Chronic lymphocytosis of functionally immature natural killer cells

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Background: The development of natural killer (NK) cells in the bone marrow is not well characterized. We recently described a mouse (referred to as an NK cell–deficient [NKD] mouse) with a selective deficiency in NK cells caused by the insertion of a transgene construct into the genetic locus for the basic leucine zipper transcription factor ATF-2. NK cells in this mouse were both phenotypically and functionally immature and accumulated in the bone marrow at a stage at which constitutive NK cell proliferation occurs in wild-type mice.
Objective: We hypothesized that excess IL-15 could potentially overcome this developmental block, allowing normal emigration of mature NK cells from the bone marrow to the periphery.
Methods: Double-transgenic mice were generated by crossing the NKD mice with transgenic mice overexpressing IL-15.
Results: The double-transgenic mice had a dramatic accumulation of phenotypically immature NK cells in the bone marrow and subsequently in the blood, liver, and spleen. NK cells from these double-transgenic mice manifested functional deficits similar to those observed in NK cells from NKD mice, as assessed by decreased cytokine production and cytotoxicity.
Conclusion: Rather than bypass the observed developmental defect in NKD mice, excess IL-15 drove a massive accumulation of phenotypically and functionally immature NK cells in the bone marrow and periphery.
Clinical implications: We propose that these double-transgenic mice will serve as a murine model of chronic NK cell lymphocytosis in human patients. (J Allergy Clin Immunol 2007;120:924-31.)

Key words: *Natural killer cells, lymphocytosis, chronic natural killer cell lymphocytosis, natural killer cell–deficient mice, IL-15, CD11b, Mac1*

Abbreviations used

bZIP:	Basic leucine zipper
CNKL:	Chronic natural killer cell lymphocytosis
IL15tg:	Transgenic IL-15
NK:	Natural killer
NKD:	Natural killer cell–deficient
NKDxIL15tg:	Double-transgenic mice
PE:	Phycoerythrin
WBC:	White blood cell

Natural killer (NK) cells are innate lymphocytes that are able to recognize and kill tumors and tumor cell lines without prior sensitization.[1-5] They play an important role in early antipathogen host defense and are believed to participate in tumor surveillance.[6-8]

The development of NK cells in the bone marrow is only beginning to be characterized. We have proposed a model of NK cell differentiation with intermediate developmental stages defined by the ordered acquisition or downregulation of a panel of informative cell-surface markers, including cytokine receptors (c-kit; CD122, IL2/15Rβ), NK receptors (NK1.1, CD94/NKG2, and Ly49 receptors), and adhesion molecules (αvβ3 integrin; α2β1 integrin, DX5; CD11b, Mac1; and CD43).[9] Of note, functional maturation and normal emigration out of the bone marrow correlates with the acquisition of Mac1 and CD43. Although the molecular basis of these putative developmental stages remains unknown, they provide a useful framework for investigating *in vivo* NK cell development.

We previously described a transgenic mouse (referred to as an NK cell–deficient [NKD] mouse) with substantially decreased numbers of peripheral NK cells that were functionally immature.[10] NK cell development in this mouse appeared blocked after the acquisition of the pan-NK cell marker DX5 and the downregulation of αvβ3 integrin but before the upregulation of Mac1 and CD43 expression, resulting in the accumulation of immature NK cells in the bone marrow. We recently reported that the molecular basis for the block at this putative developmental stage was the serendipitous insertion of the transgene (MHC-specific Ly49A inhibitory receptor under control of the granzyme A promoter) into the gene for the basic leucine zipper (bZIP) transcription factor ATF-2 on chromosome 2.[11] The observed NK cell phenotype in these mice was unrelated to the function of Ly49A as a receptor for MHC class I, as demonstrated by the persistence of the NK cell

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phenotype in transgenic mice deficient for β_2-microglobulin (which is required for normal MHC class I expression) and by the absence of a similar phenotype in 2 other independently generated Ly49A transgenic mouse lines.[12,13] Interestingly, the immature NK cell phenotype observed in the NKD mice does not appear to be directly caused by the disruption of *Atf2* itself because no NK cell maturation defects were observed in either *Atf2*-hypomorphic or *Atf2*-null allele mice.[11] The NKD mice express truncated *Atf2* transcripts, which might interfere with NK cell development through heterodimerization with other bZIP transcription factors.[11] Alternatively, the immature NK cell phenotype in the NKD mice might result from a transgene integration effect in the *Atf2* genetic locus. Although these possibilities are still under investigation, NKD mice have proved useful in elucidating NK cell development in wild-type mice and might provide clues to human conditions with abnormal NK cell development.

Because IL-15 plays a crucial role in NK cell development,[14,15] as well as in peripheral NK cell homeostasis[16-18] and proliferation,[19,20] we investigated whether excess IL-15 could overcome the apparent developmental block in NKD mice, allowing the NK cells to reach functional maturity and resume normal homeostasis from the bone marrow to the periphery. To address this hypothesis, we used transgenic IL-15 (IL15tg) mice that constitutively express murine IL-15, leading to modest increases in peripheral mature NK cells.[21,22] Crossing the NKD mice with IL15tg mice not only failed to overcome the maturational block in the NKD mice but resulted in a dramatic accumulation of phenotypically and functionally immature NK cells in the bone marrow and subsequently in the periphery. Interestingly, these double-transgenic mice exhibited a number of notable similarities to human patients with chronic NK cell lymphocytosis (CNKL). CNKL is a heterogeneous disorder defined as the persistent increase of NK cells in the peripheral blood (>40% $CD16^+/CD3^-$ lymphocytes, with an absolute number $>0.6 \times 10^9$/L) for more than 6 months without an identifiable cause of reactive NK cell lymphocytosis.[23] Patients with CNKL are reported to have relatively benign courses marked primarily by cytopenias and, in some patients, recurrent infections caused by neutropenia.[23-28] However, several patients with CNKL have been reported to have vasculitic skin lesions, arthritis, or peripheral neuropathy with NK cell infiltration of nerve fascicles, and 1 patient was reported to have progression of lymphocytosis with subsequent lung infiltration and death.[24,25,29] We propose that the double-transgenic mice described in this article, with their chronic lymphocytosis of immature NK cells, might serve as a murine model of CNKL and provide insight into this uncommon human disorder.

METHODS

Mice

C57BL/6 (B6) mice were obtained from the National Cancer Institute (Frederick, Md). IL15tg mice on a B6 background[30] were generated in the Caligiuri laboratory in a similar manner to the generation of IL15tg mice on a FVB/N background.[21,22] The generation of NKD mice on a B6 background has been described.[10] Both of these transgenic mouse lines were maintained as hemizygous mice (transgenic insert on 1 chromosome). The double-transgenic (NKDxIL15tg) mice were generated by crossing the IL15tg and NKD mice. The offspring were assessed as double-hemizygous mice by genotyping with published primers for the Ly49A[11] and IL-15[21] transgenic inserts. Additional details are included in the Online Repository at www.jacionline.org.

Cell preparation and flow cytometry studies

Single-cell suspensions were prepared from the spleen, liver, and bone marrow by using standard methods, as previously described,[11,31] and used in flow cytometry studies. Details are included in the Online Repository at www.jacionline.org.

Functional assays

Single-cell splenic suspensions were used to assess functional responses of NK cells in cytotoxicity studies, as well as in cytokine stimulation assays, as previously described.[9,11,31,32] Details are included in the Online Repository at www.jacionline.org.

In situ staining of NK cells with anti-NK1.1

NK cells were identified *in situ* by staining with phycoerythrin (PE)–conjugated anti-NK1.1 according to a modified protocol initially described by Andrews et al.[33] Details are included in the Online Repository at www.jacionline.org.

Complete blood counts

Complete blood count studies, including white blood cell (WBC) counts, hematocrit values, and platelet counts, were performed on a Baker 9000 hematology analyzer (Serono-Baker, Allentown, Pa) in the Division of Comparative Medicine at Washington University.

RESULTS

$NK1.1^+CD3^-$ cells in double-transgenic mice had an immature phenotype

To test our hypothesis that excess IL-15 might overcome the developmental block observed in NKD mice, we crossed NKD mice with IL15tg mice on a B6 background. Single- and double-hemizygous transgenic mice were analyzed. The mice all expressed the IL-15 receptor (IL2/15Rβ and common γ chain) on their bone marrow, blood, and splenic NK cells, although IL-15Rα expression on NK cells was diminished in the presence of excess IL-15 in the IL15tg and NKDxIL15tg mice (see Fig E1 in the Online Repository at www.jacionline.org). Analogous to what was observed in the NKD mice, NK cells from the double-transgenic mice were found to express only low levels of both Mac1 (CD11b) and CD43 (Fig 1). This contrasts sharply with the upregulation of these molecules on NK cells from IL15tg and wild-type mice, particularly in the spleen and liver. Mac1 and CD43 have previously been shown to be markers of NK cell maturation, and the absence or low expression of these adhesion molecules correlates with NK cell immaturity.[9] The immature phenotype of the NK cells from the double-transgenic mice was observed in the periphery (spleen and liver), as well as in