Video Article

A Novel Feeder-free System for Mass Production of Murine Natural Killer Cells *In Vitro*

Patrick Ming-Kuen Tang[1,2], Philip Chiu-Tsun Tang[2], Jeff Yat-Fai Chung[2], Jessica Shuk Chun Hung[2], Qing-Ming Wang[2], Guang-Yu Lian[2], Jingyi Sheng[2], Xiao-Ru Huang[2], Ka-Fai To[1], Hui-Yao Lan[2]

[1]Department of Anatomical and Cellular Pathology, The Chinese University of Hong Kong

[2]Li Ka Shing Institute of Health Sciences and Department of Medicine & Therapeutics, The Chinese University of Hong Kong

Correspondence to: Hui-Yao Lan at hylan@cuhk.edu.hk

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Abstract

Natural killer (NK) cells belong to the innate immune system and are a first-line anti-cancer immune defense; however, they are suppressed in the tumor microenvironment and the underlying mechanism is still largely unknown. The lack of a consistent and reliable source of NK cells limits the research progress of NK cell immunity. Here, we report an *in vitro* system that can provide high quality and quantity of bone marrow-derived murine NK cells under a feeder-free condition. More importantly, we also demonstrate that siRNA-mediated gene silencing successfully inhibits the E4bp4-dependent NK cell maturation by using this system. Thus, this novel *in vitro* NK cell differentiating system is a biomaterial solution for immunity research.

Video Link

The video component of this article can be found at https://www.jove.com/video/56785/

Introduction

Cancer progression is largely dependent on the tumor microenvironment[1,2], including host-derived immunocytes, *e.g.*, NK cells. Several studies demonstrated that intratumoral NK cells are negatively correlated with the tumor progression[3,4]. In addition, clinical studies showed that NK cell adoptive therapy is a possible strategy for cancer[5,6,7,8,9]. NK cell-based cancer immunotherapy was recently suggested as a therapeutic option for solid tumors, but challenges exist due to the secretion of immunosuppressive cytokines and downregulation of activating ligands in the microenvironment of solid tumors[10,11]. Transforming growth factor-β (TGF-β) has been suggested to play a suppressive role in carcinogenesis, but paradoxically cancer cells also produce TGF-β1 to support the tumor development[12,13,14,15]. TGF-β signaling can suppress the cytolytic activity of NK cells via down-regulating interferon responsiveness and CD16-mediated interferon-gamma (IFN-γ) production *in vitro*[16,17,18].

Although disruption of TGF-β signaling in the tumor microenvironment may be a possible way for eliminating cancers, completely blocking TGF-β signaling will cause autoimmune diseases due to its anti-inflammatory function, as evidenced by the development of adverse side effects including systemic inflammation, cardiovascular defects, and autoimmunity in mouse models[19]. Thus, understanding the working mechanism of TGF-β-mediated immunosuppression will lead to the identification of an accessible therapeutic target for treating cancer.

To elucidate the molecular events necessary for NK cell development, Williams *et al.* established an *in vitro* system for differentiating murine bone marrow hematopoietic stem cells into NK cells[20]. This system largely facilitates the mechanistic study of NK cell development, including the identification of novel progenitors of NK cells[21]. However, the bone marrow progenitors should be cultured in the system with supporting OP9 stromal cells as a feeder layer[20,21], and this heterogeneous cell population largely limits the further application of gene-disrupting tools (*e.g.*, siRNA-mediated gene silencing) specifically applied to the differentiating NK cells.

Here, we describe a feeder-free system that has been developed by further modifying the *in vitro* system of Williams *et al*[20]. In our system, the OP9 stromal feeder cells are not required, and instead OP9 conditional medium is used without affecting the differentiation of NK cells *in vitro*, and this recently lead us to uncover that TGF-β is able to promote cancer progression via suppressing E4bp4-dependent NK cell development in the tumor microenvironment[22]. This novel system successfully provides a background-free method for elucidating the molecular mechanism of NK cell development under specific conditions (*e.g.*, high TGF- β1, siRNA-mediated gene silencing, *etc.*) *in vitro*.

Protocol

The protocol for obtaining and differentiating bone marrow-derived NK cells (BM-NK) is based on previously published methods[20,21,22]. All procedures with mice have been approved by the Animal Ethics Experimental Committee (AEEC) at the Chinese University of Hong Kong.

1. Preparation of OP9 Conditional Medium

1. Culture the murine stroma cell line OP9 in alpha-MEM containing 20% FBS, 100 U/mL penicillin G and 100 µg/mL streptomycin, at 37 °C in a humidified atmosphere of air/CO_2 (95%:5%).
    1. Count the cells with a hemocytometer, then seed 2 x 10^6 of OP9 cells to each 75 cm^2 culture flask with 15 mL of medium. Culture for 48 h.
    2. When the culture reaches 80% confluence, wash the flask with 10 mL of PBS and add 20 mL of plain alpha-MEM medium (without FBS and antibiotics) per flask.
2. Collect the OP9 condition medium from the culture flask at 24 h, clean up cell debris with 0.25 µm polyethersulfone (PES) filter and preserve at 4 °C (use within 2 weeks).

2. Isolation of Mouse Bone Marrow Cells

1. Euthanize a 12-week-old C57BL/6J mouse with a pentobarbitone overdose (100 mg/kg, intraperitoneal injection). Confirm death by the lack of breathing, then harvest the femur bones with a surgical knife cutting at the junctions between bones and remove the remaining muscles with the blade by gentle scratching.
2. Place the bones in a 50 mL centrifuge tube containing chilled sterile alpha-MEM, and then perform the following steps in a biosafety cabinet.
3. Discard the alpha-MEM medium, and rinse the bones with 70% ethanol for 30 s.
4. Wash the bones with ice-cold sterile PBS twice to clean up the remaining ethanol.
5. Transfer the bones in a mortar containing 5 mL of ice-cold PBS, and gently fragmentize the bones with a pestle (do not pulverize them).
6. Agitate the bones gently by swirling the pestle to release the bone marrow cells into the PBS. Collect the PBS containing bone marrow cells into a new 50 mL centrifuge tube.
7. Repeat step 2.6 for four times until the bone fragments become solid white in color.
8. Centrifuge the tube at 465 x g for 5 min at 4 °C, then discard the supernatant.
9. Resuspend the pellet with 18 mL of sterile distilled water and let it stand for 30 s to remove red blood cells.
10. Add 2 mL of ice-cold 10X PBS to stop the reaction, then filter the mixture through a 70 µm cell strainer to remove the lysed red blood cells.
11. Centrifuge the tube at 465 x g for 5 min at 4 °C. Resuspend the pellet with 20 mL of ice-cold PBS.
12. Wash the cells by repeating step 2.11 a second time.
13. Transfer the cell pellet into a 100-mm sterile Petri dish with 10 mL of OP9 conditional medium from step 1.2 supplemented with 20% FBS and a mixture of 0.5 ng/mL murine IL-7, 30 ng/mL mouse SCF, and 100 U/mL murine flt3L.
14. Incubate the dish at 37 °C with 5% CO_2 for 2 h. Transfer the unattached cells into a new culture container at a density of 5 x 10^5 viable cells/mL (count cells by hemocytometer with trypan-blue exclusion) with the same medium formula as in step 2.13, and incubate at 37 °C with 5% CO_2.
15. On day 4, change the culture condition to OP9 conditional medium supplemented with 20% FBS and 2,000 U/mL of murine IL-2.
16. Refresh the culture medium every 3 days; mature NK cells can be obtained by Day 7 and qualified as in section 4.

3. siRNA-mediated Gene Silencing of Differentiating NK Cells

1. Premix the siRNA or nonsense control (NC) with a transfection agent according to the product manual.
2. Perform step 3.1 at any time point since day 0.
3. Add the 50 nM of siRNA or NC mixture to the cells from step 2.14.
4. Repeat step 3.1 on every medium refreshment (*e.g.*, Day 0, 4, and 7) until the experimental end point.

4. Analysis of NK Cell Differentiation Using Flow Cytometry

1. At an appropriate time point, collect the suspension of differentiating cells and wash with ice-cold PBS.
2. Fix the cells with cell fixation buffer according to the product manual.
3. Wash the fixed cells with ice-cold PBS, and then resuspend 1 x 10^6 cells in 100 µL of Flow Cytometry Staining Buffer containing Cy3-conjugated anti-mouse NKp46 and PE-conjugated anti-mouse CD244 antibodies in a dilution ratio of 1:100.
4. Stain the sample in the dark at room temperature for 2 h.
5. Resuspend the stained samples with 300 µL of PBS, then acquire FACS data and analyze with Cytobank platform[20] (http://cytobank.org/).

Representative Results

Representative results are obtained following the described protocol. Total bone marrow suspension cells were cultivated under the feeder-free differentiation system for 11 days; significant increase in proliferation rate was observed by day 7 compared with the number of total cells on day 0 (**Figure 1A**). Mature NK cells with high nuclear to cytoplasmic ratio and granule-rich cytoplasm morphology were found by day 6 in the system (**Figure 1B**).

In order to elucidate the regulatory effect of TGF-β1/Smad3 signaling in NK cell differentiation, bone marrow cells were transfected with siRNA against E4bp4 mRNA (siE4bp4, which effectively suppresses E4bp4 mRNA level as shown in **Figure 2**) or nonsense control on day 0 and day 4 in the feeder-cell free system. The differentiating cells were cultured in the system with or without Smad3 inhibitor SIS3 for 6 days. Flow analysis detected that knockdown of E4bp4 largely suppressed the NK cell maturation as shown by the reduction in $CD244^{+ve}$ $NKp46^{-ve}$ immature NK cells compared with NC-transfected control, whereas inhibition of TGF-β1/Smad3 activation significantly rescues the siRNA-mediated suppression of NK cell maturation compared with the siE4BP4-transfected group (**Figure 3**).

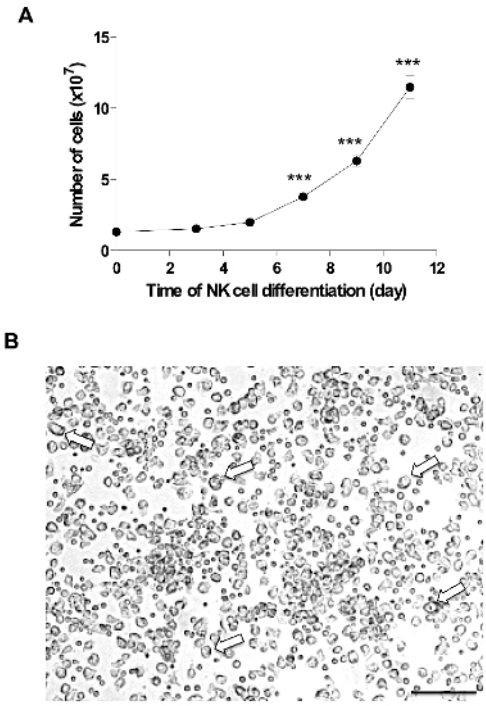


Figure 1. Image of bone marrow-derived NK cells from the feeder-free system. (**A**) Growth curve of bone marrow cells undergoing NK cell differentiation in the system until Day 11, obtained by cell counting. **(B**) Mature NK cells with high nuclear to cytoplasmic ratio and granule-rich cytoplasm morphology appear by day 6, as indicated by arrows. Magnification 200x, scale bar 50 μm. Data represent mean ± SEM for 3 independent experiments, ***$p < 0.01$. Please click here to view a larger version of this figure.

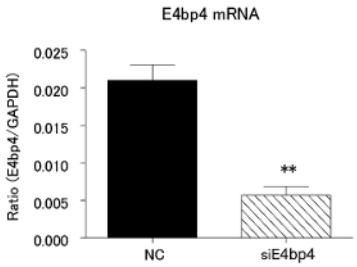


Figure 2. Effect of siRNA-mediated gene silencing on the bone marrow cells undergoing NK cell differentiation on day 6. The differentiating cells were transfected with nonsense control (NC) or siRNA targeting murine E4bp4 mRNA (siE4bp4) on day 0 and day 4. Real-time PCR shows that mRNA expression level of E4bp4 was significantly reduced in si-E4bp4 treated NK cells on day 6 compared with the NC group. Data represent mean ± SEM for 3 independent experiments, **$p < 0.05$. Please click here to view a larger version of this figure.

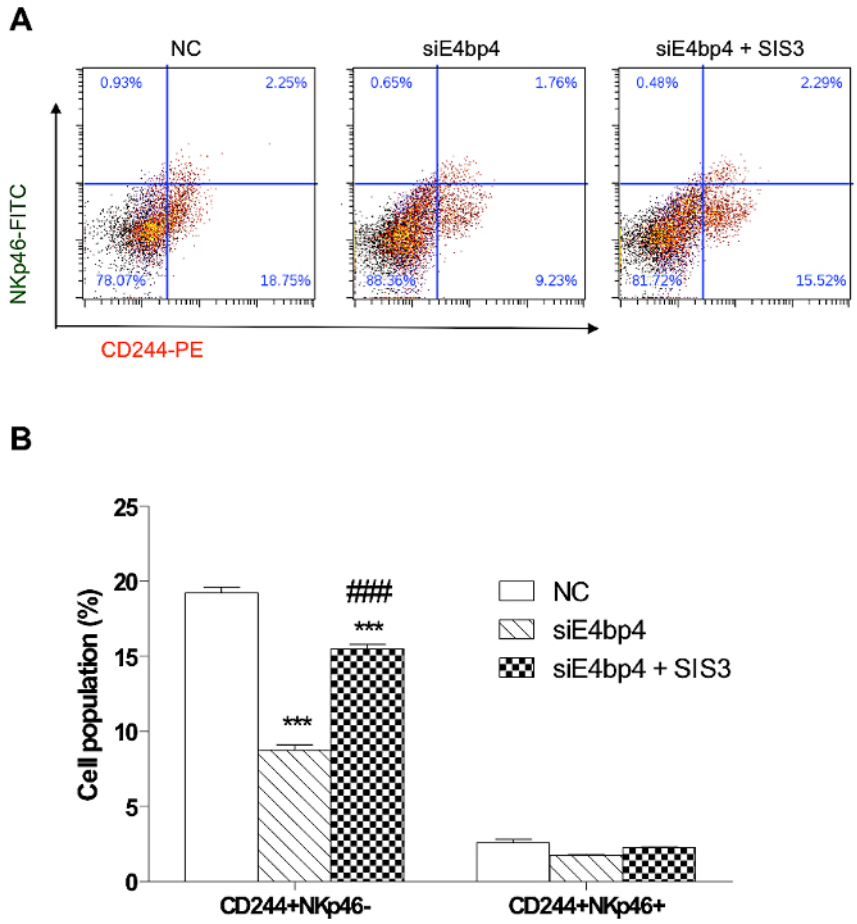
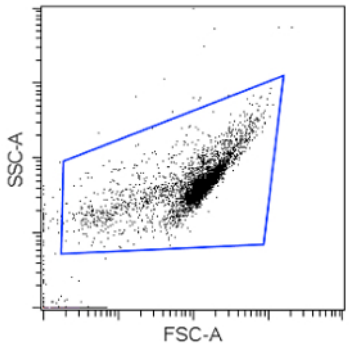


Figure 3. Silencing of E4bp4 inhibits differentiation of murine bone marrow-derived NK cells in a TGF-β1/Smad3-dependent manner. (**A**) Flow cytometry analysis shows that knockdown of E4bp4 largely decreases the production of immature ($CD244^{+ve}$ $NKp46^{-ve}$) NK cells on day 6 compared with the nonsense control (NC) group by using the feeder-free system *in vitro*, which can be significantly rescued by suppressing TGF- β1/Smad3 activation with 1 μM of Smad3 inhibitor SIS3. (**B**) Quantification of the flow analysis results, ***$p < 0.01$ compared with NC; $^{###}p < 0.01$ compared with siE4BP4-transfected group. Representative data of 3 independent experiments are shown. Please click here to view a larger version of this figure.



Supplementary Figure 1. Gating strategy for flow analysis of NK cell marker expression in total differentiating cells on day 6. Please click here to view a larger version of this figure.

Discussion

In the present work, we have described a novel method for producing bone marrow-derived murine NK cells *in vitro*. The cell feeder in the original system[21,22] is successfully replaced by the conditional medium of OP9 cells, which largely increased the stability of the differentiation

system. In addition, the system can produce high quantity and purity of mature NK cells for *in vitro* as well as *in vivo* assays, which can facilitate the mechanistic study as well as translational research of NK cells in human diseases.

The described method has been used for investigating the regulatory role of TGF-β1 signaling in NK cell suppression during cancer development[23]. As there was no influence from the feeder cells, the efficiency of siRNA-mediated gene knockdown as well as inhibitor-mediated inhibition are largely improved. The absence of the feeder cells OP9 allows us to clearly demonstrate the biological function of Smad3 in the E4BP4-mediated NK cell development, by showing the knockdown or inhibition of target genes *in vitro*[23].

More importantly, this system can be further utilized for producing a large amount of genetically modified murine NK cells for *in vivo* assays. For example, we have produced at least 1 x 10^8 mature NK cells from the bone marrow cells of a single mouse by this system, and the NK cells with knockdown of a specific gene were infused into tumor-bearing NOD/SCID mouse model to demonstrate the difference in cancer-killing effects *in vivo*[23]. Indeed, other modifications can be done on the NK cells in this system, such as viral-mediated gene overexpression, cell staining, *etc.*

However, it should be noted that a maximum 70% of mature NK cells can be produced from the system on day 9 (data not shown), which is similar to the result of the cultured system with OP9 stromal shown by Williams *et al.*[21] To overcome this limitation, the mixed NK cells can be further purified with a flow sorting machine, as well as commercial NK cell isolation kits in order to isolate the desired population.

In conclusion, a new method for differentiating NK cells from murine bone marrow cells is developed. This novel system can provide an option for obtaining high purity and quantity of mature NK cells for the study of immunity in human diseases.

Disclosures

The authors have nothing to disclose.

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H.-Y.L. designed and supervised all experiments and contributed to manuscript preparation. P.M.-K.T. performed experiments, analyzed data and contributed to manuscript preparation. P.C.-T. T., J.Y.-F.C., J.S.-C.,H., Q.-M.W., and G.-Y.L. collected animal samples and participated in animal experiments. J.S., X.-R.H., and K.-F.T. contributed to manuscript preparation.

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