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Augmentation of natural cytotoxicity by chronic low-dose ionizing radiation in murine natural killer cells primed by IL-2

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The possible beneficial effects of chronic low-dose irradiation (LDR) and its mechanism of action in a variety of pathophysiological processes such as cancer are a subject of intense investigation. While animal studies involving long-term exposure to LDR have yielded encouraging results, the influence of LDR at the cellular level has been less well defined. We reasoned that since natural killer (NK) cells constitute an early responder to exogenous stress, NK cells may reveal sentinel alterations in function upon exposure to LDR. When purified NK cells received LDR at 4.2 mGy/h for a total of 0.2 Gy *in vitro*, no significant difference in cell viability was observed. Likewise, no functional changes were detected in LDR-exposed NK cells, demonstrating that LDR alone was insufficient to generate changes at the cellular level. Nonetheless, significant augmentation of cytotoxic, but not proliferative, function was detected when NK cells were stimulated with low-dose IL-2 prior to irradiation. This enhancement of NK cytotoxicity was not due to alterations in NK-activating receptors, NK1.1, NKG2D, CD69 and 2B4, or changes in the rate of early or late apoptosis. Therefore, LDR, in the presence of suboptimal cytokine levels, can facilitate anti-tumor cytotoxicity of NK cells without influencing cellular proliferation or apoptosis. Whether these results translate to *in vivo* consequences remains to be seen; however, our data provide initial evidence that exposure to LDR can lead to subtle immune-enhancing effects on NK cells and may explain, in part, the functional basis underlying, diverse beneficial effects seen in the animals chronically exposed to LDR.

Keywords: Low-dose radiation; natural killer cells; natural cytotoxicity; innate immunity

INTRODUCTION

As innate immune effectors, natural killer (NK) cells provide a body's first line of defense against infections and cancer [1]. Through the secretion of proinflammatory cytokines and cytotoxic activity, NK cells can eliminate infected or transformed cells. Unlike T and B cells, activation of NK cells is regulated by the integration of stimulatory and inhibitory signals initiated by membrane-bound, germ line-encoded receptors [2]. Ligands for NK cell stimulatory receptors are usually poorly expressed by healthy cells but are up-regulated on 'unhealthy' cells, such as transformed, infected or stressed cells [3]. In addition to direct ligand-driven activation, NK cells can become further activated by cytokines produced by other innate immune cells such as macrophages or dendritic cells (DCs). Therefore, NK cells constitute one of the early responders of the immune system that cooperate with other cells of the immune system.

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High-dose and high-dose-rate ionizing radiation has been shown to be detrimental, causing apoptosis [4, 5] and transformation of normal cells into tumor cells [6]. High-dose radiation also impairs immune function, leading to deficient removal of damaged or malignant cells [7]. Chronic low dose radiation (LDR) exposure has been studied mainly in animal models. A low dose (<0.2 Gy) of X- or γ-ray irradiation can be beneficial to living organisms [8], as manifested by augmentation of the adaptive response [9, 10], stimulation of immunological functions [11–13], prevention and cure of disease [14, 15] and prolongation of life span [15, 16]. In addition, a series of studies demonstrated direct immune activation by chronic low-dose γ-irradiation. Ina and Sakai showed that exposure of C57BL/6 mice for 3 weeks at 1.2 mGy/h resulted in T cell activation [12] and prolongation of life span in autoimmune MRL-lpr/lpr mice for more than 5 weeks [16]. Furthermore, radiation-induced thymic lymphoma was suppressed by chronic fractionated low-dose total body irradiation [14] and further repressed by life-long exposure at 1.2 mGy/h [9]. Similarly, we found in our previous studies that AKR/J mice developing spontaneous thymic lymphoblastic lymphoma exhibited a lower tumor incidence and prolonged survival when exposed to low-dose-rate γ-irradiation at 0.7 mGy/h during their lifetime [17]. Therefore, chronic LDR appeared to limit undesired immune responses or transformation/gene mutation process, and maintain homeostatic balance of the immune system. In contrast, mice irradiated at a high dose rate (0.8 Gy/min for 4.5 Gy) developed thymic lymphoma with greater frequency and died much earlier. Interestingly, low-dose-rate γ-irradiation specifically up-regulated Jag2, a notch ligand involved in the development of NK cells, and IL-15, a cytokine that enhances NK cell function, without significantly affecting other immune-associated genes [17]. Since NK cells constitute immediate responders to exogenous stress, we hypothesized that the beneficial effects seen with LDR in tumor models might be, in part, attributable to augmented NK cell function. Therefore, this study was designed to investigate the role of LDR in the activation of NK cells upon encountering tumor targets within the cytokine-containing microenvironment.

MATERIALS AND METHODS

Isolation and culture of NK cells

Female C57BL/6 mice between 6 and 8 weeks of age were purchased from Nara Biotech (Seoul, Korea) and used under the protocol approved by the Korea University Institutional Animal Care and Use Committee (KUIACUC-2009-126). NK cells were enriched from B cell-depleted splenocytes using MACS isolation kits (Miltenyi Biotec, Bergisch Gladbach, Germany) via negative selection using a mixture of Ab including biotin-conjugated anti-CD3 (145-2C11), anti-CD4 (GK1.5), anti-CD8 (53-6.7) and anti-CD19 (MB19-1), as described previously [18]. After depletion of non-NK cells, the purity of CD3-DX5+ NK cells was approximately 80%. Enriched NK cells were cultured in RPMI-1640 (Welgene, Daegu, Korea) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Lonza Walkersville Inc., Walkersville, MD, USA) and 100 U/ml of human rIL-2 (Novartis Pharmaceuticals, East Hanover, NJ, USA). Since recombinant human IL-2 is widely available at an affordable price and exhibits similar activity to mouse IL-2 for both *in vivo* and *in vitro* experiments, we chose to use recombinant human IL-2 throughout this study [19].

Antibodies

FITC (fluoroscein isothiocyanate-conjugated anti-CD3 (145-2C11), anti-CD8 (53-6.7), anti-CD69 (H1.2F3) and anti-NK1.1 (PK136) mAbs and PE (phycoerythrin-conjugated anti-CD4 (GK1.5), anti-NK1.1 (PK136), anti-NKG2D (CX5) and anti-CD244.2 (2B4) monoclonal antibodies (mAbs) were purchased from eBioscience (San Diego, CA, USA). Purified anti-CD16/CD32 mAb (2.4G2) was purchased from Bio X Cell Co (West Lebanon, NH, USA). Annexin V-FITC and a propidium iodide (PI) apoptosis detection kit was purchased from BD Pharmingen (San Diego, CA).

Irradiation

LDR described here indicates delivery of γ-rays at a total cumulative dose of ≤0.2 Gy at a rate of ≤6 mGy/h according to the recommendation by UNSCEAR [20]. In our study, we set the total dose of radiation at 0.2 Gy over 48 h, corresponding to a rate of 4.2 mGy/h. Where indicated, cells in multi-well plates were irradiated with γ-rays (dose rate: 4.2 mGy/h) for 2 days in a CO_2 incubator at 37°C in an irradiation room equipped with a ^{137}Cs γ-ray source (IBL 437C, CIS Bio-international, France). Irradiation dose was calculated by factors including γ-ray source, distance and irradiation time. A glass dosimeter placed in the incubator was used to confirm the radiation dose for each experiment.

^{3}H-thymidine uptake assay

A total of 100 000 purified NK cells/well were incubated with 100 U/ml IL-2 in a 96-well plate as described in each figure. At the end of culture, ^{3}H-thymidine (1 μCi; Amersham Pharmacia Biotech, Seoul, Korea) was added to the well and incubated for an additional 18 h. Cells were harvested onto filter paper with a Micro96 harvester (Skatron, Lier, Norway), and dpm was counted using a γ-counter (Packard Instrument Co., Meriden, CT, USA).

Flow cytometry

Cells were resuspended in 100 µl of FACS buffer (PBS containing 2% FBS and 0.02% sodium azide) and incubated with anti-CD16/CD32 mAb to block FcγIII/II receptors. Without washing, cells were incubated with mAbs conjugated to fluorochrome as described in each figure for 20 min at 4°C. After washing with FACS buffer, cells were fixed in 200 µl of 1% paraformaldehyde in PBS. Flow cytometry was performed with a FACScalibur (BD Bioscience, San Diego, CA, USA) and the data analyzed with CellQuest software (BD Pharmingen, San Jose, CA, USA). A total of 50 000 lymphocyte populations gated by forward scatter (FCS)/side scatter (SSC) were analysed [21].

Apoptosis assay

To assess cell death, NK cells were harvested and stained with Annexin V-FITC. After washing, PI was added to the cells. Data acquisition was performed with FACScalibur and analyzed with CellQuest software. In this flow cytometry-based apoptosis assay, cells stained with Annexin V+ PI– represent cells in early apoptosis, while Annexin V+ PI+ cells represent cells undergoing late apoptosis/necrosis.

Caspase assay

Staining for active Caspase-3 on NK cells was performed according to manufacturer's instructions (BD Biosciences, San Jose, CA). Briefly, 10^6 cells were suspended in 250 µl of permeabilization/fixation buffer for 20 min, washed and then incubated with 5 µl of anti-active Caspase-3 antibody (BD 550821) conjugated to PE for 20 min at room temperature. Camptothecin was used as a positive control for active Caspase-3 staining. After a 10× volume wash, the cells were resuspended and analyzed by flow cytometry.

Cytotoxicity assay

A standard Chromium (^{51}Cr) release assay was performed with minor modifications. In brief, MHC class I-negative RMA/S (a T-cell lymphoma derived from the Rauscher murine leukaemia virus-induced RBL-5 cell line) target cells were labeled with ^{51}Cr (Perkin Elmer, Boston, MA, USA) at 50 µCi/5 × 10^5 cells. A total of 5000 RMA/S cells were mixed with serially diluted cells at the indicated E:T ratio for 4 h at 37°C [18]. The γ-scintillation of supernatant was measured by a γ-counter (Perkin Elmer). The percentage of specific lysis was calculated as follows: 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release).

Statistical analysis

All statistics were performed using non-parametric Mann-Whitney *U* test with SPSS version 12.0 software (SPSS, Chicago, IL, USA). Where the *P* value was less than 0.05, the result was considered significant.

RESULTS AND DISCUSSION

To address the direct effect of LDR on NK cells *in vitro*, NK cells were enriched up to 80% purity using MACS isolation kits prior to irradiation. Since IL-2 is widely used to assess NK cell function *in vitro* [18, 22–24] and also produced by DCs early after sensing microbial maturation-promoting stimuli or influenza viruses *in vivo* [25], we recapitulated this cytokine milieu *in vitro* by stimulating NK cells with a suboptimal dose (100 U/ml) of IL-2 as 'activation' regimen (Fig. 1A). When culturing primary NK cells, the usual loss of 50% after 3 days of culture in low-dose IL-2 at 100 U/ml is not uncommon. Under ideal culture conditions, using high-dose IL-2 (1000 U/ml or more), greater viability is observed than low-dose IL-2, but still cells undergo apoptosis as shown [22]. This likely resulted from the combination of activation-induced cell death as well as death from IL-2 unresponsiveness. We performed a chromium release assay to demonstrate the cytolytic effect of NK cells activated with varying doses of IL-2 in Fig. 1B. We chose a dose that was relatively suboptimal in order to be able to detect possible synergy between IL-2 and LDR. Since a high dose of IL-2 is less physiologic and the effect of LDR on high-dose IL-2-induced cytotoxicity was found to be minimal, we did not pursue the remainder of experiments with high-dose IL-2.

When purified NK cells were cultivated with 100 U/ml of IL-2 and exposed to LDR simultaneously for 2 days, no significant change in cell numbers was observed (Fig. 2A). The $FSC^{hi}SSC^{lo}$-gated live population was approximately ~52% for the Sham and ~49% for the LDR group after culture in IL-2 for 3 days (Top panels, Fig. 2B). The fraction of cells undergoing apoptosis/necrosis, as defined by Annexin V/PI staining, was similar between the two groups in the total ungated population (no gate, middle panels). The gated live population also demonstrated no significant difference in the late apoptotic/necrotic cells between the Sham and LDR groups (R1 gate, bottom panels). To further confirm the rate of cell death in these cultures, we analyzed cells for expression of active caspase 3. As shown in Fig. 2C, the percentage of active caspase 3+ cells was also comparable in the Sham and LDR groups among ungated total (middle panels) or gated live populations (bottom panels). These data demonstrate that LDR did not significantly affect the viability or apoptosis/necrosis processes of purified NK cells in cultures with low dose IL-2.

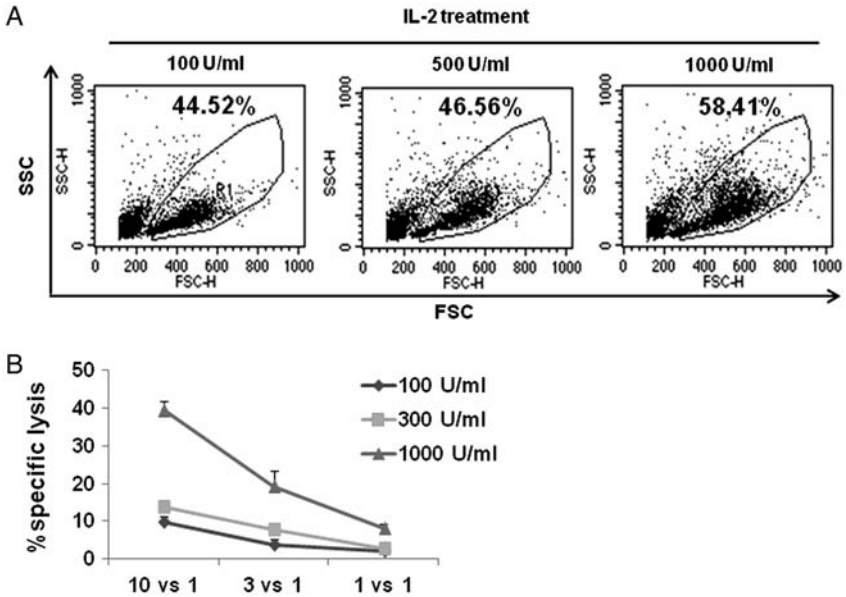

Fig. 1. The effect of suboptimal dose of IL-2 on NK killing activity. (**A**) Purified NK cells cultured with 100, 500 and 1000 U/ml of IL-2 after 6 days were shown as forward and side scatter plots. (**B**) Cytotoxicity against RMA/S tumor targets by NK cells is shown following 6 days culture with a low, medium and high dose of IL-2 (100, 300 and 1000 U/ml, respectively). Suboptimal killing is observed with 100 and 300 U/ml of IL-2.

The rate of cell proliferation, measured at the end of incubation period using ^{3}H-thymidine incorporation assay, demonstrated significantly elevated levels of DNA synthesis in response to IL-2 in both Sham ($19.76 \pm 14.52 \times 10^4$ dpm) and LDR ($14.93 \pm 15.38 \times 10^4$ dpm) groups (Fig. 3A). However, LDR did not appear to alter NK cell proliferation since little or no statistical difference was observed between the Sham and LDR group ($P > 0.05$). Upon IL-2 activation, NK cells develop natural cytotoxicity against tumor targets, such as class I-negative RMA/S T cell lymphoma lines, as seen in Fig. 3B. Thus, we examined whether LDR could influence NK natural cytotoxicity. Anti-tumor effector function of NK cells, as measured by a standard ^{51}Cr release assay against RMA/S cells, was found to be similar in both the Sham and LDR group (Fig. 3B, at the E:T ratio of 10:1 and 3:1, $P > 0.05$). Taken together, these data corroborate the fact that direct exposure of LDR to NK cells was not sufficient to trigger signaling pathways associated with apoptosis, proliferation or cytotoxic function.

The results presented above demonstrate that neither LDR alone nor LDR with suboptimal levels of IL-2 were sufficient to augment NK cell activation *in vitro*. Since healthy individuals often experience prior exposure to low levels of viral or bacterial infections that lead to cytokine activation and generally rapid resolution through NK cell activity, we next questioned if LDR exposure enhances the function of previously cytokine-activated NK cells. For this, purified NK cells were pre-cultured with 100 U/ml of IL-2 for 3 days prior to LDR exposure and NK cell proliferation was assessed 2 days following LDR (Fig. 4A). The $FSC^{hi}SSC^{lo}$-gated live population was comparable between the Sham and LDR groups. When the rate of cell proliferation was measured at the end of incubation, significant levels of DNA synthesis in activated NK cells was observed in response to IL-2 (Fig. 4B). However, exposure of LDR on activated NK cells did not alter the level of proliferation in NK cells ($17.79 \pm 7.59 \times 10^4$ dpm in the Sham vs. $18.80 \pm 4.42 \times 10^4$ dpm in the LDR group, $P > 0.05$, Fig. 4B), similar to those observed in cultures simultaneously treated with LDR + IL-2 (Fig. 3A). In the absence of IL-2 or LDR, NK cells did not undergo proliferation. Strikingly, NK cells activated with IL-2 demonstrated significant enhancement of cytotoxicity, up to 2.1-fold higher ($P = 0.014$ at 10:1 E:T ratio), against RMA/S tumor targets. At an E:T ratio of 10:1, the percentage of lysis in the Sham group was $8.57 \pm 3.40\%$, while that in the LDR group was $17.94 \pm 2.81\%$. Therefore, LDR appeared to specifically enhance anti-tumor cytotoxic function of NK cells but only when NK cells were activated with IL-2. Since increased cytotoxicity by LDR could be partially attributed to the up-regulation of NK cell surface receptors, we next examined the level of NK activation receptors, CD69, and 2B4, NK1.1, NKG2D by flow cytometry. As can be seen in Fig. 4D, all NK cells expressed comparable levels of NK1.1, NKG2D, CD69, 2B4, in both the Sham and LDR groups. Therefore, the enhancement of NK cytotoxicity by LDR was not found to be associated with alteration of

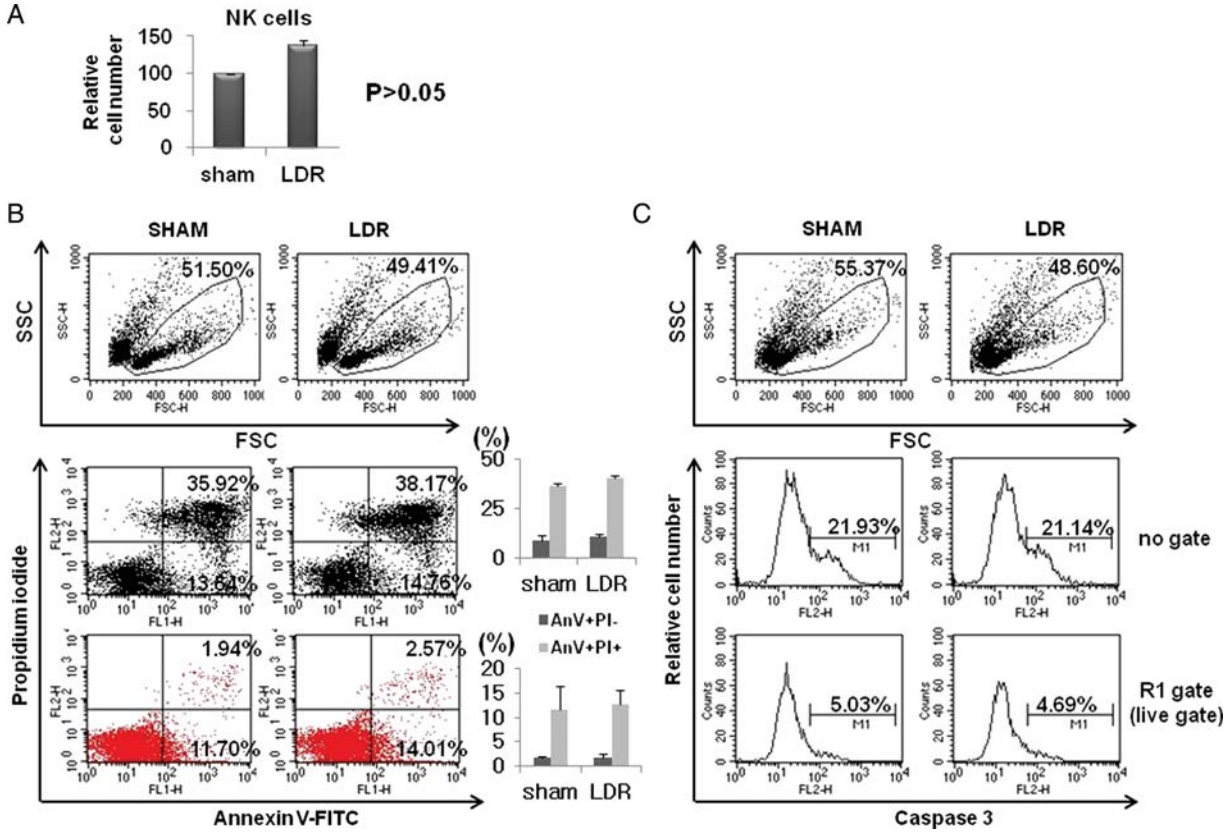

Fig. 2. The effect of LDR on NK cell viability. (**A**) Relative cell numbers at the end of culture were depicted as bar graphs. (**B**) Top, The rates of early and late apoptosis/necrosis of NK cells at the end of culture are shown as Annexin V+ PI– and Annexin V+ PI+ cells in total ungated (no gate) and gated (live gate, R1) populations. Middle, The percentage of early apoptotic (Annexin V+ PI–) cells was found to be 8.85 ± 2.81% in the Sham and 10.92 ± 1.14% in the LDR group, while that of late apoptotic/necrotic (Annexin V+ PI+) cells was 36.55 ± 0.95% and 40.43 ± 1.24% in the Sham and LDR group, respectively (all $P > 0.05$). Bottom, When cells were gated on live, the percentage of early apoptotic (Annexin V+ PI–) cells was found to be 1.67 ± 0.32% in the Sham and 1.78 ± 0.71% in the LDR group, while that of late apoptotic/necrotic (Annexin V+ PI+) cells was 11.74 ± 4.66% and 12.75 ± 2.91% in the Sham and LDR group, respectively (all $P > 0.05$). The data shown as bar graphs are means of three independent experiments. **(C)** Active caspase staining of ungated (no gate) and gated (live gate, R1) populations at end of 6 days of culture for Sham and LDR-treated cells. The data shown are representative of three independent experiments. Error bars represent SD.

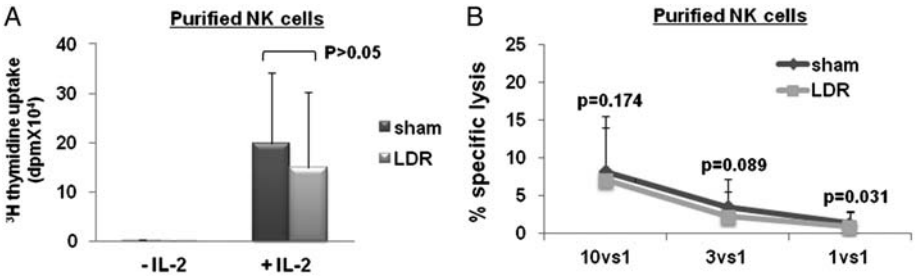

Fig. 3. Neither effector function nor proliferation in response to IL-2 was altered in NK cells exposed to LDR. (**A**) NK cells were subjected to ^{3}H-thymidine incorporation assay to monitor the level of DNA synthesis. The data shown are averaged from three independent experiments. (**B**) Purified NK cells were subjected to ^{51}Cr release assay using RMA/S cells as targets, as described in Materials and Methods. The data shown are representatives of four independent experiments. Error bars represent SD.

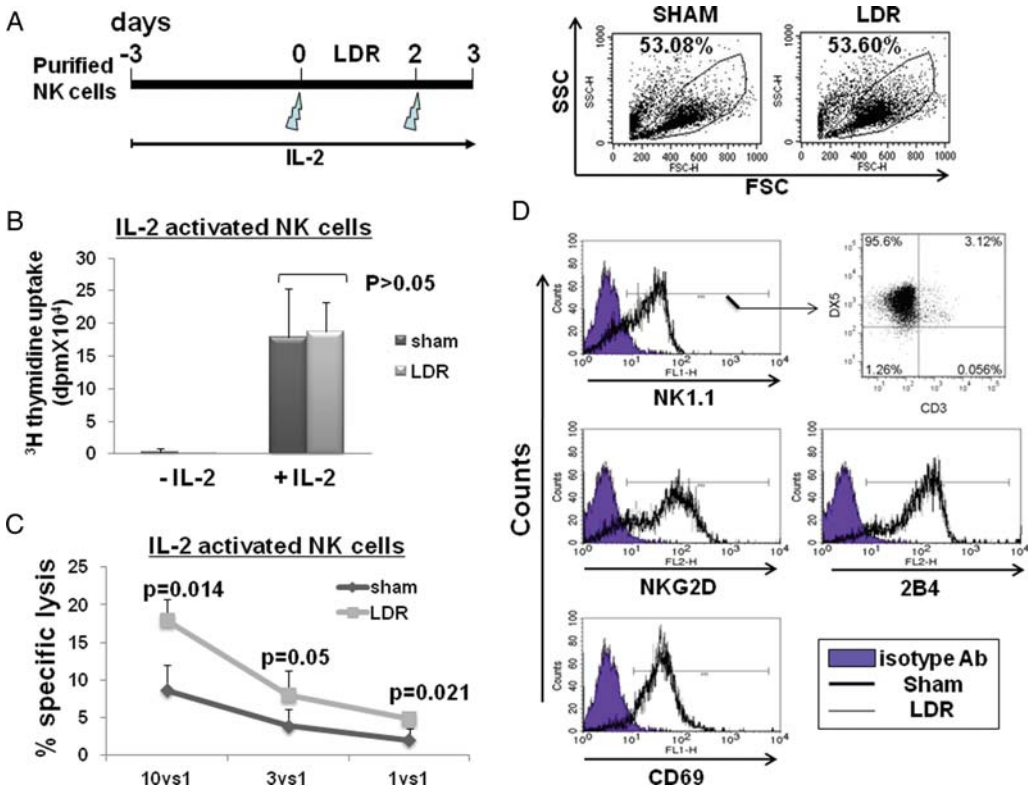

Fig. 4. Exposure to LDR significantly enhances anti-tumor cytotoxicity of NK cells when cells were activated by IL-2. (**A**) Purified NK cells were incubated with 100 U/ml of IL-2 for 3 days prior to the exposure with LDR. Cells were analyzed 1 day after 2-day LDR exposure. On Day 6 of low-dose IL-2 (100 U/ml), both Sham and LDR cultures were evaluated for fraction of viable NK cells. Both cultures showed similar percentages of viable cells by FSC/SSC. The level of DNA synthesis (**B**) and cytotoxicity against RMA/S targets (**C**) were measured, similar to those described in Fig. 3. The data shown are means of three independent experiments (B) and representative of four independent experiments (C). (**D**) Surface expression of NK1.1, NKG2D, CD69 and 2B4 on NK cells at the end of culture was measured by flow cytometry. NK1.1 and NKG2D staining demonstrate near identical populations among Sham and LDR cultures suggesting that the fractions of NK cells in both cultures are equivalent. Nearly all NK1.1^{hi} cells are CD3–, DX5+.

surface activating receptors. Nevertheless, regulation of NK cytotoxicity by LDR, occurring independently of cell proliferation, apoptosis, or surface receptor expression, might provide insights into the functional benefits of LDR at the cellular level.

While it is well established that exposure to sufficiently high doses of radiation can lead to DNA damage, necrotic and apoptotic cell death, and alteration of immune functions [4–7], very little is known regarding the physiological consequences of low-dose or low-dose-rate ionizing radiation. One of the main obstacles to studying the direct effect of LDR comes from the fact that irradiation alone did not generally induce visible changes both at the cellular or whole body levels. Consistent with this, we found no substantial effect of LDR on innate immune NK cells in their viability, apoptosis, proliferation or cytotoxic functions when LDR was given alone or simultaneously with IL-2. To circumvent this hurdle, we attempted to recapitulate *in vivo* conditions following minimal pathogen exposure prior to LDR exposure and decided to prime NK cells with low-dose cytokine, i.e. IL-2. In a series of preliminary experiments, we activated NK cells with 100 U/ml of IL-2 from 1 to 3 days *in vitro* and found that 3-day activation of NK cells ensured sub-optimal cytokine-mediated NK cell activation. In this setting, we found that significant augmentation of anti-tumor cytotoxicity was observed in IL-2-primed NK cells upon irradiation, compared with those not irradiated. However, the non-NK cell population did not contribute significantly to the killing of RMA/S tumor targets as depletion of NK cells from the culture abrogated any detectable tumor target lysis in both groups (data not shown). Therefore, LDR was capable of synergizing NK cytotoxicity among NK cells previously exposed to cytokines or foreign pathogens. This effect was most profound when cells were activated with the suboptimal, and not maximal [23], level of IL-2, demonstrating the co-stimulatory feature of LDR in the presence of weak activating signals. Therefore, the synergism seen between LDR

and cytokines in NK cells may explain, in part, the functional basis underlying diverse beneficial effects seen in the disease animals chronically exposed to LDR.

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