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# Stage-dependent gene expression profiles during natural killer cell development

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**Abstract**

Natural killer (NK) cells develop from hematopoietic stem cells (HSCs) in the bone marrow. To understand the molecular regulation of NK cell development, serial analysis of gene expression (SAGE) was applied to HSCs, NK precursor (pNK) cells, and mature NK cells (mNK) cultured without or with OP9 stromal cells. From 170,464 total individual tags from four SAGE libraries, 35,385 unique genes were identified. A set of genes was expressed in a stage-specific manner: 15 genes in HSCs, 30 genes in pNK cells, and 27 genes in mNK cells. Among them, lipoprotein lipase induced NK cell maturation and cytotoxic activity. Identification of genome-wide profiles of gene expression in different stages of NK cell development affords us a fundamental basis for defining the molecular network during NK cell development.


*Keywords:* NK cells; Development; SAGE; LPL

Natural killer (NK) cells are crucial cellular mediators of innate defense against infectious pathogens and cancers [1,2]. Upon activation, NK cells release cytokines and chemokines that regulate inflammatory responses, hematopoiesis, and monocyte functions. Recent studies indicate that NK cells can modulate the outcome of adaptive immune responses by inducing T-cell-mediated memory [3] and B-cell-mediated autoimmunity [4]. In addition, NK cells are involved in the pathogenesis of diseases such as allergen-induced airway inflammation [5], graft-versus-host disease [6], Hodgkin disease [7], and systemic lupus erythematosus [8].

NK cells, like other hematopoietic cells, are derived from pluripotent hematopoietic stem cells (HSCs) [9,10]. NK cells can be generated from fetal thymus, fetal liver, cord blood, and bone marrow (BM) HSCs [11]. NK cell development occurs primarily extrathymically, with critical steps occurring in the BM. In fetal thymus, restricted NK/T cell progenitors such as Fc$\gamma$III$^+$CD117$^+$ cells give rise to NK cells or TCR$\alpha\beta^+$ T cells. In vitro NK cell development can be divided into three stages [12]. In the initial stage, HSCs give rise to NK precursor (pNK) cells in the presence of cytokines, such as stem cell factor (SCF), Fms-like tyrosine kinase 3 ligand (Flt3L), and IL-7. pNK cells

*Abbreviations:* BM, bone marrow; Flt3L, Fms-like tyrosine kinase ligand; HSC, hematopoietic stem cell; IRF-1, interferon regulatory factor; LPL, lipoprotein lipase; LT;, lymphotoxin; mNK, mature NK; NK, natural killer; pNK, NK precursor; SAGE, serial analysis of gene expression; SCF, stem cell factor.

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express IL-2Rβ (CD122), but do not have mature NK (mNK) cell markers (NK1.1, DX5, and Ly49). They express the receptors for IL-7, IL-15, and c-Kit as well as transcription factors such as PU.1, GATA3, ID2, and Ets-1. Deficiency of Ikaros [13], PU.1 [14], and ID2 [15] reduced the numbers of pNK in mice. Next, pNK cells are induced to become immature NK cells in the presence of IL-2 or IL-15 alone. They express NK1.1, but do not express DX5 and Ly49. In humans, immature NK cells are negative for CD56 and KIR. Prolonged culture of pNK cells with IL-15 alone generates NK1.1, DX5, CD94-NKG2 positive pseudomature NK cells (mNK (−OP9)), which lack Ly49 receptors.

NK cells express a heterogeneous arsenal of surface receptors that allow them to respond to microbial products, cytokines, and stress signals [16,17]. mNK cells express the IL-15Rα, IL-2/15Rβ receptor and the common γ chain. IL-15 is necessary for maturation of functional NK cells in vitro from $CD117^+$ BM progenitors. The expression and establishment of the repertoire of major histocompatibility (MHC) class I-specific receptors during NK cell development are a very interesting issue. CD94-NKG2 inhibitory receptors are induced by cytokines, particularly by IL-15. The ontogeny of CD94-NKG2 expression is not known fully in mice, but it is feasible that functional CD94-NKG2 receptors are expressed early in NK cell development [18,19]. The other known family of murine MHC-specific inhibitory receptors, the Ly49 receptors, is expressed relatively late in NK cell development and appears to require signals other than IL-15. In vivo, its expression occurs gradually over the first few weeks of life, reaching a peak by 1 month of age. Interaction between NK cells and stromal cells is important for proper NK cell maturation [20]. This interaction is bidirectional as shown in the cases of lymphotoxin-deficient mice and interferon regulatory factor (IRF)-1-deficient mice [21–23]. Mouse immature NK cells are induced to express the DX5 and Ly49 following culture with stromal cells. Expression of Ly49 receptors is reported to be a cumulative process in vitro. Examination of clonal populations revealed that CD94-NKG2, NKG2C, and Ly49B were expressed first followed by Ly49G, then Ly49C and I, and finally Ly49A, D, E, and F [24].

The successful development of in vitro culture systems for NK cell maturation from HSCs and the application of in vivo knockout models have revealed the key roles of several cytokines, transcription factors, and receptors in NK cell development. But, little is known about the sophisticated profile of molecular events in NK cell development. To identify changes in gene expression profiles during NK cell development, we performed serial analysis of gene expression (SAGE) for mouse BM HSC, pNK cells, and mNK cells cultured without stromal OP9 cells (−OP9) and with stromal OP9 cells (+OP9). These data allow us to understand the gene expression during NK cell development at the genome level and in a quantitative manner.

## Results

### *SAGE libraries of different stages in NK cell differentiation*

To obtain the stage-specific subsets of NK cells, lineage $(Lin)^-$ c-$kit^+$ HSCs (>95%) purified from mouse BM cells were incubated in the presence of SCF, Flt3L, and IL-7 for 6 days (Fig. 1A). After incubation, $CD122^+$ pNK cells (95%) were further purified and analyzed by flow cytometry. Then, mNK cells (−OP9 or +OP9) were obtained after incubation with IL-15 alone or IL-15 plus OP9 stromal cells for another 6 days. As expected, many more mNK cells (−OP9 94% and +OP9 > 95%) were positive for the mNK cell marker NK1.1 compared with pNK cells. It was known that signals from stromal cells are required for final maturation of NK cells [20,23]. The NK cells generated in vitro under stromal-free conditions expressed CD94/NKG2 receptors, but not Ly49 receptors. Ly49 receptors were induced when pNK cells were cultured with BM-derived stromal cells (OP9) as well as IL-15, suggesting that Ly49 induction requires a direct interaction with the BM microenvironment. Ly49C/I expression between mNK (−OP9) and mNK (+OP9) was examined by flow cytometry (Fig. 1B). Consistent with previous reports [24,47], Ly49C/I was not expressed on mNK cells (−OP9), but it was induced on mNK cells (+OP9). Ly49A and Ly49G2 were also induced on mNK (+OP9), but not on mNK (−OP9) (data not shown). The expression of surface molecules in pNK and mNK cells was further analyzed. pNK cells were negative for DX5 and CD94-NKG2, but mNK cells were positive for both of them as reported previously [24]. The gene expression pattern of CD122 and perforin in NK cell-stage-specific subsets confirmed the proper maturation of NK cells during development (Fig. 1C).

Four independent SAGE libraries of different stages in NK differentiation were constructed and the results are summarized in Table 1 ($p < 1 \times 10^{-5}$). Collectively, 170,464 total individual tags were identified from four SAGE libraries, and 35,385 of 59,657 distinct transcripts were unique genes. Among 59,657 distinct transcripts, 77.9% were single copy, 16.8% existed as 2 to 4 copies, 3.2% 5 to 9 copies, 1.9% 10 to 99 copies, and only 0.2% of tags were present in more than 100 copies. Then, a set of known stage-specific markers in each SAGE library was identified (Supplementary Table 1). Granzyme and mNK cell receptors such as NKG2A, 2B4, Ly49Q, and CD94 were highly present in mNK cells, but absent from HSCs and pNK cells. IL-15 was detected only in HSCs and pNK cells, and ID2 was detected from the pNK cell stage. These data further support the identity of the purified stage-specific subsets in NK cell development.

### *Analysis of genes that were expressed stage-specifically during NK cell development*

It is believed that a distinct pattern of gene expression occurs at each stage of NK cell development. In this regard,