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

Shinsuke Nakajima, Shigeaki Hida and Shinsuke Taki*

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

*Corresponding author; Shinsuke Taki, Department of Immunology and Infectious Diseases, Shinshu University Graduate School of Medicine, 3-1-1 Asahi, Matsumoto 390-8621, Japan.

E-mail: shin-t@sch.md.shinshu-u.ac.jp, FAX: +81-263-37-2610.

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.

Key words: natural killer cells, interferon-γ, type 1 interferons, pre-B cells, interleukin-7, interleukin-15, hematopoiesis

Introduction

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 of host hematopietic system 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) that recognize 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 (“licencing”) [3]. Similarly as natural cytotoxicity, production of the potent immunoregulatory cytokine interferon (IFN)-γ also seemed to require “licencing” [3]. In accordance with the hypothesis, NK cells in β2-microglobulin-deficient ($\beta 2m^{-/-}$) 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, presumably 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 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 "unlicenced" NK cells derived from mice lacking β2m [4,5] could mediate such a pre-B cell inhibition as efficiently as did 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 hours. BrdU-labelled 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 (CD19$^+$ B220$^+$, Fig.1A) when cultured *in vitro* in the presence of IL-7. 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. On the other hand, NK (NK1.1$^+$TCRβ$^-$) cells were expanded only in IL-15-supplemented cultures (Fig.1B, C). Importantly, IL-15-expanded NK cells were uniformly B220$^+$ and expressing Mac-1, albeit at slightly lower levels than those on fresh NK cells (Fig.1D), closely resembling recently identified NK cells presumably equivalent to human CD56$^+$ NK cells [14,15]. They were also positive for c-kit, CD51 and CD69 (Nakajima, unpublished observations), suggesting that IL-15 expands not all but a subset of NK cells.

Pivotal role for IFN-γ produced by NK cells in the inhibition of pre-B cell proliferation.

BM cells depleted of NK cells by means of anti-NK1.1 (Fig.2A) or anti-CD122 (Nakajima, unpublished observation) antibodies exhibited normal IL-7-driven pre-B cell proliferation even in the presence of IL-15. Since NK cell depletion did not increase, if slightly decreased, CD19$^+$ cell recovery in cultures without IL-15 (Fig.2A, open columns), NK cells appeared to inhibit pre-B cell proliferation only in the presence of IL-15. This conclusion was supported further by the observations that BM cells

isolated from IL-15$^{-/-}$ mice, which lacked mature Mac-1^{+}B220^{+} NK cells [14] were resistant to IL-15-mediated inhibition (Fig.2B). In addition, exogenous NK cells added to the IL-15$^{-/-}$ BM cultures inhibited pre-B cell proliferation in a cell number dependent manner (Fig.2B). Thus, NK cells were not only required but also sufficient to mediate the inhibition by IL-15.

BM cells from mice lacking IFN-α/β receptors (IFNAR1$^{-/-}$ mice) were as sensitive to the inhibition by IL-15 as wild-type BM cells (Fig.2C). Notably, also in the absence of IL-15, wild-type and IFNAR1$^{-/-}$ pre-B cells proliferated at equal efficiencies (Fig.2C, open columns), indicating that IFN-α/β were dispensable to inhibit pre-B cell proliferation under the culture conditions. We observed that IFN-γ was indeed produced within the BM cultures in a manner dependent on both IL-15 and mature NK cells (Fig.2D). IFN-γ thus produced appeared to almost exclusively mediate the NK cell function to inhibit pre-B cell proliferation, as BM cells isolated from mice lacking IFN-γ receptors (IFNGR1$^{-/-}$) restored dramatically the recovery of CD19^{+} cells (Fig.2E). In agreement with this conclusion, neutralizing antibodies against IFN-γ also increased CD19^{+} cell recovery significantly (Fig.2F). Taken together, IFN-γ induced by IL-15, but not IFN-α/β, appeared to be the major, if not exclusive, mediator of NK cell inhibition of pre-B cell proliferation. Other functions of NK cells such as natural cytotoxicity seemed to play minor, if any, roles in the inhibition.

IFN-γ inhibited pre-B cell cycles.

Compared with B-lineage cells recovered from BM cultures containing IL-7 alone, those recovered from cultures containing both IL-7 and IL-15 exhibited reduction in the incorporation of BrdU (Fig.3A). In contrast, IFNGR1$^{-/-}$ cells did not show such a

reduction in BrdU incorporation (Fig.3A). Furthermore, as revealed by the dilution of CFSE labels, $CD19^{+}$ cells that had undergone cell divisions were less frequent, and instead in cultures containing IL-15 than in cultures without IL-15, whereas $IFNGR1^{-/-}$ BM cells showed cell divisions even in the presence of IL-15 as efficiently as in the absence of IL-15 (Fig. 3B). These results indicated that NK cell-derived IFN-γ acted on pre-B cells to reduce the efficiencies of their cell cycling.

NK cells from $\beta 2m^{-/-}$ mice were as potent inhibitor of pre-B cell proliferation as wild-type NK cells.

BM cultures established from $\beta 2m^{-/-}$ mice exhibited reduction in $CD19^{+}$ cells in cultures containing IL-15 to an extent similar to that in wild-type BM cultures (Fig.4A). Depletion of NK cells from $\beta 2m^{-/-}$ BM cells resulted in restoration of $CD19^{+}$ cells in cultures with IL-15 (Fig.4A). Furthermore, the amounts of IFN-γ produced in $\beta 2m^{-/-}$ BM cultures were comparable to those in wild-type BM cultures and IFN-γ appeared to be derived largely from NK cells (Fig. 4B). These observations indicated that NK cells in $\beta 2m^{-/-}$ mice acted as an inhibitor of pre-B cell proliferation through the mechanism identical to that of wild-type NK cells.

Discussion

It has been shown that IL-15 acted directly on activated mature B cells to induce proliferation and plasma cell differentiation [23]. We showed here that IL-15 could also influence B cell expansion *in vitro* through inhibiting IL-7-driven pre-B cell proliferation. Such an inhibitory function was indirect and mediated by NK cell-derived IFN-γ. Exogenously supplemented IFN-γ was previously shown to inhibit pre-B cell proliferation, but it is not fully clear if the mechanism for such an IFN-γ function involve the induction of apoptosis by this cytokine [19,20]. We observed that few pre-B cells were undergoing apoptosis (Nakajima, unpublished observation) and IFN-γ seemed rather to reduce cell cycling efficiencies of pre-B cells (Fig.3). Although the absence of apoptotic pre-B cells might possibly be due to the rapid disappearance of these cells, these observations were consistent with a previous report [20]. Notwithstanding the apparent potential of IFN-γ in pre-B cell inhibition, the cellular source(s) of IFN-γ has not been identified. Our current study demonstrated in this regard that Mac-1^{+}B220^{+} NK cells selectively expanded by IL-15 (Fig.1D), likely representing the activated NK cell subset with potent IFN-γ producing activity [14,15], appeared to be one of the major sources of IFN-γ for pre-B cell inhibition.

B lymphopenia appears to be a complex process in which distinct mechanisms seemed to be operating individually or in concert according to the sorts of stimuli and timing. Thus, in mice suffering from inflammations and infections, B cell generation was known to be inhibited by IFN-α/β [16], although it was not fully clear if IFN-α/β acted directly on pre-B cells, as well as by mobilizing pre-B cells to the periphery from BM through reducing local concentrations of CXCL12 [24]. In our *in vitro* culture system, however, IFN-α/β did not affect pre-B cell proliferation supported by IL-7 (Fig.2C),

presumably because the amounts of IFN-α/β produced *in situ* under our cultures were too low, compared with those used in previous studies [17,18]. Since IFN-α/β induced IL-15 during infections [25] and IFN-α/β were also a potent activator of NK cells [26], IL-15 and NK cells could mediate the inhibitory activity of IFN-α/β on B lymphopoiesis *in vivo* [16,18]. It was indeed known that virus infection induced IL-15 production rapidly and augments cytotoxic activity of NK cells [27]. Our *in vitro* culture system might mimic the *in vivo* process taking place after IL-15 was produced. On the contrary, however, a previous report claimed that B cell engraftment upon autologous BM transplantation was accelerated by co-transplantation of IL-2-activated NK cells [28]. Although IFN-γ production was not examined in that report, IFN-γ might not be produced by those NK cells efficiently under such “non-inflammatory” conditions where neither IL-2 and IL-15 nor other proinflammatory cytokines such as IL-12 and IL-18 could be supplied sufficiently [29,30]. A speculation, based on the observations in the current study, would be that NK cells are kept quiescent in steady states through inhibitory signals delivered by KIRs, whereas they can exert immunoregulatory functions over autologous cells, including the inhibition of B lymphopoiesis in BM, by producing IFN-γ, once inflammations are initiated. Future studies will be needed to identify under what kind of conditions B lymphopoiesis in BM is inhibited by the IL-15-NK cell-IFN-γ axis *in vivo*.

IL-15 seemed to help a specialized subset, if not all, of NK cells overcome the negative signals likely delivered through their abundantly expressed Ly49 receptors [14] engaged by self MHC class I molecules on neighboring cells or NK cells themselves. Another interesting finding in this regard was that even “unlicenced” NK cells from $\beta 2m^{-/-}$ mice normally mediated IL-15-induced inhibition of pre-B cell proliferation (Fig.4A) by

producing as much IFN-γ as did wild-type NK cells (Fig.4B). In line with this observation, it was shown that NK cells in $\beta 2m^{-/-}$ mice were as competent as wild-type NK cells in controlling mouse cytomegalovirus infections *in vivo* [31]. These observations seemed to suggest that inflammatory conditions discharge NK cells from not only "self tolerance" but also the lack of "licencing" imposed upon recognition of self MHC molecules. It is of interests if such liberation is confined to the $Mac\text{-}1^{+}B220^{+}$ NK cells.

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Figure legends

Figure 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 ($NK1.1^{+}TCR\beta^{-}$) cells within $CD19^{-}$ cells recovered from the same cultures as in A. (C) Summary for the numbers of $CD19^{+}$ B cells and $NK1.1^{+}TCR\beta^{-}$ 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 $RAG\text{-}1^{-/-}$ BM cells. Shown are the expression patterns of the indicated markers on gated $NK1.1^{+}TCR\beta^{-}$ cells. In A, B and D, the numbers indicate the percentages of cells within the gates or the quadrants.

Figure 2.

IFN-γ derived from NK cells mediated IL-15-induced inhibition of pre-B cell proliferation. $CD19^{+}$ cell recoveries (A, B, C, E, F) and the amounts of IFN-γ in the supernatants (D) from cultures containing IL-7 alone (open columns) or IL-7 plus IL-15 (filled columns) are shown. (A) Cultures of BM cells depleted of $NK1.1^{+}$ cells (NK(-)). (B) $IL\text{-}15^{-/-}$ BM cell cultures supplemented with the indicated numbers of purified wild-type NK cells. (C) Control and $IFNAR1^{-/-}$ BM cell cultures ($AR1^{-/-}$). (D) IFN-γ produced in the cultures established from control, NK cell-depleted wild-type (NK(-)) or $IL\text{-}15^{-/-}$ BM cells. (E) Cultures established with whole or NK cell-depleted (NK(-)) BM cells from control and $IFNGR1^{-/-}$ mice ($GR1^{-/-}$). (F) Wild-type BM cell cultures containing the indicated concentration of anti-IFN-γ antibodies. All data

shown in this figure are the means and SDs of duplicate cultures. Representative of three independent experiments.

Figure 3.

IFN-γ inhibits pre-B cell cycling. (A) The percentage of BrdU-labeled cells within $CD19^+$ B cells recovered from the cultures of wild-type (open symbols) and $IFNGR1^{-/-}$ (filled symbols) BM cells pulsed with BrdU. Each symbol represents the value of an individual culture and the horizontal bars are the means. Asterisks indicate significant differences ($P<0.05$) with or without IL-15. N.S., not significant. (B) CFSE dilution profiles of gated $CD19^+$ B cells recovered from BM cultures. Data are representative of three separate experiments. The numbers indicated the percentages of cells undergone cell divisions.

Figure 4.

NK cells from $\beta 2m^{-/-}$ mice were as potent inhibitor of pre-B cell proliferation as wild-type NK cells. (A) BM cells with (Whole) or without (NK(-)) NK cells from $\beta 2m^{-/-}$ mice were cultured with IL-7 (open columns) or IL-7 and IL-15 (filled columns). The mean numbers of recovered $CD19^+$ B cells and SDs are shown. (B) The amounts of IFN-γ produced in the cultures of wild-type and $\beta 2m^{-/-}$ BM cells with IL-7 (open columns, invisible) or IL-7 and IL-15 (filled columns). In both A and B, the means and SDs are shown for duplicate cultures. Representative of three separate experiments.

Figure 1

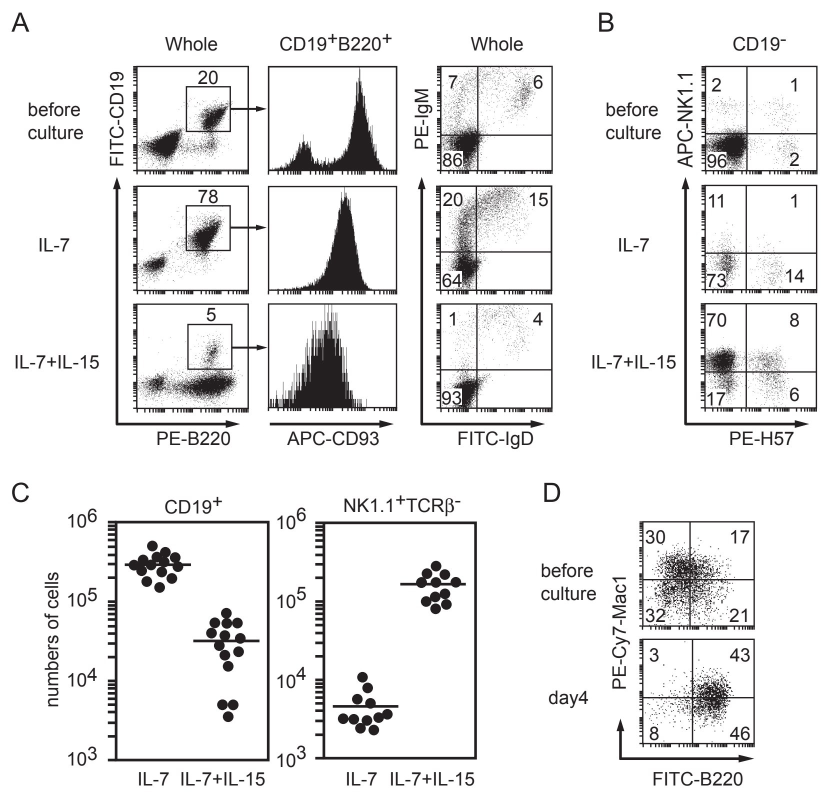

Figure 2

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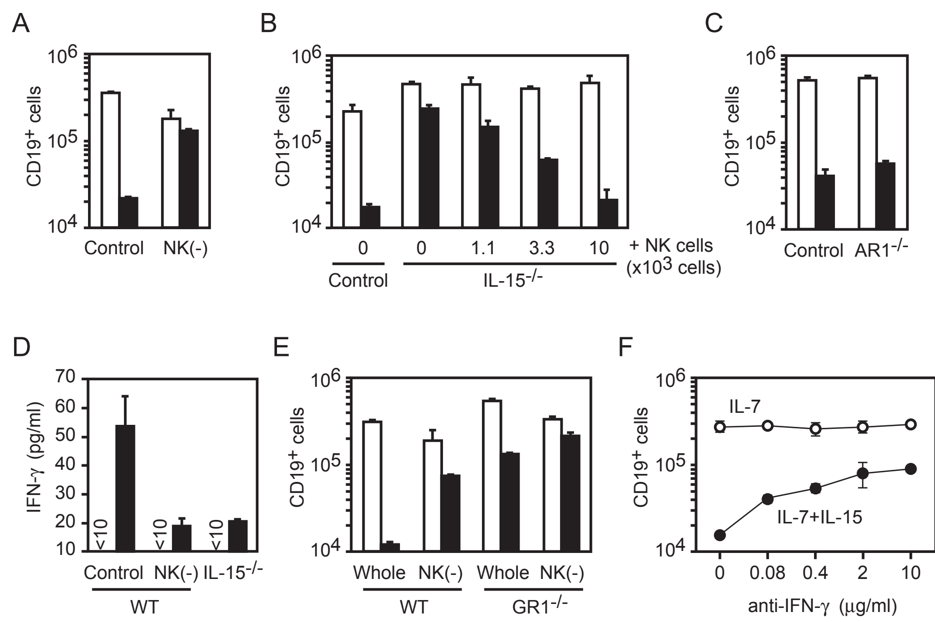

Figure 3

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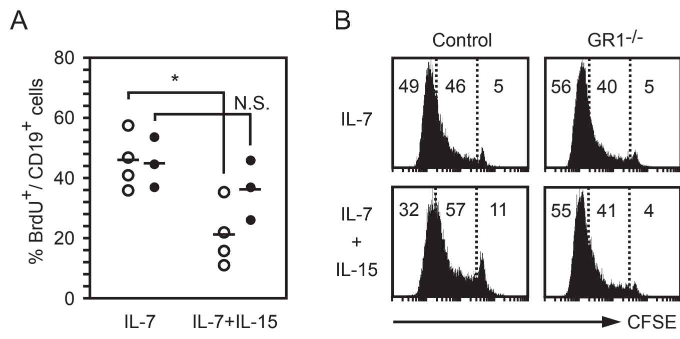

Figure 4

Figure 4.