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Eur J Immunol. 2013 Jan;43(1):249-57

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URL: http://dx.doi.org/10.1002/eji.201242735

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Activated monocytes augment TRAIL-mediated cytotoxicity by human NK cells through release of IFN-gamma

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Keywords: Natural killer cells, Zoledronic acid, IFNγ, monocytes, Immunotherapy

Article type: Full length article

Abbreviation list: Zoledronic acid (ZA); NK group 2 member D (NKG2D); MHC class I chains A and B (MICA/B); Concanamycin A (CMA); DNAX accessory molecule-1(DNAM-1); 3,5-bis[(4-nitrophenyl)methylidene]-1-prop-2-enoylpiperidin-4-one (b-AP15); Pamidronate (pam).

Summary

Natural killer (NK) cells are innate lymphocytes that are able to directly kill tumor cells through different mechanisms including ligation of TNF-related apoptosis-inducing ligand (TRAIL) receptors. Zoledronic acid (ZA) is a bisphosphonate known to up-regulate the expression of TRAIL on human gamma-delta T cells. Here we investigated whether exposure to ZA would up-regulate TRAIL expression on human NK cells and augment their cytotoxicity against tumor cells. When co-cultured with monocytes, treatment with ZA and IL-2 resulted in a significant up-regulation of TRAIL expression on human NK cells ($p=0.002$). Consequently, ZA-primed NK cells were significantly more cytotoxic against TRAIL sensitive tumor cells ($p<0.0001$). In presence of ZA and IL-2, monocytes produced high levels of IFNγ and when cultured in presence of neutralizing antibodies to IFNγ, TRAIL expression ($p=0.005$) and TRAIL-mediated cytotoxicity ($p=0.004$) of NK cells were significantly reduced. Furthermore, in tumor-bearing SCID/Beige mice, a significant delayed tumor progression ($p=0.015$) and prolonged survival ($p=0.03$) was observed after infusion of ZA-primed NK cells compared to mice infused with unprimed NK cells. These findings represent a novel approach to potentiate TRAIL-mediated apoptosis by adoptively infused NK cells that could improve the outcome in patients with cancer.

Introduction

Natural killer (NK) cells are cytotoxic lymphocytes able to kill virus infected cells and tumor cells without prior antigen stimulation. With recently developed expansion protocols, the effect of NK cells is being increasingly used in clinical studies in patients with cancer. NK cell function is regulated through a balance of inhibitory and activating receptors [1]. The largest group of inhibitory receptors belongs to the Killer Immunoglobulin-like Receptor (KIR) family, which ligate MHC class I molecules. Upon binding of inhibitory KIRs, NK cells are inhibited and unable to induce cell death in target cells. NK cells are therefore predominantly cytotoxic against target cells with absent expression of MHC class I. Ligation of natural killer group 2, member D (NKG2D), DNAX accessory molecule-1 (DNAM-1), and the natural cytotoxicity receptors (NCRs), NKp30, NKp44, and NKp46 to their respective ligands transmits an activating signal to NK cells [1-6]. Upon activation, NK cells kill their targets by the release of perforin and granzymes or through ligation of death receptors [7]. Such receptors belong to the TNF-receptor superfamily and include Fas (CD95) and Tumor Necrosis Factor-Related Apoptosis-Inducing ligand (TRAIL) receptors. We have previously demonstrated that peripheral blood NK cells express low levels of TRAIL and are unable to kill TRAIL-sensitive tumors [8].

Zoledronic acid (ZA) is a bisphosphonate that is used to prevent the loss of bone mass and to lower the risk of skeletal complication in patients with bone metastasis [9]. ZA has also been shown to play a role in activation of gamma-delta T cells in an antigen-dependent manner [10]. Furthermore, treatment with ZA and interleukin-2 (IL-2) results in increased serum levels of TRAIL in patients with prostate cancer [11]. In these patients, high serum levels of TRAIL correlated with improved prognosis. Although ZA has been shown to up-regulate

NKG2D expression on NK cells *in vitro* [12], it has not been studied whether ZA treatment can augment the expression of TRAIL on human NK cells.

Here we investigated if exposure to ZA would increase the expression of TRAIL on human NK cells and lead to increased TRAIL-mediated killing of tumor cells. When co-cultured with monocytes, ZA induced an IFNγ-dependent up-regulation of TRAIL expression on NK cells. As a result, these NK cells displayed increased cytotoxic potential against TRAIL-sensitive tumors *in vitro* and *in vivo*. Our findings suggest that adoptive infusion of ZA-primed NK cells with an improved targeting of TRAIL-sensitive tumors may lead to improved outcome in patients with cancer.

Results

Treatment with ZA induces changes in phenotype of NK cells

NK cells were isolated from healthy donors and co-cultured with irradiated PBMC and IL-2 with (ZA-primed) or without ZA (unprimed). Following exposure to ZA at concentrations ranging from 1 to 10μM, surface expression of TRAIL was up-regulated compared to NK cells not treated with ZA. However, no changes in surface expression of NKG2D, DNAM-1, FasL, perforin, granzymeB, NKp30, or NKp46 were observed. A minor up-regulation in expression of CD69 and NKp44 was detected on NK cells following exposure to ZA (Figure 1A). In contrast, when purified NK cells were treated with ZA in absence of feeder cells, no changes in TRAIL expression were observed (Supplemental Figure 1A), suggesting a requirement for PBMC feeder cells to be present during culturing to induce up-regulation of TRAIL on NK cells.

ZA up-regulates TRAIL expression on NK cells indirectly via monocytes

To determine what cells within the PBMC population were responsible for the increased TRAIL expression on NK cells following treatment with ZA; experiments using different feeder cells were performed. In experiments where NK cells were co-cultured with monocytes, but not T or B cells, treatment with ZA resulted in a significant up-regulation of TRAIL (monocytes, p=0.01; T cells, p=0.1, B cells, p=0.4) (Supplemental Figure 1B). In contrast, no change in NKG2D expression on NK cells was observed in presence or absence of ZA upon co-culture with monocytes (Supplemental Figure 1C). The fold change in TRAIL expression following treatment with ZA was higher when NK cells were co-cultured with purified monocytes (3.5-fold, p=0.02) compared to PBMC (1.2-fold, p=0.04) (Figure 1B). In addition, following co-culture with purified monocytes, ZA induced a significantly higher expression of TRAIL on NK cells compared to when co-cultured with PBMC feeders (p=0.0005). Moreover, production of soluble TRAIL, but not soluble Fas ligand, was significantly (p=0.02) increased in ZA-primed but not unprimed NK cells (Figure 1C).

ZA treatment augments TRAIL-mediated antitumor effects of NK cells

To investigate whether the increase in TRAIL expression on NK cells after exposure to ZA would result in augmented TRAIL-mediated killing; J82 cells were treated with the proteasome inhibitor b-AP15 and used as target. Indeed, ZA-primed NK cells displayed an increased cytotoxicity against b-AP15 treated J82 tumor cells compared to unprimed NK cells (Figure 2A). Although an increased killing by ZA-primed NK cells was observed against untreated J82 tumor cells compared to unprimed NK cells, the difference in killing was not significant. In addition, ZA-primed NK cells had increased ability to kill TRAIL-sensitive tumor cells of other origin including breast cancer (MCF-7), prostate cancer (PC3) and melanoma (EST025) (Figure 2B). Despite an increased TRAIL expression in the absence of

ZA, NK cells co-cultured with either PBMC or monocytes did not vary in their ability to kill b-AP15 treated J82 cells. In contrast, when exposed to ZA, monocytes stimulated a superior ($p=0.01$) NK cell-mediated killing compared to NK cells co-cultured with PBMC (Figure 3A). In presence of anti-TRAIL antibodies, cytotoxicity by ZA-primed NK cells was significantly reduced. Although the overall level of NK cell-mediated killing was reduced in presence of neutralizing antibodies to NKG2D or FasL or in presence of CMA, ZA-primed NK cells still displayed a significantly higher cytotoxicity against J82 tumor cells compared to unprimed NK cells. The level of NK cell-mediated cytotoxicity did not change in presence of neutralizing antibodies to NKp44 compared to isotype control (Figure 3B). Collectively, these data demonstrate that monocytes induce TRAIL expression on NK cells in presence of ZA leading to an increased TRAIL-mediated killing of tumor cells *in vitro*.

ZA-treated monocytes augment TRAIL-mediated cytotoxicity by human NK cells through release of IFNγ

To investigate whether the mechanism of NK cell activation was dependent on cell-contact, ZA was added to transwell cultures of NK cells and monocytes. When added to transwell cultures, ZA still induced an up-regulation of TRAIL expression on NK cells (Figure 4A). We examined the supernatant of untreated and ZA-treated monocytes for changes in cytokine production in a multiplex Luminex assay. Several cytokines including; IL-15, MIP-1α, MIP-1β, and IFNγ was detected at higher levels in supernatant of ZA-treated monocytes compared to untreated monocytes. The most pronounced effect was observed in levels of IFNγ with a fold change of 72 between untreated and ZA-treated monocytes. Fold-change between untreated and ZA-treated monocytes in production of IL-15, MIP-1α, and MIP-1β was 1.95, 2.7, and 3.6 respectively. The increased levels of IFNγ were confirmed by ELISA where ZA-treated monocytes produced 60.5-fold more IFNγ compared to untreated monocytes (Figure 4B).

Interestingly, high production of IFNγ was only observed in presence of IL-2. When monocytes were cultured in presence of ZA alone, only a minor increase in IFNγ was observed. Production of IFNγ did not change after culture in IL-2 and in absence of ZA. We next investigated whether the bisphosphonic compound pamidronate (pam) would induce similar induction of IFNγ in monocytes. Monocytes exposed to pam showed an increased level of IFNγ compared to untreated monocytes. Nevertheless, monocytes cultured in presence of IL-2 and pam produced significantly lower amount of IFNγ ($p= 0.03$) compared to monocytes cultured in presence of ZA (Figure 4C).

Given this major increase in IFNγ production in ZA-treated monocytes we next investigated whether blocking of IFNγ reduced TRAIL expression and TRAIL-mediated killing by NK cells. Monocytes were cultured in presence or absence of neutralizing anti-IFNγ antibodies and supernatant from these cultures was transferred to purified NK cells. Transfer of IFNγ-depleted supernatant significantly reduced TRAIL expression on NK cells compared to non-depleted supernatant ($p=0.005$, Figure 5A). Similarly, reduced NK cell TRAIL expression was observed when co-cultured with monocytes in presence of ZA and neutralizing anti-IFNγ antibodies (Figure 5B). Consequently, NK cell-mediated killing of b-AP15 treated J82 cells was reduced to base-line when NK cells were cultured with IFNγ-depleted supernatant (Figure 5C). Furthermore, NK cell TRAIL expression as well as cytotoxicity against b-AP15-treated J82 tumor cells significantly increased in presence of recombinant IFNγ (Figure 5D and E).

Adoptive infusion of ZA-primed NK cells results in delayed tumor progression *in vivo*

We next evaluated whether the augmented killing by ZA-primed NK cells observed *in vitro* would result in improved anti-tumor effects *in vivo* following infusion of NK cells in tumor-bearing mice. J82 tumor-bearing SCID/beige mice were infused with either ZA-primed or

unprimed NK cells for a total of four cycles. In mice bearing TRAIL-resistant J82 tumors, no change in tumor progression or survival was observed between groups receiving unprimed or ZA-primed NK cells (Figure 6A). However, following b-A15 treatment to sensitize J82 tumors to TRAIL-mediated killing, a significant delayed tumor progression ($p=0.015$) and prolonged survival ($p=0.03$) was observed in mice infused with ZA-primed NK cells compared to mice infused with unprimed NK cells (Figure 6B and C).

Discussion

Here we show that treatment with ZA increases IFNγ production by monocytes resulting in up-regulation of TRAIL expression on human NK cells. These ZA-primed NK cells displayed an increased ability to kill TRAIL-sensitive tumors *in vitro* and *in vivo*. The effects of ZA on hematopoietic cells have previously been investigated. Upon treatment of PBMC with ZA and IL-2, a selective expansion of γδ–T cells has previously been reported [13]. Such γδ–T cells express higher intracellular levels of TRAIL and secrete higher levels of soluble TRAIL [11].

Recently, Maniar *et al.* showed an increased expression of the activating receptor NKG2D on human NK cells following co-culture with ZA-treated γδ–T cells [12]. Furthermore, ZA has been shown to activate human NK cells and cytokine-induced killer cells (CIK) in a dendritic cell (DC)-dependent manner. ZA-treated DC-stimulated NK cells to produce higher levels of IFNγ [14] and increased NKG2D expression and anti-tumor cytotoxicity of CIK cells [15]. However, cell-cell contact was required to induce NK cell activation by ZA-treated γδ–T cells [12]. In agreement with these studies, we found no change in NK cell activity or phenotype when purified NK cells were treated with IL-2 and ZA, confirming that accessory cells need to be present for an efficient activation of NK cells following exposure to ZA. However, in the presence of PBMC, a significant up-regulation of TRAIL expression was observed.

Likewise, when co-cultured in the presence of immature DC, exposure to ZA also resulted in a significant up-regulation of TRAIL expression on NK cells (data not shown). When monocytes were depleted from co-cultures, the effects of ZA were abolished suggesting that ZA induced expression of TRAIL on NK cells indirectly via monocytes. Indeed, co-culture with purified monocytes induced a superior up-regulation of TRAIL expression on NK cells. Compared to when co-cultured with PBMC and ZA, culture with monocytes in presence of ZA resulted in a significantly increased expression of TRAIL on NK cells ($p=0.0005$). Furthermore, ZA-primed NK cells also secreted higher levels of soluble TRAIL, but not Fas ligand.

In contrast to earlier reports, we were unable to show that activation of NK cells is dependent on cell-contact. Rather we show that NK cell activation by ZA-treated monocytes is not cell-cell contact dependent. We demonstrate that the increase in TRAIL expression on NK cells and concurrent increase in TRAIL-mediated tumor killing is induced by IFNγ from ZA-treated monocytes. There is ample literature on how IFNγ can induce TRAIL expression and how IFNγ and TRAIL synergize to eradicate tumors. Treatment with IL-12, a powerful inducer of IFNγ production by NK cells, has previously shown to up-regulate TRAIL expression on murine NK cells [16]. Immature myeloid monocyte-like cells have been shown to produce IFNγ and protect against streptococcus infection and prevent tumor outgrowth in mice [17, 18] Taieb et. al identified a subset of dendritic cells with NK cell properties. These cells produced IFNγ and mediated TRAIL-dependent lysis of tumor cells [18]. Two separate studies demonstrated that TRAIL and IFNγ cooperate in anti-tumor effects *in vivo* [19, 20]. Upon engagement of the T cell receptor, triggering of the TRAIL signaling pathway result in an augmented proliferation and IFNγ production in antigen specific T cells [21]. Furthermore, a recent study in bisphosphonate-naive postmenopausal women with low bone mass showed elevated serum levels of IFNγ after a single infusion of ZA [22]. We further show that direct

treatment of purified NK cells with recombinant IFNγ resulted in an increased TRAIL expression on NK cells. Taken together, our *in vitro* findings provide a novel mechanism of TRAIL regulation in human NK cells.

Purified monocytes cultured in ZA alone produced higher levels of IFNγ compared to untreated monocytes. However, in presence of both IL-2 and ZA, a much higher production of IFNγ was observed. ZA treatment resulted in higher expression of IL-2Rα and IL-2Rβ transcripts in monocytes compared to untreated monocytes suggesting that ZA treated monocytes may respond stronger to IL-2 stimulation (data not shown). Indeed, monocytes treated with ZA and IL-2 displayed an increased phosphorylation of p38 compared to monocytes treated with either IL-2 or ZA alone (data not shown). Moreover, SOCS3 inhibition by ZA [23, 24] resulting in enhanced STAT1/3 signaling may contribute to increased IFNγ production when IL-2 was added to cultures.

It is well known that isopentenyl pyrophosphate (IPP) accumulates in monocytes when treated with ZA [25]. Monocytes that accumulate IPP become antigen-presenting cells and stimulate gamma-delta T cells in peripheral blood. We found that IFNγ production by monocytes was not affected by statins (Simvastatin), that act upstream of IPP production (data not shown). Furthermore, treatment of monocytes with pamidronate, that like ZA acts downstream of IPP production, resulted in an increased production of IFNγ. However, production of IFNγ was consistently higher following treatment with ZA.

Although TRAIL was significantly up-regulated on ZA-primed NK cells, we were not able to detect any changes in expression of NKG2D either in presence or absence of anti-IFNγ (Supplemental Figure 1C) and confirm previous reports. Neither did we observe any differences in cytotoxicity against tumors cells between ZA-primed nor unprimed NK cells in presence of neutralizing antibodies to NKG2D. In the study by Maniar, the increased NKG2D

expression was dependent on 4-1BB ligand interaction on gamma-delta T cells following treatment with ZA. Su *et al* observed increased NKG2D expression following co-culture with DC and treatment with ZA. Both gamma-delta T cells and DC express high levels of 4-1BB ligand whereas monocytes typically express low levels of 4-1BB ligand [12, 26]. Thus, low expression of 4-1BB ligand expression on monocytes could possibly explain why no change in NKG2D expression was observed in our experiments. Secondly, differences in culture conditions may explain why no change in NKG2D expression was detected. In our experimental system NKG2D was expressed at high levels following seven-day culture of IL-2, even in the absence of ZA.

Previous reports have shown that treatment with proteasome inhibitors increase surface expression of TRAIL receptors on tumor cells and render them more sensitive to TRAIL-mediated killing [27, 28]. To investigate whether the increase in TRAIL expression on NK cells would correlate with an increased ability to kill TRAIL-sensitive tumors, the TRAIL resistant J82 cell line was treated with the proteasome inhibitor b-AP15 and used as targets in cytotoxicity assays. Indeed, a significantly higher killing of b-AP15 treated J82 cells was observed by ZA-primed NK cells. In presence of neutralizing antibodies to TRAIL, cytotoxicity by ZA-primed NK cells was significantly reduced. The increased capacity of ZA-primed NK cells to kill tumors *in vitro* also translated to augmented anti-tumor effects *in vivo*. When tumor-bearing mice were treated with the proteasome inhibitor b-AP15 followed by adoptive infusion of ZA-primed, but not unprimed NK cells, a significant delay in tumor progression was observed ($p=0.015$). In addition, a significant survival advantage ($p=0.03$) was observed in mice treated with ZA-primed NK cells, compared to mice treated with unprimed NK cells.

In conclusion, we show that ZA and IL-2-treated monocytes augment TRAIL-mediated cytotoxicity by human NK cells. We further identify IFNγ as the main factor responsible for

the increased TRAIL expression on human NK cells. In a recent randomized phase III study, adjuvant therapy with ZA showed no clinical benefit in patients with breast cancer [29]. In contrast, Dieli *et al* showed that treatment with ZA in combination with IL-2 resulted in sustained serum levels of TRAIL, which correlated with improved clinical outcome in patients with hormone-refractory prostate cancer [30]. Although we used the J82 cell line as a model cell line, several other tumor cell lines of different origin including breast cancer, prostate cancer, and melanoma also display increased susceptibility to ZA-primed, but not unprimed NK cells.

Our findings suggest a new strategy to augment NK cell anti-tumor activity by *ex vivo* exposure to ZA and IL-2, and infusion of such NK cells could potentially result in improved clinical outcome in patients with cancer.

Materials and Methods

Cells and reagents

Human NK cells were isolated from peripheral blood mononuclear cells (PBMC) by negative immuno-magnetic bead depletion (Miltenyi Biotech, Bergisch Gladbach, Germany). Human monocytes, T cells and B cells were isolated from PBMC by immuno-magnetic positive selection (Miltenyi Biotech). Following isolation, cells were analyzed for purity by flow cytometry (Supplemental Figure 2A-C). Purified NK cells were expanded with 25Gy irradiated autologous feeder cells (PBMC, monocytes, T or B cells) at a 1:10 cell ratio (NK: feeder) in X-VIVO 20 media (Lonza, Verviers, Belgium) supplemented with 5% heat inactivated human AB serum (Karolinska Hospital, Sweden) for eight days. ZA (Novartis Pharma GmbH, Nurnberg, Germany) was added to cultures (ZA-primed) on days 0, 4 and 7 at 10µM and IL-2 (Novartis pharma GmbH, Nurnberg, Germany) was added on days 0 and 4 at

500U/ml. NK cells were analyzed for proliferation, apoptosis and expression of cell surface markers on day 7. NK cell-mediated tumor cytotoxicity against J82 (bladder cancer, ATCC), PC3 (prostate cancer, ATCC), MCF7 (breast cancer, ATCC), EST025 (melanoma, IPD - ESTDAB), was analyzed on day 8-11. STR Identifier (Applied Biosystems, UK) was used to verify the origin of the J82, MCF7 and EST025 cell lines. All cell lines were maintained in RPMI1640 media with 10% fetal calf serum (Invitrogen, Eugene, Oregon, USA), 50U/ml penicillin, and 50mg/ml streptomycin (Invitrogen). Where indicated, 500pg/ml IFNγ (Peprotech, NJ, USA) was added to NK cells every 2 days for 7 days.

Flow cytometry, ELISA, Luminex, and proliferation assays

Freshly isolated NK cells were plated at a density of $2x10^5$ per well and incubated with irradiated feeder cells in 24-well tissue culture plate for seven days and analyzed by flow cytometry (LSR II, BD Biosciences, San Jose, California, USA) for and phenotype. NK cells were stained with fluorochrome-conjugated antibodies against CD56, CD16, CD3, CD14, CD20, TRAIL, NKG2D, DNAM-1, FasL, CD69, NKp44, NKp30, NKp46, AnnexinV, 7-AAD, and intracellular staining for perforin and, granzyme B (Biolegend, San Diego, USA). Purified monocytes were cultured in 24-well plate in presence or absence of ZA for 4 days and their activation status was evaluated by staining for CD54 (ICAM-1) [31] (Supplemental Figure 3A). Acquired flow cytometry data was analyzed using FlowJo software (Ashland, USA). Supernatant from NK cell cultures was collected on day 8 of expansion and analyzed for secretion of soluble TRAIL and FasL by sandwich enzyme-linked immunosorbent assay (ELISA) (R&D systems, Minneapolis, USA).

Quantification of analytes produced by monocytes was performed by Luminex assay. Purified monocytes (≥98%) were cultured in a 24 well-plate in serum free medium (X-vivo 20) and

treated with 500U/ml IL-2 and 10uM ZA or left untreated. Supernatant was collected on day 4 and analyzed for different cytokines (multiplex Luminex, Millipore AB, Solna Sweden) (Eotaxin, G-CSF, GM-CSF, IFN-α2, IFNγ, IL-10, IL-12(p40), IL-12(p70), IL-13, IL-15, IL-17, IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IP-10, MCP-1, MIP-1α, MIP-1β, TNFα and TNFβ). Alternatively, supernatant from untreated, ZA and pamidronate (pam)-treated monocytes in absence or presence of IL-2 was analyzed for IFNγ by ELISA.

Cytotoxicity assay

NK cell-mediated killing was measured in ^{51}Cr-release assays. J82 tumor cells were labeled with (30uCi) ^{51}Cr (PerkinElmer, Groningen, The Netherlands) for 1 hour at 37ºC. Prior to labeling, J82 tumor cells were incubated for 3 hours with 1µM of the proteasome inhibitor b-AP15 to sensitize tumor cells to TRAIL-mediated killing [32] (OncoTargeting AB, Uppsala, Sweden) (Supplemental Figure 3B). Labeled tumor cells were co-cultured with NK cells at various effector-to-target ratios for 18 hours. Following co-culture, supernatants (25µl) were transferred onto 96 well-Luma plates (PerkinElmer) and analyzed using Micro-Beta scintillation counter (TRILUX 1450, PerkinElmer). Where indicated ^{51}Cr-release assays were performed in presence of neutralizing antibodies against TRAIL (10ug/ml), NKG2D (10ug/ml), NKp44 (10ug/ml), FasL (2.5ug/ml) (Biolegend). Concanamycin A (CMA, 100nM, Sigma, Schnelldorf, Germany) was added to cytotoxicity assays to degrade perforin.

Transwell assay

Transwell assays were performed to determine whether ZA-treated monocytes could up-regulate TRAIL expression on NK cells in absence of cell contact. Purified NK ($3x10^5$) cells were plated in 1ml in the lower chamber and monocytes ($2x10^6$) were added to the upper

insert (0.4µm pore size) (Corning, NY, USA) in 200µl. Cell cultures were treated with ZA and IL-2 as above. Alternatively, monocytes were cultured in 24 well-plates treated with IL-2 and ZA as above and 500µl supernatant was transferred daily to pure NK cells. Where indicated, monocytes were treated with neutralizing anti-IFNγ antibodies (0.3ug/ml) (R&D systems, Minneapolis, USA) once every day for 7 days prior to transfer to purified NK cells. Alternatively co-cultures of NK cells and monocytes were treated with neutralizing anti-IFNγ antibodies (0.3ug/ml).

***In vivo* xenograft study**

The animal study (N42/10) was approved by the ethical review board at Karolinska Institutet. SCID/Beige mice were purchased from Charles River (Sulzfeld, Germany) and bred at the department of Microbiology, Tumor and Cell Biology (MTC), Karolinska Institutet. Mice were injected subcutaneously (s.c) with J82 tumors ($2.5X10^6$/mouse) in the right flank of 8-12 weeks old mice. After ten days when tumors were palpable (>$1mm^3$), mice were treated with intraperitoneal (i.p) injection of b-AP15 (2,5mg/kg) to sensitize J82 tumors to TRAIL-mediated killing. One day following b-AP15 injection, mice were infused with either ZA-primed or unprimed NK cells. NK cells were injected intravenously (i.v) at $0.5\text{-}3.5 \times 10^6$ /mouse. Mice were treated for a total of four cycles with b-AP15 treatment on days 10, 21, 24, and 30 and NK cells on days 11, 22, 25, and 31.

Statistical analysis

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. p-values below 0.05 were considered statistically significant between treatment groups.

Acknowledgments

We would like to acknowledge Dr. Rolf Kiessling, Mao Yumeng and Juan Castro at the department of Oncology-Pathology, Stockholm, Sweden. We would also like to thank staff at the animal facility at the department of Microbiology, Tumor and Cell Biology, Karolinska Institutet and staff at the cytokine analyzing facility at the department of Clinical Immunology / Transfusion Medicine (Luminex Core facility), Karolinska Institutet.

This work was supported by funding from The Swedish Research Council, The Swedish Cancer Society, The European Research Council Society, Karolinska Institutet, Jeanssons Stiftelser, Åke Wibergs Stiftelse, Magnus Bergvalls Stiftelse, Fredrik och Ingrid Thurings Stiftelse, Stiftelsen Clas Groschinskys Minnesfond, and The Swedish Society of Medicine

Conflict of interest

None of the authors have any conflict of interest to declare.

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Figure legends

Figure 1. ZA up-regulates TRAIL expression on NK cells indirectly via monocytes. A) NK cells were co-cultured with irradiated PBMC for seven days with or without ZA and stained for cells surface and intracellular markers and analyzed by flow cytometry. One of three independent experiments with similar results is shown. B) NK cells were cultured with irradiated PBMC or monocytes for seven days in presence or absence of ZA. Expression of TRAIL was analyzed by flow cytometry on day seven of culture. Results are shown from four experiments. Lines represent average mean fluorescence intensity. C) Secretion of soluble TRAIL (S-TRAIL) or FasL (S-FasL) from NK cells was analyzed by ELISA. Results from three pooled experiments are shown (mean ± SEM). p-values were calculated by paired, between same feeder or unpaired, between different feeders, two-tailed student's t-test.

Figure 2. ZA treatment augments NK cell cytotoxicity against different tumor cell lines.

A) NK cells were co-cultured with irradiated monocytes in presence or absence of ZA and analyzed for cytotoxicity in a ^{51}Cr release assay against untreated or b-AP15 treated J82 tumor cells. Three pooled experiments are shown (mean ± SEM), p-values were calculated from two-tailed Student's t-test. B) The ability of ZA-primed NK cells to kill b-AP15 treated J82 and PC3 tumor cells or untreated MCF7 and EST025 were analyzed in a ^{51}Cr release assay at E:T ratios of 1:1 to 6:1. NK cells and tumor cells were washed before co-culture. One of three independent experiments with similar results is shown.

Figure 3. ZA treatment augments TRAIL mediated anti-tumor effects of NK cells.

A) NK cells were co-cultured with irradiated PBMC or monocytes and tested for cytotoxicity against b-AP15 treated J82 cells. Result from six pooled NK cell cultures is shown. p-values were calculated by paired in between same feeder cell groups or unpaired between different

feeder cell groups two tailed student's t-test. B) NK cells (four pooled) were co-cultured with irradiated monocytes in presence or absence of ZA, washed and tested for cytotoxicity against b-AP15 treated J82 cells in presence of neutralizing antibodies against TRAIL, NKG2D, NKp44, FasL or CMA. p-values were calculated by paired student's t-test.*p-value is less than 0.04 compared between unprimed and ZA-primed NK cells.

Figure 4. Bisphosphonates and IL-2 induce IFNγ production in monocytes. A) Purified NK cells (n=6, mean ± SEM) were cultured in the lower chamber of 24 well-plates in presence of IL-2 (500U/ml) and monocytes were added to the upper insert in presence or absence of ZA for 6 days. TRAIL expression was analyzed by flow cytometry. B) Monocytes (n=3, mean ± SEM) (Mono) were cultured in 500U/ml IL-2 in presence or absence of ZA for 4 days and production of IFNγ was analyzed by ELISA. p-values were calculated by paired two tailed student's t-test. C) Purified monocytes cultured for 4 days in presence of 10µM ZA or pamidronate (Pam) or left untreated and production of IFNγ was analyzed by ELISA. Data from two pooled experiments is shown (mean ± SEM).

Figure 5. ZA-treated monocytes augment TRAIL-mediated cytotoxicity of human NK cells through release of IFNγ

Purified NK cells cultured with supernatant from ZA-treated or untreated monocytes (n=6) A) or co-cultured with ZA-treated or untreated monocytes (n=3) B) in presence or absence of IFNγ neutralizing antibodies (α-IFNγ) for 6 days and analyzed for TRAIL expression. C) NK cells (n=6) from A) were analyzed for cytotoxicity against b-AP15 treated J82. NK cells (n=4) were treated with 500U/ml IL-2 on days 0 and 4 and 500pg/ml recombinant human IFNγ (hIFNγ) every other day for 6 days or left untreated and analyzed for TRAIL expression by flow cytometry D) and cytotoxicity against b-AP15-treated J82 tumor cells by ^{51}Cr-release

assay E). Data shown from n pooled experiments (mean ± SEM) and p-values were obtained from paired two tailed student's t-test.

Figure 6. Adoptive infusion of ZA-primed NK cells reduces tumor progression in tumor bearing mice.

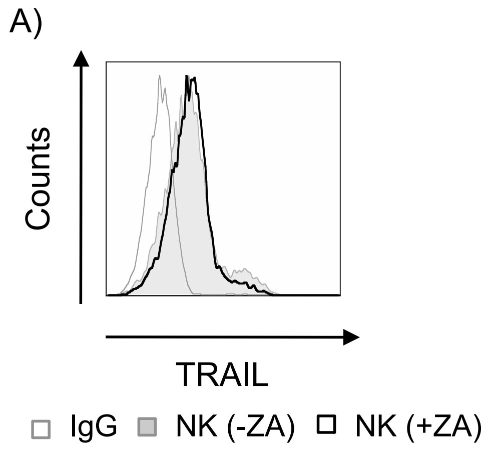
SCID/beige mice were injected with J82 cells and A) left untreated or B) were treated with b-AP15 on days 10, 21, 24 and 31 followed by infusion of ZA-primed or unprimed NK cells on days 11, 22, 25, and 31. Tumor size was analyzed by caliper measurement twice weekly. p-values for tumor progression B) and survival C) were analyzed by two-way ANOVA and log-rank tests respectively. Result from two pooled experiments with a total of seven mice per group is shown.

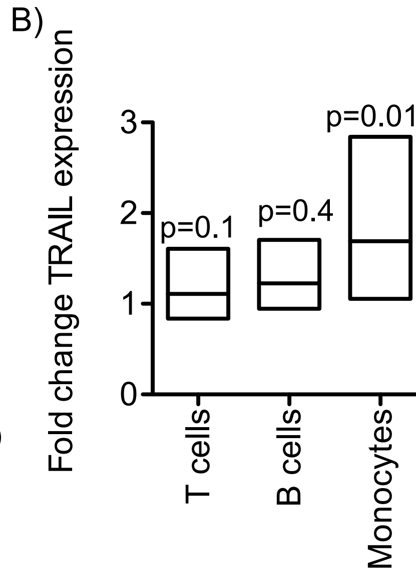
Supplemental Figure 1. NK cells were cultured for 7 days without feeder cells A) or co-cultured with T cells, B cells or monocytes B) in presence or absence of ZA and stained for CD56 and TRAIL (n=3). One of 3 independent experiments is shown for A. The fold-change in TRAIL expression was calculated between ZA-treated and untreated cultures. Boxes represent fold change values ranging from min to max and lines represent mean values. p-values were calculated by paired student's t-test. C) NK cells were co-cultured with monocytes for 7 days in presence or absence of ZA and neutralizing antibodies against IFNγ (αIFNγ) and stained for NKG2D (n=2). Data from three and two independent experiments are pooled in B and C respectively.

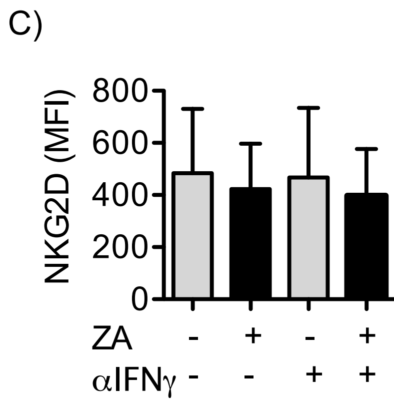
Supplemental Figure 2. A) A representative flow cytometry plots for gating strategy is shown. Monocytes, NK or T cells were isolated by magnetic beads and stained for CD56, CD3, CD14 to assess their purity on day 0 B) or day 7 after co-culture C).

Supplemental Figure 3. A) Purified monocytes were cultured for 4 days in presence or absence of ZA and stained for CD54. B) *In vitro* 18 hour ^{51}Cr-release assay by recombinant TRAIL [100ng/ml] (r-TRAIL) against untreated (Untr) or b-AP15-treated J82 tumor cells.

Supplemental figure 1. Sarhan et al.







Supplemental figure 2. Sarhan et al.

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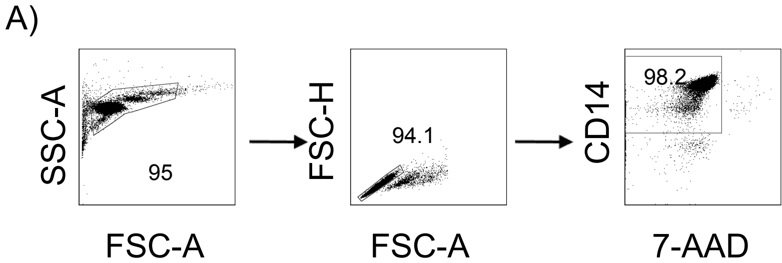

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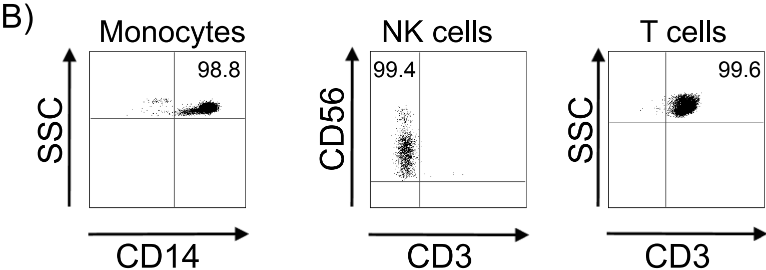

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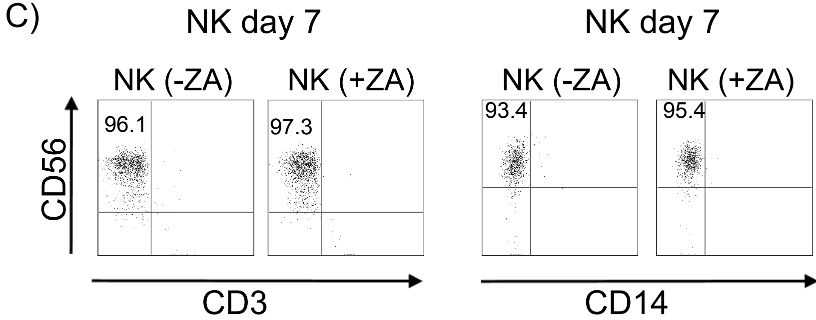

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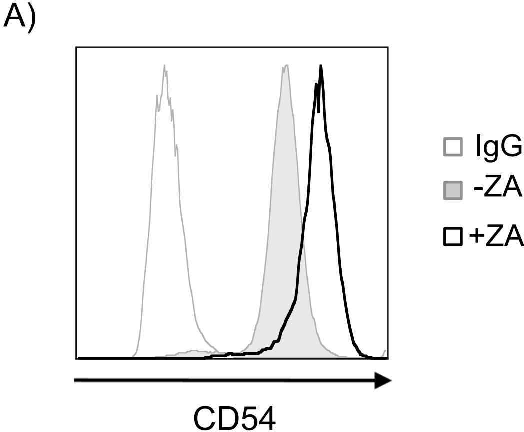

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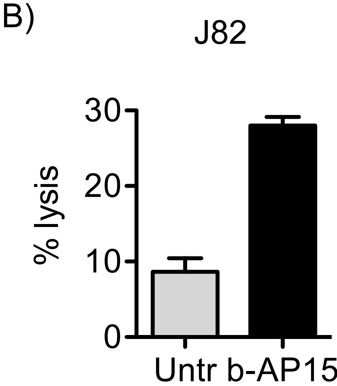

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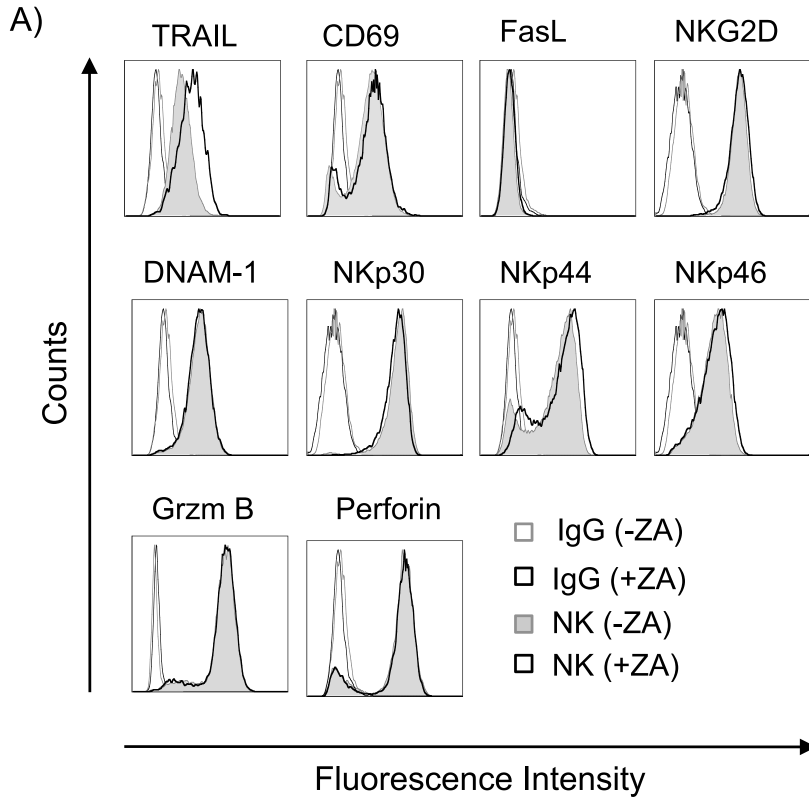

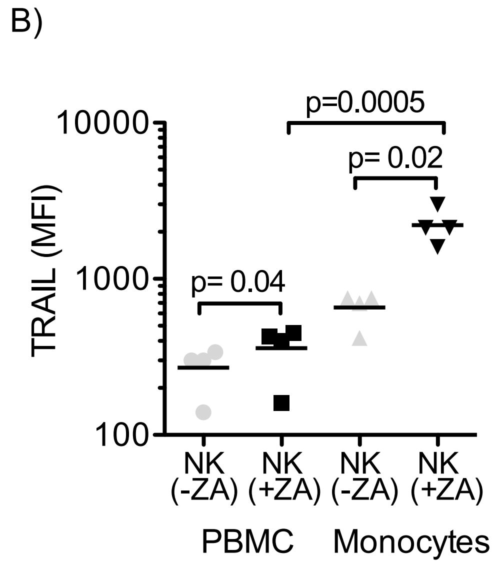

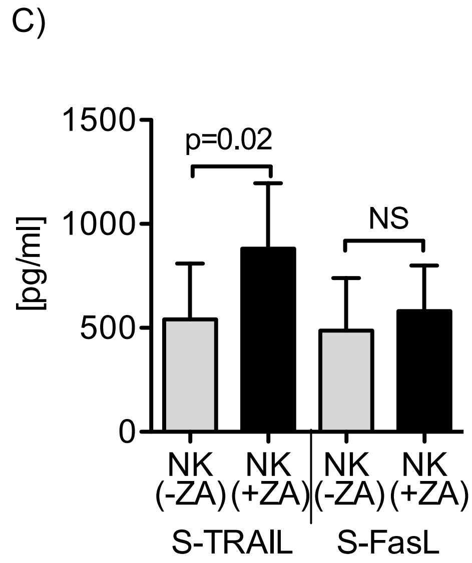

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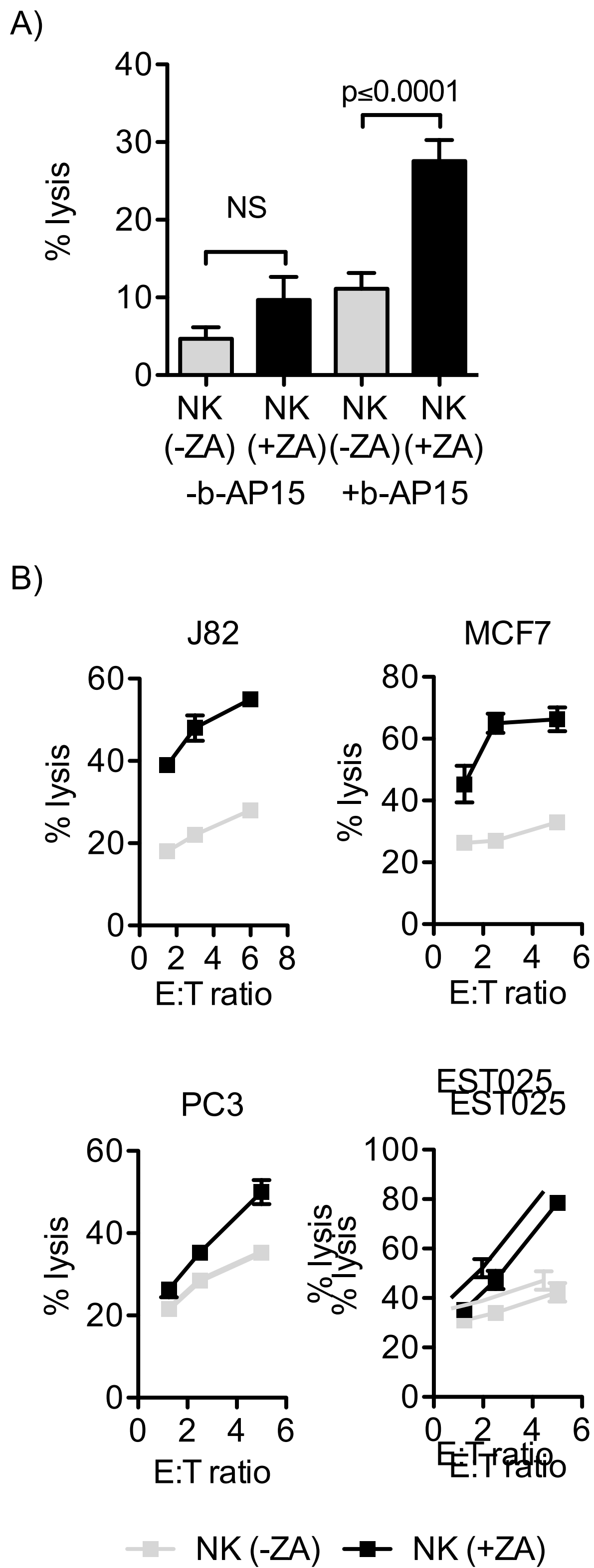

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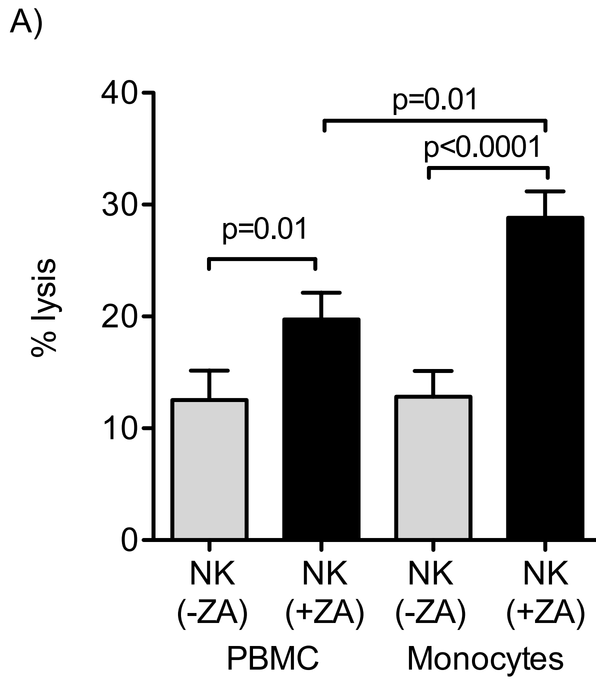

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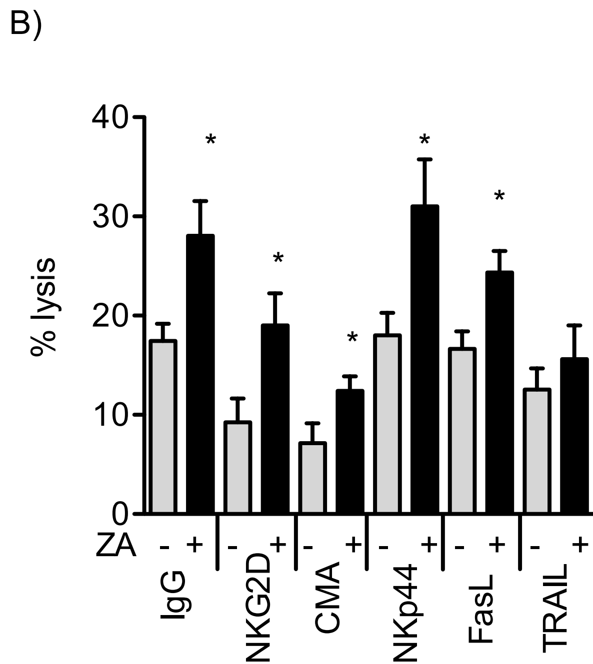

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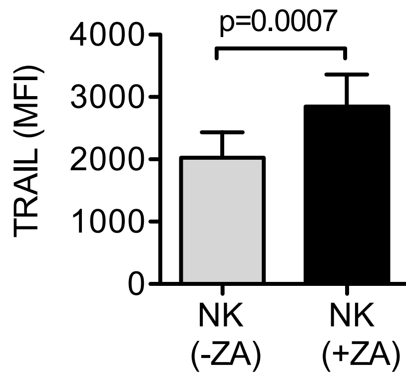

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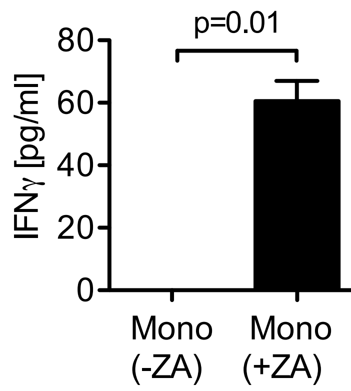

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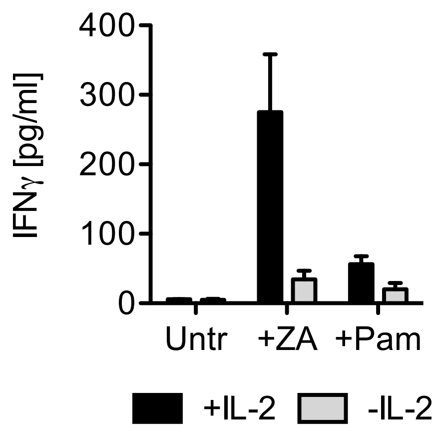

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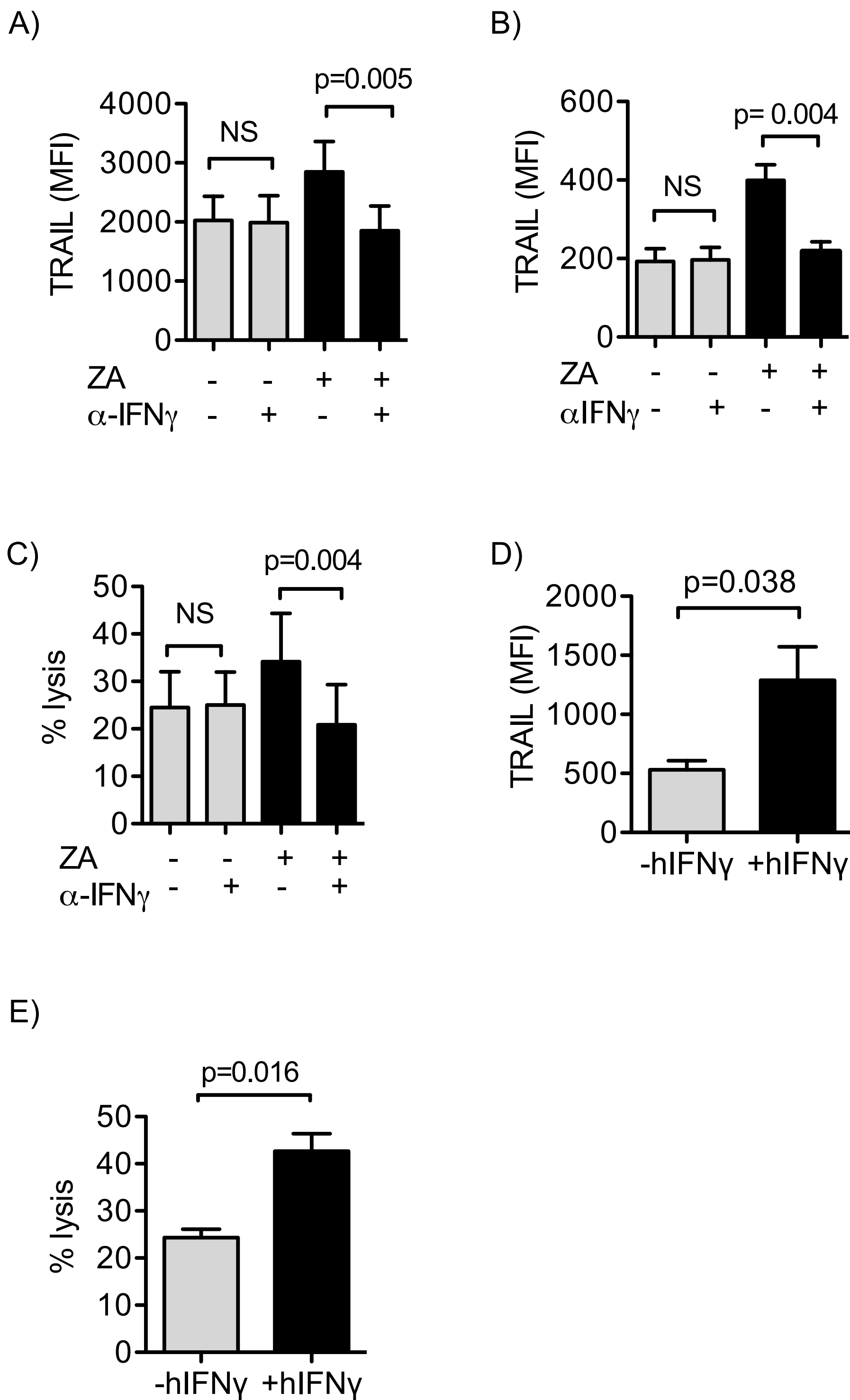
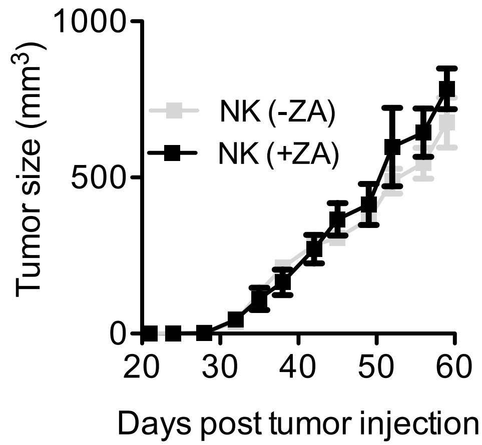


Figure 6. Sarhan et al.

A)



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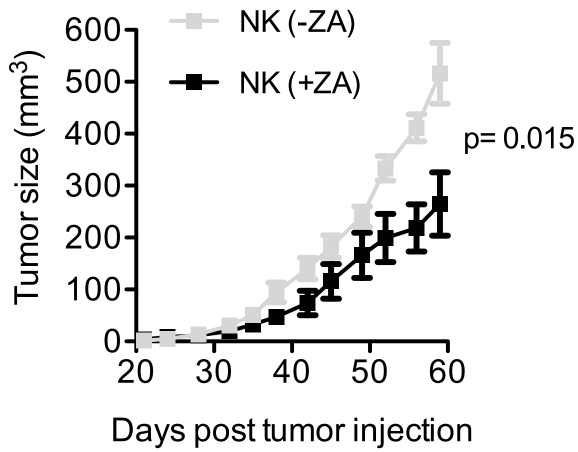

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