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Novel strategies to target cancer stem cells by NK cells; studies in humanized mice

Anna K. Kozlowska[1,3], Kawaljit Kaur[1], Paytsar Topchyan[1], Anahid Jewett[1,2]

[1]Division of Oral Biology and Oral Medicine, The Jane and Jerry Weintraub Center for Reconstructive Biotechnology, UCLA, Los Angeles, CA, USA, [2]The Jonsson Comprehensive Cancer Center, UCLA School of Dentistry and Medicine, Los Angeles, CA, USA, [3]Department of Tumor Immunology, Chair of Medical Biotechnology, Poznan University of Medical Sciences, Poznan, Poland

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1. ABSTRACT

We have previously shown, that following selection, natural killer (NK) cells differentiate cancer stem-like cells (CSCs)/poorly differentiated tumors via secreted and membrane bound IFN-gamma and TNF-alpha, leading to prevention of tumor growth and remodeling of the tumor microenvironment. Since conventional therapeutic strategies, including chemotherapy and radiotherapy remain unsuccessful in treating stem-like tumors, there has been increasing interest in NK cell-targeted immunotherapy for the treatment of aggressive malignacies. In our recent studies, we used a humanized (hu-BLT) mouse model with transplanted human bone marrow, liver and thymus to demonstrate the efficacy of adoptive transfer of *ex vivo* expanded, super-charged NK cells in selection and differentiation of stem-like tumors within the context of a fully reconstituted human immune system. We have also shown that CSCs differentiated with split anergized NK cells prior to implantation in humanized mice did not grow or metastasize. In this review, we present current advances in NK cell detection, expansion and therapeutic delivery methods, and discuss the utility of different humanized mouse models in studying NK cell-based therapies in the preclinical setting.

2. INTRODUCTION: NATURAL KILLER CELLS KILL AND DIFFERENTIATE CANCER STEM-LIKE CELLS

Natural killer (NK) cells constitute 10% to 15% of human peripheral blood lymphocytes, and represent the first line of defense against virally infected cells and transformed cells. However, decreased NK cell cytotoxicity in the tumor microenvironment and peripheral blood of cancer patients, as well as down-modulation of CD16 receptors on the surface of NK cells, have been reported and thought to contribute to cancer progression (1, 2). Increasing evidence indicates that the majority of human cancer originates and is maintained by proliferating stem-like populations, known as cancer stem-like cells (CSCs), which are capable of self-renewal. Even though CSCs are highly susceptible to NK cells, they maintain resistance to chemotherapeutic drugs and radiation through increased expression of DNA mismatch repair genes and augmented multi-drug resistance genes (3, 4).

We have previously shown that NK cells cytotoxicity is suppressed after interaction with healthy hematopoietic stem cells and malignant stem-like cells (5-11). It has also been demonstrated that NK cells lose the ability to mediate cytotoxicity and down-modulate CD16 receptor expression upon interaction with CSCs, while maintaining increased secretion of IFN-gamma, a functional outcome that we have previously coined as split anergy (5-11). In contrast, differentiated tumor cells do not down-modulate CD16 expression (7, 8).

We have found that CSCs of different origins display low levels of MHC class I, CD54 and programmed cell death protein ligand 1 (PD-L1), also known as

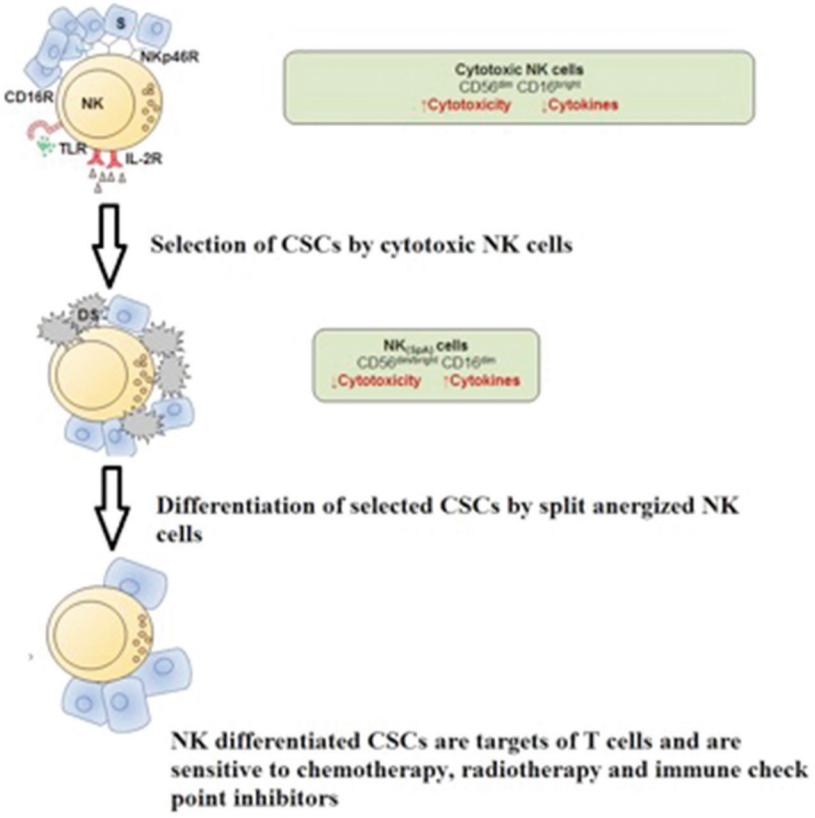

Figure 1. Hypothetical representation of osteoclast-expanded super-charged NK cell function in selection and differentiation of CSCs. Recognition of CSCs through CD16 and NKp46 NK cell receptors in a combination with Toll-like or cytokine receptors triggering on NK cells results in selection of CSCs subpopulation and differentiation of selected stem cells by split-anergized NK cells. $CD56^{dim}CD16^{bright}$ NK cells are cytotoxic and are capable of selecting CSCs, whereas $CD56^{dim/bright}CD16^{dim}$ split anergized NK cells lack cytotoxicity and are able to secrete high levels of cytokines (IFN-gamma and TNF-alpha) to support differentiation of selected stem cells. Please note, the receptors shown on NK cells are a partial list of receptors important for the activation of NK cell function. *NK,* NK cells*; NK(SpA) cells,* split anergized NK cells*; TLR,* toll-like receptors*; S,* stem cells*; CD16R,* CD16 receptors*; NKp46R,* NKp46 receptors*; IL-2R,* IL-2 receptors*; DS,* dead stem cells.

B7H1. A subset of CSCs become targets of cytotoxic NK cells, while the remaining CSCs are differentiated by split anergized/regulatory NK cells through cell-cell contact and secretion of membrane bound and secreted forms of TNF-alpha and IFN-gamma (12). In addition, differentiation by split anergized NK cells leads to inhibition of CSCs growth and upregulation of MHC class I, CD54 and PD-L1 expression, resulting in resistance of differentiated tumor cells to NK cell-mediated cytotoxicity (12, 13). Overall, our studies indicated that NK cells target and differentiate both healthy and transformed stem-like cells, resulting in the maturation of target cells and shaping of their microenvironment (Figure 1) (14-17).

Data obtained from *in vitro* cell culture studies often do not mirror the behavior of primary tumors in patients. Mice are chosen as the experimental model in cancer immunology and preclinical testing of cancer immunotherapeutics as they provide valuable insights into the behavior of tumors in the context of full immune cell repertoire. However, the major limitations of translating data obtained from the mouse model to the human system are structural and functional differences between human and murine immune systems. The major cell population in human blood is represented by neutrophils (50-70%), whereas lymphocytes constitute only 30-50%. Murine blood contains mainly lymphocytes (75-90%) and B cells are the most abundant type of lymphocyte (18). Differences in human and mouse innate immune systems include the lack of defensin expression by murine granulocytes, expression of different Toll-like receptors on various immune subsets, and phenotypic differences in monocytes, DCs and MDSCs (reviewed in (18)). Despite similar proportions of NK cells in murine and human blood, their phenotypes and activation dynamics are quite different (18, 19). $CD3^-/CD16^+CD56^{dim}$ phenotype is typical for cytotoxic NK cells, whereas the regulatory $CD3^-/CD16^{neg}CD56^{bright}$ NK cells mediate differentiation of stem cells in humans (20, 21). Murine NK cells can be characterized by DX5 expression and $CD11b^+/CD27^-$ or $CD11b^-/CD27^+$ phenotypes, respectively (19, 22, 23). We have recently shown that primary non-activated human NK cells mediate low-level

cytotoxicity, but upon short-term IL-2 activation, they display significant cytotoxicity against a number of human stem-like tumors. In contrast, naïve murine NK cells do not display any cytotoxicity and require a much longer IL-2 activation period to induce cytotoxicity against target cells (24). Human NK cells utilize KIR proteins as inhibitory receptors for MHC class I molecules, whereas murine NK cells use highly divergent structures of Ly49 protein family members. In addition, human NKG2D receptors bind to MICA, MICB or ULBP 1-6 ligands, whereas mouse ligands for NKG2D are different and include, HAE60 and Rae1beta (18, 19). Therefore, these differences should be considered and caution should be exercised when translating results of mouse tumor models to human cancer.

In this review, we discuss the utility of humanized mouse models as a preclinical platform to develop and test novel NK cells based cancer immunotherapies. Additionally, we discuss the use of osteoclast-expanded NK cells in the elimination of cancer stem-like tumors in bone marrow, liver, thymus humanized (hu-BLT) mice.

3. STUDIES OF NK CELLS IN XENOGENEIC IMPLANTATION OF HUMAN CELLS INTO IMMUNODEFICIENT MOUSE STRAINS

There have been numerous attempts to establish an appropriate small animal model to study the complex interactions between immune cells and human tumors. Immunocompetent mouse strains are useless as hosts for human tumors because they mount a xenogeneic immune response against human cells, resulting in the failure of tumor engraftment. Several xenograft mouse models have previously been developed and are of high interest since they provide information regarding the pathophysiology of human tumors in animals; however, the options to study the interaction of human cancer cells with human immune system remain limited. As human HLA molecules are not cross-reactive with murine NK cell receptors, in the earliest xenograft models, certain human cells were likely recognized as MHC class I negative cells, and were eliminated by murine NK cells (18, 25). The first immunodeficient strains used for xenogeneic transplantation were T- cell deficient athymic nude (*Foxn1*) mice and severe combined immunodeficiency (*scid*) mice (26-28). The major disadvantages of nude mice were their intact humoral adaptive immune system and high activity of murine NK cells, which prevented most primary human solid tumors from seeding or growing, and virtually disqualified engraftment of human normal or malignant hematopoietic cells (29).

The discovery of the spontaneous "*scid*" mutation in C.B17 strain (26), which destabilized mouse hematopoietic stem cells (30) and prevented T and B cells development enabled a broader range of human solid tumor engraftment compared to nude mice (31). Lapidot *et al*. demonstrated that low engraftment levels of human bone marrow cell suspensions were present, but as human cells represented only 0.5%-5% of the total *scid* recipient marrow population (32), a more effective model was still needed. The low xenograft reconstitution levels could not only be explained by the lack of critical human cytokines but also by the enhanced function of the innate immune system. Despite the block in lymphoid differentiation, C.B17-*scid* mice develop NK cells, as well as myeloid cells (33). It has been shown that, C.B17-*scid* NK cells could be stimulated with poly I: C to a higher extent than NK cells from wild type mice, and displayed cytotoxicity against human mesenchymal stem cells (MSCs), human embryonic stem cells (hESCs) and tumor initiating cells (TICs) (34-36). This enhanced NK cell activity was reported in strains carrying *scid* mutations and was considered a compensation mechanism for the lack of adaptive immunity. Similarly, we have recently shown that NK cells purified from *Cox-2*$^{flox/flox}$*;LysM*$^{Cre/+}$ mice have heightened cytotoxic activity when compared to those obtained from control littermates (24). In addition, NK cells cultured with autologous *Cox-2*$^{flox/flox}$*;LysM*$^{Cre/+}$ monocytes, DCs and mouse embryonic fibroblasts (MEFs) isolated from global COX-2 knockout mice, had increased cytotoxic function as well as augmented IFN-gamma secretion when compared to NK cells from control littermates cultured with monocytes. The list of genes that augment NK cell function when knocked out in neighboring cells is still increasing, and may point to the fundamental function of NK cells in targeting less differentiated cells in order to aid in their differentiation (13, 15, 24, 37).

The rejection of tumor and hematopoietic xenografts was partially alleviated by the development of the NOD-*scid* strain via introduction of the Prdkc^{scid} mutated gene from C.B17-*scid* mice into a NOD inbred strain with several impairments in innate immunity (38). The resulting NOD-*scid* mice were deficient in NK cells, displayed reduced development and function of macrophages and dendritic cells and the absence of hemolytic complement (39, 40). In contrast to *scid* mice, activity of NK cells remained low in NOD-*scid* strain; thus pretreatment with poly I: C was needed to detect active NK cells previously reported as NK1.1. negative (39). NOD-*scid* mice expressed polymorphic variant of Sirp-alpha, which prevented activation of murine macrophages against human CD47^{+} cells (41, 42). Human hematopoietic stem cells express the CD47 surface marker, which binds to Sirp-alpha protein during engraftment in the bone marrow (42). High affinity binding of the Sirp-alpha to human CD47 molecule prevents mouse macrophages from engulfing the human hematopoietic stem cells. Although NOD-*scid* mice support the growth of a large numbers of solid tumors and hematological malignancies, still a portion

of tumors fails to engraft or grow efficiently, because of the remaining NK cell activity and other residual innate immune functions (34, 39).

The most recent introduction of a genetically engineered complete null mutation of the gamma chain of interleukin 2 receptor (IL2rg) into NOD-*scid* mice gave rise to one of the most immunodeficient mouse strains known to date - NOD-*scid* IL2Rg^{null} (NSG) (43). IL2rg is a common component of cell surface receptors for six different interleukins (IL-2, IL-4, IL-7, IL-9, IL-15, and IL-21). Although, the cytokines are still present, IL2rg knockout blocks molecular pathways for these cytokines, resulting in the absence of functional NK cells since they require IL-15 signaling for their development. The remaining components of mouse innate immune cells include monocytes and neutrophils, as well as defective macrophages and dendritic cells. NSG similarly to NOD-Rag1^{null}IL2Rgamma^{null} (NRG) strains are profoundly immunodeficient, which is the key feature that supports the growth of various malignancies, and most importantly, differentiation of human hematopoietic stem cells into multi-lineage subsets (41, 44).

4. USE OF IMMUNODEFICIENT MOUSE STRAINS IN THE STUDIES OF CANCER IMMUNITY

Differences in the ability of CSCs to give rise to human tumors in different immunodeficient strains could be explained by different levels of NK cells impairment and/or deletion in nude, NOD-*scid* and NSG strains (45). As shown by Quintana *et al.,* approximately 25% of melanoma cells were able to initiate tumor growth in NSG mice that were incapable of mediating any immune function against injected stem-like cells, whereas only 0.1.% of melanoma cells could give rise to tumors in NOD-*scid* animals with reduced but detectable NK cell activity (46).

Results of studies performed using immunodeficient animals have raised many questions about the role of certain immune subsets in controlling cancer initiation, growth and metastasis. As it is nearly impossible to assess and compare the aggressiveness and metastatic potential of primitive CSC-like tumors using immunodeficient mouse strains, it has been concluded that humanized mice with restored human immune system would be the most suitable platform to conduct such studies.

5. HUMANIZED MICE AS PRECLINICAL MODELS TO STUDY THE COMPLEXITY OF HUMAN IMMUNE SYSTEM INTERACTIONS

Numerous attempts have been made to generate mice with a fully reconstituted human immune system. Based on current evidence, mouse strains differ in their ability to support reconstitution of the human immune system, and since a profound immunodeficiency in the background strain is critical, the strain of choice can either be NSG or NRG (47, 48). There are three major humanized mouse models, which differ in the source and donor cell type, injection route and recipient age. The most straightforward humanization model requires adoptive transfer of human peripheral blood mononuclear cells (PBMCs) obtained from adult healthy donors or patients into the NSG mice (49, 50). The advantages of such an approach are simplicity, immediate presence of mature cells circulating in recipients and the possibility to study immune cells from patients in personalized mouse systems. However, the utility of this model is limited to short term experiments due to mature human immune cells initiating graft versus host (GvHD) disease against their host (51). Another strategy involves prior isolation of CD34$^+$ progenitor cells from peripheral blood, cord blood or fetal liver and injection into myeloablated NSG mice. This model requires 8-12 weeks as HSCs need to engraft bone marrow, differentiate and mature into the various hematopoietic lineages of the human immune system. After 8-12 weeks, mature human immune subsets can be easily detected in peripheral blood and other lymphoid tissues. The major limitation of the CD34$^+$ humanization model is that due to the lack of human thymus, T cells undergo selection in the context of the mouse histocompatibility complex (41). In order to overcome this limitation, surgical implantation of human fetal liver and thymus fragments under the renal capsule of NSG mice is performed and followed by the intravenous injection of autologous CD34$^+$ hematopoietic cells to provide a complete human microenvironment for immune cell development (52, 53). As a result, developing T cells undergo positive and negative selection in a human thymic organoid and become functional CD4$^+$ helper and CD8$^+$ cytotoxic T cells after restriction based on HLA molecules in bone marrow, liver, thymus humanized (hu-BLT) mice (41, 54). Hu-BLT is the only known humanized mouse model that displays mucosal immunity (55). HSCs develop, at least to some extent, into T, B, NK cells, monocytes, MDSCs, macrophages, DCs, erythrocytes, and platelets in tissues of hu-BLT mice (44, 56-58). Stable human CD45 cell engraftment in hu-BLT immune compartments is confirmed by flow cytometry and contitutes approximately for 50% to 80% of cells in peripheral blood (manuscript in preparation). Human immune cells are also found to populate the reproductive tract of females, intestines, rectum (59, 60), pancreas and gingiva (manuscript in prep). The broad use of hu-BLT in research may be limited by the availability of fetal tissue and skilled surgical procedures. Moreover, since T cell education occurs in the context of human thymus, T cells with affinity to murine MHC may still be present and as a result hu-BLT may develop GvHD-like symptoms after 20 weeks post engraftment shortening the available experimental period (19). In contrast to PBMC humanized mice, the hu-BLT model does not allow

for personalized disease or genetic mutation study based on cells obtained from patients. Despite such limitations, hu-BLT is currently the best available model for studying human immunity.

6. POTENTIAL LIMITATIONS OF ALLOGENEIC TUMOR TRANSPLANTATION IN HUMANIZED MICE

The humanized mouse model may be considered the best and closest mouse model to reflect the human immune system. The analysis of tumor-immune cell interactions within the tumor microenvironment including systemic tumor effects, as well as testing the efficacy of anti-cancer therapies are attractive features of these mice. The major presumed limitation of studies using humanized mice appears to be the necessity to match HLA of the immune graft to the injected cancer cells. Thus, the initial questions to be addressed were whether allogeneic tumors were likely to grow and if so, how important HLA-matching was in mounting an effective immune response in humanized mice. HLA mismatch between injected cancer cells and mature immune cells populating humanized mice has been expected to cause xenograft-allograft rejection or GvHD. Wege *et al.* proposed simultaneous co-engraftment of newborn NSG mice with $CD34^+$ HSCs and human breast cancer cells to potentially avoid allograft rejection by mature immune cells (61). In such mice, human breast tumors were able to grow in the presence of maturating immune system, and generation of specific T and NK cells responses as well as infiltration of tumors with NK cells was observed (61).

Based on our previous data, we selected oral squamous cancer stem cells (OSCSCs) and pancreatic stem-like tumor cells as candidates to test tumor initiation and growth in the presence of non-HLA matched mature immune system in hu-BLT mice. We have previously shown that OSCSCs and pancreatic stem-like tumors expressed very low levels of MHC class I, CD54 and PD-L1, and they were highly susceptible to NK cell lysis, whereas T cells were not able to target such tumors. Furthermore, we demonstrated that oral and pancreatic stem-like tumors differentiated upon interaction with split-anergized NK cells and their secreted factors, IFN-gamma and TNF-alpha (Figure 1). Differentiation with split-anergized NK cell supernatants increased expression of MHC class I, CD54, and PD-L1 on cancer cells and rendered the tumors resistant to NK cell mediated cytotoxicity (14, 62). We demonstrated that non-HLA matched OSCSCs and pancreatic stem-like cancer cells were able to form tumors in the oral cavity and pancreas of hu-BLT animals and were not rejected in the presence of competent T and B cells when injected after full reconstitution of the human immune system. We also observed infiltration of major human immune subsets including NK cells, T cells, B cells and monocytes in the tumor microenvironment. Similar to *in vitro* observations, OSCSCs and pancreatic stem-like cancer cells differentiated with split anergized NK cell supernatants, grew much slower when injected at an orthotopic site in hu-BLT mice ((12, 63) and manuscript in preparation).

Similar data was obtained with melanoma cells in hu-BLT mice (manuscript in preparation). Our data is consistent with the observations from the JAX laboratories with Patient-Derived Xenografts (PDXs) of different origin in $CD34^+$ hu-mice without prior HLA matching (64). However, because differentiation stages of the PDXs were not known, it is likely that the injected tumors contained CSC populations capable of giving rise to primary tumors in the presence of competent T cells. Two different patient-derived melanoma cell lines have been shown to be successfully implanted in hu-BLT mice and treated with T cell based immunotherapy (65). Melanoma tumors could only be cleared by MART-1 transgenic T cells, HLA-matched with cancer cells, whereas non-matched MART-1 T cells did not show any therapeutic effect against melanoma. We have found that these melanoma tumor cells had stem-like/poorly differentiated tumor cell phenotypes and were sensitive targets for cytotoxic NK cells (unpublished data).

Interestingly, in our studies, NSG immunodeficient mice developed larger tumors at a faster rate when compared to hu-BLT mice. This result may account for the severe lack of immune defense and control over tumor growth in NSG mice (manuscript in preparation). Similarly, mice bearing K562 erythroleukemia tumors and injected with $CD34^+$ HSC progenitors survived significantly longer in comparison to their K562 bearing immunodeficient Balb/c $Rag2^{-/-}$ gammac $^{-/-}$ counterparts suggesting growth inhibition of malignant cells by reconstituted human immune cells (66).

We have previously demonstrated that aggressive stem-like tumors, including melanoma, oral and pancreatic CSCs, are characterized by low classical MHC class I expression and could likely be resistant to recognition and lysis by reconstituted or engrafted autologous or allogeneic T cells which could, in part, explain the lack of rejection of such cells by non-HLA-matched T cells ((12, 13, 62), manuscript in preparation). On the other hand, cancer cells lacking MHC class I expression should be susceptible to lysis by NK cells. However, the data that we, and others, have obtained suggest that autologous NK cells reconstituted in humanized mice are less potent to effectively prevent CSCs from establishing and growing in BLT mice.

7. ARE NK CELLS IN HUMANIZED MICE OF SUFFICIENT QUANTITY AND QUALITY?

Frequencies of human T cells and their subsets circulating in humanized mice have been intensely

investigated and proven to be similar to those of human peripheral blood. T cells functionality was also tested, however mostly in the context of HIV infection or other infectious diseases (67-70). As education and selection of developing T cells in the context of human thymus is critical for the generation of a broad repertoire of HLA-restricted T cells capable of mounting effective immune response, hu-BLT mice have become the model of choice for HIV studies.

Much less is known about the phenotype and function of NK cells in humanized mice. In humans, studies on NK cells are mostly limited to those isolated from peripheral blood and thus are less representative of NK cell repertoire present in human tissues. NK cells are also found in secondary lymphoid organs, with $CD56^{bright}$ population being enriched, especially in lymph nodes (71, 72). Given that over 40% of human lymphocytes are found in secondary lymphoid organs, whereas only 2% circulate in peripheral blood, it can be assumed that such $CD56^{bright}$ populations significantly contribute to NK cell-mediated innate responses in humans; therefore, a careful analysis of NK cell numbers and functions in all immune compartments of humanized mice is the key to our understanding of NK function (71, 73). Accumulated evidence suggests that NK cell numbers and functions in both $CD34^{+}$ and hu-BLT mouse models do not precisely reflect those observed in humans (74-78). Along with others, we have also observed low frequencies of NK cells in hu-BLT mice based on the use of current NK detection markers. In addition, we observed decreased cytotoxicity of NK cells in peripheral blood, bone marrow, spleen and liver of humanized animals in comparison to those obtained from human peripheral blood. Implantation of stem-like tumors further decreased cytotoxicity and cytokine secretion of reconstituted NK cells (manuscript in preparation).

NK cell development and maintenance is regulated by a variety of factors including IL-15 (79-81). Decrease in IL-15 expression has been shown to result in the absence of mature NK cells in both mice and humans (82, 83). It has been demonstrated that NK cells developed in $CD34^{+}$ Balb/c $Rag2^{-/-}$ $IL2Rgamma^{-/-}$ humanized mice in the absence of IL-15, however, they were detected in much lower numbers, mainly in the thymus and lymph nodes (74). These NK cells displayed $CD56^{bright}/CD16^{-}$ phenotype similar to NK cells isolated from human lymph nodes. In addition, similar to our previously defined split anergy in NK cells, Ferlazzo *et al.,* demonstrated secretion of IFN-gamma in the absence of cytotoxicity by $CD56^{bright}/CD16^{-}$ NK cells after 24-hour activation with IL-2 (84) (13). Upon IL-15 treatment increased numbers of NK cells in thymus, lymph nodes, spleen, bone marrow and liver could be observed (58, 74, 75). Chen *et al.* also showed that human NK cells were detected at very low percentages in blood, bone marrow, lung, spleen and liver of $CD34^{+}$ NSG hu-mice, however, the number of $CD56^{+}$ cells increased in all tissue compartments with plasmid delivered IL-15, and the effect was more dramatic with combined treatment of IL-15 and Flt-3/Flk-2. However, frequency of NK cells in blood decreased by 2-fold a month after cytokine injection, suggesting the need for repeated delivery of IL-15 to maintain a functional pool of NK cells (75). In contrast, humanized mice supplemented with IL-7 were unable to develop functional NK cells and CD8+ T cells from hematopoietic cells isolated from juvenile patients with various malignancies (78).

Binding of IL-15 to IL2rg receptor requires trans-presentation in complex with IL-15Ralpha, of a different source than the target cell (85) and simultaneous expression of IL-15 and IL-15Ralpha, within the same cell is needed for this process (86). In general, IL-15Ralpha protein is as ubiquitously expressed as its reported transcript expression, whereas IL-15 protein expression is much more restricted. In mice, myeloid or DC cells are shown to be the major sources of IL-15Ralpha. It is not clear which cells support human NK cell homeostasis *in vivo*. Recently, DCs have been proposed to utilize cross-presentation (87). Lucas *et al.* suggested that human DCs might serve as a source of IL-15Ralpha, which prime and activate NK cells in lymph nodes or at sites of inflammation; such cytotoxic $CD56^{dim}/CD16^{bright}$ NK cells might then migrate to the periphery (88, 89). IL2rg knockout mice have defects in IL-2 and IL-15 signaling. Although, there is cross reactivity between mouse and human IL-15, poor response of human immune cells to mouse IL-15 were reported and it was suggested to account for extrinsically low reconstitution of NK cells in humanized mice (77). Huntington and colleagues found similarly low levels of reconstituted NK cells in secondary lymphoid organs of $CD34^{+}$ reconstituted humanized mice that ranged from 0.3-1.5.% of human lymphocytes present in those tissues (77). Thus, reconstitution of NK cell levels in secondary lymphoid organs of humanized mice was much lower in comparison to the levels observed in spleens and lymph nodes from healthy humans, which are 5% and 7-50%, respectively (71). As expected, NK cell development in bone marrow of humanized mice was improved upon delivery of human IL-15/IL-15Ralpha. Interestingly, IL-15 in the absence of receptor was not as effective indicating the requirement for both the ligand and the receptor (77).

Although NK cells reconstituted from human HSCs in NSG mice could be detected in all blood-perfused organs, at least one half displayed NKp46/$CD56^{-}$ phenotype and exhibited decreased function resembling human cord blood (CB) immature NK cells (58). Similar findings reported by several groups might explain differential behavior of reconstituted NK cells in comparison to human NK cells obtained from blood, and collectively suggest the pre-activation requirement for reconstituted NK cells in humanized

mice in order to obtain fully functional and potent NK cells that can mimic human adult peripheral blood NK cells (61, 77, 90). Whether NKp46^{+}/CD56- subsets are immature NK populations or those that have interacted *in vivo* with the targets and subsequently down-modulated CD56 surface receptors requires further investigation.

8. NK CELL RECEPTOR DOWNREGULATION AS A POTENTIAL MECHANISM FOR THE DETECTION OF LOW NK CELL FREQUENCIES *IN VIVO*

Interaction of NK cells with CSCs results in downregulation of CD16 receptor, as well as other NK cell activating receptors, which usually correlates with cancer progression (91-94). In this regard, we have also analyzed the frequencies of circulating CD3^{-}/CD14^{-}/CD19^{-}/CD16^{-}/CD56$^{dim/neg}$ NK cells in stage III and IV progressing melanoma patients, and found higher percentages of CD16 and CD56 receptor low/negative NK cells in these patients in comparison to stage II melanoma patients responding to therapy, as well as healthy individuals (manuscript in preparation). The increased numbers of such receptor low/negative NK cells in patients with advanced stages of cancer might be the consequence of prior receptor signaling leading to down-modulation of key NK cell receptors by CSCs. As a result such NK cells become virtually undetectable if these receptors are used for their detection. Similar receptor down-modulation may also occur for NK cells in the xenogeneic environment of humanized mice. It has been reported that reconstituted NK cells display “anergized” phenotype that can be characterized by downregulation of several receptors including CD16 (58, 75, 78).

The downregulation of receptors commonly used for detection and phenotyping of NK cells raises a question regarding the true frequencies of NK cells in cancer patients as well as in humanized mice. In our recent *in vivo* studies, we have observed that autologous human NK cells isolated from hu-BLT mice with or without tumors expressed very low levels of common NK cell receptors and displayed poor cytotoxicity. Similar receptor down-modulation to those of autologous NK cells was also observed when osteoclast-expanded NK cells were injected into BLT humanized mice (manuscript in preparation). Interestingly, even though we could not see detectable numbers of NK cells using common NK cell markers at the initiation of *in vitro* cultures, we detected significant numbers of NK cells after a week of culture with IL-2. Further *in vitro* expansion and IL-2 activation of such cells not only led to restoration of NK cell receptor expression but also augmented NK cell function.

In order to track distribution of osteoclast-expanded NK in various tissue compartments, NK cells were labeled with PKH-26 dye prior to intravenous injection to tumor bearing BLT mice. Red stained NK cells could be detected in various tissue compartments including those in the tumor microenvironment whereas lower frequencies of NK cells were identified when cells isolated from the same tissues were stained with common NK cell receptor antibodies (manuscript in preparation). Thus, such observations underscore the need for a novel or improved detection methods to determine the true frequencies of receptor-low NK cells *in vivo*.

9. ADOPTIVE THERAPY WITH OSTEOCLAST-EXPANDED NK CELLS ELIMINATES CANCER STEM-LIKE CELLS IN HUMANIZED MICE

Currently, adoptive NK cell therapy has been less efficient than T cell therapy in treating cancer patients due to a number of limitations. With existing strategies, fewer numbers of potent NK cells can be expanded and used for cancer immunotherapy compared to T cells. Cytokine and tumor activated NK cells neither survive long enough nor maintain their cytotoxic function to effectively eliminate tumors, due to rapid NK cell inactivation by the suppressive microenvironment in cancer patients. To circumvent such limitations we have recently established a strategy to expand large numbers of functionally potent super-charged NK cells using specific strains of sonicated probiotic bacteria in a combination with osteoclasts (95). This strategy has not only allowed for greater expansion of activated NK cells accompanied by much higher levels of cytotoxicity and cytokine secretion, but also prevented NK cells from undergoing cell death when compared to NK cells expanded by conventional strategies (manuscript in preparation). We have previously shown that human osteoclasts produced IL-15, IL-12, IL-18 and IFN-alpha, and displayed low expression of MHC class I and II, CD14, CD11b and CD54 (95). The use of osteoclasts as feeder cells preferentially expanded high numbers of functionally potent NK cells that were maintained for a longer period of time in comparison to NK cells expanded by dendritic cells, monocytes, K562 cells and CSCs. Even though dendritic cells were initially able to expand NK cells, they could not maintain the expansion and function of NK cells after the initial burst in NK cell activation. Dendritic cells preferentially expanded T cells, whereas osteoclasts specifically maintained expansion of NK cells with high cytotoxicity and cytokine secretion capabilities ((95) and manuscript in preparation).

Hu-BLT mice, which exhibit lower frequencies of NK cells, serve as the best humanized mice for experimental NK cell therapy with *ex vivo* osteoclast-expanded human NK cells in human stem-like oral and pancreatic tumor models. Indeed, *ex vivo* osteoclast-expanded human NK cells were effective in tumor cell lysis and limiting tumor growth in hu-BLT mice (manuscript in preparation). Significant reduction of tumor burden by expanded NK cells combined with whole cell cancer vaccine was also observed in the hu-BLT model of advanced melanoma and the effect was accompanied

by a dramatic change of cytokine secretion profiles by both immune and tumor cells (manuscript in preparation). Interestingly, NK cells isolated from hu-BLT mice that received osteoclast-expanded NK cells adoptive transfer displayed higher cytotoxicity and proliferation rate in comparison to autologous NK cells that were initially reconstituted in hu-BLT mice.

It is possible that the maintenance of the increased function of osteoclast-expanded, super-charged NK cells after *ex vivo* re-stimulation from mice is a result of prior cytokine activation and NK cell receptor binding and signaling in the *in vivo* microenvironment, which imparts continued priming/signaling and the acquisition of memory-like functions to NK cells. Even though NK cells may not meet the criterion for classical memory that is reserved for T cells, it has been demonstrated that NK cells display higher function when reactivated after prior exposure to activation signals including cytokines or via engagement of activating NK receptors (96). It is known that freshly isolated "naïve" NK cells neither mediate cytotoxicity nor spontaneously produce cytokines, however cytokine pre-activated NK cells are capable of secreting IFN-gamma upon transfer into naïve mice. Functions of such NK cells were reduced after a week following transfer but could be restored after cytokine treatment or by the engagement of their NK cell receptors, suggesting the existence of primed/memory-like NK cells (96).

Importantly, since no side effects were observed after delivery of allogeneic NK cells within the xenogeneic host microenvironment of hu-BLT mice, osteoclast-expanded, super-charged NK cells may be safely used as a potential immunotherapy for human cancer patients. Indeed, we were able to optimize our expansion protocol in order to obtain high numbers of super-charged NK cells required for repeated adoptive therapy of cancer in humans by continuous expansion of NK cells for a prolonged period of time using an optimized mixture of cytokines and receptor mediated signals. Contrary to all previous expansion protocols used in clinical trials, we could continuously expand the same donor's NK cells for an extended period of time with no functional loss. In addition, unlike cytokine-activated primary NK cells which exhibited significant loss of numbers and function upon freezing, osteoclast-expanded, super-charged NK cells effectively tolerated storage temperature and exhibited no significant reduction of cell viability or function after long term storage in liquid nitrogen. Such observations are critical for successful future immunotherapy, since patient-derived NK cells can be expanded with our method and stored in liquid nitrogen for multiple future use.

10. FUTURE OF NK CELL MEDIATED IMMUNOTHERAPY

Based on our recent studies and those of others, we suggest that the hu-BLT mouse model is the best available preclinical model to study novel immunotherapeutic approaches in the context of the reconstituted immune repertoire. Thus far, hu-BLT animals provide the best available model for studying the intricate human immune cell interactions with human tumors upon immunotherapeutics delivery, and determining the molecular and cellular mechanisms of tumor resistance leading to therapy failure. Moreover, this model can further be refined by the delivery of *ex vivo* derived, osteoclast-expanded, functionally potent human NK cells in order to overcome the observed limitations in the numbers and function of autologous NK cells found in humanized mice.

Our previous studies (12, 13, 97) and current *in vivo* studies indicate that NK cells are the main immune effectors that select and differentiate CSCs, resulting in tumor growth inhibition and cessation of chronic inflammation (13) (Figure 1). We have also demonstrated that NK cell-differentiated cancer cells become sensitive targets to chemotherapeutic and radiotherapeutic strategies (12) (Figure 1). In addition, our studies indicate that CSCs have, in general, low levels of MHC class I and therefore, are likely poor targets for T cells. NK cells have the key role in differentiating such primitive cancer cells to provide differentiated targets for T cell-mediated effects.

Our recent findings show great promise for the use of NK cells in cancer immunotherapy since both autologous and allogeneic NK cells can be expanded *ex vivo* using osteoclasts, in order to not only substantially increase their numbers but also their functional potency for successful treatment of cancer. Moreover, our data also indicates that the adoptive transfer of osteoclast-expanded NK cells into hu-BLT mice initiates and enhances recruitment of $CD8^+$ T cells to the tumor microenvironment (Figure 1) (manuscript in prep). Thus, adoptive transfer of super-charged NK cells may provide the basis for successful T cell based immunotherapies including tumor cell susceptibility to immune checkpoint inhibitors and the use of peptides and cancer vaccines to boost immunity. Therefore, such combinatorial approaches that target both CSCs as well as their more differentiated counterparts should result in better control of tumor growth, inhibit invasion, and prevent tumor immune escape and metastasis (Figure 1).

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Send correspondence to: Anahid Jewett, 10833 Le Conte Ave, UCLA School of Dentistry, Los Angeles, CA 90095, USA**,** Tel: 310-206-3970, Fax: 310-794-7109**,** E-mail: ajewett@ucla.edu