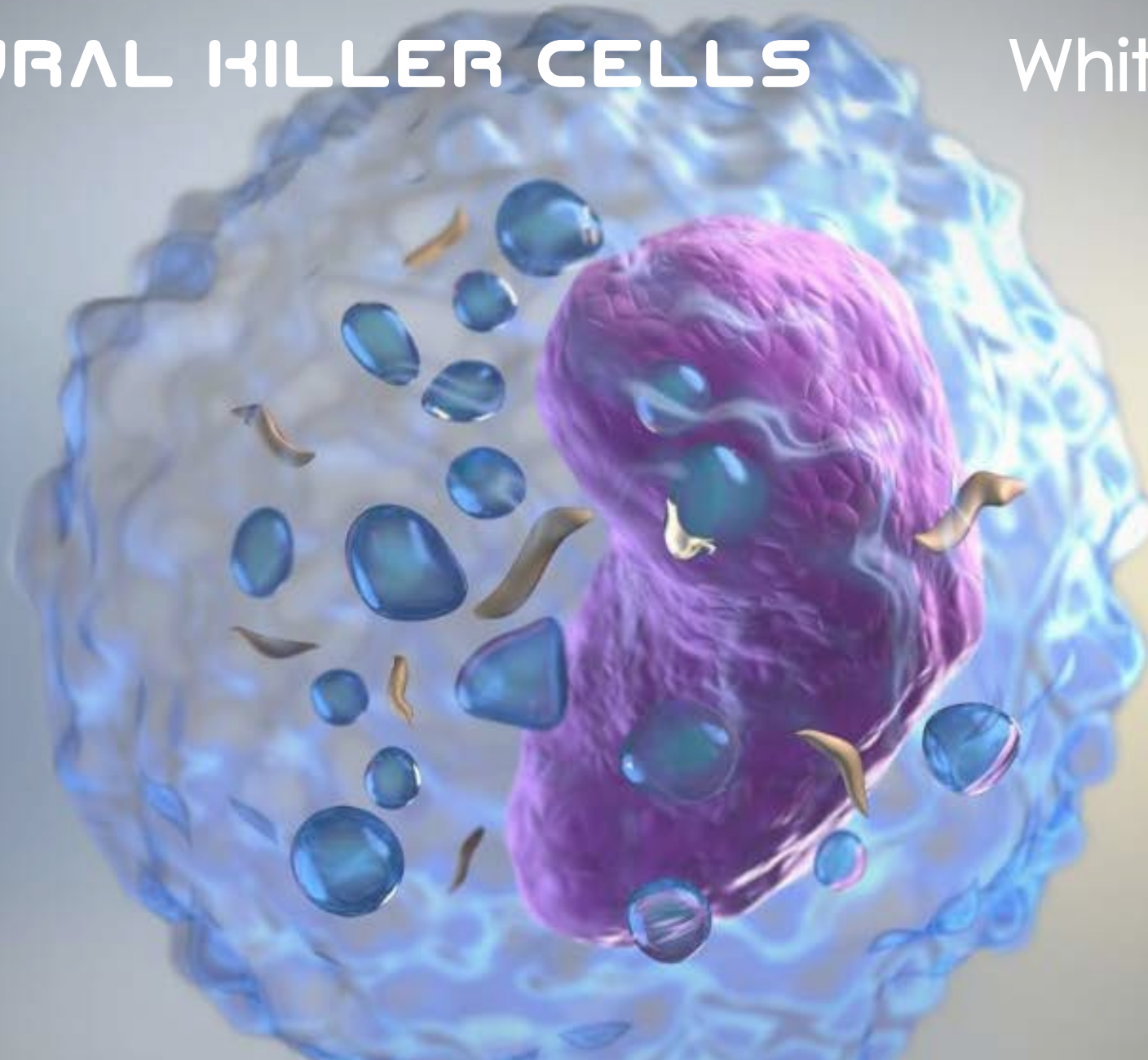


NATURAL KILLER CELLS

White Paper



UMBILICAL CORD BLOOD FOR HAPLOIDENTICAL USE

IMMUNE SYSTEM AND CANCER

Cancer formation and progression rely on cancer cell ability to bypass the immune response. This ability depends on genetic, epigenetic and phenotypic changes that mask tumor cells from immune system recognition. This is why, even if the presence of tumor-infiltrating lymphocytes (TIL) was demonstrated in many tumors, cancer development is not taken under control by these immune cells, as highlighted by tumor growth.

Nevertheless, the immune system remains a potent inhibitor of cancer growth. In recent years oncologists have been taking advantages from its curative potential, developing several kinds of cancer immune-therapies, such as monoclonal antibodies targeting “checkpoint receptors” that suppress immune response; treatment with such antibodies allowed unprecedented rates and durations of clinical responses. Among other approaches, adoptive cell therapy (ACT) utilizes autologous immune cells – that is, immune cells derived from the patient – which are transferred to the patient to elicit an antitumoral effect.

In classical ACT, a biopsy procured from the patient tumor site is grown *in vitro* with interleukine-2 (IL-2), a lymphocytes growth promoting substance. Lymphocytes obtained in such a way are then expanded to larger quantities and re-infused to the patient. At high doses, IL-2 alone is able to elicit a response in the patient; however, such a treatment is highly toxic. An IL-2 and TIL combined therapy can double the response rate achieved by IL-2 alone, but ACT efficacy could be improved further and procedures could be simplified.

In ACT, peripheral blood (PB) lymphocytes are utilized too; they can be engineered conferring them tumor-specificity, for example by means of the expression of chimeric antigen receptors (CARs). CARs are molecules characterized by an intracellular domain providing T cell receptor signaling to activate T cells, and an extracellular antigen-detecting domain of an antibody; this allows lymphocytes recognize tumor-specific or abundant antigens that could not be otherwise detected by T cell receptors, that normally recognize only non-modified, short peptides. CARs are being investigated for both hematological and solid cancers, but the identification of cancer-exclusive shared antigens, which lack in most solid tumors, represents a significant hindrance to their application. Moreover, the generation of autologous products is complicated: several weeks are often required to manufacture CAR T

cells, and it is not always possible to generate clinically relevant doses of them from heavily pre-treated patients. Unfortunately, even if human leukocyte antigen (HLA)-matched (a prerequisite for transplantation compatibility) the use of allogeneic T cells (that is, T cells from a genetically similar, but not identical, donor) carries a risk of graft-versus-host disease (GvHD), a complex reaction of donor's immune cells against recipient's tissues and organs.

UMBILICAL CORD BLOOD AND CANCER

Nowadays, in hematological cancer treatment umbilical cord blood (UCB) transplantation is an established strategy to expand the potential hematopoietic stem cell (HSC) donors pool. This approach offers several advantages: low risk of viral transmission; rapid availability; less stringent requirements for HLA matching; a lower risk of relapse, suggesting a strong graft-versus-leukemia/lymphoma (GvL) effect (that is, the capability of donor's immune cells to eliminate residual recipient's cancer cells); and a reduction in the incidence and severity of GvHD, which frequently develops in allogeneic HSC transplantation. Besides acute GvHD, chronic GvHD can result in life-threatening diseases many months post-transplantation. Reduced proliferation, cytokine production and cytotoxicity that characterize UCB lymphocytes could account for the lower incidence and severity of GvHD in UCB recipients. Moreover, UCB procurement is non-invasive and its availability is more rapid, eliminating the lengthy process of screening and obtaining stem cells from a matched unrelated donor.

If stored at birth, UCB units are readily available. The total cell numbers in UCB are lower than those in bone marrow or other peripheral stem cell source, but the substantial proliferative and engraftment potential of UCB CD34+ cells (multipotent stem cells that can give rise to all blood cell types) can counterbalance this disadvantage. Moreover, *ex vivo* (that is, outside the organism) expansion of UCB cells can overtake this problem.

Clinical findings suggest that immunocompetent cells other than T cells may mediate UCB transplantation effect. Among cells in UCB are progenitors of so-called natural killer (NK) cells, versatile lymphocytes with a role in both innate and adaptive immunity which are able to kill cancer cells without needing prior activation. NK cells can be divided in two main subsets. CD56dim NK cells are mature cytotoxic cells that mediate direct killing of target cells via exocytosis of granules, activation of cell death or antibody-dependent cytotoxicity; they correspond to

up to 90% of peripheral blood NK cells and have a poor capacity to proliferate in response to cytokines – small molecules, such as IL-2, that regulate interactions and communications between cells. CD56bright NK cells are instead less differentiated, can produce cytokines and have a tremendous proliferative capacity in response to IL-2; most NK cells in lymph nodes are CD56bright NK cells.

NK cell dysfunction was associated with impaired cytotoxicity against cancer cells. Conversely, donor-versus recipient NK cells alloreactivity was found to improve outcomes in some settings of HSC transplantations. Moreover, NK cells have shown a great potential in eliminating different types of cancer cell *in vitro* and in animal models.

The adoptive transfer of NK cells represents an attractive strategy for cancer patient treatment. CAR modification for NK cells has opened a new way to cancer treatment, and today NK cells based immunotherapy is among the most promising treatments under development for so far incurable forms of cancer. Several studies showed NK cells therapies to be feasible and safe. They have been tested, among others, on breast and ovarian cancer, Hodgkin, non-Hodgkin and refractory lymphoma, acute myeloid leukemia, melanoma, renal cell carcinoma, glioma and neuroblastoma. Infusion trials and HSC transplantations results suggest that NK cells could be the cells of choice in cellular therapies against tumor not displaying GvHD.

NK CELLS IN UMBILICAL CORD BLOOD

UCB is a source of NK cells progenitor populations that can differentiate into NK cells and that are absent in PB. Given the possibility to easily collect and cryopreserve UCB, it could be an off-the-shelf source for NK cell immunotherapy. Cells retrieval from UCB is limited by the low number of nucleated cells it contains (10-100-fold fewer than other sources of HSC), but NK cells are the first lymphocytes to recover after UCB transplantation, and it is likely that they are the main effector of the GvL effect in the first year after such a transplantation. Moreover, up to 30% of UCB lymphocytes are NK cells, compared to a more limited 10% of lymphocytes in PB, and UCB contains unique NK cell progenitor cell populations absent (or present only in a limited number) in PB. UCB contains higher percentages of NK cells than bone marrow too. Finally, NK cells isolation from UCB can be a one-step method because UCB contains only small percentages of NKT cells – a heterogeneous group of cells sharing properties of both T cells and NK cells, more abundant in PB, thus needing a two-step method for NK cells purification.

Compared to PB NK cells, UCB NK cells possess an immature phenotype, a reduced cytotoxicity and other features that have been associated to a less mature stage of differentiation; in fact, some groups reported a higher frequency of CD56bright NK cells in UCB. These differences may be related to modulation by other cells, such as IL-12 (interleukine-12) and IL-15 (interleukine-15)- producing white blood cells. In fact, cytotoxicity of UCB NK cells after incubation with IL-15 is similar to adult one, and percentages of NK cells expressing proteins that are essential for cytotoxicity are higher in UCB NK cells. Cytokines such as IL-2 or IL-15 make UCB NK cells proliferate too; IL-15 enhances NK cells migration and ability of growing into a colony, thus positively impacting engraftment. The expression of CD69 (an activation marker) appreciably increases after IL-12 and IL-18 (interleukine-18) stimulation in UCB NK cells but not in PB NK cells, and activation with interleukines (in particular, with IL-2, IL-15, IL-2 plus IL-15 or IL-15 plus IL-18) restores or enhances UCB NK cells cytotoxicity to PB NK cells one. Moreover, it seems that after IL-12 and IL-18 stimulation UCB CD56bright NK cells produce significantly more cytokines (namely, interferon- γ) compared to peripheral blood NK cells; this could compensate for UCB T cells hypofunctionality, contributing to a lower GvHD risk and to GvL effect too. UCB NK cells express also higher levels of CXCR4, the bone marrow homing receptor; that means they may be characterized by a greater capability to home to the bone marrow. Finally, UCB NK cells can be expanded to obtain sufficient numbers for clinical application from the small volume of a UCB unit. *Ex vivo* expansion techniques can increase NK cell numbers by about 1800 to 2400 fold from either fresh or cryopreserved UCB; expanded NK cells acquire functional competence and similar activity to *ex vivo* activated PB NK cells.

Expansion of UCB NK cells can be achieved using artificial antigen-presenting cells (aAPCs) or cytokines (including IL-2, IL-15 and/or FLT-3 ligand). A good manufacturing practice (GMP)-grade strategy based on aAPCs expressing membrane-bound IL-21 yielded a 2,389-mean fold expansion of NK cells from frozen UCB; it allowed to obtain NK cells displaying efficient killing ability against multiple myeloma *in vitro* and *in vivo* with a purity greater than 95%. Another strategy employing aAPCs expressing membrane-bound IL-15 and activated T lymphocytes receptor 41BBL to expand CB mononuclear cells yielded NK cells characterized by increased cytotoxicity against B-cell non-Hodgkin lymphoma both *in vitro* and *in vivo*. And expanding NK cells from frozen UCB by using irradiated Epstein-Barr virus-transformed lymphoblastoid cell lines and IL-2 gave NK cells showing higher cytotoxicity against leukemic cells than PB-derived expanded NK cells. Finally, using a cytokines cocktail it is possible

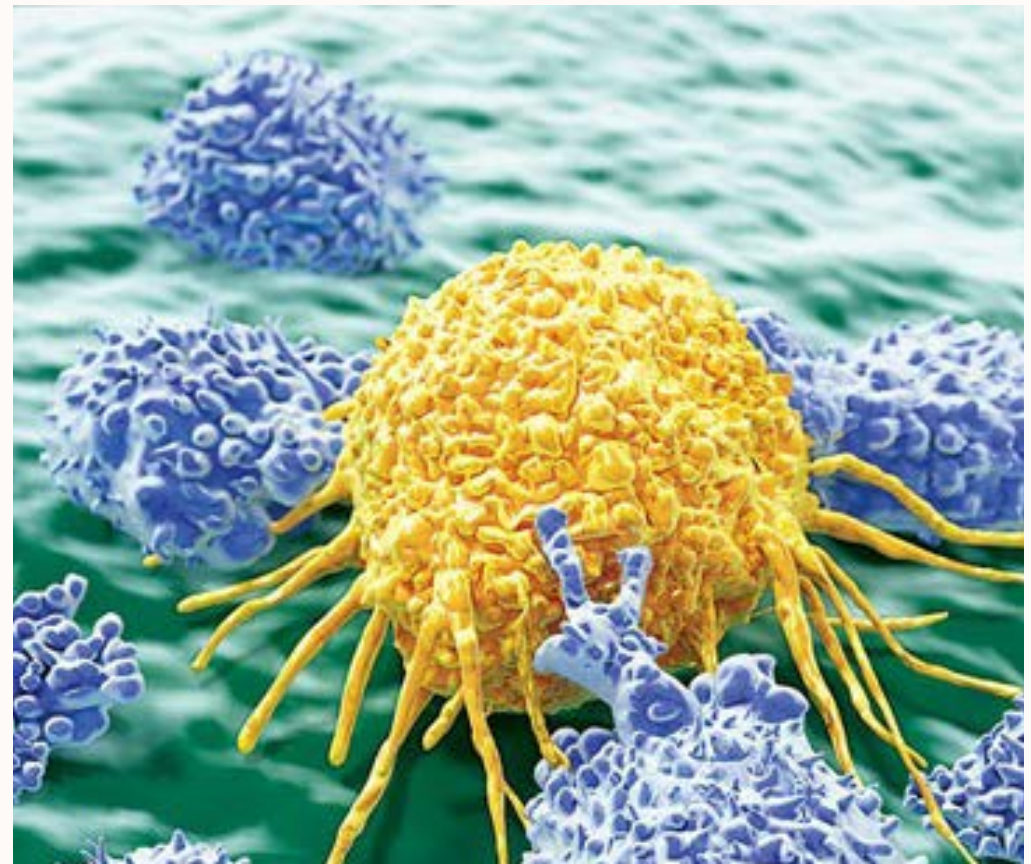
to obtain large numbers of NK cells from fresh and frozen UCB CD34+ cells too. These NK cells express low levels of inhibitory receptors, but are able to kill leukemic cell lines and patient cells both *in vitro* and *in vivo*. A greater expansion is obtained after cryopreservation, and frozen UCB CD34+ cells were found to be a NK cells source better than fresh UCB CD34+ cells and frozen PB CD34+. Thus, frozen UCB CD34+ cells could be a readily available product for NK cell immunotherapy.

Expansion of clinical-grade NK cells requires up to 28 days of culture (7-21 days on average). The final product can be characterized based on essential criteria such as purity and viability of the target cell population, contamination with undesirable cells and sterility. Additional characteristic helping identifying the most effective product are phenotype and tumor cytotoxicity.

NK CELLS AND CANCER

Among white blood cells, NK cells have long been considered potential candidates for cancer immunotherapy. They are highly cytotoxic effectors that kill their target in a non-antigen specific manner. Their activity is regulated through activating and inhibiting receptors without prior priming or antigen presentation. The overexpression of molecules induced by cellular stress on cancer cells surface triggers activation; receptors such as NCRs (natural cytotoxicity receptors) recognize such molecules, triggering NK cells response. Moreover, NK cells are activated by the interaction between CD16 receptors and antibody-coated targets too. Conversely, most KIRs (killer-cell immunoglobulin-like receptors) act as inhibitory receptors; they recognize MHC class I (MHC-I) molecules on target cells, inhibiting NK cell-mediated lysis of self, normal, cells.

NK cells stand out by their positive effects and safety demonstrated in most clinical trials. The ones produced *in vitro* have been shown to be safe and demonstrated a potent anti-tumor activity in both *in vitro* and *in vivo* setting, and cryopreservation of thawed NK cells would render multiple rounds of NK adoptive cell therapy feasible. In the early days, NK cell based cancer therapies focused on improving their antitumor activity or promoting their expansion with IL-2; however, high-dose IL-2 has significant side effects *in vivo*. Later, adoptive transfer of autologous NK cells failed to provide the expected results, probably because many tumors expressing high levels of MHC-I and/or low levels of activating receptors ligands are largely resistant to NK cells activity. However, allogeneic mature NK cells can be "re-educated" by recipient MHC-I and acquire cytotoxicity against recipient tumor



and haploidentical (that is, partially HLA-mismatched) hematopoietic stem cell transplantation used in combination with chemotherapy to treat different forms of leukemia.

Today, it is known that NK cell-mediated lysis of target cells is also involved in the mechanisms of action of monoclonal antibodies such as trastuzumab, which is utilized in breast cancer treatment. It could be possible to exploit such an involvement by targeting NK cells to cancer cells by means of monoclonal antibody already approved for clinical application, such as rituxumab for B cell leukemia or cetuximab against colon and head and neck cancers.

Moreover, NK cells represent an attractive alternative to CAR T cells. They do not cause GvHD, and as opposite to T cells, NK cells do not need prior stimulation to kill target cells; however, cytokines stimulation activates NK cells and enhances their proliferation and their cytolytic activity.

cells without causing GvHD. Evidence for their anticancer efficacy comes from both allogeneic. They do not cause GvHD, and as opposite to T cells, NK cells do not need prior stimulation to kill target cells; however, cytokines stimulation activates NK cells and enhances their proliferation and their cytolytic activity. They have the potential to exert cytotoxicity through mechanisms other than that determined by CAR specificity, reducing in principle the risk of relapse mediated by CAR-target antigen loss reported for CAR T cell therapy.

Another approach involves UCB-derived NK cells transplantation. Cancer cells often down-regulate MHC-I molecules to escape immune system eliminating NK cell inhibition and triggering NK cell-mediated lysis of cancer cells. However KIR and HLA genes (which encode MHC proteins) are independently inherited; that means that in an UCB transplantation with HLA-matched donor and recipient mismatch between KIRs and their ligand is possible, thus making possible transplanted HLA-matched NK cells trigger tumor cell lysis. This phenomenon was proposed to be beneficial in reducing relapse after HSC transplantation, but the outcome could be influenced also by KIR haplotype; its influence on UCB transplantation outcome needs to be investigated. The impact of NK cell licensing (that is, the process by which NK cells become functional and tolerant to self-HLA) needs to be investigated too; but even if licensed NK cells are significantly more cytotoxic than unlicensed one and are more likely to mediate a strong GvL response, it has been reported that unlicensed NK cells can kill neuroblastoma cells in human.

Following UCB transplantation, NK cells display features associated with maturity and are fully functional against leukemic blasts. Unfortunately, cancer cells

can shape NK cell populations with altered reactivity and suppress NK cells too. They can down-regulate tumor antigens and NCR-activating molecules. They can upregulate inhibitory MHC class I molecules too, and immunosuppressive cytokines or enzymes further impair antitumor NK cells activity. Overcoming these problems is possible by ex vivo modulation of NK cell receptor. This modulation can be achieved by interleukines (IL-2, IL-12, IL-15, IL-18 or IL-21) and human type I interferons stimulation, and culturing NK cells among accessory or feeder cells that provide additional stimulatory signals necessary for NK cell proliferation allows to reach efficient expansion rates. Finally, it is possible to cross-linking NK cells to cancer cells by multivalent reagents and to genetically engineer NK cells with natural molecules or with CAR, further empowering them with improved immune function and augmenting their activity against tumor.

NK cells engineered with a CAR against CD19, IL-15 and an inducible suicide gene effectively kill CD19-expressing leukemia/lymphoma cell lines and primary chronic lymphocytic leukemia cells; they also exert cytotoxicity non-CAR.CD19-mediated. IL-15 production leads to more robust activation of NK cells with enhanced in vivo proliferation, persistence and anti-tumor activity without the significant toxicity associated with even low dose of externally administered IL-15. Finally, the inducible suicide gene counteracts the severe toxicity of CAR T cell therapy allowing a rapid and efficient elimination of gene-modified NK cells. Clinical trials of these CAR-NK cells already begun at the Anderson Cancer Center in Houston (Texas, Usa). That is, ex vivo expansion of UCB NK cells is not only a way to obtain larger cells numbers, but a strategy to modify their antitumor feature too. In general, their



cytotoxicity increases, and they may become responsive against target previously resistant to NK cell lysis.

Actually, *ex vivo* expansion of NK cells can be achieved by many different protocols, all characterized by different features and capacities. Automated processes exist that represent highly efficient tools to standardize NK cell processing for therapeutic use. For example, using UCB and GMP-compliant procedures for robust expansion it is feasible to generate multiple clinical doses of CAR-NK cells from a single UCB unit.

UCB BANKING FOR NK CELLS HAPLOIDENTICAL USE

Donor selection can affect NK cell therapy outcome because of receptor polymorphisms influencing NK cell function. The best results of allogeneic transplantations are obtained when the donor is a HLA-matched sibling; a 75% probability that any sibling is not fully HLA-matched with the recipient, coupled with some small family size, make difficult to find HLA-matched siblings. A particularly difficult situation is in the field of HSC transplantation, where only 30% of patients have an HLA-matched sibling. HLA-haploidentical donors can be a solution, and given UCB-derived NK cells potential, Bioscience Institute offers the chance of store UCB for autologous and haploidentical use of NK cells.

Several efforts are being made to reactivate and redirecting patient's NK cells to kill his tumor. Strategies include antibodies that either stimulate or block, respectively, activating or inhibitory NK cells receptors.

In the haploidentical setting, KIR-mismatch showed a favorable effect and prompted a number of studies on allogeneic NK cells proving that haploidentical, related-donor NK-cell infusion can be safely administered. Moreover, *in vivo* expansion and persistence can be achieved following lympho-depleting chemotherapy regimens. UCB can be used even when not fully matched to the recipient, and significant progress has been made in NK cell based therapies in haploidentical stem cell transplantation. Preclinical validation studies achieved the production of significant numbers of functional NK cells either from complete UCB units or minute UCB sample, opening the way to post transplant immune cellular therapies and off-the-shelf haploidentical NK cellular therapies. With the use of haploidentical family donors growing, UCB is a possible source of NK cells for haploidentical use. The advantage of being a readily available off-the-shelf frozen source of NK cells is bolstered by methods to generate large numbers of functional NK cells from frozen UCB units *ex vivo* through good manufacturing practice (GMP)-compliant techniques.



REFERENCES

- Ayello J et al. Genetically re-engineered K562 cells significantly expand and functionally activate cord blood natural killer cells: potential for adoptive cellular immunotherapy. *Exp Hematol* (2017) 46:38–47. doi:10.1016/j.exphem.2016.10.003
- Balassa K, Rocha V. Anticancer cellular immunotherapies derived from umbilical cord blood. *Expert Opin Biol Ther*. 2018 Feb;18(2):121-134. Doi: 10.1080/14712598.2018.1402002
- Beano A et al. Correlation between NK function and response to trastuzumab in metastatic breast cancer patients. *J Transl Med*. 2008 May 16;6:25. doi: 10.1186/1479-5876-6-25
- Beziat V et al. Fully functional NK cells after unrelated cord blood transplantation. *Leukemia*. 2009 Apr;23(4):721-8. doi: 10.1038/leu.2008.343
- Brown JA, Boussiotis VA. Umbilical cord blood transplantation: basic biology and clinical challenges to immune reconstitution. *Clin Immunol*. 2008 Jun;127(3):286-97. doi: 10.1016/j.clim.2008.02.008
- Chabannon C et al. Manufacturing Natural Killer Cells as Medicinal Products. *Front Immunol*. 2016 Nov 15;7:504.
- Cohen JE et al. Adoptive cell therapy: past, present and future. *Immunotherapy*. 2017 Jan;9(2):183-196. doi: 10.2217/imt-2016-0112
- Dalle JH et al. Characterization of cord blood natural killer cells: implications for transplantation and neonatal infections. *Pediatr Res*. 2005 May;57(5 Pt 1):649-55
- Granzin M et al. Fully automated expansion and activation of clinical-grade natural killer cells for adoptive immunotherapy. *Cytotherapy*. 2015 May;17(5):621-32. doi: 10.1016/j.jcyt.2015.03.611
- Granzin M et al. Shaping of Natural Killer Cell Antitumor Activity by Ex Vivo Cultivation. *Front Immunol*. 2017 Apr 26;8:458. doi: 10.3389/fimmu.2017.00458
- Hofer E, Koehl U. Natural Killer Cell-Based Cancer Immunotherapies: From Immune Evasion to Promising Targeted Cellular Therapies. *Front Immunol*. 2017 Jul 12;8:745. doi: 10.3389/fimmu.2017.00745
- Koehl U et al. Advances in clinical NK cell studies: Donor selection, manufacturing and quality control. *Oncoimmunology*. 2015 Nov 11;5(4):e11115178
- Koo KC et al. Reduction of the CD16(-)CD56bright NK cell subset precedes NK cell dysfunction in prostate cancer. *PLoS One*. 2013 Nov 4;8(11):e78049. doi: 10.1371/journal.pone.0078049
- Li Y et al. NK cell-based cancer immunotherapy: from basic biology to clinical application. *Sci China Life Sci*. 2015 Dec;58(12):1233-45. doi: 10.1007/s11427-015-4970-9
- Lim O et al. GMP-compliant, large-scale expanded allogeneic natural killer cells have potent cytolytic activity against cancer cells *in vitro* and *in vivo*. *PLoS One*. 2013;8(1):e53611. doi: 10.1371/journal.pone.0053611

Liu E et al. Cord blood NK cells engineered to express IL-15 and a CD19-targeted CAR show long-term persistence and potent antitumor activity. *Leukemia*. 2018 Feb;32(2):520-531. doi: 10.1038/leu.2017.226

Lu X et al. CD16+ CD56- NK cells in the peripheral blood of cord blood transplant recipients: a unique subset of NK cells possibly associated with graft-versus-leukemia effect. *Eur J Haematol*. 2008 Jul;81(1):18-25. doi: 10.1111/j.1600-0609.2008.01073.x

Luevano M, Madrigal A, Saudemont A. Generation of natural killer cells from hematopoietic stem cells *in vitro* for immunotherapy. *Cell Mol Immunol* (2012) 9:310–20. doi:10.1038/cmi.2012.17

Mehta RS et al. Cord Blood as a Source of Natural Killer Cells. *Front Med (Lausanne)*. 2016 Jan 5;2:93. doi: 10.3389/fmed.2015.00093

Sarvaria A et al. Umbilical Cord Blood Natural Killer Cells, Their Characteristics, and Potential Clinical Applications. *Front Immunol*. 2017 Mar 23;8:329. doi: 10.3389/fimmu.2017.00329

Shah N, Martin-Antonio B, Yang H, Ku S, Lee DA, Cooper LJ, 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):e76781. doi:10.1371/journal.pone.0076781

Sungur CM, Murphy WJ. Positive and negative regulation by NK cells in cancer. *Crit Rev Oncog*. 2014;19(1-2):57-66

Veluchamy JP et al. In Vivo Efficacy of Umbilical Cord Blood Stem Cell-Derived NK Cells in the Treatment of Metastatic Colorectal Cancer. *Front Immunol*. 2017 Feb 6;8:87. doi: 10.3389/fimmu.2017.00087

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