shum BP, et al. CS1, a potential new therapeutic antibody target for the treatment of multiple myeloma. Clin Cancer Res (2008) **14**:9. doi: 10.1158/1078-0432.CCR-07-4246

63. Lokhorst HM, Schattenberg A, Cornelissen JJ, van Oers MH, Fibbe W, Russell I, et al. Donor lymphocyte infusions for relapsed multiple myeloma after allogeneic stem-cell transplantation: predictive factors for response and long-term outcome. J Clin Oncol (2000) **18**:3031–7.

64. Salama M, Nevill T, Marcellus D, Parker P, Johnson M, Kirk A, et al. Donor leukocyte infusions for multiple myeloma. Bone Marrow Transplant (2000) **26**:11. doi: 10.1038/sj.bmt.1702685

65. Zeiser R, Bertz H, Spyridonidis A, Houet L, Finke J. Donor lymphocyte infusions for multiple myeloma: clinical results and novel perspectives. Bone Marrow Transplant (2004) **34**:11. doi: 10.1038/sj.bmt.1704670

66. Shi J, Tricot G, Szmania S, Rosen N, Garg TK, Malaviarachchi PA, et al. Infusion of haplo-identical killer immunoglobulin-like receptor ligand mismatched NK cells for relapsed myeloma in the setting of autologous stem cell transplantation. Br J Haematol (2008) **143**:5. doi: 10.1111/j.1365-2141.2008.07340.x

67. Szmania S, Lapteva N, Garg T, Greenway A, Lingo J, Nair B, et al. Ex vivo-expanded natural killer cells demonstrate robust proliferation in vivo in high-risk relapsed multiple myeloma patients. J Immunother (2015) **38**:1. doi: 10.1097/CJI.0000000000000059

68. Whiteside TL, Sung MW, Nagashima S, Chikamatsu K, Okada K, Vujanovic NL. Human tumor antigen-specific T lymphocytes and interleukin-2-activated natural killer cells: comparisons of antitumor effects in vitro and in vivo. Clin Cancer Res (1998) **4**:1135–45.

69. Vitale M, Cantoni C, Pietra G, Mingari MC, Moretta L. Effect of tumor cells and tumor microenvironment on NK-cell function. Eur J Immunol (2014) **44**:6. doi: 10.1002/eji.201344272

70. Vesely MD, Kershaw MH, Schreiber RD, Smyth MJ. Natural innate and adaptive immunity to cancer. Annu Rev Immunol (2011) **29**:235–71. doi: 10.1146/annurev-immunol-031210-101324

71. Gras Navarro A, Björklund AT, Chekenya M. Therapeutic potential and challenges of natural killer cells in treatment of solid tumors. Front Immunol (2015) **6**:202. doi: 10.3389/fimmu.2015.00202

72. Iliopoulou EG, Kountourakis P, Karamouzis M V, Doufexis D, Ardavanis A, Baxevanis CN, et al. A phase I trial of adoptive transfer of allogeneic natural killer cells in patients with advanced non-small cell lung cancer. Cancer Immunol Immunother (2010) **59**:12. doi: 10.1007/s00262-010-0904-3

73. Krause SW, Gastpar R, Andreesen R, Gross C, Ullrich H, Thonigs G, et al. Treatment of colon and lung cancer patients with ex vivo heat shock protein 70-peptide-activated, autologous natural killer cells: a clinical phase i trial. Clin Cancer Res (2004) **10**:11. doi: 10.1158/1078-0432.CCR-03-0683

74. Geller MA, Cooley S, Judson PL, Ghebre R, Carson LF, Argenta PA, et al. A phase II study of allogeneic natural killer cell therapy to treat patients with recurrent ovarian and breast cancer. Cytotherapy (2011) **13**:1. doi: 10.3109/14653249.2010.515582

75. Rosenberg SA, Restifo NP, Yang JC, Morgan RA, Dudley ME. Adoptive cell transfer: a clinical path to effective cancer immunotherapy. Nat Rev Cancer (2008) **8**:4. doi: 10.1038/nrc2355

76. Rosenberg SA, Dudley ME. Adoptive cell therapy for the treatment of patients with metastatic melanoma. Curr Opin Immunol (2009) **21**:2. doi: 10.1016/j.coi.2009.03.002

77. Brugmann W, Laureano A, Michel K, Tao R-H, Kennis B, Somanchi S, et al. IM-07 * NK cell immunotherapy for pediatric brain tumors: overcoming resistance to expand therapeutic success. Neuro Oncol (2015) **17**:3. doi: 10.1093/neuonc/nov061.62

78. Lapteva N, Durett AG, Sun J, Rollins LA, Huye LL, Fang J, et al. Large-scale ex vivo expansion and characterization of natural killer cells for clinical applications. Cytotherapy (2012) **14**:9. doi: 10.3109/14653249.2012.700767

79. Fujisaki H, Kakuda H, Shimasaki N, Imai C, Ma J, Lockey T, et al. Expansion of highly cytotoxic human natural killer cells for cancer cell therapy. Cancer Res (2009) **69**:9. doi: 10.1158/0008-5472.CAN-08-3712

80. Sutlu T, Stellan B, Gilljam M, Quezada HC, Nahi H, Gahrton G, et al. Clinical-grade, large-scale, feeder-free expansion of highly active human natural killer cells for adoptive immunotherapy using an automated bioreactor. Cytotherapy (2010) **12**:8. doi: 10.3109/14653249.2010.504770

81. Meyer-Monard S, Passweg J, Siegler U, Kalberer C, Koehl U, Rovó A, et al. Clinical-grade purification of natural killer cells in haploidentical hematopoietic stem cell transplantation. Transfusion (2009) **49**:2. doi: 10.1111/j.1537-2995.2008.01969.x

82. Al-Ali HK, Bourgeois M, Krahl R, Edel E, Leiblein S, Poenisch W, et al. The impact of the age of HLA-identical siblings on mobilization and collection of PBSCs for allogeneic hematopoietic cell transplantation. Bone Marrow Transplant (2011) **46**:10. doi: 10.1038/bmt.2010.310

83. Shah N, Martin-Antonio B, Yang H, Ku S, Lee D a., Cooper LJN, et al. Antigen Presenting Cell-mediated expansion of human umbilical cord blood yields log-scale expansion of Natural Killer cells with anti-myeloma activity. PLoS One (2013) **8**:10. doi: 10.1371/journal.pone.0076781

84. Glienke W, Esser R, Priesner C, Suerth JD, Schambach A, Wels WS, et al. Advantages and applications of CAR-expressing natural killer cells. Front Pharmacol (2015) **6**:21. doi: 10.3389/fphar.2015.00021

85. Woll PS, Grzywacz B, Tian X, Marcus RK, Knorr DA, Verneris MR, et al. Human embryonic stem cells differentiate into a homogeneous population of natural killer cells with potent in vivo antitumor activity. Blood (2009) **113**:24. doi: 10.1182/blood-2008-06-165225

86. Spanholtz J, Tordoir M, Eissens D, Preijers F, Van Meer Der A, Joosten I, et al. High log-scale expansion of functional human natural killer cells from umbilical cord blood CD34-positive cells for adoptive cancer immunotherapy. PLoS One (2010) **5**:2. doi: 10.1371/journal.pone.0009221

87. Cany J, van der Waart AB, Tordoir M, Franssen GM, Hangalapura BN, de Vries J, et al. Natural killer cells generated from cord blood hematopoietic progenitor cells efficiently target bone marrow-residing human leukemia cells in NOD/SCID/IL2Rgnull mice. PLoS One (2013) **8**:6. doi: 10.1371/journal.pone.0064384

88. Kao IT, Yao CL, Kong ZL, Wu ML, Chuang TL, Hwang SM. Generation of natural killer cells from serum-free, expanded human umbilical cord blood $CD34^+$ cells. Stem Cells Dev (2007) **16**:6. doi: 10.1089/scd.2007.0033

89. Boissel L, Tuncer HH, Betancur M, Wolfberg A, Klingemann H. Umbilical cord mesenchymal stem cells increase expansion of cord blood natural killer cells. Biol Blood Marrow Transplant (2008) **14**:9. doi: 10.1016/j.bbmt.2008.06.016

90. Romee R, Leong JW, Fehniger TA. Utilizing cytokines to function-enable human NK cells for the immunotherapy of cancer. Scientifica (2014) **2014**:205796. doi: 10.1155/2014/205796

91. Fehniger TA. Interleukin 15: biology and relevance to human disease. Blood (2001) **97**:1. doi: 10.1182/blood.V97.1.14

92. Fehniger TA, Cooper MA, Caligiuri MA. Interleukin-2 and interleukin-15: immunotherapy for cancer. Cytokine Growth Factor Rev (2002) **13**:169–83.

93. Becknell B, Caligiuri MA. Interleukin-2, interleukin-15, and their roles in human natural killer cells. Adv Immunol (2005) **86**:209–39.

94. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat Rev Immunol (2006) **6**:8. doi: 10.1038/nri1901

95. Rosenberg SA. Interleukin-2 and the development of immunotherapy for the treatment of patients with cancer. Cancer J Sci Am (2000) **6**:S2–7.

96. Seidel MG, Freissmuth M, Pehamberger H, Micksche M. Stimulation of natural killer activity in peripheral blood lymphocytes of healthy donors and melanoma patients in vitro: synergism between interleukin (IL)-12 and IL-15 or IL-12 and IL-2. Naunyn Schmiedebergs Arch Pharmacol (1998) **358**:382-389.

97. DeBlaker-Hohe DF, Yamauchi A, Yu CR, Horvath-Arcidiacono JA, Bloom ET. IL-12 synergizes with IL-2 to induce lymphokine-activated cytotoxicity and perforin and granzyme gene expression in fresh human NK cells. Cell Immunol (1995) **165**:1. doi: 10.1006/cimm.1995.1184

98. Waldmann TA. The biology of interleukin-2 and interleukin-15: implications for cancer therapy and vaccine design. Nat Rev Immunol (2006) **6**:8. doi: 10.1038/nri1901

95. Rosenberg SA. Interleukin-2 and the development of immunotherapy for the treatment of patients with cancer. Cancer J Sci Am (2000) **6**:S2–7.

96. Seidel MG, Freissmuth M, Pehamberger H, Micksche M. Stimulation of natural killer activity in peripheral blood lymphocytes of healthy donors and melanoma patients in vitro: synergism between interleukin (IL)-12 and IL-15 or IL-12 and IL-2. Naunyn Schmiedebergs Arch Pharmacol (1998) **358**:382-389.

97. DeBlaker-Hohe DF, Yamauchi A, Yu CR, Horvath-Arcidiacono JA, Bloom ET. IL-12 synergizes with IL-2 to induce lymphokine-activated cytotoxicity and perforin and

granzyme gene expression in fresh human NK cells. Cell Immunol (1995) **165**:1. doi: 10.1006/cimm.1995.1184

102. Rosenberg SA. Cancer immunotherapy comes of age. Nat Clin Pract Oncol (2005) **2**:3. doi: 10.1038/ncponc0101

103. Robinson BW, Morstyn G. Natural killer (NK)-resistant human lung cancer cells are lysed by recombinant interleukin-2-activated NK cells. Cell Immunol (1987) **106**:215–22.

104. Rabinowich H, Sedlmayr P, Herberman RB, Whiteside TL. Increased proliferation, lytic activity, and purity of human natural killer cells cocultured with mitogen-activated feeder cells. Cell Immunol (1991) **135**:454–70.

105. Fuchshuber PR, Lotzová E, Pollock RE. Antitumor activity, growth, and phenotype of long-term IL-2 cultures of human NK and T lymphocytes. Lymphokine Cytokine Res (1991) 10:**51**–59.

106. Sutlu T, Alici E. Natural killer cell-based immunotherapy in cancer: current insights and future prospects. J Intern Med (2009) **266**:2. doi: 10.1111/j.1365-2796.2009.02121.x

107. Perussia B, Ramoni C, Anegon I, Cuturi MC, Faust J, Trinchieri G. Preferential proliferation of natural killer cells among peripheral blood mononuclear cells cocultured with B lymphoblastoid cell lines. Nat Immun Cell Growth Regul (1987) **6**:171–188.

108. Robertson MJ, Cameron C, Lazo S, Cochran KJ, Voss SD, Ritz J. Costimulation of human natural killer cell proliferation: role of accessory cytokines and cell contact-dependent signals. Nat Immun (1997) **15**:213–26.

109. Miller JS, Oelkers S, Verfaillie C, McGlave P. Role of monocytes in the expansion of human activated natural killer cells. Blood (1992) **80**:2221–2229.

110. Numbenjapon T, Serrano LM, Singh H, Kowolik CM, Olivares S, Gonzalez N, et al. Characterization of an artificial antigen-presenting cell to propagate cytolytic CD19-specific T cells. Leukemia (2006) **20**:10. doi: 10.1038/sj.leu.2404329

111. Singh H, Manuri PR, Olivares S, Dara N, Dawson MJ, Huls H, et al. Redirecting specificity of T-cell populations for CD19 using the Sleeping Beauty system. Cancer Res (2008) **68**:8. doi: 10.1158/0008-5472.CAN-07-5600

112. Davies JK, Singh H, Huls H, Yuk D, Lee DA, Kebriaei P, et al. Combining CD19 redirection and alloanergization to generate tumor-specific human T cells for allogeneic cell therapy of B-cell malignancies. Cancer Res (2010) **70**:10. doi: 10.1158/0008-5472.CAN-09-3845

113. Singh H, Figliola MJ, Dawson MJ, Huls H, Olivares S, Switzer K, et al. Reprogramming CD19-specific T cells with IL-21 signaling can improve adoptive

immunotherapy of B-lineage malignancies. Cancer Res (2011) **71**:10. doi: 10.1158/0008-5472.CAN-10-3843

114. Imai C, Iwamoto S, Campana D. Genetic modification of primary natural killer cells overcomes inhibitory signals and induces specific killing of leukemic cells. Blood (2005) **106**:1. doi: 10.1182/blood-2004-12-4797

115. Liu Y, Wu H-W, Sheard MA, Sposto R, Somanchi SS, Cooper LJN, et al. Growth and activation of natural killer cells ex vivo from children with neuroblastoma for adoptive cell therapy. Clin Cancer Res (2013) **19**:8. doi: 10.1158/1078-0432.CCR-12-1243

116. Oyer JL, Igarashi RY, Kulikowski AR, Colosimo DA, Solh MM, Zakari A, et al. Generation of highly cytotoxic Natural Killer cells for treatment of AML using feeder-free, particle based approach. Biol Blood Marrow Transplant (2015) **21**:4. doi: 10.1016/j.bbmt.2014.12.037

117. Rauch C, Feifel E, Amann E-M, Spötl HP, Schennach H, Pfaller W, et al. Alternatives to the use of fetal bovine serum: human platelet lysates as a serum substitute in cell culture media. ALTEX (2011) **28**:305–316.

118. BRASIL. ANVISA. Agência Nacional de Vigilância Sanitária. Resolução RDC nº 9 , de 14 de março de 2011(2011). Available from: http://bvsms.saude.gov.br/bvs/saudelegis/anvisa/2011/rdc0009_14_03_2011.pdf

119. Valim V, Amorin B, Pezzi A, Aparecida M, Alegretti AP, Silla L. Optimization of the cultivation of donor mesenchymal stromal cells for clinical use in cellular therapy. CellBio (2014) **3**:1. doi: 10.4236/cellbio.2014.31003

120. Blande IS, Bassaneze V, Lavini-Ramos C, Fae KC, Kalil J, Miyakawa AA, et al. Adipose tissue mesenchymal stem cell expansion in animal serum-free medium supplemented with autologous human platelet lysate. Transfusion (2009) **49**:12. doi: 10.1111/j.1537-2995.2009.02346.x

121. Hildner F, Eder MJ, Hofer K, Aberl J, Redl H, van Griensven M, et al. Human platelet lysate successfully promotes proliferation and subsequent chondrogenic differentiation of adipose-derived stem cells: a comparison with articular chondrocytes. J Tissue Eng Regen Med (2013). doi: 10.1002/term.1649

122. Brown RL, Ortaldo JR, Griffith RL, Blanca I, Rabin H. The proliferation and function of human mononuclear leukocytes and natural killer cells in serum-free medium. J Immunol Methods (1985) **81**:207–214.

123. McKenna DH, Sumstad D, Bostrom N, Kadidlo DM, Fautsch S, McNearney S, et al. Good manufacturing practices production of natural killer cells for immunotherapy: a six-year single-institution experience. Transfusion (2007) **47**:3. doi: 10.1111/j.1537-2995.2006.01145.x

124. Huijskens MJAJ, Walczak M, Sarkar S, Atrafi F, Senden-Gijsbers BLMG, Tilanus MGJ, et al. Ascorbic acid promotes proliferation of natural killer cell populations

in culture systems applicable for natural killer cell therapy. Cytotherapy (2015) **17**:5. doi: http://dx.doi.org/10.1016/j.jcyt.2015.01.004

125. Carlens S, Gilljam M, Chambers BJ, Aschan J, Guven H, Ljunggren H-G, et al. A new method for in vitro expansion of cytotoxic human CD3−CD56$^+$ natural killer cells. Hum Immunol (2001) **62**:10. doi: 10.1016/S0198-8859(01)00313-5

126. Arai S, Meagher R, Swearingen M, Myint H, Rich E, Martinson J, et al. Infusion of the allogeneic cell line NK-92 in patients with advanced renal cell cancer or melanoma: a phase I trial. Cytotherapy (2008) **10**:6. doi: 10.1080/14653240802301872

127. Pittari G, Filippini P, Gentilcore G, Grivel JC, Rutella S. Revving up natural killer cells and cytokine-induced killer cells against hematological malignancies. Front Immunol (2015) **6**:230. doi: 10.3389/fimmu.2015.00230

128. Iyengar R, Handgretinger R, Babarin-Dorner A, Leimig T, Otto M, Geiger TL, et al. Purification of human natural killer cells using a clinical-scale immunomagnetic method. Cytotherapy (2003) **5**:6. doi: 10.1080/14653240310003558

129. Passweg JR, Tichelli A, Meyer-Monard S, Heim D, Stern M, Kühne T, et al. Purified donor NK-lymphocyte infusion to consolidate engraftment after haploidentical stem cell transplantation. Leukemia (2004) **18**:11. doi: 10.1038/sj.leu.2403524

130. Lang P, Pfeiffer M, Handgretinger R, Schumm M, Demirdelen B, Stanojevic S, et al. Clinical scale isolation of T cell-depleted CD56$^+$ donor lymphocytes in children. Bone Marrow Transplant (2002) **29**:6. doi: 10.1038/sj.bmt.1703406

131. Sarkar S, van Gelder M, Noort W, Xu Y, Rouschop KMA, Groen R, et al. Optimal selection of natural killer cells to kill myeloma: the role of HLA-E and NKG2A. Cancer Immunol Immunother (2015). doi: 10.1007/s00262-015-1694-4

132. Arai S, Klingemann H-G. Natural killer cells: can they be useful as adoptive immunotherapy for cancer? Expert Opin Biol Ther (2005) **5**:2. doi: 10.1517/14712598.5.2.163

133. Jang YY, Cho D, Kim SK, Shin DJ, Park MH, Lee JJ, et al. An Improved flow cytometry-based natural killer cytotoxicity assay involving calcein AM staining of effector cells. Ann Clin Lab Sci (2012) **42**:42–49.

134. Cholujová D, Jakubíková J, Kubes M, Arendacká B, Sapák M, Ihnatko R, et al. Comparative study of four fluorescent probes for evaluation of natural killer cell cytotoxicity assays. Immunobiology (2008) **213**:8. doi: 10.1016/j.imbio.2008.02.006

135. Kane KL, Ashton FA, Schmitz JL, Folds JD. Determination of natural killer cell function by flow cytometry. Clin Diagn Lab Immunol (1996) **3**:295–300.

136. Mhatre S, Madkaikar M, Ghosh K, Desai M, Pujari V, Gupta M. Rapid flow cytometry based cytotoxicity assay for evaluation of NK cell function. Indian J Exp Biol (2014) **52**:983–988.

137. Kim GG, Donnenberg VS, Donnenberg AD, Gooding W, Whiteside TL. A novel multiparametric flow cytometry-based cytotoxicity assay simultaneously immunophenotypes effector cells: comparisons to a 4 h 51Cr-release assay. J Immunol Methods (2007) **325**:1-2. doi: 10.1016/j.jim.2007.05.013

138. Porter DL, Levine BL, Kalos M, Bagg A, June CH. Chimeric antigen receptor-modified T cells in chronic lymphoid leukemia. N Engl J Med (2011) **365**:8. doi: 10.1056/NEJMoa1103849

139. Grupp SA, Kalos M, Barrett D, Aplenc R, Porter DL, Rheingold SR, et al. Chimeric antigen receptor-modified T cells for acute lymphoid leukemia. N Engl J Med (2013) **368**:16. doi: 10.1056/NEJMoa1215134

140. Maude SL, Frey N, Shaw PA, Aplenc R, Barrett DM, Bunin NJ, et al. Chimeric antigen receptor T cells for sustained remissions in leukemia. N Engl J Med (2014) **371**:16. doi: 10.1056/NEJMoa1407222

141. Hermanson DL, Kaufman DS. Utilizing chimeric antigen receptors to direct natural killer cell activity. Front Immunol (2015) **6**:195. doi: 10.3389/fimmu.2015.00195

142. Zhang J, Sun R, Wei H, Zhang J, Tian Z. Characterization of interleukin-15 gene-modified human natural killer cells: implications for adoptive cellular immunotherapy. Haematologica (2004) **89**:338–347.

143. Jiang W, Zhang J, Tian Z. Functional characterization of interleukin-15 gene transduction into the human natural killer cell line NKL. Cytotherapy (2008) **10**:3. doi: 10.1080/14653240801965156

144. Goding SR, Yang Q, Knudsen KB, Potter DM, Basse PH. Cytokine gene therapy using adenovirally transduced, tumor-seeking activated natural killer cells. Hum Gene Ther (2007) **18**:8. doi: 10.1089/hum.2007.052

145. Müller T, Uherek C, Maki G, Chow KU, Schimpf A, Klingemann HG, et al. Expression of a CD20-specific chimeric antigen receptor enhances cytotoxic activity of NK cells and overcomes NK-resistance of lymphoma and leukemia cells. Cancer Immunol Immunother (2008) **57**:3. doi: 10.1007/s00262-007-0383-3

146. Boissel L, Betancur M, Wels WS, Tuncer H, Klingemann H. Transfection with mRNA for CD19 specific chimeric antigen receptor restores NK cell mediated killing of CLL cells. Leuk Res (2009) **33**:9. doi: 10.1016/j.leukres.2008.11.024

147. Esser R, Müller T, Stefes D, Kloess S, Seidel D, Gillies SD, et al. NK cells engineered to express a GD2 -specific antigen receptor display built-in ADCC-like activity against tumour cells of neuroectodermal origin. J Cell Mol Med (2012) **16**:3. doi: 10.1111/j.1582-4934.2011.01343.x

148. St. Jude Children's Research Hospital. Genetically Modified Haploidentical Natural Killer Cell Infusions for B-Lineage Acute Lymphoblastic Leukemia. Clinical Trials (2009). Available from: https://clinicaltrials.gov/ct2/show/NCT00995137#wrapper

149. Tonn T, Schwabe D, Klingemann HG, Becker S, Esser R, Koehl U, et al. Treatment of patients with advanced cancer with the natural killer cell line NK-92. Cytotherapy (2013) **15**:12. doi: 10.1016/j.jcyt.2013.06.017

150. Gong JH, Maki G, Klingemann HG. Characterization of a human cell line (NK-92) with phenotypical and functional characteristics of activated natural killer cells. Leukemia (1994) **8**:652–658.

151. Yan Y, Steinherz P, Klingemann HG, Dennig D, Childs BH, McGuirk J, et al. Antileukemia activity of a natural killer cell line against human leukemias. Clin Cancer Res (1998) **4**:2859–2868.

152. Tam YK, Miyagawa B, Ho VC, Klingemann HG. Immunotherapy of malignant melanoma in a SCID mouse model using the highly cytotoxic natural killer cell line NK-92. J Hematother (1999) **8**:281–290.

153. Maki G, Klingemann HG, Martinson JA, Tam YK. Factors regulating the cytotoxic activity of the human natural killer cell line, NK-92. J Hematother Stem Cell Res (2001) **10**:3. doi: 10.1089/152581601750288975

154. Tonn T, Becker S, Esser R, Schwabe D, Seifried E. Cellular immunotherapy of malignancies using the clonal natural killer cell line NK-92. J Hematother Stem Cell (2001) **10**:4. doi: 10.1089/15258160152509145

155. Cheng M, Chen Y, Xiao W, Sun R, Tian Z. NK cell-based immunotherapy for malignant diseases. Cell Mol Immunol (2013) **10**:3. doi: 10.1038/cmi.2013.10

156. Shahabi V, Postow MA, Tuck D, Wolchok JD. Immune-priming of the tumor microenvironment by radiotherapy. Am J Clin Oncol (2015) **38**:1. doi: 10.1097/COC.0b013e3182868ec8

157. Erba HP. Finding the optimal combination therapy for the treatment of newly diagnosed AML in older patients unfit for intensive therapy. Leuk Res (2015) **39**:2. doi: 10.1016/j.leukres.2014.11.027

158. Lotze MT, Thomson AW. *Natural Killer Cells: Basic Science and Clinical Application*. London: Academic Press (2009). 660 p.

159. Loza MJ. Expression of type 1 (interferon gamma) and type 2 (interleukin-13, interleukin-5) cytokines at distinct stages of natural killer cell differentiation from progenitor cells. Blood (2002) **99**:4. doi: 10.1182/blood.V99.4.1273

160. Mocellin S, Panelli M, Wang E, Rossi CR, Pilati P, Nitti D, et al. IL-10 stimulatory effects on human NK cells explored by gene profile analysis. Genes Immun (2004) **5**:8. doi: 10.1038/sj.gene.6364135