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A novel immunomodulatory mechanism of ribavirin in suppressing natural killer cell function

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ABSTRACT

Ribavirin, a broad-spectrum anti-viral drug, exhibits immunomodulatory activities. To study direct effects of ribavirin on natural killer (NK) cell effector functions and signaling, resting NK cells and interleukin (IL)-15-activated NK cells were treated for 5 days with therapeutic ribavirin concentrations ranging from 5 μg/ml to 20 μg/ml. Both resting and IL-15-activated NK cells that were not treated with ribavirin were used as control. Cytotoxicity assays, flow cytometry, enzyme linked immunosorbent assays, and Western blot experiments were performed to elucidate ribavirin effect on NK cells. Results showed that ribavirin (not toxic at concentrations tested; $IC_{50} > 80$ μg/ml) had no influence on lysis of target cells by freshly isolated NK cells. Conversely, ribavirin dose-dependently inhibited lysis of target cells by up to 66% and impaired interferon gamma production when IL-15-activated NK cells were used. IL-15-induced increased expression and hence function of NK cell activating receptors including NKp30, NKp44, NKp46 and NKG2D were selectively down-regulated and impaired. These inhibitory effects were associated with the down-regulation of IL-15 receptor beta and gamma expression. Accordingly, downstream events involved in NK cell signaling via IL-15 receptors including the activation of Janus kinase (Jak)-1, signal transducer and activator of transcription STAT-1, STAT-3, and STAT-5 as well as pathways responsible for NK cell degranulation including extracellular signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK) were impaired. These results reveal a novel mechanism by which ribavirin exerts its immunomodulatory activities.

1. Introduction

Ribavirin (1-β-D-ribofuranosyl-1,2,4-triazole-3-carboxamide), a synthetic nucleoside analog, is a broad-spectrum inhibitor of RNA and DNA viruses that is currently licensed for the treatment of severe respiratory syncytial virus (RSV) disease [1] and chronic hepatitis C virus (HCV) infection [2,3]. Anti-viral properties of ribavirin were ascribed to inhibition of inosine monophosphate dehydrogenase, interference with viral RNA capping reactions, inhibition of viral polymerase and induction of error catastrophe resulting from the accumulation of lethal mutations in viral genome [4].

Apart from direct anti-viral effects, ribavirin was reported to have immunomodulatory effects on different constituents of the immune system. Ribavirin induced a switch in T-helper (Th) cell phenotype from type 2 to type 1 [5]. In patients with chronic HCV infection, ribavirin was shown to exert anti-inflammatory effect by reducing the blood levels of interferon (IFN)-γ and the expression of IFN-γ by activated T cells [6], thus thereby helping to suppress IFN-γ-driven T cell activation and liver damage [6]. In macrophages, ribavirin inhibited the induction of pro-inflammatory cytokines like interleukin (IL)-1β and tumor necrosis factor (TNF)-α [7]. In dendritic cells, ribavirin suppressed the production of TNF-α, IL-10, and IL-12(p70) [8]. In murine natural killer (NK) cells, ribavirin inhibited IFN-γ production in a dose-dependent manner [9].

NK cells provide the first line of defense and substantially contribute to the elimination of virus-infected cells as well as antitumor immune response [10]. Moreover, IL-15 is known to play a pivotal role in NK cell development *in vivo* as well as in NK cell activation and proliferation and is secreted in response to infectious pathogens to mediate NK cytotoxicity [11]. IL-15 and IL-2 receptor gamma (IL-2Rγ) genes, both involved in lymphocyte activation and signaling, were reported to be down-regulated by ribavirin in RSV-infected human A549 pulmonary type II epithelial cells [12]. In NK cells, the IL-15 receptor includes IL-15Rα, IL-2/15Rβ and γ chain subunits (β and γ_c subunits are shared with IL-2) [11]. As IL-2 and IL-15 share common signaling components, most evidence suggests that the interaction of IL-2 or IL-15 with their respective receptor complex in various cell types leads to a series of

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similar if not identical signaling events [13]. IL-15Rβ is associated with Janus kinase (Jak)-1 and the γ_c is associated with Jak-3, resulting in signal transducer and activator of transcription (STAT)-3 and STAT-5 phosphorylation respectively, following ligation with IL-15 [13]. IL-15 also activates the phosphorylation of STAT-1 [14,15]. In addition to signaling via IL-15/IL-15R, NK cell activation is also tightly regulated by a delicate balance between signaling through inhibitory [killer immunoglobulin-like receptors (KIR), CD94-NK group 2, member A (NKG2A)] and activating receptors [natural cytotoxic receptors (NCRs-NKp30, NKp44 and NKp46), NK group 2, member D (NKG2D) and DNAX accessory molecule-1 (DNAM-1)] [10]. The expressions of these receptors are up-regulated upon treatment with IL-15 [16,17]. Inhibitory receptors consist of a single polypeptide containing cytoplasmic immunoreceptor tyrosine-based inhibitory motifs that recruit tyrosine phosphatases and abort signaling. On the other hand, activating receptors typically couple to signaling adaptors that contain either an immunoreceptor tyrosine-based activating motif (ITAM, such as DAP12, FcRγ or CD3ζ) or 'YXNM' motif, where 'X' is any amino acid (such as the signaling adaptor DAP10) through interactions specified by their transmembrane regions [18]. Interestingly, signaling through DAP10, an adaptor that transmits important signals for NKG2D [19], was shown to be coupled to IL-15R signaling pathway [20]. Here, DAP10 was shown to specifically bind β- and γ-chains of IL15R [20].

In this report, we have studied the direct effect of ribavirin on effector functions (including cytolytic activity and IFN-γ production) of IL-15-activated human NK cells as well as molecular mechanisms by which ribavirin influences NK cell functions. We observed that ribavirin exerts immunomodulatory activities on NK cells by down-regulating the expression of IL-15Rβ and γ and thus inhibiting downstream events involved in NK cell signaling (IL-15-activation of Jak/STAT pathway) and degranulation [extracellular signal-regulated kinase (ERK1/2) and c-Jun N-terminal kinase (JNK) signaling pathways].

2. Materials and methods

2.1. Reagents and monoclonal antibodies

Ribavirin (Virazole®) was obtained from Valeant Pharmaceuticals Germany GmbH (Eschborn, Germany), DETA NONO-ate and tetrahydrobiopterin were purchased from Sigma–Aldrich (Taufkirchen, Germany), recombinant human IL-15 was from Cell Concepts (Umkirch, Germany). The following phycoerythrin (PE)-conjugated anti-human monoclonal antibodies (mAbs) were used: NKp30, NKp44, NKp46, NKG2D, DNAM-1, KIR- KIR2DL2/DL3 and KIR3DL1, all from Miltenyi Biotec (Bergisch Gladbach, Germany), NKG2A, IL-2Rβ and IL-2Rγ were from R&D systems (Wiesbaden, Germany), perforin and granzyme B were from Abcam (Cambridge, UK). Unconjugated IL-15Rα was from R&D systems (Wiesbaden, Germany). Fluorescein isothiocynate (FITC)-conjugated lymphocyte function antigen (LFA)-1 was from BD Pharmingen (San Diego, CA, USA). For redirected killing experiments, purified NKp30, NKp44, NKp46, NKG2D, DNAM-1 mAb (Miltenyi Biotec, Bergisch Gladbach, Germany) were used.

2.2. Cells

Human erythroleukemic K562 cell line was obtained from American type culture collection (ATCC; Manassas, VA, USA). P815 FcγR$^+$ murine cell line was from DSMZ, Braunschweig, Germany. K562 cell line was grown in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20% fetal calf serum (FCS), while P815 cell line was grown in IMDM supplemented with 10% FCS. Media and supplements were from Seromed (Berlin, Germany).

2.3. Polyclonal NK cell preparation

Human PBMCs were isolated from the blood of healthy volunteers by Ficoll–Hypaque centrifugation followed by separation using the MACS NK cell isolation kit II (Miltenyi Biotec, Bergisch Gladbach, Germany) according to manufacturer's protocol. Flow cytometric analysis to determine purity of NK cells showed that more than 95% of the cells were CD56+CD3− (not shown). NK cells were cultured using IMDM containing 10% FCS, with or without 40 U/ml recombinant IL-15. For all experiments, freshly isolated NK cells or IL-15-activated NK cells were simultaneously treated with or without ribavirin at indicated concentration for 5 days.

2.4. Cytotoxicity assay and flow cytofluorometric analysis

Cytotoxicity of NK cells was determined by a 4 h coupled luminescent method using the "ACELLA-TOX™" kit (Cell Technology, Mountain View, CA, USA), as described in Ref. [21]. K562 and P815 FcγR$^+$ cell lines were used as target cells. For redirected killing experiments, 1 μg/ml of the corresponding purified mAb was used. Flow cytometry (FACS Calibur; Becton Dickinson, Mountain View, CA, USA) was used for cell surface expression analysis.

2.5. Cell cycle analysis

Cell cycle was determined using a commercial kit (BD Biosciences, San Jose, CA, USA) following the manufacturer's instructions as described previously [22].

2.6. Viability assay

The effect of ribavirin on viability of IL-15-activated NK cells was assessed using CELL TITRE-GLO™ luminescent cell viability assay (Promega, Mannheim, Germany). This assay is a homogenous method of determining the number of viable cells in culture based on the quantitation of ATP present, which signals the presence of metabolically active cells. After 5 days treatment with ribavirin 100 μl cell suspension was incubated with 100 μl of CELL TITRE-GLO™ reagent and contents were allowed to mix on an orbital shaker in accordance with the assay protocols, this resulted in cell lysis and generation of a luminescent signal proportional to the amount of ATP present. The amount of ATP is proportional to the number of cells present in culture. The luminescence signal was recorded with a luminometer (Glomax; Promega, Mannheim, Germany). Trypan blue exclusion assay was additionally used to assess the amount of dead cells.

2.7. NK cell degranulation experiment

NK cells were stimulated by mAb cross-linking as previously described [15,23]. Briefly, after 5 days of culture in 40 U/ml IL-15 with or without ribavirin, cells were labeled with 1 μg/ml appropriate mAbs for 30 min at 4 °C. After washing, cells were stimulated with 10 μg/ml AffiniPure F(ab′)$_2$ Fragment Goat Anti-Mouse IgG (Jackson ImmunoResearch, West Grove, PA, USA) for 5 min at 37 °C. Reaction was stopped with ice-cold PBS. After overnight incubation at 37 °C, supernatants were collected for analysis and quantification of granule release by ELISA assay (Perforin/Granzyme B-ELISA kit, Diaclone Research, Besancon Cedex, France) according to manufacturer's instructions.

2.8. Western blot

Cells were cultured in IL-15 and in the presence or absence of ribavirin for 18 h and 5 days. Afterwards, Western blot analysis

was performed as previously described [24]. Briefly, cell lysates were subjected to SDS-PAGE before transfer to nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany) using the Mini-Protean II system (Bio-Rad, Munich, Germany). After transfer, blots were blocked in tris-buffered saline (TBS) blocking buffer containing 3% bovine serum albumin for 1 h at room temperature to saturate the non-specific protein-binding sites on the nitrocellulose membrane. The following primary rabbit polyclonal Abs were used: Jak-1, phospho-Jak-1 (tyrosine (Tyr)1022/1023), Jak-3, STAT-1, phospho-STAT-1 (Tyr701), STAT-3, phospho-STAT-3 (Tyr705), STAT-5, phospho-STAT-5 (Tyr694), ERK1/2, phospho-ERK1/2, JNK, phospho-JNK—all from Cell Signaling (Beverly, MA, USA). Phospho-Jak-3 (Tyr980) was from Santa Cruz biotechnology (Santa Cruz, CA, USA), mouse polyclonal beta actin Ab was from Sigma–Aldrich (Taufkirchen, Germany). The blots were incubated overnight with the primary Ab diluted in TBS at 4 °C with gentle agitation. Following a 1 h incubation period with peroxidase-conjugated secondary Ab at room temperature visualization was performed by enhanced chemiluminescence using a commercially available kit (Amersham, Liverpool, UK).

2.9. Measurement of IFN-γ production

A total of 2×10^4 K562 cells were cocultured for 24 h with 1×10^5 NK cells cultured in IL-15 and in the presence or absence of ribavirin. Both ribavirin-treated and untreated IL-15-activated NK cells alone or K562 cells were used as control. Supernatants were collected and tested for production of IFN-γ. The amounts of IFN-γ were determined using the Quantikine Human IFN-γ ELISA kit (R&D Systems, Wiesbaden, Germany) according to manufacturer's protocol.

2.10. Statistics

Values presented are the mean ± standard error of mean (S.E.M.) of at least three experiments. Comparisons between two groups were performed using Student's *t*-test. *P*-values lower than 0.05 were considered to be significant.

2.11. Measurement of transcription factor activation

The activation of nuclear factor kappa B (NFκB) p50, NFκB p65, T-box expressed in T cells (T-bet), activator protein-1 (AP-1) (c-Jun), and nuclear factor of activated T cells (NFAT) in ribavirin-treated and untreated IL-15-activated NK cells was determined using TRANSAM™ ELISA kits (Active Motif, Carlsbad, CA, USA) for the corresponding transcription factor, according to manufacturer's protocols.

3. Results

3.1. Effect of RBV on viability of NK cells

We previously demonstrated that ribavirin neither induced a block in the cell cycle progression nor exerted cytotoxic effects in primary HUVEC at concentrations up to 20 μg/ml and 100 μg/ml respectively [25]. We therefore studied effects of ribavirin at concentrations ranging from 5 μg/ml to 80 μg/ml on viability of IL-15-activated NK cells. For this purpose, IL-15-activated NK cells were treated for 5 days with ribavirin. Dead cells were identified with fractional DNA content ("sub-G1 fraction"). Untreated NK cells were used as control. Results revealed ribavirin to be non-toxic to IL-15-activated NK cells at concentrations up to 40 μg/ml. At a concentration of 80 μg/ml, ribavirin induced about 40% cell death in IL-15-activated NK cells. Ribavirin also induced a G1 block in the cell cycle progression of the cells at concentrations ranging between 20 μg/ml and 80 μg/ml (Fig. 1A). Trypan blue and ATP release assays also revealed that ribavirin did not exert significant cytotoxic effect in IL-15-activated NK cells at concentrations up to 20 μg/ml (Fig. 1B). The cell viability (ATP release) was however reduced to 59% at 80 μg/ml ribavirin concentration.

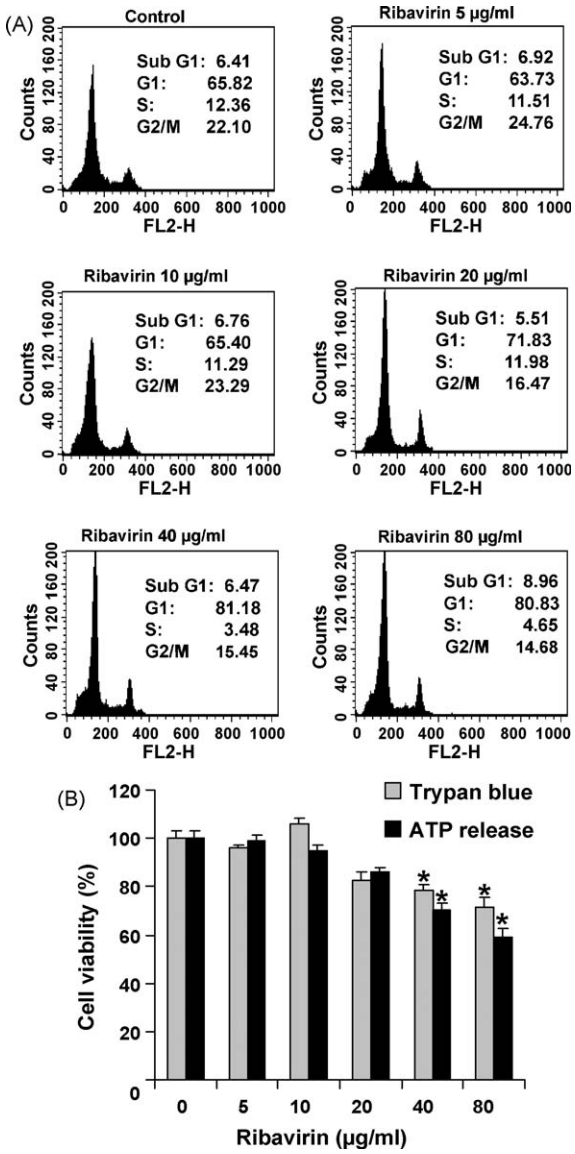

Fig. 1. Influence of ribavirin on cell cycle and viability of NK cells. Primary NK cells from healthy donors were cultured in 40 U/ml IL-15 and in the presence or absence of ribavirin at indicated concentrations for 5 days. (A) The influence of ribavirin on cell cycle was determined by staining cells with propidium iodide. Values represent percentage of cells in the different phases. The percentages of cells in G1, S and G2/M phases were deduced from the number of viable cells (set to 100%) after subtracting the dead cells (sub G1) from total gated cells. One representative of 3 different experiments is shown. (B) Concentration-dependent influence of ribavirin on NK cell viability using both trypan blue exclusion and ATP release assays. Columns represent means of triplicate of one representative experiment; error bars indicate ±S.E.M.; $^*P < 0.05$ relative to control.

3.2. Ribavirin suppresses NK cell cytotoxicity and IFN-γ production

The role of ribavirin in NK cell cytotoxicity was next investigated. Both freshly isolated NK cells and IL-15-activated NK cells were treated with 5 μg/ml, 10 μg/ml, and 20 μg/ml ribavirin for 5 days after which the cytotoxicity against K562 cells was determined using a 4 h coupled luminescent method [21]. The

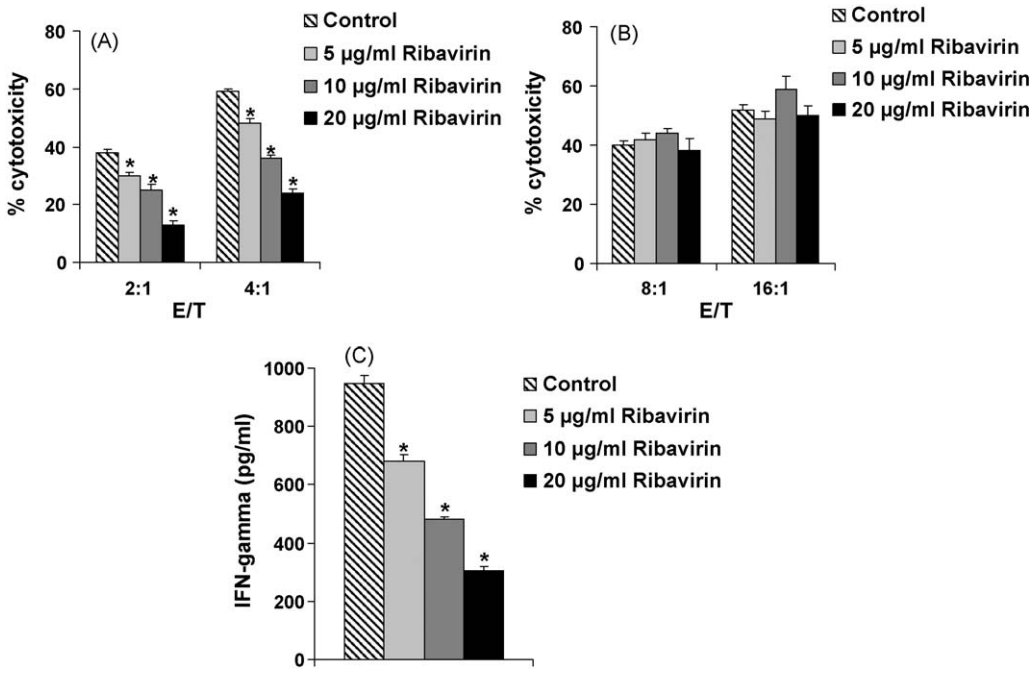

Fig. 2. Effect of ribavirin on NK cell cytotoxicity and IFN-γ production. A 4 h cytotoxicity assay against 5000 K562 target cells was performed at indicated *E*/*T* ratios using (A) ribavirin-treated and untreated (control) IL-15-activated NK cells and (B) ribavirin-treated and untreated (control) NK cells. (C). IFN-γ production in ribavirin-treated and untreated (control) IL-15-activated NK cells was measured as described in Section 2. Columns represent means of triplicate of one representative experiment; error bars indicate ±S.E.M.; $^{*}P < 0.05$ relative to control.

results obtained show that ribavirin significantly suppressed IL-15-activated NK cell lysis of K562 in a dose-dependent manner (Fig. 2A). The % inhibition in comparison to control were 21%, 34.2%, and 65.8% respectively for 5 μg/ml, 10 μg/ml, and 20 μg/ml ribavirin at an effector/target (E/T) ratio of 2:1. At *E*/*T* ratio of 4:1, the % inhibition was 18.6%, 39%, and 59.3% respectively for 5 μg/ml, 10 μg/ml, and 20 μg/ml RBV (Fig. 2A). On the other hand, ribavirin did not influence lysis of K562 target cells by freshly isolated NK cells (Fig. 2B). Since IL-15-activated NK cell cytotoxicity was inhibited upon simultaneous addition of IL-15 and ribavirin for 5 days, we tested the effect of ribavirin on IL-15-activated NK cells after 18 h ribavirin treatment. Here, NK cells were stimulated for 5 days with IL-15 (10 ng/ml) prior to addition of 20 μg/ml ribavirin. Results show a 23.8% reduction in lysis of K562 target cells in ribavirin-treated NK cells compared to untreated control (E/T ratio 4:1) but no ribavirin-induced cytotoxicity in NK cells (data not shown). The inhibition of NK cytotoxicity is known to be associated with inhibition of IFN-γ production [26,27]. The production of INF-γ by IL-15-activated NK cells was therefore measured upon coculture of IL-15-activated NK cells and ribavirin-treated IL-15-activated NK cells with K562 target cells. Ribavirin dose-dependently suppressed IFN-γ production in IL-15-activated NK cells. The % inhibition in comparison to control was 28.1%, 49.1%, and 67.7% respectively for 5 μg/ml, 10 μg/ml, and 20 μg/ml ribavirin (Fig. 2C). As more than 50% inhibition of NK lytic capacity and cytokine production was achieved by 20 μg/ml ribavirin treatment, and at this concentration ribavirin was not toxic to the cells, further experiments to clarify the mechanisms by which ribavirin inhibits IL-15-activated NK cell function were performed using 20 μg/ml.

3.3. Ribavirin suppresses NK cell cytotoxicity by mechanisms other than tetrahydrobiopterin or NO inhibition

Previous studies indicated that ribavirin acts by inhibition of tetrahydrobiopterin, a cofactor of NO synthase enzymes, thereby leading to suppression of NO synthesis [25,28]. We therefore investigated whether ribavirin suppressed IL-15-activated NK cell cytotoxicity by tetrahydrobiopterin inhibition. For this purpose, we exogenously added tetrahydrobiopterin or NO to ribavirin-treated and untreated IL-15-activated NK cells to demonstrate if inhibitory effects on NK cell cytotoxicity induced by ribavirin can be reversed by tetrahydrobiopterin or NO addition. The results obtained show that addition of tetrahydrobiopterin or NO did not reverse the inhibitory effects of ribavirin on IL-15-activated NK cell cytotoxicity (Fig. 3). While treatment of IL-15-activated NK cells with tetrahydrobiopterin alone did not influence NK cell lytic capacity treatment with NO alone significantly suppressed cytotoxicity of NK cells (Fig. 3).

3.4. Ribavirin interferes with IL-15R expression and its associated downstream signaling events

The results from NK cell cytotoxicity showed that only IL-15-activated NK cells were inhibited by ribavirin treatment. It is therefore likely that ribavirin interferes with signaling pathways involved in IL-15 activation of NK cells. We therefore analyzed for the expression of IL-15Rα, β, and γ by flow cytometry as well as the Tyr phosphorylation of Jak-1, Jak-3, STAT-1, STAT-3, and STAT-5 by Western blot in NK cells cultured in IL-15 and in the presence or absence of ribavirin. As shown in Fig. 4A, ribavirin inhibited IL-15Rβ and γ expression. IL-15Rα expression was not changed (not shown). Also, Jak-1, STAT-1 and STAT-3 Tyr phosphorylation were sharply inhibited by ribavirin, while a weak inhibition of STAT-5 was observed (Fig. 4B). Ribavirin did not inhibit Tyr phosphorylation of Jak-3 (Fig. 4B). We also studied effect of ribavirin on IL-15 signaling after 18 h IL-15/ribavirin treatment of NK cells. The results obtained revealed the inhibition of Jak-1, STAT-1, STAT-3, but not of Jak-3 and STAT-5 phosphorylation (Fig. 4C). IL-2Rβ and γ expression was also suppressed (not shown) after 18 h ribavirin treatment.

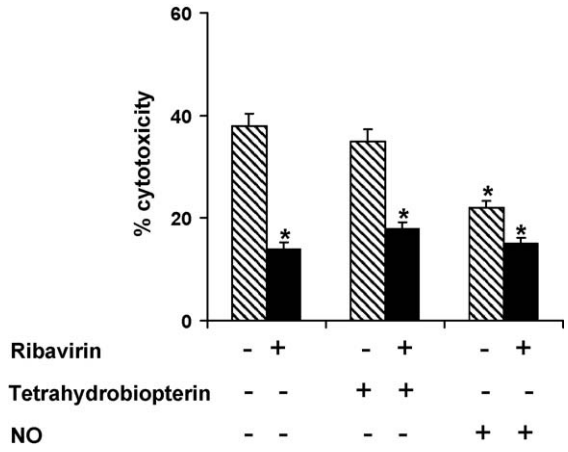

Fig. 3. Ribavirin does not suppress NK cytotoxicity by inhibiting tetrahydrobiopterin or NO. Primary NK cells from healthy donors were cultured in 40 U/ml IL-15 and in the presence or absence of 20 μg/ml ribavirin for 5 days. Tetrahydrobiopterin (10 μM) or DETA NONO-ate (used as NO donor; 5 μM) was added to ribavirin-treated or untreated IL-15-activated NK cells for the 5 days incubation period. IL-15-activated NK cells were used as control. A 4 h cytotoxicity assay against 5000 K562 target cells was performed at an *E*/*T* ratio of 4:1. Columns represent means of triplicate of one representative experiment; error bars indicate ±S.E.M.; $^{*}P < 0.05$ relative to control.

3.5. Ribavirin down-modulates NK cell activating receptor expression

NK cell cytotoxicity is critically dependent on signaling through its receptors, leading to granule polarization and exocytosis. Since IL-15 enhances NK cell cytotoxicity by up-regulating the expression of NK receptors, it is conceivable that the inhibition of IL-15 signaling via IL-15Rβ and γ by ribavirin might lead to a suppressed expression of NK cell receptors responsible for cytotoxicity signals. To address these issues, we investigated the surface expression patterns of NK cell triggering receptors NKp30, NKp44, NKp46, NKG2D and DNAM-1 as well as NK cell inhibitory receptors NKG2A, KIR3DL1 and KIR2DL2/DL3 in both untreated and ribavirin-treated IL-15-activated NK cells. A correlation between NK cell cytotoxicity and NK cell receptor expression pattern was observed. The lytic capacity of ribavirin-treated IL-15-activated NK cells was associated with a decreased surface expression of NKp30, NKp44, NKp46 and NKG2D in comparison to untreated control. No changes were observed in the surface expression of DNAM-1, NKG2A, KIR3DL1 and KIR2DL2/DL3 (Fig. 5A). Although not shown, ribavirin did not suppress the surface expression of triggering and inhibitory receptors in NK cells cultured without IL-15. Basically NK cell activating or inhibitory receptor expression levels were 3–4 folds higher in IL-15 cultured NK cells than in NK cells cultured without IL-15 (not shown). Since NK cytolysis also depends on binding mediated by adhesion molecules like LFA-1, we determined effects of ribavirin on LFA-1 surface expression. Ribavirin did not modify the expression of LFA-1 (Fig. 5A). These results suggest that ribavirin acts directly on selected NK cell receptors rather than by interfering with NK cell binding to target cells.

3.6. Ribavirin suppresses NK cell receptor function

To assess whether ribavirin-induced down-modulation of NK cell activating receptors correlated with an alteration of receptor function, ribavirin-treated and untreated NK cells were compared

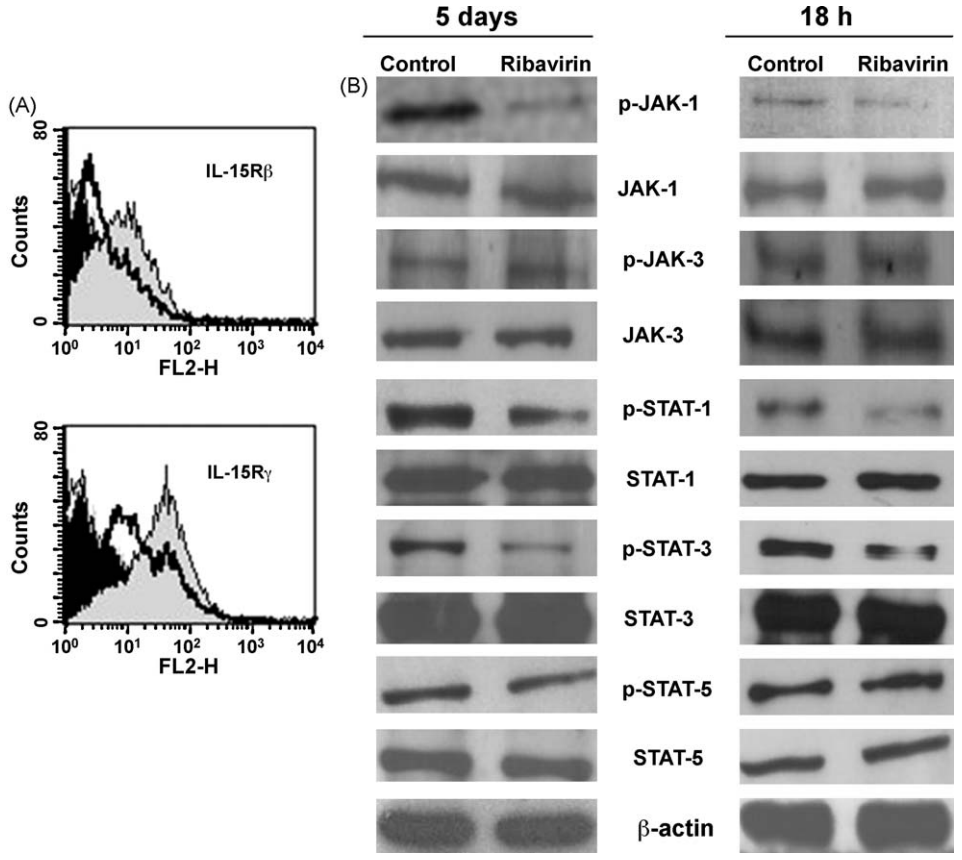

Fig. 4. Ribavirin interferes with IL-15 signaling. NK cells were treated for 18 h or 5 days with IL-15 and in the presence or absence of 20 μg/ml ribavirin. (A) Flow cytometric analysis for the expression of IL-15 receptors in NK cells treated with IL-15/ribavirin for 5 days. Grey line and black filled histogram indicate isotype control staining for untreated and ribavirin-treated IL-15-activated NK cells respectively; grey filled histogram and black line indicate antibody staining for untreated and ribavirin-treated IL-15-activated NK cells respectively. One representative of at least 5 separate experiments is shown. (B) The same amount of protein extracts prepared from ribavirin-treated and untreated IL-15-activated NK cells was used for Western blot analysis of the indicated signaling proteins. Data are representative of at least three experiments.

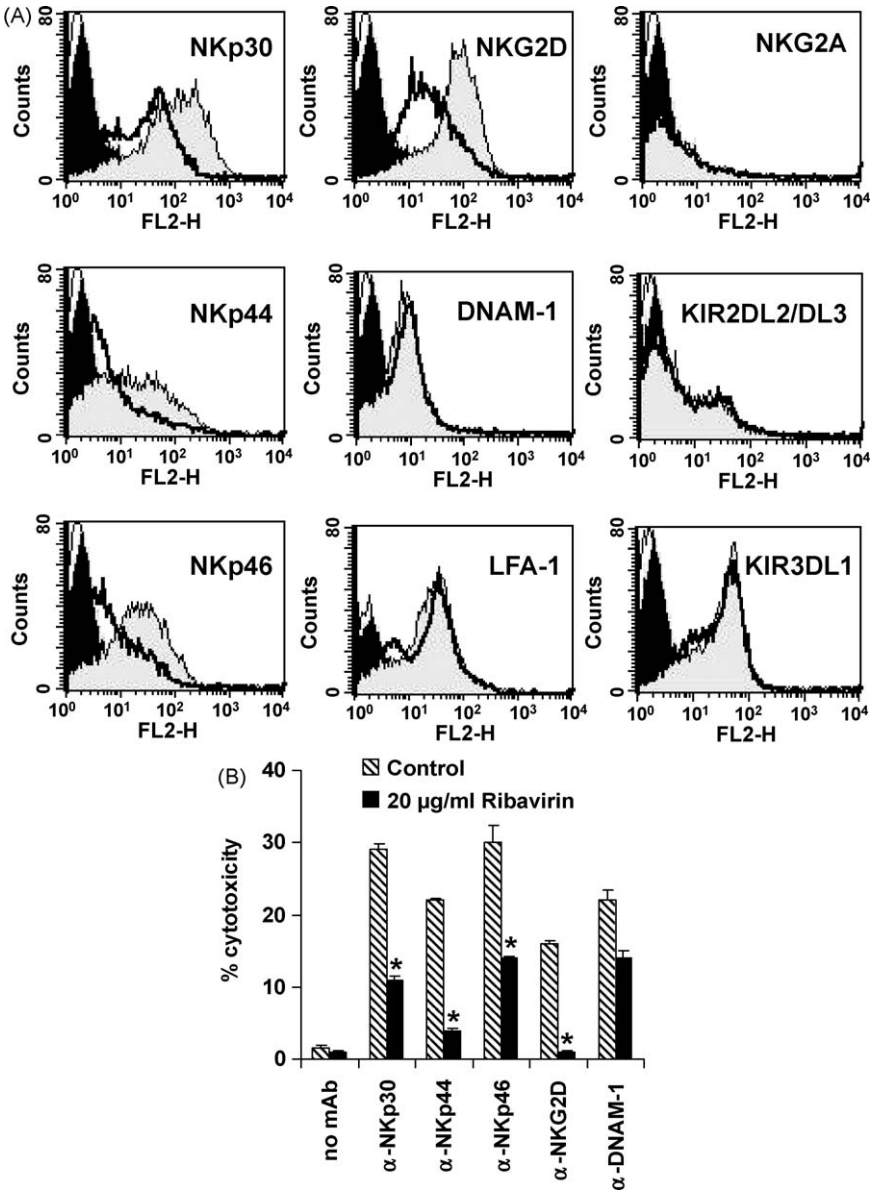

Fig. 5. Effect of ribavirin on NK cell receptor expression and function. NK cells were treated with IL-15 and in the presence or absence of 20 μg/ml ribavirin. (A) Flow cytometric analysis for the expression of NK activating and inhibitory receptors. Grey line and black filled histogram indicate isotype control staining for untreated and ribavirin-treated IL-15-activated NK cells respectively; grey filled histogram and black line indicate antibody staining for untreated and ribavirin-treated IL-15-activated NK cells respectively. One representative of at least 3 separate experiments is shown. (B) A 4 h NK cell cytotoxicity was assessed in a redirected killing assay against the FcγR$^+$ P815 target cell line either in the presence or absence of mAbs to the indicated receptors. Columns represent means of triplicate of one representative experiment; error bars indicate ± S.E.M.; $^*P < 0.05$ relative to control.

in a redirected killing assay against FcγR$^+$ P815 target cell line. The FcγR$^+$ P815 cell line has been extensively used for mAb-mediated redirected killing assays using NK cells and mAbs capable of triggering their cytolytic functions [23,29]. This would allow us assess in a cytolytic assay the direct effect of ribavirin on the specific activity of NK triggering receptors. As shown in Fig. 5B, treatment of IL-15-activated NK cells with 20 μg/ml ribavirin significantly reduced the ability of anti-NKp30 (19.3- vs. 11-fold increase for untreated and ribavirin-treated IL-15-activated NK cells respectively compared to control), anti-NKp44 (14.7- vs. 4-fold increase for untreated and ribavirin-treated IL-15-activated NK cells respectively compared to control), anti-NKp46 (20- vs. 14-fold increase for untreated and ribavirin-treated IL-15-activated NK cells respectively compared to control) and anti-NKG2D (10.7- vs. 1-fold increase for untreated and ribavirin-treated IL-15-activated NK cells respectively compared to control) mAbs to induce NK cell-mediated lysis. The ability of anti-DNAM-1 mAb to induce NK cell lysis was not significantly impaired by ribavirin treatment (14.7- vs. 14-fold increase for untreated and ribavirin-treated IL-15-activated NK cells respectively compared to control). These findings suggest that ribavirin impairs NK cell cytotoxicity by interfering with the expression and function of NKp30, NKp44, NKp46 and NKG2D triggering receptors.

3.7. Ribavirin interferes with pathways involved in NK cell degranulation and impairs NK cell granule release

Two downstream signaling pathways, ERK and JNK, have been implicated to be responsible for lytic granule polarization in NK cells [15,30–34]. We therefore analyzed by Western blot the phosphorylation of ERK1/2 and JNK in NK cells cultured in IL-15 and in the presence or absence of ribavirin. As revealed in Fig. 6A,

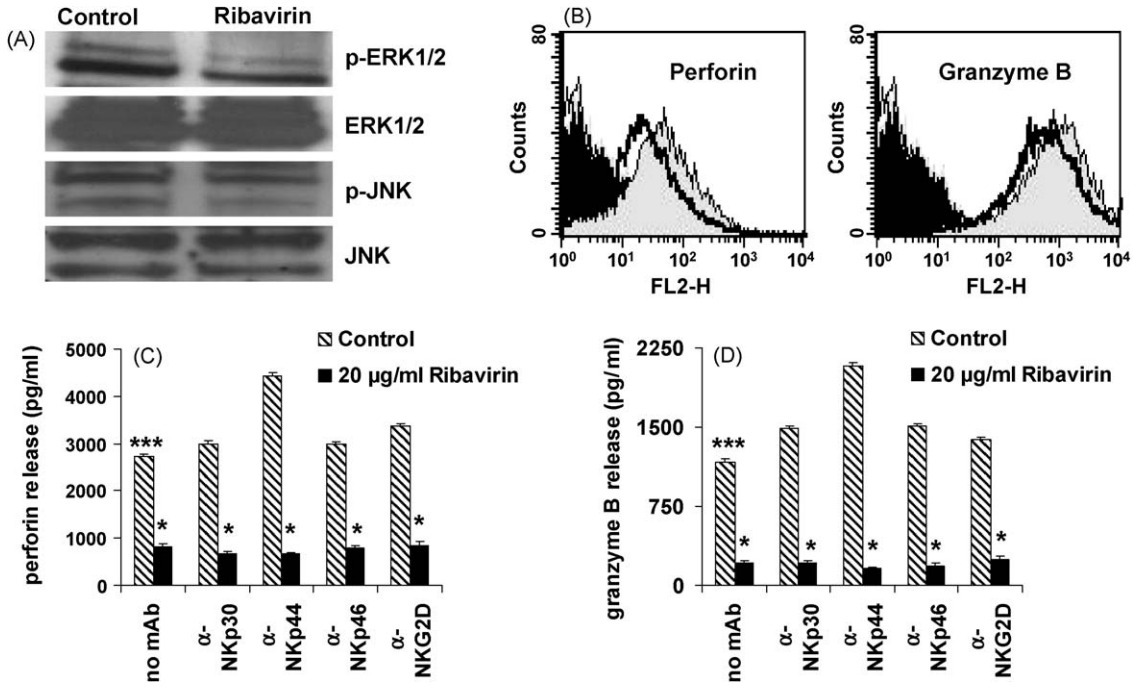

Fig. 6. Ribavirin interferes with NK cell degranulation pathway and granule exocytosis. NK cells were treated with IL-15 and in the presence or absence of 20 μg/ml ribavirin. (A) The same amount of protein extracts prepared from ribavirin-treated and untreated IL-15-activated NK cells was used for Western blot analysis of the indicated signaling proteins. Data are representative of at least three experiments. (B) Flow cytometric analysis for the expression of perforin and granzyme B lytic granules in ribavirin-treated and untreated IL-15-activated NK cells. Grey line and black filled histogram indicate isotype control staining for untreated and ribavirin-treated IL-15-activated NK cells respectively; grey filled histogram and black line indicate antibody staining for untreated and ribavirin-treated IL-15-activated NK cells respectively. One representative of at least 3 experiments is shown. (C and D) Ribavirin-treated and untreated IL-15-activated NK cells were stimulated by cross-linking NK receptors with indicated mAb. After overnight incubation at 37 °C in IMDM+10% FCS alone, supernatants were collected and analyzed in an ELISA assay specific for *in vitro* quantitative determination of perforin and granzyme B release. Columns represent means of triplicate of one representative experiment; error bars indicate ±S.E.M.; $^{*}P < 0.05$ relative to control; $^{***}P < 0.05$ relative to mAb control.

ERK1/2 and JNK phosphorylation was inhibited by ribavirin. From the above results, it was therefore necessary to clarify whether the inhibition of ERK and JNK phosphorylation by ribavirin correlated with reduced granule expression and exocytosis. To this end, intracellular perforin and granzyme B expression were measured by flow cytometry in both untreated and ribavirin-treated IL-15-activated NK cells. Also perforin and granzyme B degranulation after stimulation of cells with mAb as described was analyzed. As shown in Fig. 6B, ribavirin inhibited both perforin and granzyme B expression in IL-15-activated NK cells. A sharp impairment of perforin and granzyme B release was also observed upon treatment of IL-15-activated NK cells with ribavirin (Fig. 6C and D).

4. Discussion

The data presented in this study show for the first time that ribavirin inhibits human NK cell function. Moreover, evidence is presented about the molecular mechanisms that underlie the immunomodulatory activities of ribavirin in NK cells. NK cells mediate host cell defenses by direct lysis of target cells and/or by production of IFN-γ [30]. Suppression of NK-cell cytotoxicity may be associated with the inhibition of IFN-γ production [26,27]. Our results show that while ribavirin had no effect on lytic activity of NK cells that are not treated with IL-15, it exerted a dose-dependent inhibition on the lytic activity of IL-15-treated NK cells. Ribavirin also dose-dependently inhibited the production of IFN-γ in IL-15-activated NK cells. Although not shown, ribavirin also suppressed the function of IL-2-activated NK cells. Remarkably, we found that NK cells from different donors can display different susceptibilities to ribavirin treatment in the presence of IL-15. Thus, in 2 of 15 donors, cytotoxicity and IFN-γ production was not inhibited by ribavirin treatment.

In an attempt to identify the possible mechanism by which ribavirin inhibits cytotoxicity of IL-15-activated NK cells, we investigated a mode of action that has been ascribed to ribavirin—the inhibition of tetrahydrobiopterin synthesis and hence NO synthesis inhibition [25,28]. NO is a highly reactive free radical synthesized in vessel endothelium, immune cells, brain, and other tissues. NO-mediated cell death is prominent among the many paths the immune system uses to kill target cells [28,35]. While some studies suggest that NO blocks activation of NK and cytotoxic T lymphocyte cells [36–38], another report suggests that NO has no effect on lymphokine activated killer (LAK) cells [39]. Some more studies however showed that NO increases LAK activity [40–42]. Results presented in this report show that addition of tetrahydrobiopterin or exogenous NO to ribavirin-treated IL-15-activated NK cells did not prevent ribavirin-induced inhibition of NK cell cytotoxicity. This indicates that ribavirin suppresses IL-15-activated NK cell cytotoxicity by a mechanism other than tetrahydrobiopterin or NO depletion. While treatment of IL-15-activated NK cells with tetrahydrobiopterin alone did not influence NK cell lytic capacity treatment with NO alone significantly suppressed cytotoxicity of NK cells. These experiments were performed with highest non-toxic concentrations of tetrahydrobiopterin (10 μg/ml) and NO (5 μg/ml). Higher concentrations (tetrahydrobiopterin (20 μg/ml), NO (10 μg/ml)), turned out to be cytotoxic in NK cells.

Since inhibition of NK cell activity by ribavirin occurred when NK cells were treated with IL-15, it is conceivable that the inhibition of NK cell cytotoxicity and IFN-γ production was due to the interference of ribavirin with IL-15 receptor expression and/or signaling in NK cells. IL-15 signaling via its receptors in NK cells involves the Tyr phosphorylation of Jak-1, Jak-3, STAT-1, STAT-3, and STAT-5 [13,15,43,44]. The results presented in this report show

that ribavirin suppressed expression of IL-15Rβ and γ in IL-15-activated NK cells. It also inhibited Tyr phosphorylation of Jak-1, STAT-1, STAT-3, but not Jak-3 and STAT-5 after 18 h IL-15/ribavirin treatment of NK cells. The inhibition of Jak-1, STAT-1 and STAT-3 phosphorylation remained after 5 days IL-15/ribavirin treatment of NK cells. In contrast to the previous report showing maximal STAT-5 activation after 18 h IL-15 stimulation of NK cells, and then abolishment of STAT-5 phosphorylation after 48 h IL-15 stimulation [45], we still found phosphorylated p-STAT-5 as late as 5 days after start of IL-15 stimulation. Ribavirin caused a weak inhibition of STAT-5 phosphorylation after 5 days. The reasons for the differences in kinetics of IL-15-induced STAT-5 phosphorylation observed by Pillet et al. [45] and us remain obscure. However, there are distinct differences in the experimental procedures applied that may contribute to the varying results. While Pillet et al. [45] precultured human PBMC in the presence of 150 pM IL-15 for 18 h or 48 h and then restimulated with 500 pM IL-15 for 15 min, we treated NK cells with 10 ng/ml IL-15 for 5 days.

It is well established that IL-15Rβ binds to Jak-1, which in turn, activates STAT-3 [13]. In this study, both Jak-1 and STAT-3 activation were inhibited by ribavirin. Therefore, ribavirin-induced inhibition of IL-15Rβ expression may lead to a weak/impaired IL-15Rβ/Jak-1 association and subsequently to blocking of Jak-1 and STAT-3 activation. Also, IL-15Rγ binds to Jak-3, which in turn, activates STAT-5 [13]. It appears that ribavirin does not impair the activation of Jak-3 by IL-15Rγ despite reduced expression of IL-15Rγ. We have shown that STAT-5 phosphorylation was only weakly inhibited by ribavirin without a corresponding inhibition of Jak-3. A likely explanation for this phenomenon might be that signaling via IL-15Rβ was directly responsible for STAT-5 phosphorylation and that STAT-5 phosphorylation may have been uncoupled from Jak-3 in ribavirin-treated IL-15-activated NK cells. This is in line with some reports that demonstrated that IL-15Rβ directly phosphorylates STAT-5 [46,47] and that neither the four cytoplasmic tyrosine residues of IL-15Rγ nor Jak-3 are required for STAT-5 induction [46]. It is not known whether IL-15 activation of Jak-1 or Jak-3 results in downstream phosphorylation of STAT-1. However, it has been reported that IL-15 activates STAT-1 phosphorylation [14,15].

Different transcription factors are known to regulate the expression of IL-15Rβ and γ in NK cells. For instance, T-bet and Runx proteins were shown to regulate IL-15Rβ expression [48–50], while NF-κB was shown to be involved in IL-15Rα and γ expression [51]. It appeared possible that ribavirin might interfere with any of these transcription factors. To clarify this hypothesis, we simultaneously treated NK cells with IL-15 (10 ng/ml) and ribavirin (20 μg/ml) for 18 h and 5 days and thereafter performed transcription factor activation experiments to detect NF-κBp50, NF-κBp65, T-bet, AP-1 (c-Jun), and NFAT activation. Although not shown, ribavirin did not interfere with the activation of NF-κBp50, NF-κBp65, T-bet, AP-1 (c-Jun), and NFAT. This indicates that mechanisms other than inhibition of NF-κBp50, NF-κBp65, T-bet, AP-1 (c-Jun) and NFAT activation might be responsible for the reduced expression of IL-15Rβ and γ in ribavirin-treated NK cells.

NK cells treated with IL-15 generally exhibit increased cytotoxic potential when compared to resting NK cells because of increased expression of NK cell receptors and granules [14]. Since IL-15 signaling via its receptors was impaired by ribavirin, other downstream events involved in NK cytotoxicity such as NK cell receptors expression, NK cell signaling via its receptors, lytic granule expression, and lytic granule polarization and release, may also be suppressed. It is generally thought that integration of positive and negative signals from activating and inhibitory receptors determines downstream signaling events. Signals emanating from activating and inhibitory receptors determine the repertoire of NK receptors expressed on developing NK cells and regulate effector functions such as cytotoxicity and the production of IFN-γ [52]. In this regard, several reports demonstrated that inhibition of NK cytotoxicity is associated with suppression of activating receptor expression on the cell surface of NK cells [15,23,29,53]. The results presented in this study show that ribavirin selectively down-regulated NK triggering receptors like NKp30, NKp44, NKp46, and NKG2D and hence their function, while DNAM-1 and the adhesion molecule LFA-1 expression did not change. The inhibitory receptors like NKG2A, KIR2DL2/DL3, and KIR3DL1 were also not affected by ribavirin treatment. Although not shown, we did observe a 3 to 6-fold increase in the expression of activating receptors upon IL-15 treatment of NK cells for 5 days. This increased expression was reduced 2 to 3-fold by ribavirin, however not to the level of expression in non-activated NK cells.

The ligation of NK cell with its target rapidly causes a transient activation of ERK, which apparently control lytic granule movement [31]. Indeed, the activation of the mitogen-activated protein kinase (MAPK) pathways, ERK1/2 and JNK, by activating receptors like NKp46, CD16, 2B4, and NKG2D [15,33] has been reported to play a pivotal role in NK cell cytotoxicity and granule polarization. ERK2 in particular was reported to be the final mediator of perforin and granzyme B granule polarization towards target cells [31]. JNK was shown to be implicated in the prevention of down-regulation of NKG2D expression in NK cells by transforming growth factor beta [34]. Notably, in the presence of ribavirin, the inhibition of ERK1/2 activation was observed. JNK activation was only moderately inhibited by ribavirin. Although IL-15 mainly phosphorylates ERK1/2, it also does phosphorylate JNK. In T cells, IL-15 phosphorylation of ERK and JNK was shown to be mediated by NKG2D, an NK activating receptor which is up-regulated by IL-15 [54,55]. Furthermore NKG2D signaling in NK cells is known to be coupled to IL-15 signaling [20]. It is therefore conceivable that inhibition of NK activating receptors expression, which in turn leads to impaired receptor signaling, may delineate how ribavirin blocks ERK1/2 and JNK activation. Additionally, inhibition of expression as well as a sharp decrease in release of perforin and granzyme B was detected in ribavirin-treated cells.

Ribavirin effects on IFN-γ production were measured in NK cells stimulated by co-incubation with target K562 cell line. In this case, IFN-γ production relies on signaling pathways triggered by engagement of NK cell activating receptors. Interestingly, although many NK cell activating receptors signal through ITAM containing transmembrane adaptors (Dap12, FcRγ, and CD3ζ) distinct signaling intermediates within the ITAM pathway are variably involved in cytotoxicity and/or IFN-γ secretion [56–58]. Early signaling events through ITAM which require activation of protein tyrosine kinases Syk and ZAP70, or downstream events including activation of phosphoinositol 3-kinase (PI3K) and MAPKs including ERK and JNK are important for IFN-γ production. Moreover, NK cell activating receptors requires protein kinase C-θ (PKC-θ) to generate sustained intracellular ERK and JNK signals that reach the nucleus and promote transcriptional activation, ultimately inducing IFN-γ production [59]. In the present study, effects of ribavirin on pathways upstream of MAPKs were not investigated. However, the inhibition of ERK1/2 and JNK phosphorylation in ribavirin-treated NK cells may be relevant for inhibition of IFN-γ production.

Other clinically used substances such as steroids have been shown to exert similar inhibitory effects on NK cell cytotoxicity like those described here for ribavirin. For instance, methylprednisolone down-regulated and impaired function of triggering receptors involved in NK cytotoxicity [15,29]. It also inhibited Jak/STAT and ERK1/2 signaling as well as granule release in IL-2/IL-15-activated NK cells [15]. Cortisol was also reported to inhibit NK function and repress cytokine, perforin mRNA and granzyme A

syntheses [60]. It is therefore possible that ribavirin in viral diseases may suppress inflammatory responses through this "steroid-like" effects. In fact combination therapy with ribavirin seems to decrease the stimulatory effects of INF-α on NK cells in chronic HCV patients [61]. In concordance, ribavirin was shown to exert anti-inflammatory effect and reduced IFN-γ-driven T cell activation in chronic HCV patients [6].

Taken together, this report provides novel mechanisms by which ribavirin exerts its immunomodulatory activities. Ribavirin co-ordinately suppressed NK cell effector functions by inhibiting IL-15Rβ and γ expression and signaling and thus impairing downstream events involved in NK cell cytotoxicity including decrease in NK cell activating receptors expression and function, decrease in ERK1/2 and JNK activation, as well as decrease in levels of granzyme B and perforin expression and release.

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