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A novel inhibitor of proteasome deubiquitinating activity renders tumor cells sensitive to TRAIL-mediated apoptosis by natural killer cells and T cells

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Abstract

The proteasome inhibitor bortezomib simultaneously renders tumor cells sensitive to killing by natural killer (NK) cells and resistant to killing by tumor-specific T cells. Here, we show that b-AP15, a novel inhibitor of proteasome deubiquitinating activity, sensitizes tumors to both NK and T cell-mediated killing. Exposure to b-AP15 significantly increased the susceptibility of tumor cell lines of various origins to NK ($p < 0.0002$) and T cell ($p = 0.02$) – mediated cytotoxicity. Treatment with b-AP15 resulted in increased TRAIL [tumor necrosis factor-related apoptosis-inducing ligand] receptor-2 expression ($p = 0.03$) and decreased cFLIP expression in tumor cells *in vitro*. In tumor-bearing SCID/Beige mice, treatment with b-AP15 followed by infusion of either human NK cells or tumor-specific T cells resulted in a significantly delayed tumor progression compared with mice treated with NK cells ($p = 0.006$), T cells ($p < 0.0001$), or b-AP15 alone ($p = 0.003$). Combined infusion of NK and T cells in tumor-bearing BALB/c mice following treatment with b-AP15 resulted in a significantly prolonged long-term survival compared with mice treated with b-AP15 and NK or T cells ($p \leq 0.01$). Our findings show that b-AP15-induced sensitization to TRAIL-mediated apoptosis could be used as a novel strategy to augment the anti-cancer effects of adoptively infused NK and T cells in patients with cancer.

Keywords: Natural killer cells, T cells, proteasome inhibitor, TRAIL, Immunotherapy

Précis: Treatment with the proteasome deubiquitinating inhibitor b-AP15 sensitizes tumor cells to NK and T cell –mediated killing *in vitro* and *in vivo*. Infusion of both NK and T cells following b-AP15 treatment prolongs survival in tumor-bearing hosts.

Introduction

Adoptive T cell therapy elicits long-lasting clinical responses in patients with malignant metastatic melanoma [1]. However, randomized clinical trials have failed to find similar results in other malignancies [2]. In addition the adoptive infusion of autologous natural killer (NK) cells has yet to show clinical benefit in treating cancer patients [3]. This clinical failure may be in part due to sustained immunological selection pressure on tumor cells resulting in the development of tumor escape variants that are effectively invisible to the immune system. One of the most frequent immune escape strategies tumors use to avoid being detected by cytotoxic T cells is down-regulation of MHC class I [4,5]. As a result, individual cells may display differential expression of MHC class I within a growing tumor mass [6]. While MHC class I positive tumor cells are targeted by tumor-specific T cells, MHC class I negative tumor cells can be killed by NK cells due to lack of ligation of inhibitory Killer Immunoglobulin-like receptors (KIRs) on NK cells. Simultaneous adoptive cell therapy with tumor-specific T cells and NK cells may therefore offer a strategy to generate broader anti-tumor responses compared with infusion of NK or T cells as a monotherapy. Such combined therapy could potentially result in improved clinical responses.

Besides secreting lytic granules, T and NK cells ligate death receptors on tumor cells to induce apoptosis [7-10]. We and others have recently demonstrated that exposure to the proteasome inhibitor bortezomib significantly up-regulated the expression of death receptors to TNF-related apoptosis-inducing ligand (TRAIL) [11-13]. However, bortezomib-treated tumors sensitized to NK cell apoptosis simultaneously acquire resistance to antigen-specific T cells [14]. As bortezomib predominantly inhibits the chymotryptic-like activity of the 20S

proteasome [15], impaired antigen processing and presentation prevent tumor-specific T cells from killing bortezomib-treated tumors.

We have recently identified the small molecule b-AP15 as a novel inhibitor of proteasome deubiquitinating activity [16]. Cells treated with b-AP15 accumulate polyubiquitinated proteins, with similar kinetics to cells treated with bortezomib. b-AP15-induced proteasome inhibition was concomitant with the up-regulation of apoptotic markers and the accumulation of cell-cycle regulatory proteins, leading to cell cycle arrest and apoptosis independent of the tumor suppressor p53 or overexpression of the *BCL2* oncogene, which are implicated in bortezomib resistance.

In the present study we sought to investigate whether inhibition of proteasome deubiquitinating activity sensitizes tumors to both T and NK cell-mediated killing. Similarly to bortezomib, we found that treatment with sub-apoptotic doses of b-AP15 resulted in an increased surface expression of TRAIL-R2 and decreased expression of cFLIP in tumor cells. As a consequence, b-AP15-treated tumors displayed an increased sensitivity to killing by both NK and T cells *in vitro*. Furthermore, significantly increased survival times were observed in mice treated with b-AP15 followed by combined infusion of syngeneic NK and tumor-specific T cells relative to mice treated with b-AP15 followed by infusion of either syngeneic NK or tumor-specific T cells.

Materials and methods

Human NK and T cell culture

Human NK cells were isolated from peripheral blood mononuclear cells (PBMC) of healthy donors and melanoma patients (ethical permit dnr #2009/848-3) by negative immunomagnetic bead depletion (Miltenyi Biotech, BergischGladbach, Germany). Purified NK cells ($\geq 95\%$) were expanded with 25 Gy irradiated autologous PBMC (feeder cells) at a 1:20 cell ratio (NK: feeder) in X-VIVO 20 media (Lonza, Verviers, Belgium) supplemented with 10% heat-inactivated human AB serum (Karolinska Hospital, Stockholm, Sweden) and 500 U/ml recombinant human interleukin-2 (IL-2) (Novartis pharma GmbH, Nurnberg, Germany). Fresh medium containing 500 U/ml IL-2 was added on days 5, 8 and 11, and NK cells were tested for cytotoxicity or infused into mice on days 11-14 of expansion. Contamination of CD3 positive T cells was $<1\%$.

Tumor specific T cells were isolated from tumor biopsies from HLA-A2 positive melanoma patients. Biopsies were manually dissected into small pieces and transferred to 24-well plates followed by incubation with 6,000 U/ml of IL-2 in X-vivo 20 medium supplemented with 5% AB serum for six days. Tumor infiltrating lymphocytes were screened for antigen-specificity by intracellular staining for interferon-gamma (IFN γ) and IL-2 (Biolegend, San Diego, USA) against T2 cells pulsed with HLA-A2 binding melanoma-associated peptides gp100, MAGE-1, MAGE-3, Survivin and Mart-1 (Genecust, Dudelang, Luxembourg). 8.4% of T cells stained positive for IFN γ and IL-2 after co-culture with Mart1_(ELAGIGILTV)-pulsed T2 cells, but not against any of the other peptides. These TILs were expanded in X-vivo 20 medium supplemented with 5% AB, 500 U/ml IL-2 and 50 ng/ml anti-CD3 antibody (Janssen-Cilag, Sollentuna, Sweden) and irradiated (40 Gy) allogeneic PBMC. Medium supplemented with 500 U/ml of IL-2 was replenished on days 5 and 8 of culture. Purity of CD3 positive T cells

was $\geq 94\%$ with $< 1\%$ contamination of CD56 positive NK cells. Expanded NK and T cells were tested against K562 cells (Supplementary Fig. 1A). T cells were tested negative against the HLA-A2 negative tumor cell line EST115 (Table 1).

Murine NK and T cell culture

Murine NK cells were isolated from BALB/c splenocytes by negative bead depletion (MACS, Miltenyi Biotech) and cultured in 96-U bottom plates in RPMI1640 medium supplemented with 10% FCS, 50 μM Beta-mercapethanol, 25 $\mu\text{g/ml}$ gentamycin, 2 mM L-glutamine, 1% NEAA (Invitrogen, Eugene, Oregon, USA) and 500 U/ml of human IL-2 for 5 days. Murine tumor-specific T cells were generated by whole tumor cell vaccination. Briefly, BALB/c mice received three weekly intraperitoneal (i.p) injections of 1×10^6 irradiated (10,000 Rad) D2F2 breast cancer cells. Spleen and lymphnodes were collected and re-stimulated with irradiated (5,000 Rad) D2F2 and recombinant human IL-2 (100 U/ml) *in vitro* for four days. T cells were tested for tumor reactivity in a ^{51}Cr -release assay against D2F2 cells, YAC-1 cells, and RENCA cells. Generated T cells tested negative against RENCA (Table 1) and YAC-1 tumors (Supplementary Fig. 1B).

Tumor cell lines

Tumor cell lines include: EST152, EST027, EST066, EST049, EST025, JuSo (human melanoma) MDA453, MCF-7, MDA484 (human breast cancer), PC3, DU145 (human prostate cancer), J82, RT4 (human bladder cancer), A498, JOHW (kidney cancer), HCT116 (colon cancer), SKOV3, HeLa (ovarian cancer), BJ1 (normal fibroblast), and K562. Murine cell lines include: D2F2 (breast cancer), RENCA (renal cancer), B16 (melanoma) and YAC-1 (lymphoma). STR Identifier (Applied Biosystems, UK) was used to verify the origin of human tumor cell lines; EST152, EST027, EST066, EST049, EST025, MDA453, MCF-7, MDA484, J82, HCT116, HeLa and SKOV3. The EST melanoma cell lines were provided by

Dr. Rolf Kiessling, Karolinska Institutet through the IPD-ESTDAB Database

(<http://www.ebi.ac.uk/ipd/estdab/directory.html>). All cell lines were maintained in RPMI1640 media with 10% fetal calf serum (Invitrogen, Eugene, Oregon, USA), 50 U/ml penicillin, and 50 mg/ml streptomycin (Invitrogen).

Reagents and antibodies

Tumor cells were treated *in vitro* with 1 μ M of b-AP15 (3,5-bis[(4-nitrophenyl)methylidene]-1-prop-2-enoylpiperidin-4-one, Oncotargeting AB Uppsala, Sweden) for 3 hours before analysis for phenotype by flow cytometry or susceptibility to killing in cytotoxicity assays. J82 and EST027 cells were treated with 20 nM of bortezomib (Millennium Pharmaceuticals, Inc.) for 20 hours before testing in cytotoxicity assays. To evaluate TRAIL- or Fas ligand – mediated cytotoxicity of human tumors, recombinant TRAIL or Fas ligand (Peprotech, London, UK) was added to cells in cytotoxicity assays at 100 ng/ml and 150 ng/ml respectively. Concanamycin A (CMA; Sigma, Schnellendorf, Germany) was added in cytotoxicity assays at 100 nM to degrade perforin. Isolated and expanded NK cells and TILs were assessed for purity and TRAIL expression by flow cytometry after staining with antibodies against human CD3, CD56, CD4 and CD8, TRAIL (Biolegend). Human tumors were stained for TRAIL-R1, R2, R3, Fas, MICA/B and Nectin-2, MHC class I (Biolegend). Murine BALB/c D2F2 tumor cells were stained with TRAIL-R2 (Biolegend) before and after treatment with 1 μ M b-AP15 for 3 hours. Apoptosis was analyzed by staining with Annexin-V and 7-AAD (Biolegend). All flow cytometry experiments were acquired on an LSRII (BD Biosciences, San Jose, California, USA) and data was analyzed using FlowJo software (Tree Star, Ashland, USA).

Western Blot

J82, JOHW, DU145, JuSo, A498, HCT116, HeLa and MCF-7 cells were treated with 1 μ M of b-AP15 for 3 hours or left untreated. Post-treatment cells were lysed in buffer (cellLytic M, Sigma, St. Louis, Mo, USA) containing protease inhibitor cocktail (Sigma). Equal amount of protein (20 μ g) from total cell lysates were resolved on NuPAGE gels, transferred to PVDF, blocked in 5% milk and incubated overnight incubation with rabbit anti-c-FLIP (Cell Signaling Technology, Beverly, MA), or mouse anti- β -actin (Sigma) antibodies. Membranes were incubated with HRP-linked goat anti-rabbit or anti-mouse IgG Abs (Cell Signaling Technology) for 1 hour at room temperature and developed by enhanced chemiluminescence (GE Healthcare, Fairfield, CT). The protein quantity was determined relative to β -actin and analyzed by ImageJ-software (National Institutes of Health, <http://rsb.info.nih.gov/ij>).

Cytotoxicity and cytokine production assays

In chromium51-release cytotoxicity assays, target cells were labeled with ^{51}Cr (50 μ Ci) (PerkinElmer, Groningen, The Netherlands) and seeded in 96 U-bottom plates in triplicates. Human or murine NK or T cells were added to target cells at different E:T ratios. Following 18 hour incubation, 25 μ l of supernatant was transferred onto Luma plates (PerkinElmer) and analyzed using MicroBeta scintillation (TRILUX 1450) counter (PerkinElmer). For intracellular staining of IFN γ , 10,000 tumor cells were plated with 20,000-200,000 NK or T cells in a 96 U-bottom plate and incubated for 6 hours. Staining was performed following the manufacturer's instructions (BD Biosciences). Where indicated, cytotoxicity assays were performed in presence of neutralizing antibodies against TRAIL (10 μ g/ml), NKG2D (10 μ g/ml), Fas-ligand (10 μ g/ml), DNAM-1(10 μ g/ml) (Biolegend) or CMA (100 nM,).

***In vivo* tumor models**

All animal experiments were performed at the Department of Microbiology, Tumor and Cell Biology (MTC) or the Swedish Institute for Infectious Disease Control, Karolinska Institutet

under approved study N42/10. SCID/beige mice were purchased from Charles River (Sulzfeld, Germany). SCID/Beige mice (8-12 weeks old) were injected subcutaneously (s.c) with J82 tumors (3×10^6 /mouse) in the right flank. When tumors were palpable ($>1 \text{ mm}^3$, day 10), mice were grouped to receive either vehicle (n=4) or b-AP15 (n=4) at 2.5 mg per kg of body weight (i.p). The following day, mice were injected intravenously (i.v) with 2×10^6 NK cells or 4×10^6 TILs. In cytotoxicity assays, *in vitro* killing of untreated J82 tumors was approximately 2-fold higher by NK cells compared with TILs; therefore a 1:2 ratio of infused NK cells and TILs was used. Mice were treated for a total of three cycles, receiving injections of b-AP15 on days 12, 19 and 26 and cell infusions on days 13, 20, and 27 post tumor injection.

To investigate the potential synergistic effect of combined NK and T cell therapy, immune competent male BALB/c mice were injected s.c with D2F2 cells (100,000/mouse) in the right flank. Alternatively, BALB/c mice were injected i.v with lentiviral-transduced luciferase (AMS Biotechnology, UK) D2F2 cells (100,000/mouse). Within one week, when s.c tumors were palpable ($>5 \text{ mm}^3$, day 6) or pulmonary metastases were visible by bioluminescence imaging (day 3), BALB/c mice were treated with either vehicle (PBS) or three weekly cycles of b-AP15 (2.5 mg/kg) followed by the next day infusion of NK cells ($0.75\text{-}1.5 \times 10^6$ cells) and/or tumor-specific T cells ($1\text{-}2 \times 10^6$ cells). Mice treated with the combination of both NK and T cells received the same number of total cells compared to mice receiving NK or T cells alone. Tumor progression was assessed by palpation twice weekly or by *in vivo* bioluminescence imaging. *In vivo* imaging was performed by injecting mice with luciferin (3 ug/mouse, i.p). Mice were imaged the IVIS camera platform (Caliper LifeSciences Benelux & Nordic, Belgium). The *in vivo* experiments were repeated twice with 4-6 mice per treatment group.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 5.0 software. The *in vitro* data were analyzed by paired or unpaired t-test and *in vivo* data were analyzed using unpaired t-test, two-way ANOVA and Log-rank (Mantel-Cox) test. Correlation tests were performed using Pearson's correlation test. p-values below 0.05 were considered statistically significant between treatment groups.

Results

Increased TRAIL-mediated killing of b-AP15 treated tumors

b-AP15-treated J82 tumor cells were stained with Annexin V to evaluate the level of apoptosis. No change in the percentage of dead cells was observed in cells treated with 1 μ M b-AP15 for 3 hours compared with untreated cells (untreated $7\pm 4\%$, b-AP15 $8\pm 4\%$). Under these conditions, the expression of TRAIL-R2 was significantly up-regulated ($p=0.03$) compared with untreated controls in a panel of tumor cell lines (J82, DU145, A498, HCT116, HeLa, MDA453) (Fig. 1A). In contrast, no significant change in expression of Fas was observed in these cell lines following treatment with b-AP15 (Fig. 1B). Additionally, no changes were observed in the expression of TRAIL-R1, TRAIL-R3, MHC class I, MICA/B, and Nectin-2 following treatment of J82 cells with b-AP15 (Supplementary Fig. 1C). In contrast, expression of cFLIP was down-regulated in J82 cells following exposure to b-AP15 (Supplementary Fig. 1D). When tumor cell lines were examined for susceptibility to TRAIL-induced apoptosis, b-AP15 pre-treatment resulted in significantly increased TRAIL-mediated killing in several tumor cell lines including HCT116, A498, J82, RT4, MDA453, and DU145 ($p=0.002$) (Fig. 1C). Killing by recombinant FasL was higher in b-AP15 treated A498 cells compared with untreated cells. However, no increase in FasL-mediated killing was observed in any of the other cells lines after treatment with b-AP15 ($p=0.07$) (Fig. 1D).

b-AP15 sensitizes tumor cells to TRAIL-mediated NK- and T- cell killing *in vitro*

Upon activation with IL-2, human NK and T cells express TRAIL (Supplementary Fig. 2A). We next investigated whether b-AP15-treated tumor cells would be more sensitive to killing by activated NK and T cells. Indeed, human tumor cells treated with b-AP15 were

significantly more sensitive to killing by NK ($p < 0.0002$) and tumor (Mart1) specific T cells ($p = 0.02$) (Fig. 2A and Table 1). In contrast to our previous findings, where tumor cell lines pretreated with the proteasome inhibitor bortezomib were resistant to T cell-mediated killing [14], b-AP15 pretreated J82 and EST027 cells were highly sensitized to T cell-mediated killing relative to untreated or bortezomib treated cells (Supplementary Fig. 2B). Importantly b-AP15 did not sensitize fibroblasts to NK cell-mediated killing (Table 1).

To confirm if the underlying mechanism of killing was dependent on TRAIL-R2 signaling, cytotoxicity assays were performed in the presence of neutralizing antibodies. Both NK and T cell-mediated killing of b-AP15-treated human tumors was significantly reduced in the presence of neutralizing antibodies to TRAIL. Although overall cytotoxicity was reduced in presence of the perforin inhibitor concanamycin A and neutralizing antibodies to Fas ligand, b-AP15 still sensitized tumors to NK- and T-cell-mediated killing in presence of these inhibitors (Fig. 2B). Furthermore, no significant reduction in killing were observed in presence of neutralizing antibodies to NKG2D or DNAM-1 between untreated and b-AP15 treated tumor cells (Supplementary Fig. 2C).

Given that treatment with b-AP15 results in increased TRAIL-R2 expression and decreased cFLIP expression, we next addressed which of these events are required for b-AP15-mediated sensitization to NK cell killing. An overall positive correlation in NK cell-mediated killing and expression of TRAIL-R2 was observed in tumor cell lines before and after treatment with b-AP15 ($p = 0.006$). Although an average decrease of $56\% \pm 11\%$ in the expression of cFLIP was observed following treatment with b-AP15 in several cell lines (J82, JOHW, JuSo, and HeLa), no significant overall correlation was found between the cFLIP expression and sensitivity to NK cell-mediated killing (Fig. 3A). Furthermore, increased NK cell-mediated killing was only observed in individual cell lines where expression of TRAIL-R2 was

significantly increased. In contrast, NK cell-mediated killing was independent of decreased cFLIP expression after b-AP15 treatment (Fig. 3B).

b-AP15 sensitizes tumor cells to adoptively infused NK and T cells *in vivo*.

To investigate the therapeutic impact of b-AP15 in sensitizing tumors to human NK and T cell –mediated killing, tumor-bearing immune-deficient SCID/Beige mice were pre-treated with b-AP15 and thereafter infused with either NK or tumor-specific T cells. Treatment with b-AP15, NK, or T cells alone resulted in a significantly delayed tumor progression compared with untreated mice ($p < 0.002$; data not shown). Furthermore, a further delay in tumor progression was observed upon infusions of NK or T cells in mice pre-treated with b-AP15 compared with mice treated with b-AP15, T, or NK cells alone ($p < 0.01$) (Fig. 4A, B, and C).

We next asked whether combined infusion of both NK and T cells would result in further delayed tumor progression in b-AP15 treated mice. *In vitro*, treatment with b-AP15 resulted in a minor up-regulation of TRAIL-R2 expression. Nevertheless, this increase in expression was sufficient to sensitize D2F2 tumor cells to NK and T cell –mediated killing. Furthermore, the level of murine NK cell and T cell killing of b-AP15-treated tumor cells was reduced in the presence of neutralizing antibodies to TRAIL (Supplementary Fig. 3A, B and C). In addition, RENCA, but not B16 melanoma tumor cell lines were efficiently killed by NK cells following exposure to b-AP15 compared with untreated tumor cells (Table 1). Moreover murine NK and tumor specific T cells displayed elevated IFN γ production when co-cultured with b-AP15 treated D2F2 cells compared with those co-cultured with untreated D2F2 tumors (data not shown).

In mice bearing s.c implanted syngeneic D2F2 tumors, treatment with b-AP15, NK cells, or T cells alone did not result in reduced tumor burden compared with untreated tumor-bearing

BALB/c mice. After three infusions, the median tumor sizes were 334 ± 11 (untreated mice), 326 ± 91 (b-AP15 treated mice), 340 ± 84 (NK cell treated mice), 260 ± 23 (T cell treated mice), and 363 ± 25 (NK cell + T cell treated mice). However, compared with mice treated with b-AP15 alone or NK+T cell treated mice without b-AP-15, treatment with b-AP15 followed by infusion of both NK and T cells resulted in significantly reduced tumor burden in mice bearing s.c tumors ($p\leq 0.01$; Fig. 5A).

In addition bioluminescence imaging of mice implanted with luciferase expressing D2F2 cells, showed a significant decrease in tumor burden in mice pre-treated with b-AP15 and co-infused with NK- and T-cells ($p\leq 0.002$, Fig. 5B and C). Similarly to the subcutaneous model, no change in tumor burden was observed after treatment with b-AP15, NK cells, T cells, or T cells and NK cells alone compared with untreated mice (data not shown). Moreover, the combined infusion of NK and T cells resulted in significantly prolonged long-term survival in mice bearing i.v tumors compared with mice receiving b-AP15 and NK cells ($p=0.001$) or b-AP15 and T cells ($p=0.001$) (Fig. 5D).

Discussion

Here we demonstrate that inhibition of proteasome deubiquitinating activity leads to sensitization of tumor cells to NK and T cell –mediated killing, and that the mechanism of sensitization centers upon up-regulation of TRAIL-R2. Several chemotherapeutical agents have been shown to increase TRAIL receptor expression on tumor cells [17,18]. We and others previously demonstrated that treatment with the proteasome inhibitor bortezomib increases TRAIL-R2 expression on tumor cells resulting in an increased killing by activated NK cells via TRAIL *in vitro* [12,19]. Whether bortezomib can sensitize tumor cells to T cell-mediated killing is less clear. Bortezomib was recently shown to inhibit NF- κ B activity and restore MART-1 expression and pro-apoptotic signaling, resulting in sensitization of melanoma tumors to killing by TCR-engineered CTLs [20]. We have demonstrated that bortezomib-treated tumor cells are resistant to killing by tumor-specific TILs due to impaired antigen processing and presentation [14]. Differences in T cell preparation and source may account for these differences. In accordance with our previous findings, we show that treatment with bortezomib results in increased NK but abrogated T cell-mediated killing of tumor cells. However, the same tumor cells were rendered sensitive to killing by both NK and T cells following exposure to the proteasome deubiquitinase inhibitor b-AP15. b-AP15 inhibits the deubiquitinating activity of the regulatory 19S particle of the proteasome resulting in impaired degradation of cellular proteasome substrates [16]. However, unlike bortezomib the proteolytic activities of the 20S core proteasome is not inhibited, thus immunoproteasome processing and presentation of antigenic peptides to T cells is not expected to be impaired [16]. Therefore, we hypothesized that b-AP15 may enhance NK cell killing in a manner similar to bortezomib, but without dampening antigen processing and T cell-mediated killing.

Overall, b-AP15 induced up-regulation of TRAIL-R2 and down-regulation of cFLIP in human tumor cell lines *in vitro*. We found that up-regulation of TRAIL-R2, rather than down-regulation of cFLIP, was associated with b-AP15-induced sensitization to NK cell-mediated killing. We previously demonstrated that the amplitude of NK cell-mediated lysis positively correlated with the degree of induction of TRAIL-R2 expression following treatment with bortezomib [19]. Several studies have demonstrated that down-regulation of cFLIP in tumor cells is required for sensitization to TRAIL-mediated killing [21,22]. In contrast, Ganten *et al.* showed that proteasome inhibition with MG132 sensitized hepatocellular carcinoma cells despite high levels of cFLIP [23].

Although NK and T cell –mediated killing of b-AP15 treated tumor cells was reduced in presence of neutralizing antibodies to TRAIL, killing was also reduced in the presence of neutralizing antibodies to Fas ligand or CMA. Therefore, we cannot exclude that b-AP15 sensitizes tumor cells partly to Fas ligand and perforin-mediated killing. This interpretation would be consistent with the report by Hallett *et al.* that treatment with proteasome inhibitors can sensitize tumor cells not only to TRAIL-mediated killing, but also to killing via Fas ligand *in vitro* [24].

We have previously demonstrated that treatment with b-A15 results in reduced metastases and tumor growth [16]. Similarly, in this study we observed delayed tumor progression (J82 bladder cancer) in immune deficient SCID/Beige mice upon weekly treatment with b-AP15 compared to untreated mice. Infusion of either NK or T cells significantly delayed tumor progression in tumor-bearing SCID/Beige mice following pre-treatment with b-AP15 relative to mice receiving b-AP15 only. However, we did not observe any delay in tumor progression in immune competent BALB/c mice after infusion of NK or T cells or after treatment with b-AP15. The discrepancy in results between SCID/Beige and BALB/c mice may be due to the rapid growth rate of the syngeneic D2F2 tumors (day 21; $359 \pm 27 \text{ mm}^3$) compared with the

J82 tumor xenografts (day 20; $12.5 \pm 1.5 \text{ mm}^3$). In addition, D2F2 tumors could be less sensitive to treatment due to the infusion of fewer lymphocytes (BALB/c; $0.75\text{-}2 \times 10^6$, SCID/Beige; $2\text{-}4 \times 10^6$) in this model.

Given that tumor cells within a tumor mass have a variable expression of MHC class I [6], combined adoptive cell therapy with NK and tumor-specific T cells may result in better clinical responses compared with infusion of NK or T cells alone. However, several studies have demonstrated that NK and T cells may counteract each other. NK cells play an important role in maintaining immune homeostasis by regulating expansion of activated T cells through p21-mediated cell cycle arrest [25]. Olson *et al.* demonstrated that NK cells can inhibit and kill autologous alloreactive T cells [26]. Furthermore, activated T cells can inhibit NK cell-mediated tumor rejection in mice [27]. To investigate whether combined infusion of NK and T cells would result in synergistic anti-tumor effects, tumor-bearing BALB/c mice were treated with b-AP15 followed by infusion of syngeneic NK and/or T cells. Following b-AP15 treatment, infusion of both NK and T cells showed a significantly reduced tumor burden compared with mice treated with either NK or T cells. The reduced tumor burden in b-AP15 treated mice receiving a combined infusion of NK and T cells resulted in a significantly extended survival.

Several clinical studies have evaluated TRAIL receptor targeting in solid tumors. These studies concluded that TRAIL targeting needs to be combined with chemotherapy to generate objective clinical response rates [28-30]. Combined therapy with drugs like b-AP15 and adoptive cell therapy may have additional benefits over the use of combining immunosensitizing drugs and agonistic monoclonal antibodies against TRAIL or recombinant TRAIL for several reasons. The half-life of anti-TRAIL receptor antibodies and recombinant TRAIL is approximately 17 days and less than one hour respectively [31]. Adoptively transferred T and NK cells can be detected several months after infusion potentially resulting

in sustained anti-tumor effects [3,32]. Our results indicate that following exposure to b-AP15, tumor cells remained sensitive to NK cell killing for 72 hours *in vitro*, thus providing a relatively long therapeutic window for NK cell therapy (data not shown). Infused lymphocytes upon tumor interaction, produce pro-inflammatory cytokines that contribute to tumor eradication. Such cytokines may also recruit other inflammatory cells to the tumor microenvironment further heightening the anti-tumor immune response. In addition, tumor cells can develop resistance to TRAIL targeting by monoclonal antibodies. Since NK and T cells use a variety of cytolytic mechanism to induce cell death, drug-induced sensitization combined with adoptive cell infusion may offer a broader anti-tumor effect.

Our findings demonstrate that inhibition of proteasome deubiquitinating activity with b-AP15 renders tumor cells sensitive to killing by NK and T cells. Since combined therapy with NK and T cells increase the chances of targeting tumor cells with variable MHC class I expression, b-AP15 pre-treatment followed by infusion of NK and T cells may represent a valuable therapeutic option for the treatment of a wide variety of cancers.

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Conflict of interest

None of the authors have any conflict of interest to declare. The content of this work has not been published in the form of a meeting abstract or in any journal.

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Figure legends

Fig. 1: Sub-apoptotic doses of b-AP15 increase TRAIL-R2 and sensitizes human tumor cells to TRAIL-mediated killing *in vitro*

TRAIL-R2 (A) and Fas (B) expression in untreated and b-AP15 treated [1 uM, 3 hours] J82 (■), DU145 (□), A498 (◆), HCT116 (●), HeLa (▲) and MDA453 (○) cells. *In vitro* 18 hour ⁵¹Cr-release assay of recombinant TRAIL (C) [100 ng/ml] or recombinant FasL (D) [150 ng/ml] –mediated killing of J82 (■), DU145 (□), A498 (◆), HCT116 (●), HeLa (▲) and MDA453 (○) tumor cells (n=2). Statistical analysis of mean fluorescence intensity (MFI) of TRAIL-R2 expression (n=2); was performed by student's paired T-test (mean+SEM). n= numbers of replicates.

Fig. 2: b-AP15 sensitizes human tumor cell lines to NK and T cell –mediated killing via TRAIL.

A) Human tumor cell lines J82, MDA453, HCT116, EST152, and EST027 were treated with 1 uM b-AP15 for 3 hours and tested for susceptibility to killing by human NK cells and T cells in an 18 hour ⁵¹Cr-release assay (n=3). B) J82 cells were treated with b-AP15 [1 uM, 3 hours] and tested for killing by NK or T cells in presence of neutralizing antibodies to TRAIL or FasL or in presence of Concanamycin A (CMA) (n=3, mean±SEM). The statistical significance was calculated by student's paired T-test (mean+SEM). *p<0.05.

Fig. 3: Increased TRAIL-R2 but not decreased cFLIP expression is associated with b-AP15-induced sensitization to NK cell-mediated killing.

A) The Pearson's correlation test between NK cell-mediated killing and expression of TRAIL-R2 or cFLIP expression was performed in tumor cell lines including J82, JOHW, DU145, JuSo, HeLa, A498, HCT116, MCF7, and MDA453 before and after treatment with b-

AP15 (n=2). B) J82, JOHW and HCT116 were treated with b-AP15 or left untreated and tested for susceptibility to killing by human NK cells in an 18 hour ⁵¹Cr-release assay (n=3; mean±SEM), analyzed for TRAIL-R2 (n=2) and cFLIP expression (n=2) by flow cytometry and Western Blot respectively.

Fig. 4: Adoptive infusion of human NK or T cells following b-AP15 treatment delay tumor progression in immune-deficient mice

SCID/Beige mice were injected (s.c) with 3×10^6 J82 cells and treated with b-AP15 [2.5 mg/kg] on days 12, 19, and 26 (A) followed by infusion of either NK cells (B) or Mart-1 specific T cells (C) on days 13, 20, and 27 post tumor injection. Tumor size was measured by palpation twice every week. The statistical significance was calculated by two-way ANOVA and p-values obtained from the interaction test between all groups compared to groups treated with b-AP15 and NK cells or b-AP15 and T cells.

Fig. 5: NK- and T- cell combined therapy delay tumor progression and prolong survival in b-AP15 pre-treated BALB/c mice

BALB/c mice were injected s.c (A) (n=4) or i.v (B and C) (n=5) with 0.1×10^6 D2F2 cells and treated with b-AP15 on days 7, 14, and 21 or 3, 10 and 17 respectively or left untreated followed by the next day infusion of NK cells, T cells, or NK and T cells. (A) Tumor size on day 22, (B) Bioluminescence imaging on day 27 after tumor inoculation or (C) Bioluminescent images of two representative mice of (B). (D) Survival of tumor-bearing (i.v tumors) mice. The statistical significance of A) and B) was calculated by two-way ANOVA and p-values obtained from single row test between all groups and group receiving NK cells + T cells + b-AP15. Statistical analysis for survival was calculated by Log-rank (Mantel-Cox) test (n=6). n indicate number of mice in each treatment group.

Table 1. NK and T cell –mediated killing of tumor cell lines after treatment with b-AP15.

Human and mouse tumor cell lines of different origin were treated with b-AP15 [1 uM, 3 hours] and tested for susceptibility to killing by NK or T cells in an 18 hour ⁵¹Cr-release assay at E:T ratio ranging between 0.3-3:1 or 3-9:1 respectively. p-values are calculated by student's paired T-test based on fold-change values. Experiments were repeated at least twice for every cell line.

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		Cell line	Cell type	Untr.	b-AP15	fold change	E:T ratio	
% Lysis of human tumor cell lines	NK cells	EST152	Skin	18.6	32.6	1.7	1:1	
		EST027	Skin	24.0	37.3	1.5	1:1	
		EST049	Skin	26.5	33.0	1.2	1:1	
		EST115*	Skin	32.0	39.0	1.2	2.5:1	
		EST025	Skin	22.7	46.3	2.0	2.5:1	
		JuSo	Skin	5.0	7.0	1.4	1:1	
		MDA453	Breast	8.0	17.3	2.2	1:1	
		MCF-7	Breast	31.0	31.0	1.0	3:1	
		MDA484	Breast	9.0	29.0	3.2	1:1	
		PC3	Prostate	12.0	22.0	1.8	1.25:1	
		DU145	Prostate	9.0	22.0	2.4	0.3:1	
		J82	Bladder	13.6	36.0	2.6	1:1	
		RT4	Bladder	29.0	38.0	1.3	1.25:1	
		JOHW	Bladder	12.0	12.0	1.0	2:1	
		A498	Kidney	13.0	24.0	2.4	1:1	
		HCT116	Colon	10.3	26.6	2.6	1:1	
	HEP-G	Liver	16.0	32.0	2.0	1:1		
	SKOV3	Ovarian	2.0	23.0	11.5	3:1		
	HeLa	Ovarian	24.0	89.0	3.7	3:1		
	BJ1	Fibroblast	5.0	5.0	1.0	3:1		
			p-value		=0.0002			
		T cells	EST152	Skin	12.0	35.0	2.1	5:1
			EST027	Skin	15.0	39.0	2.8	3:1
			EST049	Skin	14.0	16.0	0.0	5:1
			EST115*	Skin	6.1	6.1	1.1	5:1
			EST025	Skin	17.7	32.7	1.9	9:1
	J82		Bladder	4.0	23.0	5.75	5:1	
		p-value		=0.02				
% Lysis of murine tumor cell lines	NK cells	D2F2	Breast	14.0	32.0	2.3	2.5:1	
		B16	Melanoma	11.0	11.0	1.0	2.5:1	
		RENCA	Kidney	15.0	46.0	3.0	5:1	
	T cells	D2F2	Breast	18.0	37.0	2.1	7.5:1	
		RENCA	Kidney	6.0	6.0	1.0	5:1	

*The cell line is not HLA-matched

Figure 1.

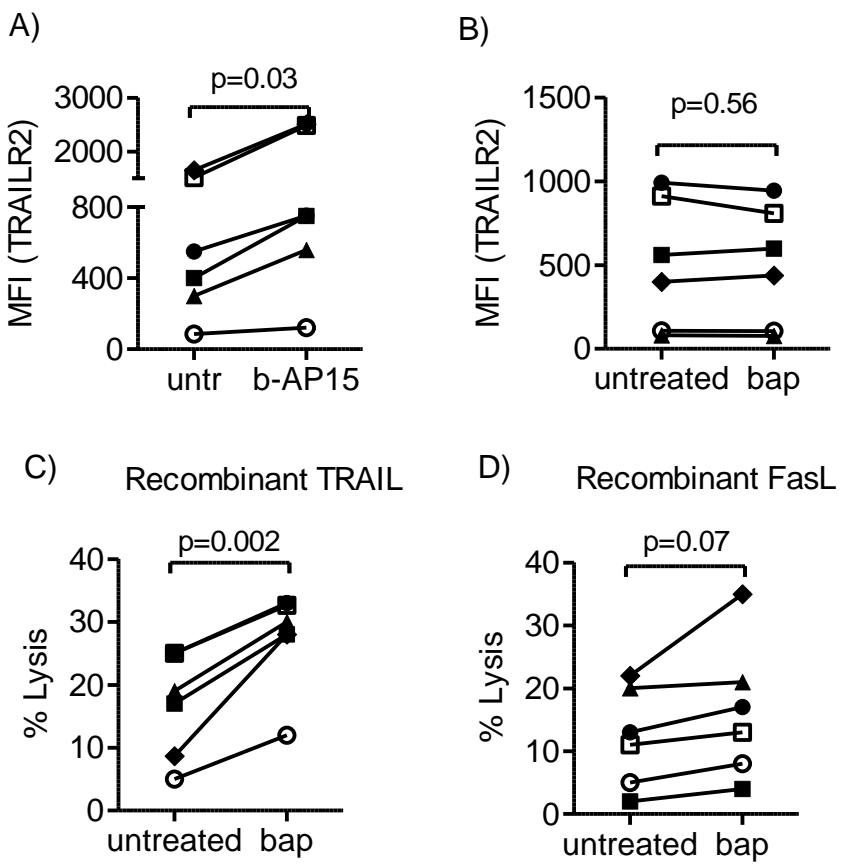


Figure 2.

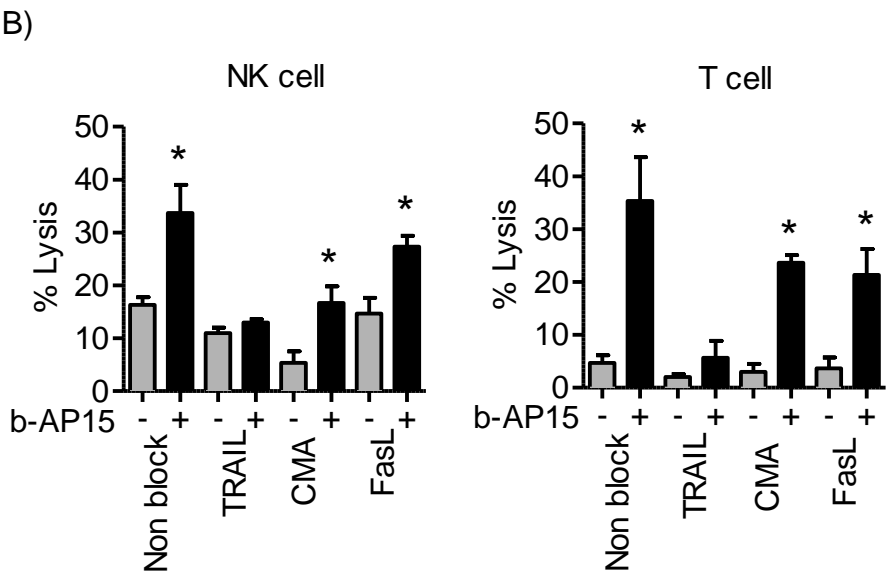
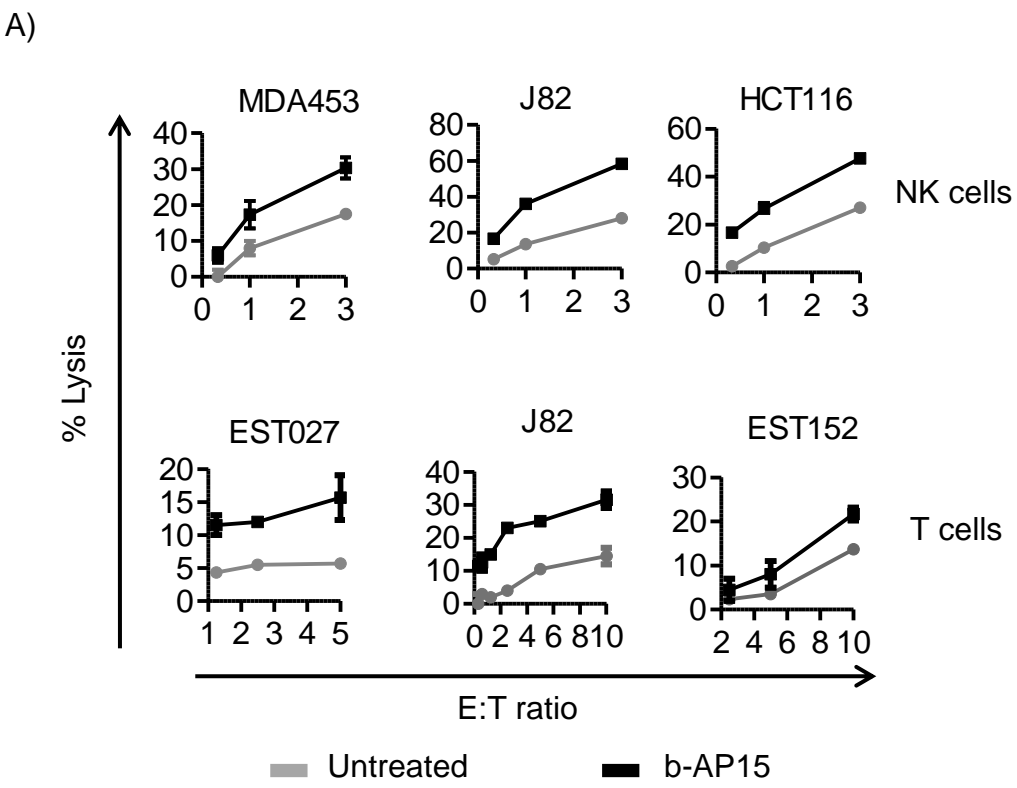
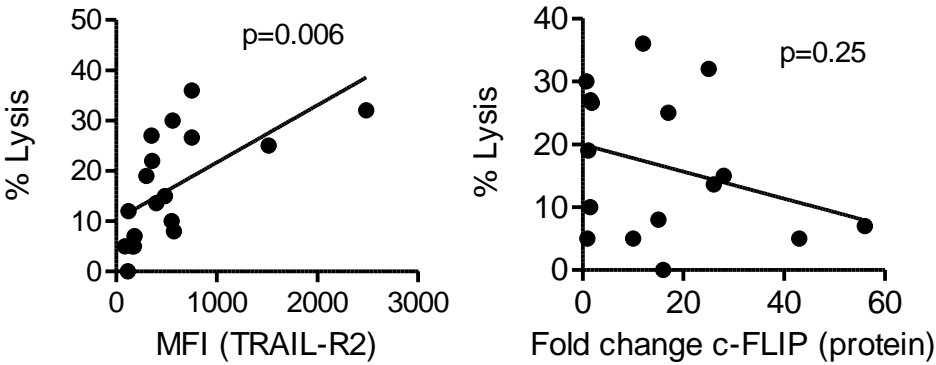


Figure 3.

A)



B)

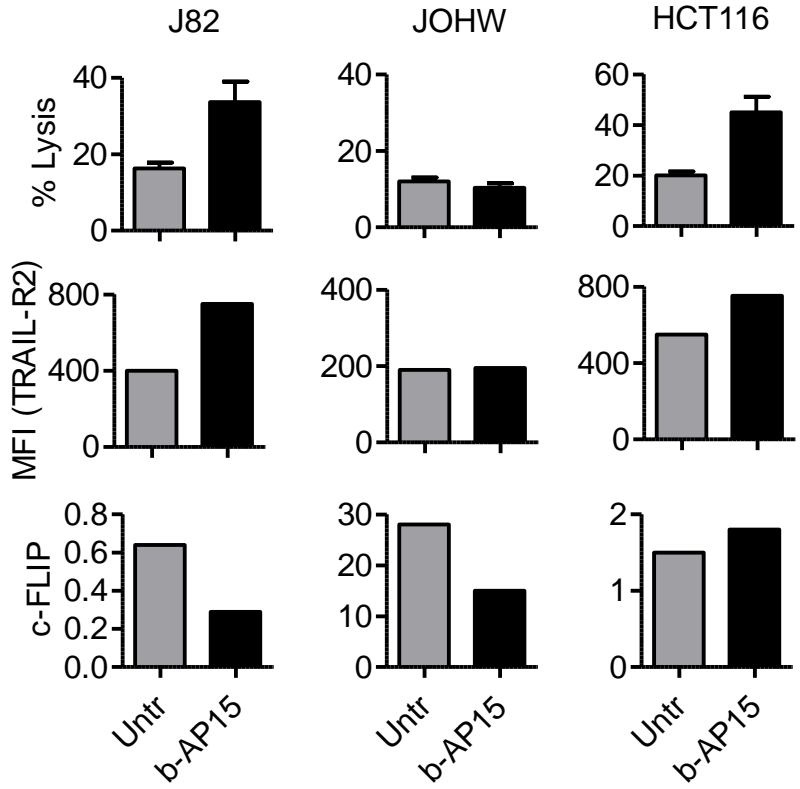


Figure 4.

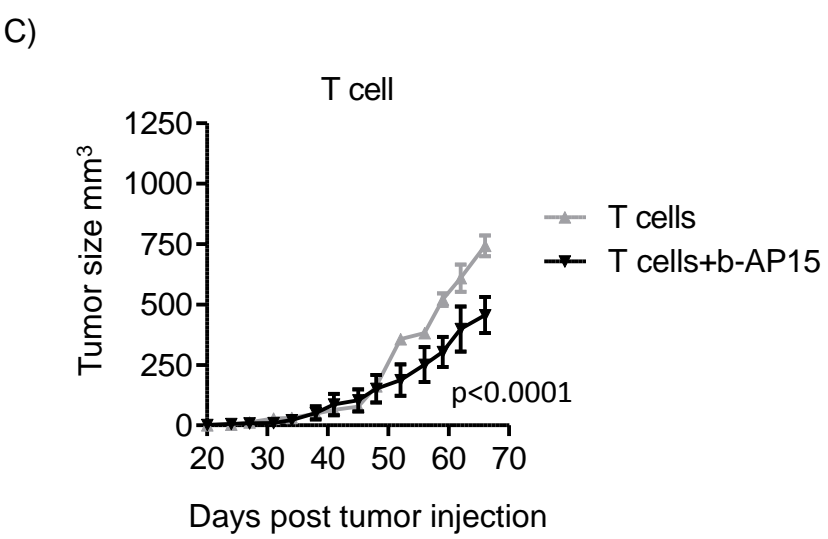
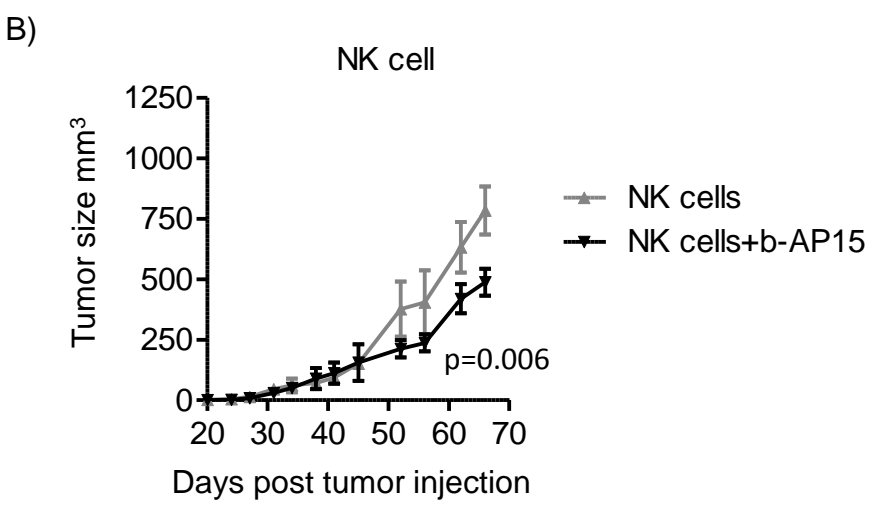
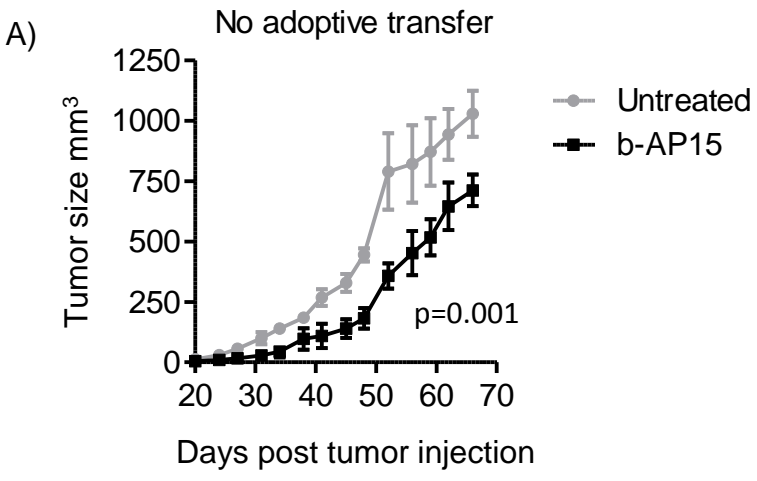
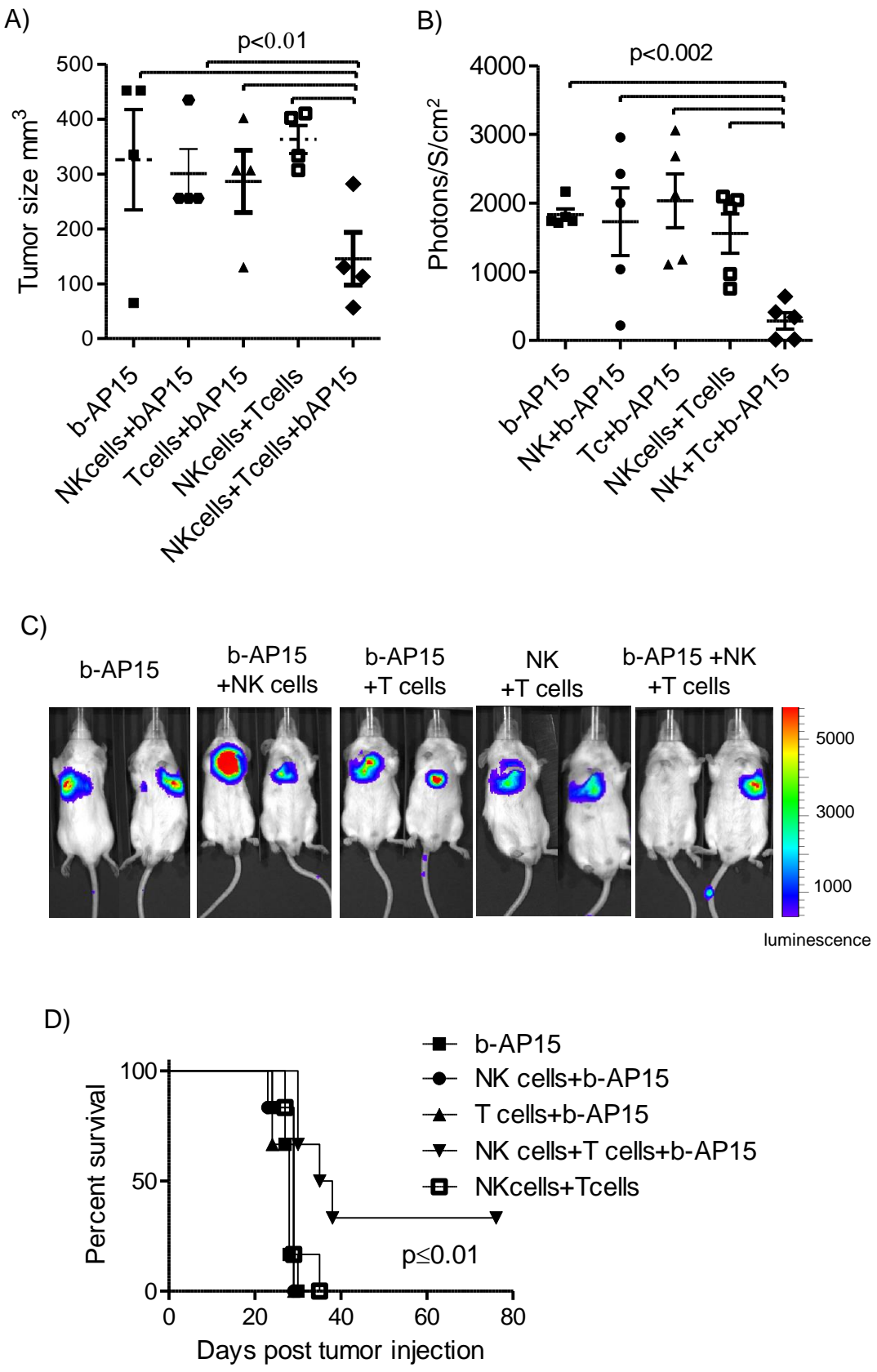
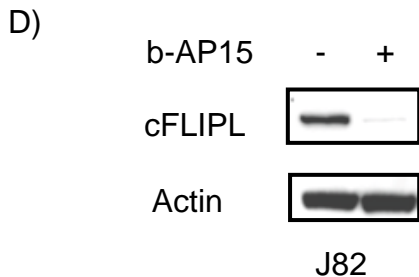
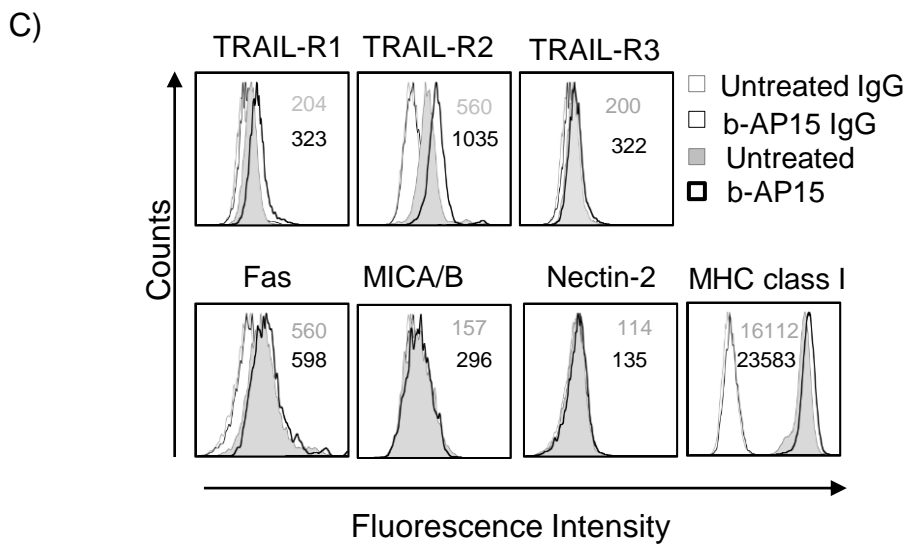
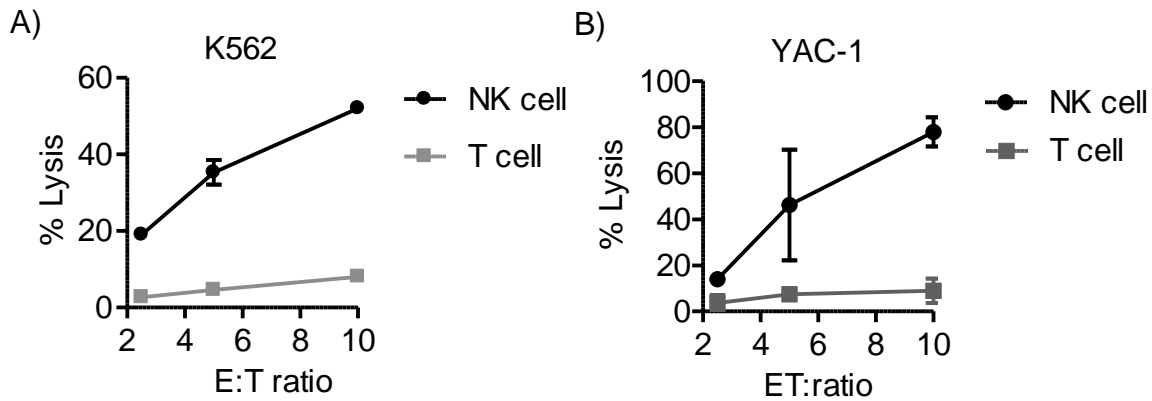


Figure 5.



Supplementary Figure 1.

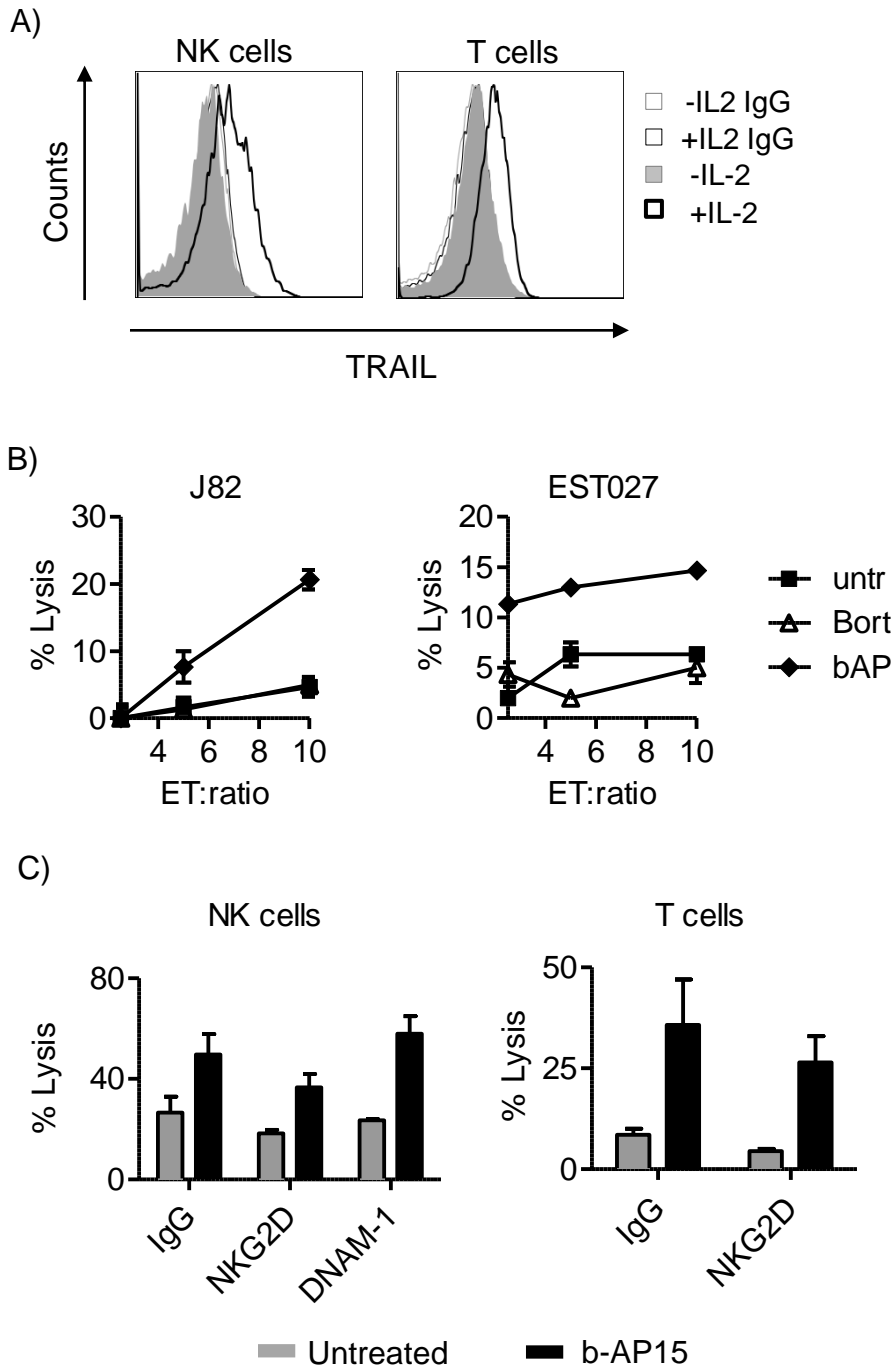
Cancer Immunology, Immunotherapy (submitted in 2013) – Dhifaf Sarhan et al.



Human and murine NK and T cell –mediated cytotoxicity of A) K562 or B) YAC-1 cells respectively. (C) Flow cytometry analysis of TRAIL-R1, -R2, -R3, Fas, MICA/B, Nectin-2 and MHC class I expression in untreated and b-AP15 treated [1 μ M, 3 hours] J82 cells. D) cFLIP expression of J82 cells was analyzed by Western Blot before and after b-AP15 treatment.

Supplementary Figure 2.

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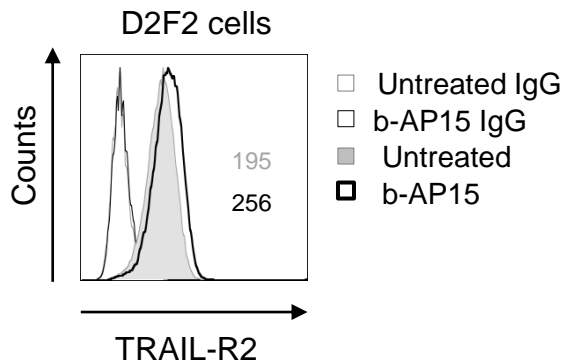


A) Flow cytometry analysis of TRAIL expression on NK- and T-cells in presence or absence of [500 U/ml] IL-2 for four days. One of two independent experiments with similar results is shown. B) J82 and EST027 cells were either treated with b-AP15 [1 μ M, 3 hours], bortezomib [Bort; 20 nM, 20 hours] or left untreated and tested in an in vitro 18 hour 51 Cr-release assay for killing by Mart-1 specific T cells. C) J82 cells were treated with b-AP15 [1 μ M, 3hours] and tested in an in vitro 18 hour 51 Cr-release assay for killing by NK cells in presence of neutralizing antibodies to NKG2D or DNAM-1 or T cells in presence of neutralizing antibodies to NKG2D.

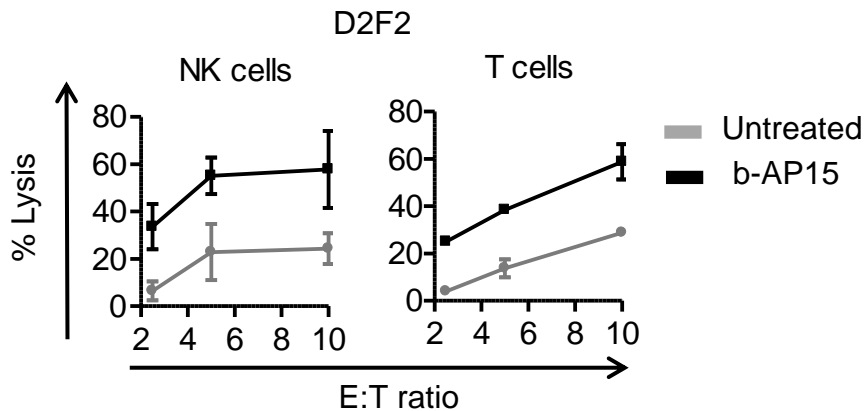
Supplementary Figure 3.

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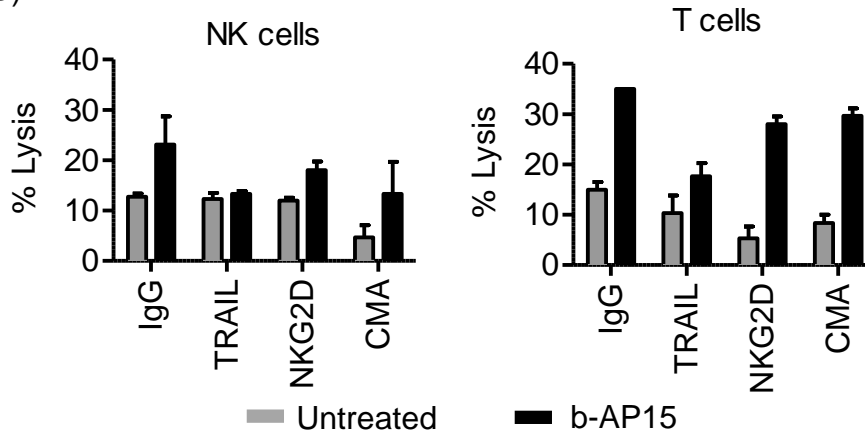
A)



B)



C)



A) Flow cytometry analysis of TRAIL-R2 expression in untreated and b-AP15 treated [1 μ M, 3 hours] D2F2 cells. Values in graph represent MFI-values of TRAIL-R2 staining. B) Murine BALB/c NK cells or D2F2-specific T cells were tested against untreated and b-AP15 treated [1 μ M, 3 hours] D2F2 tumor cells in an 18 hour 51 Cr-release assay (n=3). C) D2F2 cells were treated with b-AP15 [1 μ M, 3 hours] and tested in an in vitro 18 hour 51 Cr-release assay for killing by NK or T cells in presence of neutralizing antibodies to TRAIL, NKG2D or in presence of Concanamycin A (CMA).