Published in final edited form as:

Cancer Immunol Immunother. 2018 April ; 67(4): 675–689. doi:10.1007/s00262-018-2121-4.

An IL-15 superagonist/IL-15R α fusion complex protects and rescues NK cell cytotoxic function from TGF- β 1-mediated immunosuppression

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Abstract

Natural killer (NK) cells are innate cytotoxic lymphocytes that play a fundamental role in the immunosurveillance of cancers. NK cells of cancer patients exhibit impaired function mediated by immunosuppressive factors released from the tumor microenvironment (TME), such as transforming growth factor (TGF)- β 1. An interleukin (IL)-15 superagonist/IL-15 receptor α fusion complex (IL-15SA/IL-15RA; ALT-803) activates the IL-15 receptor on CD8 T cells and NK cells, and has shown significant anti-tumor activity in several *in vivo* studies. This *in vitro* study investigated the efficacy of IL-15SA/IL-15RA on TGF- β 1-induced suppression of NK cell-cytotoxic function. IL-15SA/IL-15RA inhibited TGF- β 1 from decreasing NK cell-lysis of four of four tumor cell lines (H460, LNCap, MCF7, MDA-MB-231). IL-15SA/IL-15RA rescued healthy donor and cancer patient NK cell-cytotoxicity, which had previously been suppressed by culture

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Compliance with ethical standards

Conflicts of Interest

Hing C. Wong is an officer and stockholder of Altor BioScience Corporation. All other authors declare that they have no conflicts of interest.

Ethical Approval

All procedures performed in studies involving human participants or human participant blood products were in accordance with the ethical standards of the National Institutes of Health Institutional Review Board and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. Human NK cells were isolated from fresh or frozen peripheral blood of anonymized healthy volunteer donors (NIH Clinical Center Blood Bank (protocol NCT00001846)). Human NK cells were isolated from frozen peripheral blood of anonymized prostate cancer patients (protocol NCT01496131).

Informed Consent

Blood donors meeting research donor eligibility criteria were recruited to donate blood by standard phlebotomy and apheresis techniques. The general investigational nature of the studies in which their blood would be used, and the risks and discomforts of the donation process were carefully explained to the donors, and a signed informed consent document was obtained.

with TGF- β 1. TGF- β 1 downregulated expression of NK cell-activating markers and cytotoxic granules, CD226, NKG2D, NKp30, granzyme B, and perforin. Smad2/3 signaling was responsible for this TGF- β 1-induced downregulation of NK cell-activating markers and cytotoxic granules. IL-15SA/IL-15RA blocked Smad2/3-induced transcription, resulting in the rescue of NK cell-cytotoxic function from TGF- β 1-induced suppression. These findings suggest that in addition to increasing NK cell function via promoting the IL-15 signaling pathway, IL-15SA/IL-15RA can function as an inhibitor of TGF- β 1 signaling, providing a potential remedy for NK cell dysfunction in the immunosuppressive tumor microenvironment.

Precis:

An interleukin (IL)-15 superagonist was utilized to overcome immunosuppression of NK cell function mediated by TGF- β 1, demonstrating a new therapeutic potential of the IL-15 superagonist for NK cells in the immunosuppressive tumor microenvironment.

Keywords

NK cells; IL-15; TGF- β 1; immunosuppression; tumor microenvironment

Introduction

Natural killer (NK) cells are innate cytotoxic lymphocytes that play a fundamental role in the immunosurveillance of cancers. They produce immunoregulatory cytokines and chemokines and directly eliminate malignant target cells that lack cognate MHC class I ligands. NK cells also kill tumor cells via antibody-dependent cellular cytotoxicity (ADCC) by the interaction between IgG1 antibody-bound tumor cells and Fc γ receptor of NK cells, which triggers activation and degranulation of NK cells (1, 2).

Tumor cells employ several mechanisms to escape from NK cell surveillance through the modulation of NK cell-targeted cell surface molecules or the release of immunosuppressive soluble factors, such as prostaglandin E2, adenosine, and transforming growth factor (TGF)- β 1 (3). Several clinical studies reported that the levels of TGF- β 1 are often elevated in the serum of cancer patients and correlate with disease progression and poor prognosis (4–6). TGF- β 1 is commonly viewed as one of the most potent immunosuppressive cytokines (7). Previous reports demonstrated that TGF- β 1 reduced cytokine production and cytolytic function of NK cells (8, 9).

In general, NK cells can be activated by several cytokines such as IL-2, IL-12, IL-15, IL-18, IL-21, and type I interferon (IFN). Therefore, the potential for utilizing these cytokines to overcome TGF- β 1-mediated NK suppression is being investigated (1). Of these, IL-2 is the only U.S. Food and Drug Administration (FDA)-approved cytokine monotherapy for cancer patients. Indeed, IL-2 has been shown to potently activate tumor-infiltrating NK cells, enhancing their ability to lyse tumor cells (10). However, its serious toxicity and low objective responsive rate have prevented the application of IL-2 treatment as standard therapy (11, 12). IL-15 also mediates the development of cytotoxicity of NK cells with less systemic toxicity compared to IL-2 and has been shown to be well tolerated in preliminary

human clinical trials (13). Despite its promising anti-tumor immune capacity, IL-15 has been shown to exhibit a short half-life and low biological activity *in vivo* (14, 15), thus resulting in limited anti-tumor responses in patients (13). To increase the therapeutic effectiveness and facilitate the use of IL-15 in the immunotherapy of cancer, an IL-15 superagonist/IL-15R α Sushi-Fc fusion complex (IL-15 N72D superagonist/IL-15R α Su-Fc; ALT-803) has been developed to address the limitations of IL-15-based therapeutics. The mutant IL-15, IL-15 N72D superagonist (IL-15SA) has an improved affinity for the IL-2 receptor β chain (16, 17), and association with a soluble IL-15R α Su-Fc (IL-15RA) enables IL-15SA to form a complex of IL-15R $\alpha\beta\gamma$ with optimized *in vivo* activity, resulting in further improved pharmacokinetics and biologic activity of IL-15 (18, 19). The IL-15SA/IL-15R α Su-Fc fusion complex (IL-15SA/IL-15RA) has shown encouraging results in several *in vivo* studies: murine multiple myeloma (20), rat bladder cancer (21), murine glioblastoma (22), murine breast and colon cancer (23), and human ovarian cancer (24), informing multiple clinical trials against hematological and solid cancers.

Here, for the first time, we evaluate the potential of IL-15SA/IL-15RA to overcome immunosuppression of NK cell function mediated by TGF- β 1. We demonstrate that (1) IL-15SA/IL-15RA protected NK cell function from TGF- β 1-induced suppression, (2) IL-15SA/IL-15RA rescued TGF- β 1-suppressed NK cell cytotoxic function, (3) Smad2/3 signaling was responsible for the TGF- β 1-downregulated expression of NK cell-activating markers and cytotoxic granules, and finally, (4) IL-15SA/IL-15RA blocked Smad2/3-induced transcription, resulting in the rescue of NK cell-cytotoxic function from TGF- β 1-induced suppression. Our findings demonstrate a new therapeutic potential of IL15SA/IL15RA for NK cells in the immunosuppressive tumor microenvironment.

Materials and Methods

Cell culture and reagents

The tumor cell lines H460 (lung), LNCap (prostate), MCF7 (estrogen receptor positive breast cancer) and MDA-MB-231 (triple negative breast cancer) were obtained from American Type Culture Collection (ATCC; Manassas, VA). All cells were passaged for fewer than 6 months. MCF7 were cultured in medium designated by the provider. H460, LNCap, and MDA-MB-231 were maintained in RPMI-1640 medium supplemented with 10% fetal bovine serum, and 1% of HEPES, penicillin/streptomycin, L-glutamine, nonessential amino acids and sodium pyruvate. For select experiments, additional lung cell lines H1703, H520, and HCC006, as well as K-562 (chronic myelogenous leukemia), were utilized (ATCC). B cells were isolated from frozen peripheral blood of healthy volunteer donors (NIH Clinical Center Blood Bank (NCT00001846)) using a negative selection Human B cell isolation kit (Miltenyi Biotech, Auburn, CA) following the manufacturer's protocol.

NK cell preparations

Human NK cells were isolated from fresh or frozen peripheral blood of healthy volunteer donors (NIH Clinical Center Blood Bank (NCT00001846)) using a negative selection Human NK Cell Isolation Kit (Miltenyi Biotech, Auburn, CA) following the manufacturer's

protocol, resulting in >80% purity (CD3-/CD56+). Each experiment and experimental repeat utilized distinct healthy donors. NK cells were treated with 50 ng/ml of IL-15SA/IL-15RA (IL-15 N72D superagonist/IL-15R α Su-Fc; ALT-803, Altor Bioscience, Miramar, FL) and/or 2 ng/ml of TGF- β 1 (R&D Systems, Minneapolis, MN), and/or 1 μ g/ml of the TGF β receptor I kinase inhibitor SD208 (Tocris Bioscience, Bristol, UK) for experiments. The concentration of IL-15SA/IL-15RA treatment was determined by previous reports (20, 25). The concentration of TGF- β 1 treatment was determined by the TGF- β 1 level in plasma of cancer patients in previous studies (4, 6). For select experiments, NK cells were isolated from frozen peripheral blood obtained from prostate cancer patients.

Flow cytometry

The anti-human mAbs used were as follows: PE-CD274, PE-EGFR, PE-CD3, PE-CD226 (DNAM-1), PerCP-Cy5.5-NKG2D, BV421-NKp30, BV510-granzyme B, PE-Cy5-CD107a, and PE-Smad2 (pS465/pS467)/Smad3 (pS423/pS425) (BD Biosciences, San Jose, CA); APC-CD56 (BioLegend, San Diego, CA); PE-Cy7-perforin (eBioscience, San Diego, CA), and PE-TGF β receptor II (R&D Systems). Samples were acquired on a FACSCalibur flow cytometer or FACSVerse (Becton Dickinson, Franklin Lakes, NJ), and analyzed using FlowJo software (TreeStar, Inc., Ashland, OR). Isotype control staining was < 5% for all samples analyzed. For human PBMC subset analysis, PBMCs from three healthy donors were obtained from the NIH Clinical Center Blood Bank (NCT00001846), as previously described (26). Frozen PBMCs were thawed and seeded at 5×10^6 per well, and treated with IL-15SA/IL-15RA at 0, 1, 5, 25, and 50 ng/ml for 72h. Cells were harvested and assessed for the frequency of immune cell subsets by multi-parametric flow cytometry as previously described (27).

CD107a degranulation assay

Freshly isolated NK cells were either untreated or treated with TGF- β 1 (2 ng/ml) and/or IL-15SA/IL-15RA (50 ng/ml) for 48 hours. 1×10^5 cells of NK cells and 1×10^4 cells of K-562 were seeded into each well of 96-well round bottom plates and incubated for 4 hours in the presence of anti-CD107a antibody. After 4-hour incubation, cells were stained with anti-CD3 and CD56 antibodies. Flow cytometric analysis was performed by FACSVerse and FlowJo.

Antibody-dependent cellular cytotoxicity (ADCC) assay

ADCC assays were performed as described previously (28) with indicated modifications. Freshly isolated NK effector cells were either untreated or treated with TGF- β 1 (2 ng/ml) and/or IL-15SA/IL-15RA (50 ng/ml) for 48 hours. Tumor cells were harvested and labeled with ^{111}In . Cells were plated as targets at 2,000 cells per well in 96-well plates. For ADCC assays, tumor cells were incubated with 2 μ g/mL of avelumab (Bavencio®, EMD Serono, Billerica, MA) or 10 μ g/mL of cetuximab (Erbix®/Erbix®, Lilly, Indianapolis, IN) or 10 μ g/mL of irresponsive rituximab (Rituxan®, Biogen, Cambridge, MA) as control isotype antibody at room temperature for 30 minutes. NK cells were added as effector cells. Various effector cell:target cell (E:T) ratios were used in the study. After 4 hours, supernatants were harvested and analyzed for the presence of ^{111}In using a WIZARD2 Automatic Gamma Counter (PerkinElmer, Waltham, MA). Spontaneous release was determined by incubating

target cells without effector cells, and complete lysis was determined by incubation with 0.05% Triton X-100. Experiments were carried out in triplicate. Specific ADCC lysis was determined using the following equation: Percent lysis = [(experimental cpm – spontaneous cpm) / (complete cpm – spontaneous cpm)] x 100.

RNA isolation and quantitative real-time PCR

Total RNA was isolated from the NK cells using the RNeasy Extraction Kit (Qiagen, Valencia, CA). RNA was reverse transcribed into cDNA using the Advantage RT-for-PCR Kit (Clontech, Mountain View, CA). Resultant cDNA (100 ng) was quantitated using TaqMan primers and probes as follows: *CD226* (Hs00170832_m1), *KLRK1* (Hs00183683_m1), *NCR3* (Hs01553309_g1), *GZMB* (Hs00188051_m1), *PRFI* (Hs00169473_m1), *EOMES* (Hs00172872_m1), *JUN* (Hs01103582_s1), *SERPINE1* (Hs00167155_m1), *SMAD7* (Hs00998193_m1), and *GAPDH* (Hs02786624_g1) (Life Technologies, Grand Island, NY). Each mRNA expression level was calculated as expression relative to *GAPDH*. Real-time PCR was performed on the 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, CA).

Simple Western assays

Simple Western™ (ProteinSimple, San Jose, CA), an automated capillary-electrophoresis immunoassay system, which employs high-resolution molecular weight separation, followed by target-specific immunoprobe was utilized. Analyses were performed as previously described unless stated otherwise (29).

Statistical analysis

Significant differences in the distribution of data acquired by ADCC assays and real-time PCR were determined by paired Student's *t* test with a 2-tailed distribution and reported as *P* values, using Prism 7.0f software. Significant differences in the distribution of data acquired by flow cytometry analysis were determined by the Kolmogorov-Smirnov test using FlowJo software (TreeStar, Inc.).

Results

IL-15SA/IL-15RA protected NK cell-cytotoxicity from TGF-β1-induced functional suppression

It has been previously shown that pre-treatment with IL-15 prevented NK cells from impaired function induced by co-culture with tumor cells (30). We first examined whether IL-15SA/IL-15RA inhibited TGF-β1 from suppressing NK cell-killing activity. We cultured NK cells with TGF-β1 and IL-15SA/IL-15RA for 48 hours, modeling IL-15SA/IL-15RA treatment for NK cells with normal-function, i.e., peripheral NK cells in early stage cancer patients or adjuvant therapy. Fig. 1 depicts direct NK lysis of four tumor cell lines: lung cancer cell line H460, prostate cancer cell line LNCap, estrogen receptor positive breast cancer cell line MCF7, and triple negative breast cancer cell line MDA-MB-231. IL-15SA/IL-15RA significantly increased NK cell-lysis in H460 (3.9-fold; *p* = 0.006), LNCap (1.4-fold; *p* = 0.0009), MCF7 (1.4-fold; *p* = 0.03), and MDA-MB-231 (3.5-fold; *p* = 0.0002). IL-15SA/IL-15RA significantly increased NK cell-lysis in three additional lung cancer cell

lines: H1703 (5.6-fold; $p = 0.002$), H520 (2.7-fold; $p = 0.0153$) and HCC4006 (1.4-fold; $p = 0.039$), (Supplemental Fig. 1). In agreement with previous reports (31, 32), TGF- β 1 significantly decreased NK cell-lysis in four of four cell lines. NK cells treated with TGF- β 1 in combination with IL-15SA/IL-15RA showed significantly increased lysis compared to TGF- β 1-treated NK cells in H460 (9.2-fold; $p = 0.02$), LNCap (1.3-fold; $p = 0.006$), MCF7 (1.7-fold; $p = 0.04$), and MDA-MB-231 (16.9-fold; $p = 0.002$). These results suggest that IL-15SA/IL-15RA protected NK cell-cytotoxicity from TGF- β 1-induced suppression. NK cells recognize and bind to tumor cells via multiple ligands. The tumors utilized (Fig. 1) represent a range of tumor types, each with diverse phenotypes. We have frequently observed that certain tumor cell lines are killed to a much greater degree than others, as well as seeing differences in killing of the same tumor from different healthy NK cell donors. When assaying for IL-15SA/IL-15RA mediated improvement of tumor cell killing, each cell line is controlled to itself. IL-15SA/IL-15RA was able to significantly improve the killing of TGF- β 1 inhibited NK cells, albeit not to the same level as NK cells that were not exposed to TGF- β 1. As this was a model for tumor microenvironment inhibition of NK cells (via TGF- β 1), significant improvement of TGF- β 1 exposed NK function by IL-15SA/IL-15RA may be clinically relevant.

To assess the potential activity of NK cells treated with IL-15SA/IL-15RA against normal cells, NK cells and B cells were isolated from a single (autologous) healthy donor. NK cells were either untreated or treated with IL-15SA/IL-15RA and used for killing assays against normal 'self' autologous B cells. IL-15SA/IL-15RA nominally increased NK lysis in B cells (6.2%). However, this was to a much lower magnitude compared to that of K-562 chronic myelogenous leukemia cells (positive tumor cell control; 36.3%) (Supplemental Fig. 2). These results are consistent with that of Rhode et al., where IL-15SA/IL-15RA administration to mice was well tolerated and there was no evidence of normal cell toxicity [25].

IL-15SA/IL-15RA rescued NK cell-cytotoxicity that had already been suppressed by TGF- β 1

To our best knowledge, it has been unclear whether NK cells already impaired by TGF- β 1 could be rescued. Therefore, modeling the IL-15SA/IL-15RA treatment for NK cells whose function is already suppressed by TGF- β 1, i.e., peripheral NK cells in advanced cancer patients or NK cells which already infiltrated tumor site, NK cells were treated with TGF- β 1 for 24 hours followed by culturing another 24 hours with IL-15SA/IL-15RA added to the medium containing TGF- β 1 (Fig. 2a). We first confirmed that the 24-hour treatment of TGF- β 1 significantly decreased NK lysis in H460 ($p = 0.009$) and MDA-MB-231 ($p = 0.003$) (Fig. 2b). Next, NK cells impaired by 24-hour TGF- β 1 treatment were cultured with IL-15SA/IL-15RA for another 24 hours. As shown in Fig. 2c, compared to NK cells treated with TGF- β 1 for 48 hours without adding IL-15SA/IL-15RA, NK cells treated with TGF- β 1 followed by IL-15SA/IL-15RA showed significantly higher lysis in H460 (8.5-fold; $p = 0.002$), LNCap (1.3-fold; $p = 0.003$), MCF7 (1.3-fold; $p = 0.03$), and MDA-MB-231 (11.1-fold; $p = 0.0005$). Taken together, these data indicate that IL-15SA/IL-15RA was able to substantially and significantly improve the killing TGF- β 1 inhibited NK cells, with or without cetuximab or avelumab, as well as rescue NK cell-cytotoxicity that had already been suppressed by TGF- β 1.

IL-15SA/IL-15RA inhibited TGF- β 1 from suppressing NK cell-ADCC activity

In order to measure the effect of IL-15SA/IL-15RA and TGF- β 1 on ADCC activity, an additional mechanism for NK killing, we confirmed the expression of programmed death-ligand 1 (PD-L1) and epidermal growth factor receptor (EGFR) in H460, LNCap, MCF7, and MDA-MB-231. H460, LNCap, and MDA-MB-231 expressed different levels of PD-L1 (Fig. 3a) and EGFR (Fig. 3b), while MCF-7 did not express these targets. Focusing on those three cell lines, ADCC activity mediated via PD-L1 antibody avelumab and EGFR antibody cetuximab was examined using NK cells cultured with TGF- β 1 and/or IL-15SA/IL-15RA for 48 hours. As shown in Fig. 3c, IL-15SA/IL-15RA-treated NK cells induced higher ADCC activity compared to untreated NK cells in three of three cell lines. In LNCap, avelumab-mediated ADCC lysis of NK cells treated by TGF- β 1 was significantly lower compared to that of untreated NK cells (0.7-fold; $p = 0.003$). However, avelumab-mediated ADCC lysis by NK cells treated with the combination of TGF- β 1 and IL-15SA/IL-15RA was significantly higher than that of NK cells treated with TGF- β 1 alone (1.5-fold; $p = 0.003$). Similarly, cetuximab-mediated ADCC activity by NK cells treated with the combination of TGF- β 1 and IL-15SA/IL-15RA was significantly higher than that of NK cells treated with TGF- β 1 alone (1.3-fold; $p = 0.03$). In these assays, the tumor cells express PD-L1. Avelumab (anti-PD-L1) binds to the tumor cells and the Fc portion of the IgG1 is recognized by the Fc receptor on the NK cells. The increased killing with avelumab is ADCC mediated and independent of the PD-1/PDL1 axis of NK cells. Taken together, IL-15SA/IL-15RA inhibited TGF- β 1 from suppressing NK cell-induced ADCC activity.

IL-15SA/IL-15RA inhibited TGF- β 1 from decreasing the expression of NK cell-activating receptors and cytotoxic granules

It has been shown that TGF- β 1 decreases the expression of NK-activating receptors and cytotoxic granules; this downregulation is associated with a concomitant decrease of NK cell activity (7, 31, 33). To assess whether IL-15SA/IL-15RA modulates the phenotype of NK cells modulated by TGF- β 1, the expression of select NK cell-activating receptors and cytotoxic granules was examined. As shown in Fig. 4a, the 3-hour treatment of NK cells with TGF- β 1 markedly decreased the RNA expression of *CD226*, *KLRK1*, *NCR3*, *GZMB*, *PRF1*. Compared to TGF- β 1-treated NK cells, NK cells treated with the combination of TGF- β 1 and IL-15SA/IL-15RA had significantly higher expression of *CD226* (2.4-fold; $p = 0.0009$), *NCR3* (3.1-fold; $p < 0.0001$), *GZMB* (1.2-fold; $p = 0.03$), and *PRF1* (1.4-fold; $p = 0.008$), whereas the RNA expression of *KLRK1* was decreased by IL-15SA/IL-15RA. Next, NK cells were cultured with TGF- β 1 and/or IL-15SA/IL-15RA for 48 hours followed by assessment of protein expression. As shown in Fig. 4b, the 48-hour treatment of NK cells with TGF- β 1 decreased the protein expression of CD226, NKG2D, Nkp30, granzyme B, and perforin, which was consistent with our observations of the RNA expression. IL-15SA/IL-15RA significantly increased the TGF- β 1-decreased MFI of CD226 ($p < 0.001$), NKG2D ($p < 0.001$), Nkp30 ($p < 0.001$), granzyme B ($p < 0.001$), and perforin ($p < 0.001$). Taken together, IL-15SA/IL-15RA inhibited TGF- β 1 from downregulating the expression of CD226, NKG2D, Nkp30, granzyme B, and perforin at the mRNA level and the protein level. NK cells were also obtained from two prostate cancer patients and either untreated or treated with IL-15SA/IL-15RA. These IL-15SA/IL-15RA-treated NK cells showed significantly increased lysis of H460 tumor cells (donor #1: 7.7-fold; $p = 0.0002$; donor #2:

10.8-fold; $p = 0.0003$), and MDA-MB-231 (donor #1: 14.2-fold; $p = 0.001$; donor #2: 18.5-fold; $p = 0.0005$) (Supplemental Fig. 3a). Moreover, for both donors, IL-15SA/IL-15RA significantly increased the expression of CD226, NKG2D, NKp30, granzyme B, and perforin (Supplemental Fig. 3b).

TGF- β 1-induced Smad2/3 signaling decreased the expression of NK cell-activating receptors and cytotoxic granules

TGF- β 1 activates both the canonical Smad-dependent pathway and the non-canonical Smad-independent pathway (34). It has been shown that TGF- β 1-induced Smad2/3 signaling downregulates transcription factor T-bet in NK cells, resulting in decreased expression of IFN- γ and inhibition of ADCC (9). To determine whether the canonical Smad2/3 pathway is responsible in TGF- β 1-mediated downregulation of the expression of NK cell-activating receptors and cytotoxic granules, the phenotype of NK cells was examined utilizing TGF β receptor I kinase inhibitor (SD208), which blocks phosphorylation of Smad2 and Smad3. We first confirmed that TGF β receptor I inhibitor blocked phosphorylation of Smad2/3 (Fig. 5a). As shown in Fig. 5b, blockade of phosphorylation of Smad2/3 by SD208 markedly inhibited TGF- β 1 from decreasing mRNA expression of *CD226* (2.3-fold), *KLRK1* (1.3-fold), *NCR3* (1.6-fold), *GZMB* (1.5-fold), and *PRF1* (1.4-fold). Fig. 5c shows protein expression determined by flow cytometry. The blockade of phosphorylation of Smad2/3 significantly increased the TGF- β 1-suppressed MFI of CD226 ($p < 0.001$), NKG2D ($p < 0.001$), NKp30 ($p < 0.001$), granzyme B ($p < 0.001$), perforin ($p < 0.001$). Taken together, TGF- β 1-induced Smad2/3 signaling decreased the expression of CD226, NKG2D, NKp30, granzyme B, and perforin in NK cells at the mRNA level and the protein level.

IL-15SA/IL-15RA inhibited Smad2/3-induced transcription

Our data suggested that IL-15SA/IL-15RA inhibited TGF- β 1 from decreasing the expression of NK cell-activating receptors and cytotoxic granules (Fig. 4) and that Smad2/3 signaling was responsible for the TGF- β 1 decreased expression of NK cell-activating receptors and cytotoxic granules (Fig. 5). We hypothesized that IL-15SA/IL-15RA interrupts the TGF- β 1-induced Smad2/3 pathway, and inhibits TGF- β 1 from suppressing NK cell-cytotoxicity. To examine which factors of the Smad2/3 pathway are affected by IL-15SA/IL-15RA, the expression of TGF β receptor II in NK cells was measured. The 48-hour treatment with IL-15SA/IL-15RA did not decrease the expression of TGF β receptor II (Fig. 6a). We next examined whether the phosphorylation of Smad2/3 in NK cells is affected by IL-15SA/IL-15RA. As shown in Fig. 6b, 1-hour treatment with TGF- β 1 increased phosphorylation of Smad2/3. IL-15SA/IL-15RA did not decrease the TGF- β 1-induced phosphorylation of Smad2 or Smad3 (Fig. 6b). Finally, we assessed the expression of Smad-induced genes, *EOMES*, *JUN*, *SERPINE1*, and *SMAD7* (35–38), to evaluate Smad-induced transcription activity. We confirmed that the 6-hour treatment of TGF- β 1 significantly increased the mRNA expression of four of four genes (Fig. 6c). Compared to non-treatment control, IL-15SA/IL-15RA significantly decreased the mRNA expression of *EOMES* (0.7-fold), *JUN* (0.7-fold), *SERPINE1* (0.3-fold), and *SMAD7* (0.7-fold). Moreover, compared to TGF- β 1-treated NK cells, NK cells treated with the combination of TGF- β 1 and IL-15SA/IL-15RA showed significantly decreased expression of *EOMES* (0.5-fold), *JUN* (0.7-fold), *SERPINE1* (0.2-fold), and *SMAD7* (0.7-fold). These data, taken together, indicate that

IL-15SA/IL-15RA-induced signaling interrupts TGF- β 1 signaling pathway by blocking Smad2/3-induced transcription, in turn indicating that IL-15SA/IL-15RA protects NK cells from TGF- β 1-induced immunosuppression (Fig. 6d).

***In vitro* treatment of PBMCs from healthy donors with IL-15SA/IL-15RA did not alter the frequencies of immune cell subsets**

Additional studies were carried out to determine if treatment of human PBMCs with IL-15SA/IL-15RA would adversely modulate the frequencies of immune cell subsets. PBMCs from three healthy donors were incubated for 72 hours with increasing concentrations of IL-15SA/IL-15RA, and then evaluated by flow cytometry. Of the 9 'classic' immune cell subsets analyzed, only NKT cells increased significantly after treatment (Supplemental Table 1a). Of the 'refined' immune cell subsets, there were trends for decreases in naïve and central memory CD4 and CD8 T-cells, but only central memory CD4 decreased significantly (Supplemental Table 1b).

Discussion

The clinical efficacy of select immune checkpoint inhibitors suggests that cytotoxic immune cells eliminating tumor cells play a key role in cancer immunotherapy. TGF- β 1 is a key immunosuppressive factor released within the tumor microenvironment. Several clinical studies reported that the levels of TGF- β 1 were often elevated in the serum of cancer patients and correlate with tumor progression (5, 6, 39). To investigate the efficacy of IL-15SA/IL-15RA for TGF- β 1-induced suppression of NK cell-cytotoxic function, we utilized two models of IL-15SA/IL-15RA treatment for NK cells: (1) treatment for normal function NK cells, such as peripheral NK cells in early stage cancer patients or adjuvant therapy (Fig. 1) and (2) treatment for TGF- β 1-impaired NK cells, such as tumor-infiltrating NK cells or peripheral NK cells in advanced cancer patients (Fig. 2). As shown in Fig. 1, IL-15SA/IL-15RA protected NK cell-cytotoxic function from TGF- β 1-induced suppression. Similarly, IL-15SA/IL-15RA rescued NK cell function that had already been suppressed by TGF- β 1 (Fig. 2c). Moreover, we demonstrated that the activity of cancer patient-derived NK cells was increased by treatment with IL-15SA/IL-15RA (Supplemental Fig. 3). We have observed a significant upregulation of NK ligands on NK cells following exposure to IL-15SA/IL-15RA, suggesting that enhanced NK cell-tumor cell synapse formation may be important in improved NK killing. Planned studies will examine this hypothesis. Our previous *in vivo* study showed that IL-15SA/IL-15RA induced significant anti-tumor activity against spontaneous pulmonary metastases, resulting in prolonged survival (23). Taken together, our observations indicate the utility of IL-15SA/IL-15RA for both adjuvant and advanced cancer patients.

It has been reported that TGF- β 1 suppresses the function of NK cells by inducing miRNA23a, which in turn targets the degranulation marker CD107a (LAMP1) (40, 41). To test this possibility, NK cells were isolated from fresh healthy donor PBMC and treated with IL-15SA/IL-15RA or TGF- β 1 and then incubated with K-562 cells for 4 hours in the presence of CD107a antibody, followed by flow cytometry analysis. We observed that TGF- β 1 treatment did reduce the expression of the degranulation marker CD107a as reported.

However, concurrent treatment with IL-15SA/IL-15RA and TGF- β 1 abrogated the reduced levels of CD107a. IL-15SA/IL-15RA mediated a 1.7-fold increase in MFI of CD107a in TGF- β 1-untreated NK cells and 1.6-fold increase in CD107a in TGF- β 1 treated NK cells (Supplemental Fig. 4).

NK cells also eliminate tumor cells by ADCC. As shown in Fig. 3c, avelumab, an anti-PD-L1 IgG1 antibody, increased TGF- β 1-treated NK cell-lysis in H460 (4.8-fold), LNCap (1.6-fold), and MDA-MB-231 (12.4-fold) compared to isotype control antibody. We previously reported that avelumab-induced ADCC activity has a correlation with PD-L1 expression of tumor cells (28), indicating that the lower ADCC activity against LNCap was derived from its lower PD-L1 expression (18.1%) compared to H460 (96.8%) and MDA-MB-231 (78.3%) (Fig. 3a). Moreover, against H460 and MDA-MB-231, TGF- β 1 did not decrease avelumab-mediated ADCC activity. Consistent with our results, some studies also showed that TGF- β 1 did not suppress cetuximab-mediated ADCC activity against tumor cells with high-EGFR expression (9, 42). On the contrary, in LNCap, which has low PD-L1 expression, TGF- β 1 downregulated avelumab-mediated ADCC activity (0.7-fold) (Fig. 3c). However, the addition of IL-15SA/IL-15RA to TGF- β 1 significantly enhanced ADCC lysis (1.5-fold; $p = 0.003$). We have previously showed that IL-15SA/IL-15RA enhanced rituximab-mediated ADCC activity against B cell lymphoma (43). The results of this study further demonstrate that IL-15SA/IL-15RA has efficacy to increase ADCC activity against tumors that have low expression of target receptor for IgG1 antibody.

Intratumoral and peripheral NK cells from cancer patients may display phenotype alterations compared with NK cells from healthy donors (7, 42). Lee, et al., reported that the elevated level of plasma TGF- β 1 correlated with low NKG2D expression on human NK cells from cancer patients (44). Several *in vitro* studies provided the mechanisms of TGF- β 1-mediated suppression of NK cell function. Previous reports showed that TGF- β 1 decreased the expression of NK receptors by interrupting mTOR signaling (45), and that TGF- β 1-induced Smad2/3 signaling inhibited T-bet expression of NK cells, mediating the decrease of IFN γ , granzyme B, and perforin (8, 9). In this study, we confirmed that the expression of granzyme B and perforin was downregulated by Smad2/3 signaling, and further detected that NKG2D, NKP30, CD226 were also downregulated by Smad2/3 for the first time (Fig. 5b and c).

Since it has been shown that TGF- β signaling contributes not only to suppress functions of cytotoxic immune cells, but also in generating a favorable microenvironment for tumor growth and metastasis, numerous reagents targeting the TGF- β pathway have been developed. Despite the large number of long-term clinical studies, no TGF- β inhibitors have yet been approved. It is well known that TGF- β has dual functions as a tumor suppressor in normal and early neoplastic cells and as a promoter of tumor growth and metastasis in established cancers (5). Furthermore, the crosstalk between TGF- β and other signaling pathways may lead to insufficient TGF- β signaling inhibition (34). Our data showed that IL-15SA/IL-15RA blocked Smad2/3-induced transcription (Fig. 6), resulting in IL-15SA/IL-15RA inhibition of TGF- β 1 from decreasing the expression of NK cell-activating markers and cytotoxic granules (Fig. 4). These results indicate that in addition to increased NK cell function via promoting IL-15 signaling pathway, IL-15SA/IL-15RA functions as an inhibitor of TGF- β 1 signaling, which downregulates NK cell function. IL-15 is also shown

to augment mTOR signaling which correlates with increased expression of genes related to cell metabolism, respiration, and activation (46). Thus, IL-15SA/IL-15RA could also play a role in reversing the suppression of TGF- β 1 on NK-cell cytotoxicity by activating the mTOR pathway in addition to its capability of mitigating the TGF- β 1 Smad2/3-induced transcription.

In conclusion, in this study we demonstrated that (1) IL-15SA/IL-15RA protected/rescued TGF- β 1-suppressed NK cell cytotoxic function, (2) Smad2/3 signaling was responsible for TGF- β 1-downregulated expression of NK cell-activating markers and cytotoxic granules, and finally, (3) IL-15SA/IL-15RA blocked Smad2/3-induced transcription, resulting in the rescue of NK cell-cytotoxic function from TGF- β 1-induced suppression. Our findings demonstrate that IL-15SA/IL-15RA augments NK cell function by inhibiting TGF- β 1-induced suppression, providing a potential remedy for NK cell dysfunction in the immunosuppressive tumor microenvironment. The clinical application of these data would be to treat patients with standard-of-care FDA approved avelumab (anti-PD-L1), cetuximab (anti-EGFR), or additional emerging mAbs with an IgG1 isotype, while co-administering the IL-15SA/IL-15RA. In this way, we hypothesize that mAbs would engage endogenous patient NK cells or adoptively transfused NK cells (47–49) to facilitate NK killing of tumor via ADCC, and that the tumor derived immunosuppressive TGF- β 1 effects would be abrogated by NK exposure to IL-15SA/IL-15RA.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

The authors thank Marion Taylor for excellent technical assistance, and Debra Weingarten for her editorial assistance in the preparation of this manuscript. We thank Jin-Qiu Chen and Xiaoling Luo (Collaborative Protein Technology Resource, NCI/CCR) for capillary-electrophoresis immunoassays.

Grant Support

This research was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National Institutes of Health, as well as through a Cooperative Research and Development Agreement (CRADA) between Altos BioScience and the National Cancer Institute.

Abbreviations:

ADCC	Antibody-dependent cellular cytotoxicity
EGFR	Epidermal growth factor receptor
E:T	Effector cell:target cell
FDA	Food and Drug Administration
IFN	Interferon
IL	Interleukin

IL-15SA/IL-15RA	Interleukin-15 N72D superagonist/interleukin-15 receptor α Sushi-Fc fusion complex
mAb	Monoclonal antibody
MHC	Major histocompatibility complex
mTOR	Mammalian target of rapamycin
NK	Natural killer
PBMC	Peripheral blood mononuclear cell
PCR	Polymerase chain reaction
PD-L1	Programmed death-ligand 1
TGF	Transforming growth factor
TME	Tumor microenvironment

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Fig. 1.

IL-15 super agonist/IL-15 receptor α (IL-15SA/IL-15RA) protected NK cell-cytotoxicity from TGF- β 1-induced functional suppression. NK cell-killing assays were performed using four tumor cell lines: lung cancer H460, prostate cancer LNCap, estrogen receptor positive breast cancer MCF7, and triple negative breast cancer MDA-MB-231. NK cells obtained from healthy donors were either untreated or treated with TGF- β 1 (2 ng/ml) and/or IL-15SA/IL15-RA (50 ng/ml) for 48 hours, after which NK cells were washed and used for killing assays at an effector:target (E:T) ratio of 50:1. Statistical analyses were done by

Student's t -test, * = $p < 0.05$, error bars indicate mean $\pm$ S.D. for triplicate measurements.
This experiment was repeated at least three times with similar results.

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Fig. 2.

IL-15SA/IL-15RA rescued NK cell-cytotoxicity that had already been suppressed by TGF- β 1. NK cell-killing assays were performed using four tumor cell lines: lung cancer H460, prostate cancer LNCap, estrogen receptor positive breast cancer MCF7, and triple negative breast cancer MDA-MB-231. NK cells obtained from healthy donors were either untreated or treated with TGF- β 1 (2 ng/ml) for 48 hours or treated with TGF- β 1 (2 ng/ml) for 24 hours followed by culturing of cells for another 24 hours with added IL-15SA/IL-15RA (50 ng/ml). NK cells were used for killing assays at an E:T ratio of 50:1. **(a)** Schema of the

treatment for NK cells. **(b)** NK cell-killing assays utilizing NK cells that were untreated or treated with TGF- β 1 for 24 hours. **(c)** NK cell-killing assays utilizing NK cells that were untreated or treated with TGF- β 1 for 48 hours, treated with TGF- β 1 for 24 hours followed by another 24 hours with added IL-15SA/IL-15RA. Statistical analyses were done by Student's *t*-test, * = $p < 0.05$, error bars indicate mean $\pm$ S.D. for triplicate measurements

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Fig. 3. IL-15SA/IL-15RA inhibited TGF- β 1 from suppressing NK cell-ADCC activity. **(a)** The expression of programmed death-ligand 1 (PD-L1) (solid histograms) and **(b)** epidermal growth factor receptor (EGFR) (solid histograms) in four tumor cell lines: lung cancer H460, prostate cancer LNCap, estrogen receptor positive breast cancer MCF7, and triple negative breast cancer MDA-MB-231. Dot histograms depict isotype control for each antibody. Inset numbers indicate % positive cells and mean fluorescence intensity (MFI) (parentheses). **(c)** ADCC assays were performed using three tumor cell lines. NK cells obtained from healthy

donors were untreated or treated with TGF- β 1 (2 ng/ml) and/or IL-15SA/IL-15RA (50 ng/ml) for 48 hours, then washed and used for ADCC assays at an E:T ratio of 10:1. To mediate ADCC, the following IgG1 mAbs were utilized: rituximab as an isotype control, avelumab as anti-PD-L1 antibody, cetuximab as anti-EGFR antibody. Statistical analyses were done by Student's *t*-test, * = $p < 0.05$, error bars indicate mean $\pm$ S.D. for triplicate measurements. This experiment was repeated at least three times with similar results

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a **b**

			CD226		NKG2D		NKp30		Granzyme B		perforin	
	TGF-β1	IL-15SA/IL-15RA	%	MFI	%	MFI	%	MFI	%	MFI	%	MFI
□	-	-	67	270	67	212	78	871	100	3579	100	36952
■	-	+	75	359	88	488	96	4897	100	7901	100	47041
■	+	-	35	207	43	134	42	481	100	2693	100	28266
▨	+	+	53	262	51	162	44	587	100	4739	100	44452

Fig. 4.

IL-15SA/IL-15RA inhibited TGF-β1 from decreasing the expression of NK cell-activating receptors and cytotoxic granules. **(a)** NK cells were untreated or treated with TGF-β1 (2 ng/ml) and/or IL-15SA/IL-15RA (50 ng/ml) for 3 hours. The mRNA expression relative to GAPDH was determined by real time-PCR. Statistical analyses were done by Student's *t*-test, * = *p* < 0.05, error bars indicate mean ± S.D. for triplicate measurements. This experiment was repeated at least twice with similar results. **(b)** NK cells were untreated or treated with TGF-β1 (2 ng/ml) and/or IL-15SA/IL-15RA (50 ng/ml) for 48 hours followed

by flow cytometry for CD226, NKp30, NKG2D, granzyme B, and perforin. Histograms and table depict the expression of these proteins (% positive and MFI are shown in the table). Values in bold denote a significant ($p < 0.05$) increase relative to NK cells treated with TGF- β 1.

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Fig. 5.

TGF-β1-induced Smad2/3 signaling decreased the expression of NK cell activating receptors and cytotoxic granules. **(a)** NK cells were untreated or treated with the TGF-β1 receptor inhibitor SD208 (1 μg/ml) for 1 hour, followed by treatment with TGF-β1 (2 ng/ml) for 1 hour, then stained for phosphorylated Smad2/3 (pSmad2/3). Histograms and table depict the expression of these proteins. Values in bold denote a significant ($p < 0.05$) decrease relative to NK cells treated with TGF-β1 alone. **(b)** NK cells were untreated or treated with TGF-β1 receptor inhibitor (1 μg/ml) for 1 hour, followed by treatment with

TGF- β 1 (2 ng/ml) for 6 hours; RNA was then extracted. The mRNA expression relative to GAPDH was determined by real time-PCR. Statistical analyses were done by Student's *t*-test, * = $p < 0.05$, error bars indicate mean $\pm$ S.D. for triplicate measurements. (c) NK cells were untreated or treated with TGF- β 1 receptor inhibitor (1 μ g/ml) for 1 hour, followed by TGF- β 1 (2 ng/ml) for 48 hours. Cells were then stained for flow cytometry for CD226, NKG2D, NKp30, granzyme B, and perforin. The bar graphs depict the MFI of these proteins. * = $p < 0.05$.

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Fig. 6.

IL-15SA/IL-15RA inhibited Smad2/3-induced transcription. **(a)** NK cells were untreated or treated with TGF- β 1 (2 ng/ml) and/or IL-15SA/IL-15RA (50 ng/ml) for 48 hours; cells were then stained with TGF β receptor II antibody for flow cytometry. The table shows % positive and MFI of TGF β receptor II. **(b)** NK cells were untreated or treated with TGF- β 1 (2 ng/ml) and/or IL-15SA/IL-15RA (50 ng/ml) for 1 hour. The cell lysis was extracted followed by Simple Western technology as described in Materials and Methods. Gel images of Simple Western analyses and the quantitation of relative pSmad and Smad peaks are shown. **(c)** NK

cells were untreated or treated with TGF- β 1 (2 ng/ml) and/or IL-15SA/IL-15RA (50 ng/ml) for 6 hours. The relative mRNA expression to GAPDH was determined by real time-PCR. Statistical analyses were done by Student's *t*-test, * = $p < 0.05$, error bars indicate mean $\pm$ S.D. for triplicate measurements. Each experiment was repeated at least twice with similar results. **(d)** A schema of the TGF- β 1 signaling pathway interrupted by IL-15SA/IL-15RA. IL-15SA/IL-15RA-mediated IL-15 signaling blocks SMAD2/3-induced transcription.