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IRX-2, a novel immunotherapeutic, enhances and protects NK-cell functions in cancer patients

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Abstract

Background—IRX-2 is a primary biologic which has been used for the therapy of head and neck squamous cell cancer (HNSCC) with promising clinical results. Since NK-cell function is compromised in HNSCC patients, we tested the effects of IRX-2 on the restoration of human NK-cell functions in vitro.

Methods—Peripheral blood mononuclear cells (PBMC) were isolated from 23 HNSCC patients and 10 normal controls (NC). The NK-cell phenotype and functions were compared before and after culture $\pm$ IRX-2 or $\pm$ 50 IU/ml rhIL-2. Flow cytometry was used to study the NK-cell phenotype, cytotoxic activity and cytokine expression.

Results—Impaired NK-cell cytotoxicity in HNSCC patients was related to lower expression of NKG2D, NKp30 and NKp46 receptors ($P < 0.05$) and not to a decreased frequency of NK cells.

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Incubation of patients' NK cells with IRX-2 up-regulated the percentage of receptor-positive NK cells ($P < 0.05$). It also up-regulated cytotoxicity of patients' NK cells ($P < 0.01$) more effectively than rhIL-2 ($P < 0.01$). IRX-2, but not rhIL-2, protected NK cells from suppression mediated by TGF- β , and it restored ($P < 0.05$) expression of activating NK-cell receptors and NK-cell cytotoxicity suppressed by TGF- β . Expression of pSMAD was decreased in NK cells treated with IRX-2 but not in those treated with rhIL-2.

Conclusions—IRX-2 was more effective than IL-2 in enhancing NK-cell cytotoxicity and protecting NK-cell function of HNSCC patients in vitro, emphasizing the potential advantage of IRX-2 as a component of future therapies for HNSCC.

Keywords

NK cells; Head and neck squamous cell cancer (HNSCC); IRX-2; Natural cytotoxicity receptors (NCR); Transforming growth factor β (TGF- β)

Introduction

IRX-2 is a well-defined multi-targeted biologic produced under good manufacturing procedures (cGMP) for clinical applications. IRX-2 has been used for the treatment of head and neck squamous cell cancer (HNSCC) patients in FDA-approved phase I and II clinical trials with promising clinical results [1, 2]. Patients treated with IRX-2 showed only minimal, tolerable side effects and experienced improved symptom control and prolonged survival [1, 2]. Translational studies revealed several possible mechanisms explaining the observed IRX-2-associated antitumor responses. Thus, it has been shown that IRX-2 increases the number of circulating native and memory T cells and enhances lymphocytic infiltration into tumor-draining lymph nodes [3–5]. In addition, in vitro studies showed that IRX-2 matures human monocyte-derived dendritic cells (DC) and protects T cells from tumor-induced apoptosis [6, 7].

Natural killer (NK) cells are able to lyse malignant and virally infected cells without prior antigen-specific priming and, thus, play a critical role in innate immunity [8]. In humans, NK cells encompass two functional subsets: CD56^{dim}CD16^{bright} cytotoxic NK cells, which represent 90% of all NK cells, and CD56^{bright}CD16^{neg}, cytokine-producing NK cells, which account for remaining 10% of all NK cells [9]. NK-cell function is controlled by the balance of inhibitory and activating receptors found on the cell surface. Natural cytotoxicity receptors (NCR), such as NKp46 and NKp30, or NKG2D, a C-type lectin-like receptor, are activating receptors involved in NK-cell cytotoxicity. NKG2D recognizes two distinct families of ligands: MHC class I chain-related molecules (MICA and MICB) and UL-16-binding proteins (ULBP) [10]. NK cells can recognize MICA, MICB and ULBP expression on malignant target cells and eliminate them [11]. Ligands for NCR expressed on resting and activated NK cells have not yet been well characterized, but they also enable NK cells to recognize and lyse tumor cells [12]. To maintain the balance between activation and inhibition, NK cells express inhibitory receptors, including the killer immunoglobulin-like receptors (KIRs) that bind MHC class I ligands (mainly HLA-C, but also HLA-A and HLA-B) and CD94-NKG2A, a C-type lectin receptor, which recognizes peptides presented by HLA-E molecules [9]. In consequence, normal autologous cells expressing self-MHC class I molecules are not targeted by NK cells, while cells that have reduced inhibitory receptor/ligand expression are killed [13].

Impairment in NK-cell cytotoxicity in patients with HNSCC was first reported by Schantz et al. [14] and was correlated with a higher risk of local recurrence as well as metastatic spread [14, 15]. At that time, the cause of reduced NK-cell cytotoxicity in HNSCC patients was unknown, but enhanced NK-cell functions after therapy with recombinant human (rh) IL-2

and interferon- α (IFN- α) correlated with a better response in the phase II trial for HNSCC patients [16]. In other types of cancer, TGF- β 1 seems to be the key molecule suppressing NK-cell functions [17, 18]. In addition, it has been shown in murine models that NK cells mediate the rejection of HNSCC and that TGF- β 1 protects tumors from NK-cell-mediated cytotoxicity [19, 20]. Based on these data, new therapeutic strategies are needed for the restoration and protection of NK-cell functions in HNSCC patients in order to optimize antitumor effects, perhaps in combination with conventional therapy, and thus improve outcome. To evaluate the potential of IRX-2 in up-regulating NK-cell activity and to further investigate its mode of action, we studied in vitro effects of IRX-2 on the phenotype and functions of NK cells in HNSCC patients.

Materials and methods

Blood samples

Peripheral venous blood was obtained from 23 HNSCC patients and 10 age- and sex-matched normal controls (NC). Blood was drawn at the time of diagnosis prior to therapy. All subjects signed an informed consent approved by the Institutional Review Board of the University of Pittsburgh (IRB#991206). The patients were seen at the Outpatient Otolaryngology Clinic between June 2010 and July 2011. Clinicopathological characteristics of all patients enrolled in this study are listed in Supplementary Table 1.

IRX-2

IRX-2 is a uniform, well-defined mixture of cytokines produced under current good manufacturing procedure(s) (cGMP) by the stimulation of human peripheral blood mononuclear cells (PBMC) with phytohemagglutinin (PHA) [1, 2]. PBMC are purchased from FDA-licensed blood banks as buffy coats. PHA and all cellular components are removed after stimulation by centrifugation and washing. DNA is removed by anion exchange chromatography. The cell-free product is nanofiltered and then formulated to contain a defined amount of cytokines. The lot we used for all experiments contained various cytokines at the concentrations shown in Supplementary Table 2. Testing for sterility, DNA, mycoplasma, endotoxin and testing for viral nucleic acid from cytomegalovirus and Epstein-Barr virus are also part of the cGMP process. IRX-2 has been given to more than 40 cancer patients in several FDA-approved clinical trials with tolerable minor side effects [1–5].

Isolation of PBMC, NK cells and cell culture

PBMC were isolated from heparinized blood obtained from NC and HNSCC patients by centrifugation on Ficoll-Hypaque (GE Healthcare, Uppsala, Sweden). After washing with RPMI1640 medium (Lonza, Houston, TX), PBMC were immediately used for functional and phenotypic studies. To study the effects of IRX-2 on NK cells, PBMC were incubated in a complete RPMI1640 medium containing 10% (v/v) heat-inactivated FBS, 2 mmol L-glutamine, 100 IU/ml Penicillin and 100 μ g/ml Streptomycin. An equal volume of IRX-2 or X-Vivo 10 medium (Lonza, Houston, TX) containing 100 IU/ml rhIL-2 (Peprotech, Rocky Hill, NJ) was then added. PBMC were cultured at 37°C in the presence of 5% CO₂ in air for 16 h. Where indicated, TGF- β 1 was added (R&D Systems, Minneapolis, MN) at a final concentration of 10 ng/ml.

For cytokine/chemokine measurements and analysis of SMAD signaling, NK cells were isolated by a negative selection using magnetic beads (Miltenyi, Auburn, CA) according to the manufacturer's instructions. Purity, as determined by flow cytometry, regularly exceeded 95%. For intracellular cytokine staining, cells were washed extensively after short-term culture and stimulated for 4 h with Phorbol 12-Myristate 13-Acetate (PMA), 2 μ M

Ionomycin in the presence of 10 µg/ml Brefeldin A (all from Sigma-Aldrich, St. Louis, MO). Phosphorylation of SMAD was induced by TGF- β 1 as described previously [8]. Briefly, isolated NK cells were cultured in RPMI1640 containing 2 mmol L-glutamine and 0.5% bovine serum albumin (BSA, Sigma-Aldrich, St. Louis, MO) in the presence of 10 ng/ml TGF- β 1 to induce phosphorylation of SMAD2/3 for 15 min at 37°C in the presence of 5% CO₂ in air. IL-2 or IRX-2 was added together with TGF- β 1 at the same time. The human erythromyeloblastoid leukemia cell line, K562, was purchased from ATCC (Manassas, VA). The human HNSCC cell line, PCI-13, was established and maintained in our laboratory as previously described [21]. Both cell lines were cultured in RPMI1640 containing 10% heat-inactivated FBS, 2 mmol L-glutamine, 100 IU/ml Penicillin and 100 µg/ml Streptomycin at 37°C in an atmosphere of 5% CO₂ in air. All cell cultures were routinely tested for mycoplasma by PCR and found to be negative.

Antibodies

The following fluoro-chrome-labeled mouse anti-human monoclonal antibodies (mAbs) purchased from Beckman Coulter were used: anti-CD3-ECD, anti-CD16-FITC, anti-CD56-PeCy5, anti-NKp46-PE and anti-interferon- γ -FITC. In addition, the following PE-labeled mAbs were purchased from R&D Systems: anti-NKG2A, anti-NKG2C, anti-NKG2D, anti-CCL4 as well as FITC-labeled anti-CCL3. PE-labeled anti-NKp30 was purchased from Miltenyi, and anti-TNF- α -PE was obtained from eBioscience (San Diego, CA). Unlabeled rabbit anti-human-SMAD2/3 and anti-human-phospho-SMAD2/3 were purchased from Cell Signaling, Danvers, MA. PE-labeled donkey anti-rabbit-Fab₂ was obtained from eBioscience. Functional grade antagonistic antibodies were obtained from Miltenyi (NKp30 and NKp46) and from Biolegend (NKG2D, San Diego, CA). Appropriate isotype antibodies were purchased from the same companies and used as controls in all experiments.

Staining for flow cytometry

Cells were washed after harvest with PBS-azide containing 3% FBS and incubated with labeled specific mAbs or isotype controls for 20 min on ice. After washing, cells were fixed with paraformaldehyde (PFA) 2% (w/v), stored at 4°C in the dark and were tested on the same day. For intracellular cytokine/chemokine staining, isolated NK cells were washed twice with PBS-azide containing 3% FBS, fixed and permeabilized using a Fix/Perm Kit (eBioscience) according to the manufacturer's instructions and then stained with mAb for 30 min at RT. Cells were washed and resuspended in 2% PFA and tested on the same day. For intracellular SMAD and pSMAD staining, cells were fixed using 2% PFA and permeabilized with 90% methanol. Incubation with primary antibodies was then performed for 1 h at RT followed by incubation with a relevant secondary antibody for 30 min at RT.

Flow cytometry

Flow cytometry was performed on a Beckman Coulter Epics XL. Data were analyzed using EXPO32 software.

Flow cytometry-based cytotoxicity assay

NK-cell cytotoxicity was assessed as previously described [22] with the following modifications. Briefly, K562 target cells were stained with 2 µM carboxyfluorescein succinimidyl ester (CFSE, Invitrogen, Carlsbad, CA) for 10 min at 37°C in the dark. Cells were washed extensively afterward and incubated with bulk PBMC, which were used either directly after isolation or after 16-h incubation with IRX-2 or rhIL-2, at various effector to target ratios for 3 h at 37°C and 5% CO₂. Cells were incubated for additional 20 min in the presence of 7-aminoactinomycin D (7-AAD, Invitrogen, Carlsbad, CA) used at a concentration of 1 µg/ml. Cells were acquired on a Beckman Coulter XL cytometer.

detecting CFSE on FL1 and 7-AAD on FL4. Target cells were identified as CFSE positive, and the percentage of 7-AAD⁺ target cells was determined. Target cells maintained at 37°C without PBMC for 3 h served as a negative control, and target cells incubated for 10 min at 56°C before 3-h incubation served as a positive control for 7-AAD staining. The percentage of cytotoxic activity was calculated using the following formula: % specific lysis = 7-AAD⁺ targets minus spontaneous 7-AAD⁺ targets. Lytic units (LU) were calculated as the number of NK cells needed to lyse 20% of 5×10^4 target cells and expressed as the reciprocal of this value per 10^5 NK cells [23], thus expressing cytotoxicity on a per cell basis.

Statistical analysis

Statistical analysis was performed using StatXact software (Cytel Inc., Cambridge, MA). The Friedman test and Wilcoxon signed-rank test were used for paired samples; Wilcoxon-Mann-Whitney test was used for unpaired samples. A *P* value <0.05 was considered to be statistically significant.

Results

NK cells in HNSCC patients

Using flow cytometry-based methods, we observed that the frequency of NK cells defined as CD3⁺CD56⁺ lymphocytes (Fig. 1a) and the frequency of CD3⁺CD56^{dim}CD16⁺ or CD3⁺CD56^{bright}CD16⁺ NK-cell subsets (Fig. 1b) were comparable in HNSCC patients and NC. However, the frequency of NK cells expressing activating (or inhibitory) receptors was different in HNSCC patients relative to NC (Fig. 2a). In patients, the frequency of NK cells positive for NKG2D, NKp30 and NKp46 was significantly decreased, while the frequency of NK cells expressing NKG2A was increased (Fig. 2a). Only the frequency of NKG2C⁺ cells was comparable between the two cohorts (Fig. 2a). Further, the levels of NCR expression on NK cells were decreased in HNSCC (Supplementary Figure 1). These data suggested that NCR-mediated signaling may be altered in NK cells obtained from HNSCC patients versus NC, affecting NK-cell cytotoxicity. Indeed, HNSCC patients had lower (*P* < 0.01) NK activity against K562 targets than NC as measured in flow-based cytotoxicity assays (Fig. 2b). As the frequency of NK cells was similar in both cohorts, our data suggest that NK activity on per single cell is impaired in HNSCC patients, as is also evident when cytotoxicity data are expressed in LU₂₀/10⁵ NK cells (Fig. 2c). Subdividing HNSCC patients into those with T1/T2 tumors versus those with T3/T4 tumors, we observed the lowest levels of cytotoxicity in the latter (Fig. 2b). All together, we showed that HNSCC patients have impaired NK-cell activity, although the frequency of NK cells is not different from that in NC.

IRX-2 up-regulates NCR on CD3⁺CD56⁺ NK cells

Since we found reduced expression of activating NCR and decreased cytotoxicity in the patients' NK cells, we asked whether IRX-2 could restore function in these cells. Preliminary experiments showed a dose-dependent effect of IRX-2 on the NK-cell phenotype, reaching a maximum when used at a 1:1 dilution (data not shown). Since the lot of IRX-2 used in this study contains 100 IU/ml IL-2 (Supplementary Table 2), known to activate human NK cells [24], we compared IRX-2 to an equivalent dose of rhIL-2. After culturing freshly isolated PBMC in the presence of rhIL-2 or IRX-2 for 16 h, expression of NCR was significantly increased on CD3⁺CD56⁺ NK cells from NC (data not shown) and, more importantly, in those obtained from HNSCC patients, as shown in Fig. 3a. IRX-2-treated patients' NK cells showed significantly higher (*P* < 0.05) expression of NKG2D, NKp30 and NKp46, whereas NKG2C and NKG2A expression was not significantly different in comparison with cells treated with rhIL-2 alone. Viability of NK cells, as

determined by 7-AAD staining after treatment, was similar in both groups (rhIL-2, median $90 \pm 4\%$ vs. IRX-2, median $89 \pm 5\%$).

IRX-2 increases NK-cell cytotoxicity in HNSCC patients

To determine whether the IRX-2-mediated increases in the NCR expression on CD3⁺CD56⁺ NK cells were associated with increased NK-cell cytotoxicity, we compared the ability of rhIL-2- or IRX-2-treated NK cells to kill K562 targets. As shown in Fig. 3b, the incubation of PBMC with rhIL-2 or IRX-2 increased NK activity in comparison with that seen with PBMC incubated in medium alone. NK cells +rhIL-2 showed significantly increased median cytotoxicity ($30.4 \text{ LU}_{20}/10^5$ vs. $9.5 \text{ LU}_{20}/10^5$ in medium alone). IRX-2 treatment of NK cells from the same donor resulted in higher median cytotoxicity of $58.6 \text{ LU}_{20}/10^5$ in comparison with rhIL-2 ($P < 0.01$). By using antagonistic antibodies to NKG2D, NKp30, NKp46 and NKG2D, we showed that increased cytotoxicity largely depended on NKG2D upregulation (Fig. 3c). Since K562 cells are only surrogate targets, we also tested the ability of NK cells isolated by magnetic bead separation to lyse the HNSCC cells (PCI-13). IRX-2 significantly enhanced cytotoxicity against this target as well and to a greater extent than rhIL-2 (Fig. 3d).

IRX-2 enhances NK-cell cytokine and chemokine production

In addition to killing of malignant cells, cytokine and chemokine production is an important aspect of NK-cell activity [9]. To investigate the impact of IRX-2 on this function, NK cells isolated by magnetic beads were cultured in the presence of medium, rhIL-2 or IRX-2. As shown in Fig. 4 and Supplementary Figure 2, culture in the presence of rhIL-2 and IRX-2 increased the percentage of IFN- γ ⁺ NK cells and IFN- γ expression levels as compared to medium alone ($P < 0.05$). In addition, more NK cells exposed to IRX-2 were positive for IFN- γ than those cultured in medium containing rhIL-2 ($P < 0.05$), while neither IL-2 nor IRX-2 increased TNF- α or CCL4 expression in NK cells (Fig. 4 and Supplementary Figure 2). However, the frequency of CCL3⁺ NK cells increased when NK cells were cultured in the presence of rhIL-2 and IRX-2 as compared to medium alone ($P < 0.05$). As reported for IFN- γ , IRX-2 was also able to increase CCL3 expression levels significantly better than IL-2 ($P < 0.05$, Fig. 4 and Supplementary Figure 2).

IRX-2 protects NK cells from TGF- β 1-mediated suppression

It has recently been shown that TGF- β 1 suppresses NK-cell functions in HNSCC and down-regulates NKp30 and NKG2D expression [19, 25]. We, therefore, asked whether IRX-2 can protect NK cells from TGF- β 1-mediated suppression in addition to enhancing NK-cell functions in HNSCC patients. Preliminary experiments showed a dose-dependent suppression of NK-cell cytotoxicity by TGF- β 1, that is, 90% suppression in the presence of 10 ng/ml rhTGF- β 1 (data not shown). We found that NKG2D and NKp30 expression was down-regulated by TGF- β 1 in comparison with medium alone ($P < 0.05$), while TGF- β 1 did not affect NKp46 expression (Fig. 5 and Supplementary Figure 3). More importantly, culturing patients' PBMC in the presence of TGF- β 1 and rhIL-2 completely abolished the upregulation of NKG2D, NKp30 and NKp46 seen when PBMC from the same donor were cultured with rhIL-2 alone (Fig. 5 and Supplementary Figure 3). In contrast, patients' PBMC cultured in the presence of TGF- β 1 and IRX-2 did not down-regulate expression of NKG2D, NKp30 and NKp46 as did cells from the same donor cultured in IRX-2 alone. Thus, IRX-2 was able to up-regulate NCR expression even in the presence of TGF- β 1, while rhIL-2 did not.

We next asked, whether IRX-2 is also able to prevent down-regulation of NK-cell cytotoxicity by TGF- β 1. Culture of PBMC in the presence of rhIL-2 increased NK-cell cytotoxicity from 10 to $31 \text{ LU}_{20}/10^5$ NK cells (Fig. 5), and this increase was completely abolished when NK cells were incubated in the presence of rhIL-2 and TGF- β 1. In contrast,

IRX-2 alone increased cytotoxicity to 60.4 LU₂₀/10⁵ NK cells, and in the presence of IRX-2 and TGF- β , NK cytotoxicity was 55 LU₂₀/10⁵ NK cells (Fig. 5), indicating protection of NK cytotoxicity by IRX-2 from TGF- β 1-mediated suppression.

TGF- β 1-induced SMAD phosphorylation is decreased by IRX-2

TGF- β 1-mediated suppression of NK-cell function is accompanied by phosphorylation of SMAD proteins [8, 17]. Since IRX-2 protected NK cells from a TGF- β 1-mediated loss of function, we asked whether IRX-2 also decreased TGF- β 1-mediated SMAD phosphorylation. As shown in Fig. 6, NK cells exposed to TGF- β 1 contained the phosphorylated form of SMAD2/3 (pSMAD2/3). While TGF- β 1 induced SMAD2/3 phosphorylation in NK cells, IL-2 or IRX-2 alone did not (data not shown). pSMAD2/3 was detectable in NK cells incubated with TGF- β 1 in the presence of IL-2. However, pSMAD2/3 expression decreased in NK cells incubated with TGF- β 1 in the presence of IRX-2 (Fig. 6). Thus, IRX-2 prevented TGF- β 1-mediated SMAD2/3 phosphorylation. Expression of non-phosphorylated SMAD2/3 was not affected in these experiments (data not shown).

Discussion

Over 20 years ago, Schantz et al. [14, 15] described impaired NK-cell cytotoxicity in HNSCC patients and showed that this impairment was associated with a higher risk of local recurrence as well as metastasis. These data, recently confirmed by, for example, Wulff et al. [26], suggested that NK cells play a key role in tumor control, and subsequent studies confirmed NK-cell involvement in immunosurveillance [9, 27]. Using newer flow-based technology, we also found significantly reduced NK-cell cytotoxicity against tumor targets in HNSCC patients compared to NC. In addition, we showed for the first time that NK cells of HNSCC patients express lower levels of NKG2D, NKp30 and NKp46 but higher levels of NKG2A than those seen in NK cells of NC. In regard to activating receptor expression on NK cells in NC, reports are conflicting, with older data suggesting expression of these receptors on nearly all NK cells, and newer reports indicating considerable individual-related variability [28–31]. Nevertheless, in cancer patients, expression of these receptors is low as is NK cytotoxicity [30, 31]. Low NK-cell cytotoxicity seen in HNSCC patients could be impaired due to low expression of receptors involved in target cell recognition and activation of NK cells. A recent work by Chen et al. [32] indicated that HNSCC cells express NKG2D ligands, MICA and MICB and that up-regulation of the receptor on NK cells increased their ability to recognize and kill HNSCC target cells. Other studies report that tumor-derived exosomes present in sera of cancer patients carry membrane-bound MICA/MICB and deliver them to circulating NK cells blocking their ability to recognize and eliminate tumor cells [33]. These findings emphasize the clinical relevance of enhancing expression of NKG2D on NK cells of HNSCC patients. In addition to NKG2D, NKp30 and NKp46 could also be important in the rejection of HNSCC, although it is unknown whether HNSCC cells express NKp30 or NKp46 ligands. In our hands, the novel biologic IRX-2 is able to up-regulate expression of NKG2D, NKp30 and NKp46 on NK cells of HNSCC patients and enhance cytotoxicity of patients' NK cells both against K562 and HNSCC targets.

In addition to their role in the direct elimination of target cells, NK cells contribute to innate and adaptive immune responses by cytokine and chemokine secretion [10]. It has been shown that NK cells are an early source of IFN- γ in the induction of Th1 adaptive immune responses [34], and both CCL3 and CCL4 produced by NK cells may be involved in the recruitment of dendritic cells into tissues [35]. Here, we showed that in addition to increasing NK-cell cytotoxicity, IRX-2 is more effective than rhIL-2 in priming NK cells for the production of IFN- γ and CCL3. Thus, IRX-2 improves NK-cell-dependent antitumor

responses, benefiting HNSCC patients. Based on these data, it is reasonable to suggest that the clinical responses observed in HNSCC patients treated with IRX-2 could be, at least in part, attributed to improved NK-cell functions [1]. It could be argued, however, that IL-15, a known activator of NK activity, could be responsible for IRX-2-mediated effects on NK cells [36]. We determined that IL-15 is present in IRX-2 at an extremely low concentrations (35 pg/ml), which is about 100-fold lower from that used for the modulation of NK activity ex vivo [31]. For this reason, we think that IL-2 present in IRX-2 at 5 ng/ml is more likely to be responsible for the up-regulation of NK activity. Therefore, we selected to compare IRX-2 with IL-2 in this report.

The molecular mechanisms that are responsible for protective or restorative effects of IRX on human lymphocytes have been extensively examined [37, 38]. IRX-2 likely contains an almost unlimited number of cytokines, so that selecting any one of potentially effective mediators for further studies is a gamble. But because we and others have previously reported that the down-regulation of NCR and decreased cytotoxicity of NK cells in cancer patients are mediated by TGF- β 1, it was rational to see whether IRX-2 could restore NK-cell function and protect NK cells from TGF- β 1-mediated suppression [17, 18]. Interestingly, TGF- β 1-mediated down-regulation of NKG2D expression, and NK activity was reported to be mediated by the membrane-bound, not soluble, form of this cytokine carried on exosomes in sera of cancer patients [17, 33]. To achieve protection from TGF- β 1, we incubated NK cells with rhIL-2 or IRX-2. While rhIL-2 was unable to protect HNSCC patients' NK cells against TGF- β 1-mediated suppression, IRX-2 induced protection. To investigate the mechanism responsible for IRX-2-mediated protection of NK cells, the intracellular signaling pathway involving TGF- β 1 was interrogated. In general, receptor ligation by TGF- β 1 leads to SMAD phosphorylation [39]. We looked for and were able to detect pSMAD2/3 in NK cells exposed to TGF- β 1 and observed that the addition of IRX-2, but not of rhIL-2, prevented SMAD2/3 phosphorylation. Others previously reported that IL-2 alone is not able to protect human NK cells from TGF- β 1-mediated down-regulation of NKG2D and impaired cytotoxicity [18, 40]. In contrast, IRX-2 effectively protected HNSCC patients' NK cells from TGF- β 1-mediated suppression of cytotoxicity in vitro.

In aggregate, our in vitro studies with NK cells of HNSCC patients suggest that IRX-2, which contains low doses of multiple cytokines, has stimulatory and protective effects on innate immunity. IRX-2 was more effective than rhIL-2 in increasing cytotoxicity and cytokine/chemokine production in NK cells of patients with cancer and also protected them from TGF- β 1-mediated suppression. In contrast, rhIL-2 alone was ineffective to protect NK cells from TGF- β 1-mediated suppression. These results support the strategy of incorporating IRX-2 into future therapies, including conventional chemoradiotherapy, for patients with HNSCC.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

NK cells Natural killer cells

HNSCC	Head and neck squamous cell carcinoma
NC	Normal controls
KIR	Killer immunoglobulin-like receptor
TGF-β	Transforming growth factor beta
rhIL-2	Recombinant human interleukin 2
AJCC	American joint committee on cancer
NCR	Natural cytotoxicity receptor

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Fig. 1.

NK-cell frequency in HNSCC patients and NC: **a** percentages of CD3⁻CD56⁺ NK cells in PBMC of HNSCC patients ($n = 23$) and NC ($n = 10$) were determined by flow cytometry. *Box plots* show median values, *boxes* are upper and lower quartile, and *whiskers* show the maximum and the minimum values. **b** The distribution of CD3⁻CD56^{dim}CD16⁺ (*white*) and CD3⁻CD56^{bright}CD16⁻ (*black*) NK-cell subsets among total CD3⁻CD56⁺ NK-cell fraction in HNSCC patients and NC. The frequency was determined by flow cytometry gating on lymphocytes. The data for 23 HNSCC patients and 10 NC are expressed as medians

Fig. 2.

NK-cell phenotype and cytotoxicity in HNSCC patients and NC: **a** expression of activating and inhibitory receptors on NK cells in the peripheral blood of HNSCC patients ($n = 12$) and NC ($n = 10$). Phenotype was determined by flow cytometry, and percentages of positive cells in the CD3⁺CD56⁺ lymphocytes gate are shown. Bars indicate medians. **b** Box plots show NK cytotoxicity in 16 HNSCC patients (10 patients with T1/T2 tumors and 6 patients with T3/T4 tumors) and 10 NC. Bars indicate medians. In this and all other figures, cytotoxicity is expressed in LU₂₀/10⁵ NK cells. **c** Representative histograms of NK-cell marker expression on CD3⁺CD56⁺ lymphocytes obtained from NC and HNSCC patients. Isotype controls are shown in gray

Fig. 3.

Effects of IRX-2 on the NK-cell phenotype and cytotoxicity: **a** expression of activating and inhibitory receptors on NK cells of 12 HNSCC patients after culture in the presence of IRX-2, rhIL-2 or medium. Median percentages of positive cells are shown. * $P < 0.05$. **b** Box plots showing cytotoxicity in 12 HNSCC patients after culture in the presence of IRX-2, IL-2 or medium. Bars indicate medians. **c** Cytotoxicity was determined in 5 HNSCC patients after a short-term culture in the presence of IRX-2 and the effects of NCR blocking. Blocking antibodies against NKp30 (*second column*) and NKp46 (*third column*) as well as a nonspecific isotype IgG (*last column*) do not significantly decrease cytotoxicity. In contrast, blocking of NKG2D decreases cytotoxicity ($P < 0.01$). Bars indicate medians

Fig. 4.

Effects of IRX-2 on NK-cell cytokine expression: isolated NK cells from HNSCC patients were restimulated with PMA/Ionomycin in the presence of Brefeldin A after 16-h culture in the presence of IRX-2, rhIL-2 or medium, and intracellular cytokine expression was determined by flow cytometry. Results from 6 different HNSCC patients shown, and bars indicate medians

Fig. 5.

Protective effects of IRX-2 against TGF- β -mediated suppression: **a** surface expression of NKG2D and activating NCRs on NK cells of HNSCC patients after culture in the presence of IRX-2, IL-2 or medium $\pm$ TGF- β . The phenotype was determined by flow cytometry, and percentages of positive cells gated on CD3⁺CD56⁺ lymphocytes from 6 HNSCC patients are shown. Bars indicate medians. **b** Cytotoxicity was measured with NK cells of 6 HNSCC patients after culture in the presence of IRX-2, IL-2 or medium $\pm$ TGF- β . Bars indicate medians

Fig. 6. IRX-2 prevents SMAD2/3 phosphorylation: representative data for pSMAD2/3 expression as determined by flow cytometry with NK cells obtained from a HNSCC patient after culture $\pm$ TGF- β . One set of histograms out of 5 independent experiments is shown