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Natural Killer (NK) and CAR-NK cell expansion method using membrane bound-IL-21-modified B cell line
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1   **TITLE:**
2   **Natural Killer (NK) and CAR-NK Cell Expansion Method using Membrane Bound-IL-21-Modified**
3   **B Cell Line**
4
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21
22 **SUMMARY**
23 Here, we present a method to expand peripheral blood natural killer (PBNK), NK cells from liver
24 tissues, and chimeric antigen receptor (CAR)-NK cells derived from peripheral blood mononuclear
25 cells (PBMCs) or cord blood (CB). This protocol demonstrates the expansion of NK and CAR-NK
26 cells using 221-mIL-21 feeder cells in addition to the optimized purity of expanded NK cells.
27
28 **ABSTRACT**
29 Chimeric antigen receptor (CAR)-modified immune cell therapy has become an emerging
30 treatment for cancers and infectious diseases. NK-based immunotherapy, particularly CAR-NK
31 cell, is one of the most promising 'off-the-shelf' development without severe life-threatening
32 toxicity. However, the bottleneck for developing a successful CAR-NK therapy is achieving
33 sufficient numbers of non-exhaustive, long-lived, 'off-the-shelf' CAR-NK cells from a third party.
34 Here, we developed a new CAR-NK expansion method using an Epstein-Barr virus- (EBV)
35 transformed B cell line expressing a genetically modified membrane form of interleukin-21 (IL-
36 21). In this protocol, step-by-step procedures are provided to expand NK and CAR-NK cells from
37 cord blood and peripheral blood, as well as solid organ tissues. This work will significantly
38 enhance the clinical development of CAR-NK immunotherapy.
39

INTRODUCTION

Natural killer (NK) cells are an important subset of lymphocytes that express CD56 and lack expression of the T cell marker, CD3[1,2]. Conventional NK cells are classified as innate immune cells responsible for immunosurveillance of virally infected cells and cancerous cells. Unlike T cells, NK cells recognize infected or malignant cells using CD16 or other activating receptors and do not require the formation of a complex between antigen peptides and major histocompatibility complex (MHC) class I molecules[3,4]. Recent clinical investigations using chimeric antigen receptor (CAR)-NK cells to treat relapsed or refractory CD19-positive cancers (non-Hodgkin's lymphoma or chronic lymphocytic leukemia [CLL]) showed the outstanding safety advantages of CAR-NK cells[5]. For instance, CAR-NK cell infusion is associated with minimal or negligible graft versus host disease (GVHD), neurotoxicity, cardiotoxicity, and cytokine release syndrome (CRS)[6–10]. However, conventional methods to expand human NK cells showed exhaustive phenotypes with strong fratricidal killing and telomere shortage, which presents a major challenge in obtaining an adequate number of functional NK cells for adoptive immunotherapy[11].

To overcome these challenges, a method was developed to expand primary NK cells directly from unfractionated peripheral blood mononuclear cells (PBMCs) or cord blood (CB) using an irradiated and genetically engineered 721.221 (hereinafter, 221) cell line, a human B-lymphoblastoid cell line with low expression of MHC class I molecules[3]. Previous studies showed the importance of IL-21 in NK cell expansion; therefore, a genetically engineered membrane-bound IL-21 expressing a version of the 721.221 cell line (starting now, 221-mIL-21) was developed[11–15]. The results showed that 221-mIL-21 feeder-cell-expanded primary NK cells were expanded to an average of >40,000-fold with persistent high NK cell purity for approximately 2–3 weeks. Additional information regarding the application of this protocol can be found in Yang et al.[16].

This protocol aims to demonstrate the step-by-step procedure of the novel expansion of PBNK, CBNK, tissue-derived NK, and CAR-NK cells *ex vivo*.

PROTOCOL

Human tissues and blood-related work in this protocol follows the guidelines of the Rutgers University Institutional Review Board (IRB)

1.   NK cell expansion from liver tissues (Day 0), as shown in Figure 1.

NOTE: Initial cell number and viability are strongly correlated with the time since organ removal and the initial tissue sample amount. However, if tissues are placed in 30 mL of Hank's Balanced Salt Solution (HBSS) and kept on ice or in the fridge at 4 °C overnight, NK cells can still be expanded at high purity and viability up to 24 h later.

1.1.   Identify viable tissue areas to obtain lymphocytes from tissues and sections using sterile surgical equipment.

84

85   1.2.   Place tissues in 30 mL of HBSS (w/o calcium or magnesium) and keep on ice until ready to
86   prepare for isolation.

87

88   1.3.   Mince the tissue into <0.5 cm cubes using sterile razor blades or scissors and forceps
89   inside a biosafety cabinet.

90

91   1.4.   Prepare a 1x collagenase IV solution (1 mg/mL) by diluting a 10x stock in HBSS (10x
92   Collagenase IV: 10 mg/mL or ~200 U/mL).

93

94   1.5.   Place the minced tissue pieces in the tissue dissociator tubes. Fill the tubes with no more
95   than 4 g of tissue and immerse the tissue pieces in ~10 mL of 1x collagenase IV.

96

97   NOTE: Using DNase I is not recommended as it may slightly decrease NK viability and yield. Please
98   refer to the **Table of Materials** for the specific tissue dissociator tubes used.

99

100   1.6.   Place the tissue dissociator tubes into a tissue dissociator and blend at 37 °C to mince the
101   tissue thoroughly.

102

103   NOTE: For liver tissue, this may take over 30 min. For more friable tissue, around 15 min may be
104   sufficient. Please refer to the **Table of Materials** for specific tissue dissociator tubes and the tissue
105   dissociator used.

106

107   1.7.   Remove the tissue dissociator tubes and triturate through 40 μm nylon cell strainer using
108   the backend of a 5 mL syringe. Collect the eluent and discard the large undigested fragments.

109

110   1.8.   Spin down the eluent at 400 x g for 5 min at room temperature. Aspirate the supernatant.

111

112   1.9.   Resuspend the cell pellets in 30% polyvinylpyrrolidone (PVP)-coated silica to remove fat
113   cells that will otherwise contaminate the final lymphocyte fraction.

114

115   1.9.1. To prepare 1x PVP-coated silica, use 9:1 dilution of PVP-coated silica in 10x PBS.

116

117   NOTE: Refer to the **Table of Materials** for the specific PVP-coated silica used.

118

119   1.9.2. To prepare 30% PVP-coated silica, dilute 1x PVP-coated silica with PBS/HBSS.

120

121   1.10.   Spin down the cell pellet at 400 x g for 5 min at room temperature. Aspirate the
122   supernatant.

123

124   1.11.   Resuspend the cell pellet in 9 mL of R-10 media.

125

126   NOTE: Refer to the **Table of Materials** for the composition of R-10 media

127

128 1.12. Carefully layer the cell suspension over 4 mL of Ficoll or lymphocyte separation media to
129 separate lymphocytes from red blood cells and polymorphonuclear cells.
130
131 1.13. Separate the layers by centrifuging at 400 x g for 23 min at room temperature with the
132 acceleration and brakes off or at the lowest setting. Carefully decant the upper-medium layer
133 and harvest the interphase containing tissue-infiltrating lymphocytes.
134
135 1.14. Rinse the cells with 10 mL of media and proceed to cell counting, flow cytometry,
136 aliquoting and freezing of cells, or primary NK expansion protocol.
137
138 **2. Primary NK cell expansion from PBMCs (or CB or organ tissues) (Day 0), as shown in**
139 **Figure 2.**
140
141 2.1. Thaw the frozen PBMC and the frozen, irradiated feeder cells in a 37 °C water bath.
142
143 2.2. Wash the PBMC and 100 Gamma-irradiated (Gy-irradiated) 221-mIL-21 cells by
144 centrifugation at 400 x g for 5 min with 10 mL of R-10 media separately.
145
146 2.3. Save 1 x 10^6 cells of PBMC for flow cytometry.
147
148 NOTE: The initial NK cell purity is an important factor in calculating NK cell expansion rate.
149
150 2.4. Resuspend the cells in 1 mL of R-10 media. Count the cells using Trypan Blue.
151
152 2.5. Mix 5 x 10^6 cells of PBMC with 10 x 10^6 cells of 100 Gy-irradiated 221-mIL-21 cells in a
153 special 6-well plate (see **Table of Materials**).
154
155 2.6. Add 30 mL of R-10 media supplemented with human IL-2 (200 U/mL) and human IL-15 (5
156 ng/mL) (see **Table of Materials**).
157
158 2.7. Incubate the special 6-well plate at 37 °C with 5% CO_2.
159
160 2.8. Replace the media with R-10 media supplemented with human IL-2 (200 U/mL) and
161 human IL-15 (5 ng/mL) to maintain NK cells every 3–4 days.
162
163 NOTE: Maintain less than 20 x 10^6 cells per well for further expansion at each media change. For
164 the best viability, ensure that the total cell number in each well does not exceed 100 x 10^6 cells.
165
166 2.9. Record the total cell number, viability and perform flow cytometry every 3–4 days to
167 calculate the NK cell expansion rate.
168
169 **3. Attachment of 293T cells (Day 2), as shown in Figure 2.**
170
171 3.1. Split 1.8 x 10^6 293T cells in 11 mL of D-10 media per treated 100 mm plate.

NOTE: Refer to the **Table of Materials** for the composition of D-10 media

3.2.   Incubate 293T cells at 37 °C with 5% CO_2.

4.   Retrovirus transfection (Day 3)

4.1.   In a 1.7 mL tube, mix 470 µL of reduced serum media with 30 µL of transfection reagent.

NOTE: Refer to the **Table of Materials** for the specific reduced serum media and transfection reagent used.

4.2.   In a separate 1.7 mL tube, add 2.5 µg of pRDF plasmid, 3.75 µg of Pegpam3 plasmid, and 2.5 µg of CAR construct in SFG vector into the reduced serum media so that the final volume is 500 µL.

4.3.   Mix the solutions in steps 4.1 and 4.2 dropwise.

4.4.   Incubate the tube at room temperature for 15 min.

4.5.   Add 1 mL of the mixture from step 4.4 to 293T cell plate on Day 1 in a dropwise manner.

4.6.   Incubate the plate(s) at 37 °C with 5% CO_2 for 48–72 h.

5.   Retronectin plate-coating (Day 3)

5.1.   Dilute retronectin protein with Phosphate Buffered Saline (PBS) to a final concentration of 50–100 µg/mL.

5.2.   Add 500 µL of the diluted retronectin into each well of an untreated 24-well plate (5 wells per CAR construct). Seal the plate using parafilm and incubate the plate at 4 °C overnight.

6.   Transduction (Day 4)

6.1.   Centrifuge the retronectin plate at 2103 x *g* for 30 min at 4 °C. Discard the supernatant.

6.2.   Block each well of the 24-well plate with 1 mL of R-10 medium.

6.3.   Incubate the plate at 37 °C with 5% CO_2 for 1 h.

6.4.   Pre-warm the centrifuge to 32 °C while the retronectin plate is being blocked.

6.5.   Collect the retrovirus supernatant by filtering the transfected 293T cells using a 0.45 µm filter.

216

6.6.   Aliquot 2 mL of the filtered retrovirus supernatant into each well.

6.7.   Centrifuge the 24-well plate at 2103 x g for 2 h at 32 °C.

6.8.   During plate centrifugation, collect the expanded PBNK cells from Day 0 and count the cells using Trypan Blue.

NOTE: Continue expanding PBNK cells by adding R-10 media supplemented with IL-2 (200 U/mL) and IL-15 (5 ng/mL).

6.9.   Dilute the expanded PBNK cells with R-10 media supplemented with IL-2 (200 U/mL) and IL-15 (5 ng/mL) to 2.5×10^5–5×10^5 cells/mL (0.5×10^6–1×10^6 cells per well).

NOTE: Record the total cell number, viability, and save 5×10^5 expanded PBNK cells for flow cytometry as these values are important in determining the NK cell expansion rate.

6.10.   After centrifugation, partially aspirate the retrovirus supernatant from each well.

NOTE: Do not completely aspirate, i.e., leave approximately 100 µL of retrovirus supernatant per well, as this will decrease the transduction efficiency.

6.11.   Aliquot 2 mL of the diluted expanded PBNK cells from step 6.8 to each well.

6.12.   Centrifuge the plate at 600 x g for 10 min at 32 °C. Incubate the plate at 37 °C with 5% CO_2 for 48–72 h.

NOTE: Do not parafilm the plate.

7.   CAR-NK cells collection (Day 6 or 7), as shown in Figure 2.

7.1.   Gently collect the cells from the 24-well plate and transfer the cells to a 50 mL centrifuge tube

NOTE: Try not to generate bubbles, as this will result in a decrease in cell viability.

7.2.   Centrifuge the tube at 400 x g for 5 min.

7.3.   Resuspend the pellet with 1 mL of R-10 media and count the cells using Trypan Blue.

NOTE: Save 5×10^5 cells for flow cytometry to determine the transduction efficiency.

7.4.   Transfer the resuspended cells to a special 6-well plate containing 30 mL of R-10 media supplemented with IL-2 (200 U/mL) and IL-15 (5 ng/mL).

7.5.   Incubate the special 6-well plate at 37 °C with 5% CO_2.

7.6.   Replace R-10 media supplemented with IL-2 (200 U/mL) and IL-15 (5 ng/mL) to maintain NK cells every 3 – 4 days.

NOTE: Maintain less than 20 x 10^6 cells per well for further expansion at each change. For the best viability, ensure that the total cell number in each well does not exceed 100 x 10^6 cells.

7.7.   Record the total cell number, viability, and perform flow cytometry every 3–4 days to calculate the NK cell expansion rate.

7.8.   Use the cells for appropriate *in vitro* or *in vivo* assays.

NOTE: The *ex vivo* expanded PBNK, and CAR-NK cells can be cultured in a 37 °C incubator for approximately 4 weeks.

7.9.   Examine the NK cell number and purity at day 7, day 11, day 14, day 18, and day 21 by flow cytometry.

REPRESENTATIVE RESULTS

A schematic workflow of tissue-infiltrating NK cell isolation and PBNK cell expansion using the 221-mIL-21 feeder cell methodology is shown in **Figure 1** and **Figure 2**. Expanded PBNK cells were collected every 3 or 4 days for flow cytometry to determine the NK cell purity by staining cells with anti-human CD56 and anti-human CD3. The experiment was repeated using two different donors to show the reproducibility of the expansion system (**Figure 3**). PBNK cells expanded by 221-mIL-21 were shown to expand nearly 5×10^4 folds **(Figure 3A)**. Furthermore, the NK cell purity was highly maintained, around 85% throughout the 21-day expansion **(Figure 3B)**. Using the 221-mIL-21 feeder cell expansion system, the NK cell purity consistently ranged between 85%–95%, independent of the donors (data not shown). To demonstrate the robustness of the 221-mIL-21 expansion system, PBMCs were stained for anti-CD56 and anti-CD3 prior to the expansion, which showed a cell purity of 7.09% for NK cells and a high percentage of T cells (**Figure 4A**). PBMCs were cocultured with 221-mIL-21 to expand NK cells; the NK purity was checked prior to CAR-NK transduction on Day 4 (**Figure 4A**). CAR-NK cells were collected and stained for anti-CD56, anti-CD3, and anti-hIgG(H+L) F(ab')$_2$, which showed a high NK cell population (86.9% on Day 7) and a high CAR transduction efficiency of approximately 70% (**Figure 4**). Higher transduction efficiencies (up to 95%) were also observed using the retrovirus packaging system. Altogether, these data show that the 221-mIL-21 feeder cells could successfully expand NK cells and preserve the NK cell purity *ex vivo*.

FIGURE AND TABLE LEGENDS

Figure 1: Diagram of NK cell expansion from solid human organ samples. Briefly, the obtained human liver samples are minced into small cubes for mechanical digestion. Dissociated cells are

303 then isolated using PVP-coated silica and Lymphocyte Separation Media. Further, the NK cells are
304 expanded using the expansion protocol described in **Figure 2.**

305

306 **Figure 2: Schematic workflow of CAR-NK cell generation from PBMCs.** Briefly, 221-mIL21 feeder
307 cells were irradiated at 100 Gy prior to coculturing with PBMCs supplemented with IL-2 and IL-
308 15 on Day 0. In parallel, 293T cells were transfected with the retrovirus packaging system to
309 produce CAR retrovirus that was then transduced into the expanded PBNK cells in the presence
310 of IL-2 and IL-15. Primary CAR-NK cells were harvested on Day 7 and continued expansion for 21
311 days. This figure has been modified from Yang et al.[16].

312

313 **Figure 3: Dynamic time-lapsed expansion of PBNK cells**. (**A**) Fold expansion of PBNK during a 22-
314 day time course. Cells were stained with anti-CD56 and anti-CD3 at indicated days for flow
315 cytometry. The total number of NK cells was determined by multiplying NK cell purity to the total
316 number of cells. Expansion rate was generated as follows: (Number of NK cells)$_{Tn}$/(Number of NK
317 cells)$_{T0}$, where Number of NK cells = (percentage of NK cell purity) $\times$ (total number of cells), T_0 is
318 the number of NK cells at time day 0, and Tn is the number of NK cells at time day n. (**B**) NK cell
319 purity during a 22-day time course. The NK cell expansion was repeated two times with two
320 different donors. Error bars represent ± SEM.

321

322 **Figure 4: Representative flow cytometric analysis of CAR-NK cells.** (**A**) Representative dot plots
323 showing the dynamic time-lapse of NK cell purity of the CAR-NK cells during an 18-day course.
324 Flow cytometry analysis was assessed by staining the cells with anti-CD56 and anti-CD3 at
325 indicated timepoints. Day 0 indicates pre-expansion of PBNK. Day 4 indicates post-expansion of
326 PBNK and pre-transduction of CAR-NK cells. Day 7 indicates the post-transduction of CAR-NK
327 cells. (**B**) Representative dot plots showing the transduction efficiency of CAR-NK cells using the
328 retrovirus packaging system. Cells were stained with anti-CD56, anti-CD3, and anti-hIgG(H+L)
329 F(ab')2 for flow cytometry. (**C**) Representative dot plots showing the CAR expression in various
330 subsets, including CD56$^+$CD3$^-$, CD56$^-$CD3$^+$, CD56$^+$CD3$^+$, and CD56$^-$CD3$^-$ on Day 18. Cells were
331 stained with anti-CD56, anti-CD3, and anti-hIgG(H+L) F(ab')2 (indicating CAR expression) for flow
332 cytometry.

333

334 **DISCUSSION**
335 Most of the current CAR-NK products in clinical trials utilize NK cell lines[17], such as NK-92, a cell
336 line isolated from a non-Hodgkin's lymphoma patient[18], NK-92MI, IL-2 independent NK-92 cell
337 line[19], and NKL, isolated from a large granular lymphocyte patient[20], as these cell lines are easily
338 proliferative for 'off-the-shelf' products. However, these cell lines, e.g., NK-92 cells, have
339 marginal clinical efficacies and *in vivo* expansion, as they require irradiation prior to infusion, thus
340 limiting their proliferation and cytotoxicity *in vivo*[21]. Given these reasons, various strategies are
341 currently being explored to expand primary NK cells from several sources, including peripheral
342 blood, CB, bone marrow (BM), human embryonic stem cells (HSCs), induced pluripotent stem
343 cells (iPSCs), and tumor tissues[21–23]. For instance, NK cells can be expanded *ex vivo* using
344 interleukins including IL-15, IL-18, and IL-21. Lymphoblastoid cell lines such as K562 cells or
345 Epstein-Barr Virus-transformed lymphoblastoid cell lines such as 721.221 cells, are also used for
346 NK cell expansion[16]. However, the aforementioned strategies often generate insufficient number

347 of NK cells for an adoptive transfer of CAR-NK immunotherapy[22,24]. To help solve the problem,
348 the study here shows a protocol for an *ex vivo* NK cell expansion using a genetically modified EBV-
349 transformed cell line, 221-mIL-21 feeder cells.
350
351 The expansion methodology using 221-mIL-21 feeder cells shown in this protocol is optimized to
352 expand NK cells with an expansion rate of at least 10 to 100-fold higher than other leukemia cell
353 lines, including HL-60 and OCl-AML3 expressing membrane IL-21, K562, and K652-mIL21
354 expressing OX40 ligand[22,24,25]. The CAR expression is also evaluated for approximately 2 weeks *ex*
355 *vivo.* More significantly, the 221-mIL-21 feeder cell expansion strategy can be applied to expand
356 NK cells from various sources, including PBMCs, CB, and solid organs such as the liver, without an
357 initial NK enrichment step. Although the 221-mIL-21 feeder system is not as donor-dependent as
358 the aforementioned feeder cell lines, it is not entirely independent of donors. On average, the
359 221-mIL-21 expansion system can achieve 90% of NK cell purity with a high NK cell number, with
360 approximately <5% of T cell contamination on day 14 post-expansion. Therefore, to eliminate the
361 possibilities of T cell contamination, it is necessary to isolate NK cells from obtained samples prior
362 to the *ex vivo* expansion or use a CD3$^+$ selection system to eliminate T cells after an *ex vivo*
363 expansion.
364
365 One of the criticisms in using an NK cell expansion system is that the feeder cells may not have
366 been fully eradicated after the expansion or prior to a transfusion, which may possess significant
367 regulatory concerns; therefore, complete eradication of feeder cells before a transfusion is
368 crucial. However, recent CAR-NK clinical trials in which K562-mIL21-4-1BBL feeder cells were used
369 for the *ex vivo* CBNK cell expansion[24,25] showed no concerning complications. Furthermore, our
370 preliminary data showed a gradual decrease of the irradiated 221-mIL-21 population as the
371 expansion progressed (data not shown). However, more extensive studies are required for this
372 expansion method to be implemented in a clinical setting. Collectively, the 221-mIL-21 expansion
373 system helps solve the challenge of expanding primary CAR-NK cells, and therefore will
374 significantly contribute to the broader use of CAR-NK cell-based immunotherapy in the near
375 future.
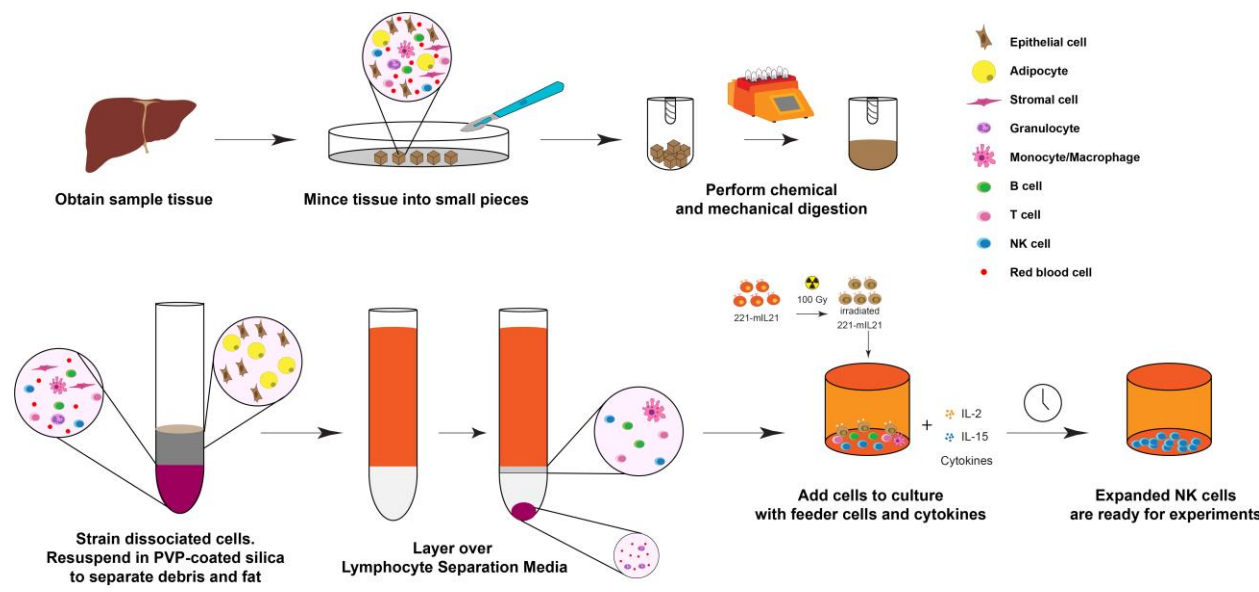376

385
386 **DISCLOSURES**
387 The authors declare no competing interests.
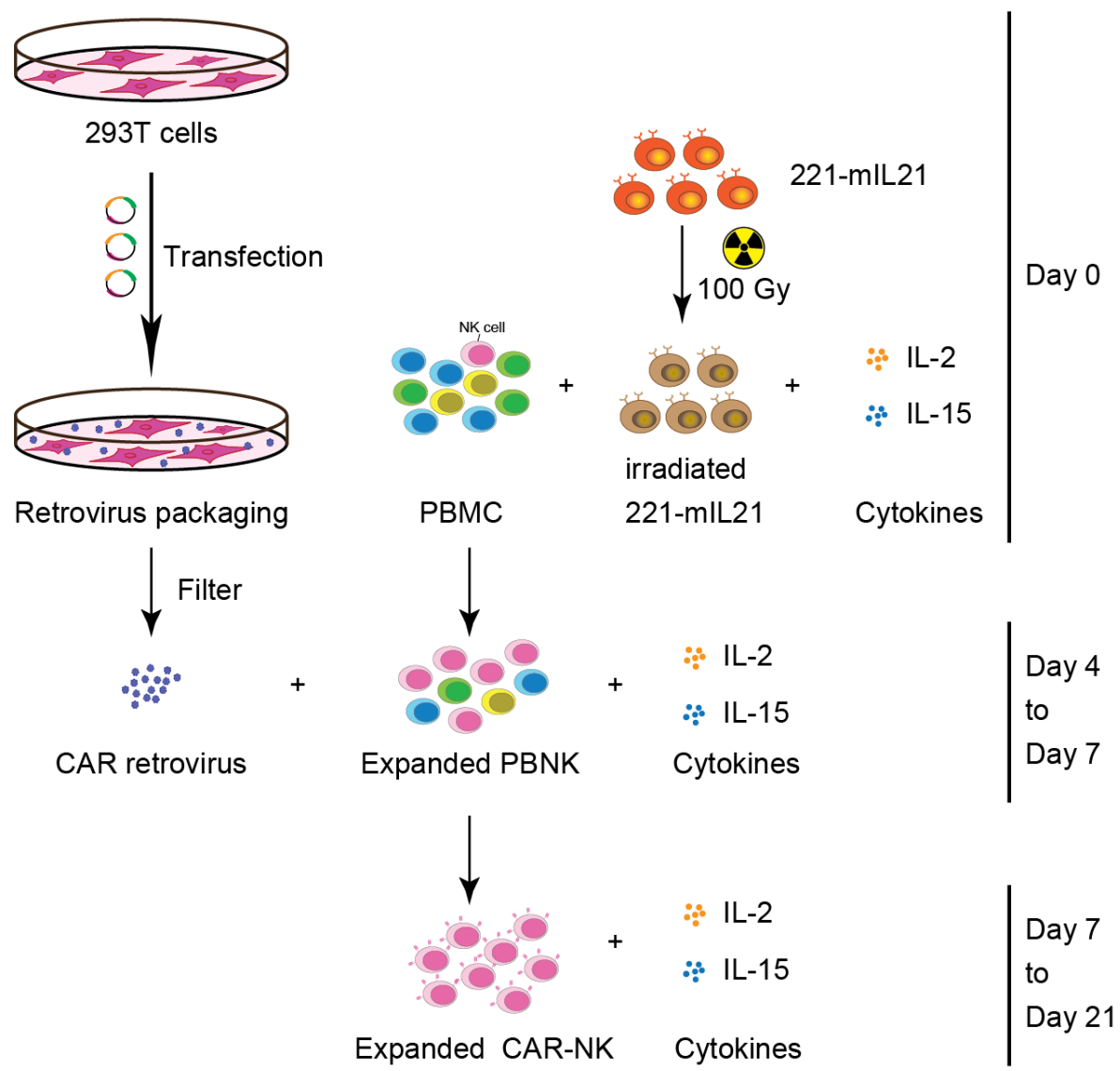388
389 **REFERENCES**
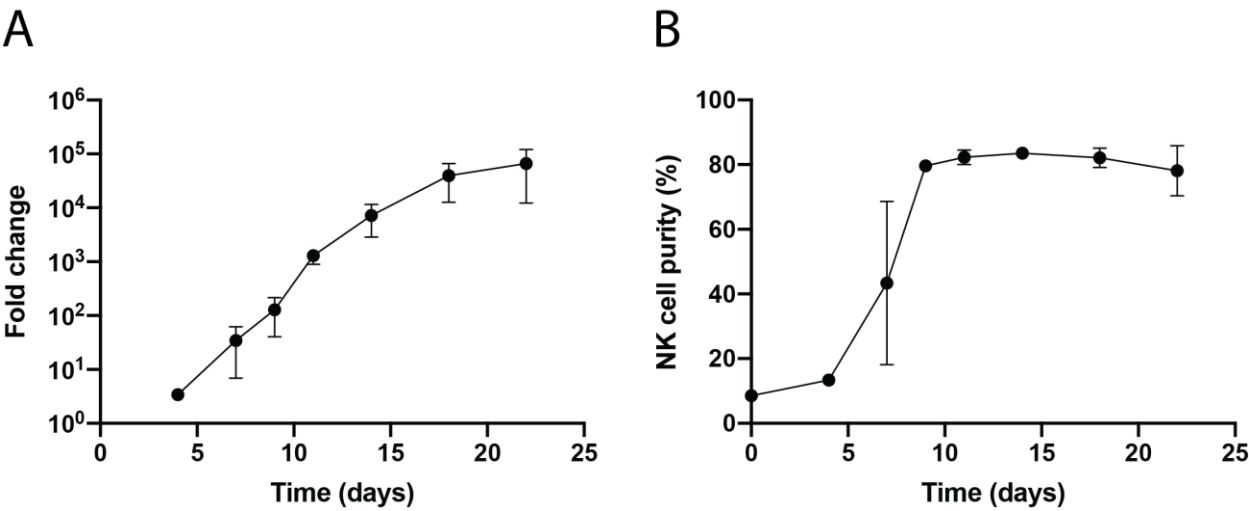
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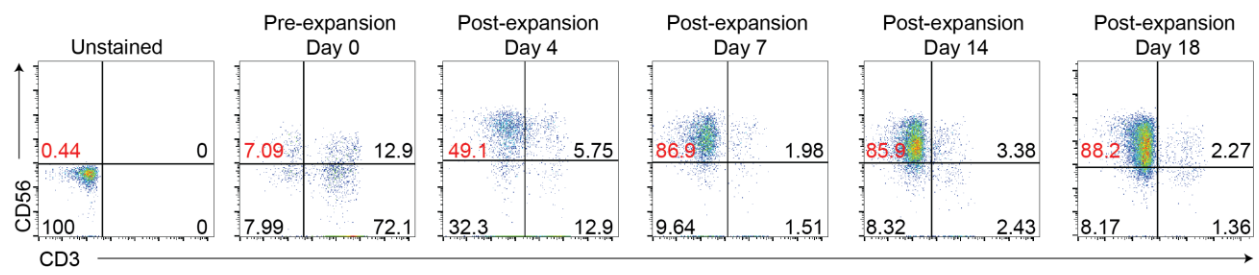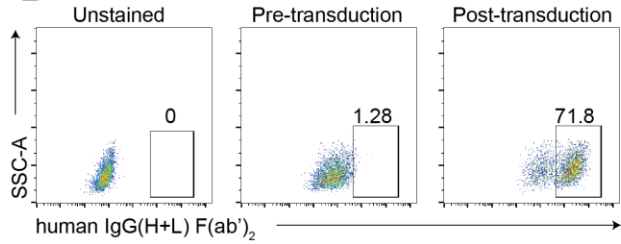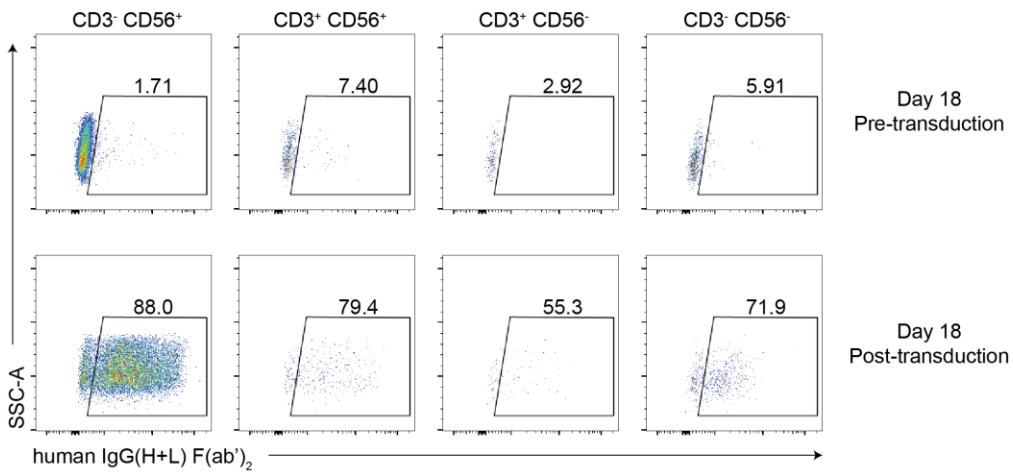447   leukemia activity. *Blood.* **126** (23) (2015).

448

Figure 2

293T cells

Transfection

221-mIL21

100 Gy

Day 0

NK cell

PBMC   +   irradiated   +   IL-2
                          221-mIL21   IL-15

Retrovirus packaging   Cytokines

Filter

CAR retrovirus   +   Expanded PBNK   +   IL-2   Day 4
                                                  IL-15   to
                                          Cytokines   Day 7

Expanded CAR-NK   +   IL-2   Day 7
                                      IL-15   to
                          Cytokines   Day 21

Figure 3

A

B

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Table of Materials

Materials list_03-09-2021_FINAL.xlsx

Editorial comments:

1. Line 39-42: Please move the lines to the introduction and cite the appropriate reference number.

Response: Lines 39-42 have been moved to the introduction and cited appropriately (highlighted in teal).

2. Please note that some of the references are duplicated (18 and 27, 6 and 30). Please replace/remove one of the references.

Response: We apologize for our carelessness. The citations have been removed.

3. Please ensure that the responses (Reviewer #3) in the rebuttal are included in the manuscript.

Response: Thank you for the editor's reminder. The questions asked by reviewer #3 had been addressed in the last 2 paragraphs in the discussion (now highlighted in teal).

_____

Reviewers' comments:

Reviewer #4:

Major Concerns:

In my opinion this protocol does not give any additional value to the known results.

Response: Thank you for the reviewer's comment. We believe that this protocol will help advance the field of adoptive NK cell transfer therapy and CAR-NK therapy as follows:

1. The expansion system used by other groups require an additional NK cell isolation step[1]. As NK cells only represent 5-10% of the total lymphocytes[2], this step will result in a significant loss in starting NK cells, and increase the cost of manufacturing process, which results in lower expansion rate with high cost. Our current system **does not** require NK cell isolation step, which (1) simplifies the protocol and (2) reserves the limited amount of NK cells.

2. We and other groups (industry and academia) have compared our 221-mIL21 feeder cells with the currently well-known K562-mIL21-41BBL feeder cells and the results showed that the 221-mIL21 feeder cells out-perform K562-mIL21-41BBL feeder cells in both NK cell purity and expansion rate from in vitro and in vivo studies. This will significantly contribute to the field in making adoptive NK cell transfer therapy and CAR-NK therapy more affordable.

3. K562 is used to expand NK cells *ex vivo* because it does not express HLA molecules[1, 3]. However, the interaction between HLA molecules and NK cell receptors is necessary for NK cell "licensing", i.e., training NK cells to appropriately activate[4]. Therefore, NK cells expanded with K562 feeder cells may remain unlicensed and could result in hyporesponsiveness[5]. 221 cell line, however, expresses low amount of HLA ligands for

NK cell "licensing"[6], and therefore may prevent activation-induced NK cell death, as well as licensed NK and CAR-NK cells.

4. A current bottleneck in the field is that expanded NK cells by cytokine addition are phenotypically exhausted[7]. By using the 221-mIL21 feeder cell system, we not only demonstrated that the expanded NK cells are not exhausted but also have higher cytolytic functions compared to that of NK cells expanded by K562-mIL21[8].

5. To our knowledge, we are the first group to have demonstrated the antitumor responses of expanded NK cells *in vivo* using different feeder cell lines, and the results showed that NK cells expanded with 221-mIL21 have better anti-tumor responses compared to that of NK cells expanded with K562-mIL21[8].

Collectively, the expansion system described in this protocol adds substantial value to the adoptive NK cell transfer and CAR-NK therapies by providing (1) more simplified protocol, (2) robust and reproducible protocol, (3) higher NK cell purity and expansion rate, (4) less exhaustive NK cells, (5) more affordable adoptive NK cell transfer and CAR-NK therapies in the near future.

Reviewer #5:
Manuscript Summary:
Ma et al accurately explain the protocol of expanding CAR NK cells from liver tissue. The protocol is nice, clean and easy.

Major Concerns:
None

Minor Concerns:
None

Reviewer #6:
Manuscript Summary:
The protocol to expand NK and CAR-NK using 221-mil-21 feeder cells is described clearly and in details.

Major Concerns:
None

Minor Concerns:
please make sure of the formular listed in line 325. I think the expansion rate should be

calculated by dividing the number of NK cells after T days of culture by the respective number on day 0.

Response: Thank you for the reviewer's comment. We have changed the equation in the main text (highlighted in teal).

References

1.   Liu EL, Tong YJ, Dotti G, Savoldo B, Muftuoglu M, Kondo K, et al. Cord Blood Derived Natural Killer Cells Engineered with a Chimeric Antigen Receptor Targeting CD19 and Expressing IL-15 Have Long Term Persistence and Exert Potent Anti-Leukemia Activity. Blood. 2015;126(23). doi: DOI 10.1182/blood.V126.23.3091.3091. PubMed PMID: WOS:000368020103286.

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The recent publication by Yang et. al. is an open access article under the CC BYNC-ND license (http://creativecommons.org/licenses/by-nc-nd/4.0/). We also cited the manuscript accordingly. In the Figure legend, we also added the sentence –" This figure has been modified from (citation)."