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IL-15 inhibits pre-B cell proliferation by selectively expanding Mac-1$^+$B220$^+$ NK cells

Shinsuke Nakajima, Shigeaki Hida, Shinsuke Taki *

Department of Immunology and Infectious Diseases, Shinshu University Graduate School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan

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ABSTRACT

Natural killer (NK) cells are the cells critical for inhibition of repopulation of allogenic bone marrow cells. However, it is not well known if NK cells affect autologous lymphopoiesis. Here, we observed that NK cells could inhibit pre-B cell proliferation *in vitro* driven by interleukin (IL)-7 in a manner dependent on IL-15. Interestingly, the great majority of expanding NK cells were Mac-1$^+$B220$^+$, a recently identified potent interferon (IFN)-γ producer. Indeed, IFN-γ was produced in those cultures, and pre-B cells lacking IFN-γ receptors, but not those lacking type I IFN receptors, were resistant to such an inhibition. Furthermore, even NK cells from mice lacking β2-microglobulin, which were known to be functionally dampened, inhibited pre-B cell proliferation as well. Thus, activated NK cells, which were expanded selectively by IL-15, could potentially regulate B lymphopoiesis through IFN-γ beyond the selection imposed upon self-recognition.

It has been well documented that natural killer (NK) cells are a critical parameter for successful bone marrow (BM) transplantation [1]. Natural cytotoxicity of NK cells played an essential role in the inhibition of reconstitution by allogenic BM transplants. In contrast, NK cells are unable to kill autologous cells. This "self tolerance" was considered to be mediated through inhibitory signals delivered by multiple killer inhibitory receptors (KIRs) recognizing self major histocompatibility complex-encoded class I (MHC-I) molecules [2]. On the other hand, self MHC-I molecules were proposed to play critical roles in the acquisition of functional competence by developing NK cells ("licensing") [3]. Similarly as natural cytotoxicity, production of a potent immunoregulatory cytokine interferon (IFN)-γ also seemed to require "licensing" [3]. In accordance with the hypothesis, NK cells in β2-microglobulin-deficient (β2m$^{-/-}$) mice fail to produce IFN-γ efficiently in response to the stimuli through activating NK receptors as well as to exhibit natural cytotoxicity [3–5].

In addition to the effects on allogenic cells, NK cells were reported to have potential to functionally inhibit autologous dendritic cells under certain circumstances [6,7], suggesting that NK cells could act beyond "self tolerance" to exert their immunoregulatory functions. However, questions are to be addressed as to under what conditions and how NK cells escape "self tolerance". Cytokines such as interleukin (IL)-2 might enable NK cell to overcome "self tolerance" [3,8]. Unlike IL-2, IL-15 is well known not only to be essential for NK cell development and proliferation, but also to play important roles in inflammation, acting in conjunction with other proinflammatory cytokines such as IL-12 and IL-18 [9–11]. Its potential to enable NK cells to overcome "self tolerance" is to be examined.

NK cells were heterogeneous both in mice and humans, comprising of mature NK cell subsets as well as cells of various maturation stages [12]. In contrast to the well known mature NK cell subsets in humans distinguished by CD56 expression, murine NK cell subsets have just recently been identified using various cell surface markers. Thus, Mac-1/CD11b$^+$ mature NK cells could be subdivided into CD27$^+$ and CD27$^-$ populations, with the former cells displaying greater effector functions than the latter [13]. More recently, B220$^+$NK1.1$^+$ cells with variable CD11c expression were identified as activated NK cells belonging to the Mac-1$^+$CD27$^+$ NK cell subset [14,15]. Although Mac-1$^+$CD27$^+$B220$^+$ NK cells were shown to produce greater amounts of IFN-γ, questions remain as to what roles these cells would play.

Supply of immune cells such as T and B cells from BM in steady states or under ongoing immune responses is believed to be regulated by various cytokines. Considering negative influence alone, type I IFNs (IFN-α/β) and IFN-γ could inhibit pre-B cell proliferation, possibly mediating a part of virus-induced pan-cytopenia [16–20]. However, the cell types producing those cytokines have not been clearly identified. Here, we found that IL-15 inhibited IL-7-driven pre-B cell proliferation *in vitro*. Such an inhibition acted

* Corresponding author. Fax: +81 263 37 2610.
E-mail address: shin-t@sch.md.shinshu-u.ac.jp (S. Taki).

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not directly on pre-B cells but was mediated by NK cells and IFN-γ, but not IFN-α/β. Notably, the vast majority of NK cells expanded by IL-15 expressed Mac-1 and B220, representing activated NK cells. Furthermore, even "unlicensed" NK cells derived from mice lacking β2m$^{-/-}$ [4,5] could mediate such a pre-B cell inhibition as efficiently as wild-type NK cells, indicating that IL-15 could empower the Mac-1^{+}B220^{+} NK cell subset to overcome the lack of "licensing" and participate potentially in regulating autologous B lymphopoiesis during inflammation.

Materials and methods

Mice. C57BL/6 mice were purchased from SLC (Shizuoka, Japan). Mice lacking the α chain of the receptors for IFN-α/IFN-β or IFN-γ (IFNAR1$^{-/-}$ or IFNGR1$^{-/-}$ mice, respectively) were purchased from B&K Universal. IL-15$^{-/-}$ mice [9] were from Taconic Farm. β2m$^{-/-}$ mice and RAG-1$^{-/-}$ mice were described previously [21]. All these mice were backcrossed at least 10 times to C57BL/6 mice and kept under specific pathogen-free conditions in the animal facility of Shinshu University. All animal experiments were approved by the Committee for Animal Experimentation of Shinshu University and conducted according to the guideline.

Reagents. FITC-, PE- or biotin-conjugated monoclonal antibodies (mAb) for CD19, B220/CD45R, and CD49b/DX5 were purchased from BD Pharmingen. PE-anti-T cell receptor β-chain (TCRβ), APC-anti-CD93, APC-anti-NK1.1, biotin-anti-TER-119 and PE-Cy7-anti-Mac-1/CD11b mAbs were from eBioscience. FITC-anti-δ mAb and PE-goat anti-μ antibody were from Southern Biotech and Biomeda, respectively. Biotin-labeled mAbs was developed with PE-Cy7-streptavidin (BD Pharmingen). Anti-CD16/32 mAb was used to block Fc-mediated binding of antibodies during staining and cell sorting. Recombinant mouse IL-7 and IL-15 were purchased from PeproTech. Recombinant human IL-2 was provided by Ajinomoto Co., Inc. (Kawasaki, Japan). Anti-IFN-γ (XMG1.2) mAb was from BD Pharmingen.

Cell culture, flowcytometry, and ELISA. BM cells were plated at a concentration of 10^6 cells/ml into a 48-well plate in RPMI 1640 supplemented with 10% fetal calf serum, 50 μM 2-mercaptoethanol and 5 ng/ml IL-7 for 5 days. IL-15 was used at the concentration of 50 ng/ml. For NK cell phenotyping, RAG-1$^{-/-}$ BM cells were similarly cultured for 4 days in the presence of IL-15 alone. Cells recovered from those cultures were stained and analyzed on the Cytomics FC500 flowcytometer (Beckman Coulter). Data analysis was carried out with the RXP software (Beckman Coulter). The amounts of IFN-γ in the culture supernatants were measured by Mouse IFN-γ ELISA kits (eBioscience) according to the manufacturer's instructions.

Cell sorting. For NK cell enrichment, RAG-1$^{-/-}$ spleen cells were treated with anti-CD16/32 mAb and stained with biotin-conjugated anti-TER-119 mAb. Depletion of TER-119^{+} cells was performed by magnetic-activated cell sorting (MACS) using anti-biotin MicroBeads (Miltenyi Biotec). After depletion, cells were stained with PE-conjugated anti-DX5 mAb and NK cells were positively selected using anti-PE MicroBeads on an AutoMACS. Enriched NK cell preparations were at least 92% pure. For depletion of NK cells, BM cells were stained with PE-anti-NK1.1 mAb, and NK1.1^{+} cells were depleted on an AutoMACS. The frequencies of NK cells in those preparations were lower than 0.1%, compared with >1% before depletion.

Cell proliferation assays. BM cells were cultured for 3 days as described above. On day 3, bromodeoxyuridine (BrdU, 10 μM, BD Pharmingen) was added for further 6 h. BrdU-labeled cells were then processed using the BrdU Flow kit (BD Pharmingen) according to the manufacturer's instructions, and analyzed on the Cytomics FC500 flowcytometer. In other experiments, freshly isolated BM cells were labeled with 1 μM CFSE (Molecular Probe), cultured for 3 days and analyzed by flowcytometry.

Results

IL-15 expanded B220^{+} NK cells and inhibited pre-B cell proliferation

BM cells isolated from wild-type mice exhibited vigorous proliferation of B-lineage cells when cultured *in vitro* in the presence of IL-7 (CD19^{+}B220^{+}, Fig. 1A). More than a half of surviving cells were pre-B cells expressing CD93 but not surface IgM and IgD (Fig. 1A) [22]. Notably, when IL-15 was supplemented, the numbers of surviving CD19^{+}CD93^{+} as well as CD19^{+}B220^{+} cells were severely reduced (Fig. 1A, C). CD93 expression was lower in IL-15-containing cultures (Fig. 1A), suggesting that CD93^{high}CD19^{+} cells were lost preferentially in those cultures. NK (NK1.1^{+}TCRβ^{-}) cells were

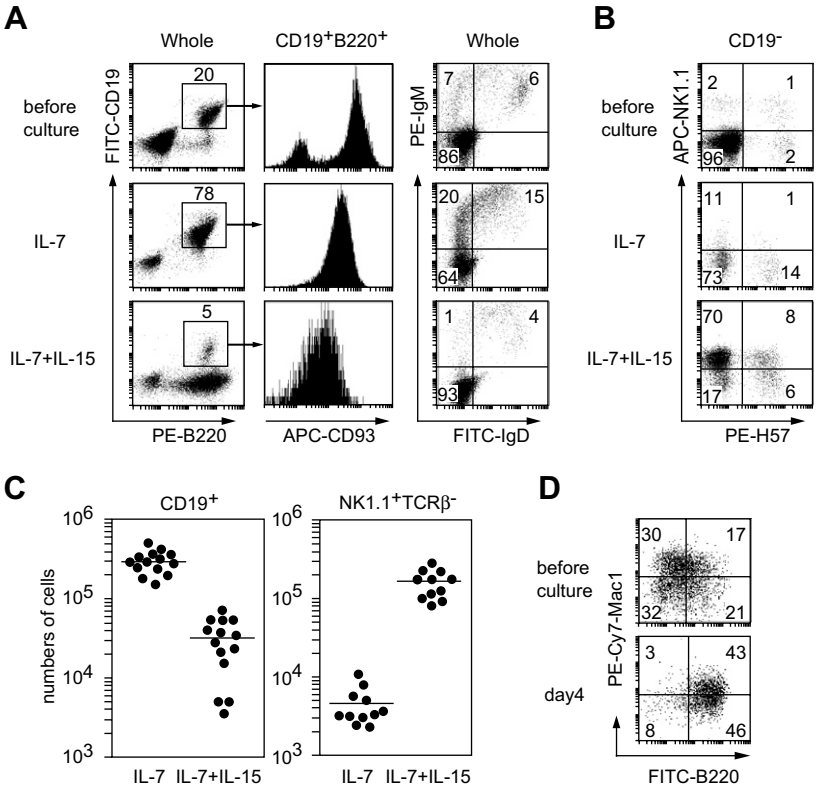

Fig. 1. IL-15 inhibited pre-B cell proliferation and expanded an NK cell subset *in vitro*. (A) Expressions of B cell markers before and after culture with the indicated cytokines. Histograms denote expression of CD93 marker on CD19^{+}B220^{+} cells as gated on the left most dot plots. (B) The frequencies of NK (NK1.1^{+}TCRβ^{-}) cells within CD19^{-} cells recovered from the same cultures as in (A). (C) Summary for the numbers of CD19^{+} B cells and NK1.1^{+}TCRβ^{-} cells. Each symbol in C represents the value from an individual culture with the means (horizontal bars). (D) Phenotypes of NK cells recovered from IL-15-supplemented cultures of RAG-1$^{-/-}$ BM cells. Shown are the expression patterns of the indicated markers on gated NK1.1^{+}TCRβ^{-} cells. In (A), (B), and (D), the numbers indicate the percentages of cells within the gates or the quadrants.