Potential for enhanced therapeutic activity of biological cancer therapies with doxycycline combination

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Despite significant strides made in the clinical translation of adoptive immune cell therapies, it is apparent that many tumors incorporate strategies to avoid recognition by receptors expressed on the immune cells, such as NKG2D. Strategies that stabilize the expression of ligands for these receptors may enhance the therapeutic potential of these and related therapies. Doxycycline inhibits matrix metalloproteinases (MMPs) that act to cleave the extracellular domain of MICA/B, ligands for the NKG2D receptor. Doxycycline treatment blocked shedding of MICA/B from a panel of human tumor cells, but also acted to increase their expression and cell surface translocation, possibly through its action on ATM. This meant that many tumor cells displayed increased MICA/B expression and enhanced susceptibility to CIK cells. Interestingly, doxycycline also selectively enhanced the replication of oncolytic vaccinia in many tumor cell lines, leading to increased sensitivity to these therapies. Combination (CIK-oncolytic vaccinia) therapies used in conjunction with doxycycline led to increased anti-tumor effects. The unexpected and pleiotropic beneficial anti-tumor effects of doxycycline on both immune cell and oncolytic viral therapies make it an excellent candidate for rapid clinical testing.

INTRODUCTION

Adoptively transferred immune cells have made significant clinical strides in recent years in the treatment of multiple cancer types. However several limitations remain, and approaches that increase either the targeting or the potency of these immune cells are needed. One such approach that we have previously described uses some immune cell populations (including Cytokine Induced Killer, CIK cells6–7) as carrier vehicles to deliver oncolytic viruses to tumors, so increasing their potency. Oncolytic viruses are viral therapies whose replication is restricted to tumor cells, and strains of engineered vaccinia virus have displayed encouraging pre-clinical and clinical responses. The capacity to utilize CIK cells as delivery vehicles significantly enhances their therapeutic capabilities. CIK cells, like Lymphokine Activated Killer (LAK), and some experimental cell lines (TALL-104, NK-92) express the receptor NKG2D on their surface that recognizes stress response ligands, such as MICA and MICB that are frequently expressed on cancer cells. However many tumors have also developed strategies to evade detection by these and related therapies. In particular NKG2D ligands may be downregulated from the surface of cancer cells2, 12 or their expression may be downregulated. Strategies that increase and stabilize NKG2D-ligand expression on tumor cells would therefore increase the potency of many immune cell therapies.

It has been found that some histone deacetylase inhibitors can upregulate expression of MICA/B, however this frequently leads to further increased shedding of the extracellular domains of these surface proteins. Shedding of soluble MICA/B is typically mediated by the action of matrix metalloproteinases (MMP). Although MMP inhibitors can block this shedding, no targeted MMPi are currently approved for clinical use. Because the commonly used antibiotic doxycycline has known MMPi activity, it was decided to test the applicability of using doxycycline in combination with CIK cell therapy. However, the pleiotropic functions of tetracyclines such as doxycycline29 meant that a thorough examination of the effects of this treatment on CIK cell therapy and CIK cell-oncolytic vaccinia virus combination therapy was required. Especially as we observed that doxycycline apparently enhanced vaccinia replication in at least some cell types. Initial experiments determined that doxycycline treatment not only reduced the shedding of sMICA/B, it actually enhanced the levels of surface expression of MICA and MICB on tumor cells through multiple mechanisms, leading to greatly stabilized surface expression across many cell lines. This observation, coupled with the observation that doxycycline also increased vaccinia replication in tumor cells led us to try to define the mechanisms behind these effects and to determine the effects of doxycycline treatment on anti-tumor activity of these therapies used alone or in combination in different mouse models.

RESULTS

Doxycycline treatment reduces shedding of soluble MICA and MICB and increases surface expression in cancer cells

A panel of 12 human tumor cell lines, (primarily ovarian, colorectal and breast cancers) and several non-tumor cell lines were grown in culture and the levels of soluble MICA and MICB released into the media after 24 h, were quantified by ELISA. Only 4 cell lines released detectable amounts of soluble MICA/B under these conditions (HeLa; UCI-101; UCI-107 and MDA-MB-231). The effects of exposing these cells to a pan MMP inhibitor or doxycycline for
this period was examined (Figure 1a). In most cases either treatment significantly reduced shedding \( (P < 0.05) \), typically by 2–5-fold. There was only one cell line where only one of the treatments reduced shedding of either sMICA or sMICB; for sMICB release from UCI-101, where only MMPi reduced shedding significantly (HeLa cells displayed very low shedding levels \( (< 25 \text{ pg/ml/24 h}) \) and neither treatment had any significant effect). Combining both treatments did not produce additional benefits (data not shown).

Further, when the overall level of MICA/B on the surface of all 13 cell lines were assayed by flow cytometry (Figure 1b), doxycycline was found to significantly increase the level of surface MICA/B expression both on those cell lines found to shed the ligands (UCI-101; UCI-107; MDA-MB-231), as well as other cell lines that did not shed, and in which MMPi had no effect (Ovarcar4, DLD1, MCF-7). The only cell lines in which doxycycline had no effect were those with very low \( (< 10\%) \) background level of MICA/B (Skov3; Ovarcar8, HT-29; HS96) and in the non-tumor cell line MRC-5. It therefore appears that doxycycline is able to stabilize MICA/B surface expression through additional mechanisms beyond inhibition of MMPs. Histone deacetylase inhibitors (HDACi) are also known to enhance MICA/B surface expression levels, and so a panel of HDACi (Trichostatin A (TSA), Valproic acid (VPA), PXD101) were also tested. These also enhanced MICA/B surface levels in many of the cell lines, occasionally to even greater levels than doxycycline (Ovarcar4, MCF-7) as well as in HS96 cells where neither MMPi nor doxycycline had any effect. However, the increased surface expression levels of MICA/B after HDACi treatment also appeared to come with the cost of increased shedding in some cases (Figure 1c), indicating that the increased MICA/B levels after HDACi treatment may not translate into increased sensitivity to NKG2D expressing immune cells.

Doxycycline treatment increases overall MICA/B levels, movement to the surface and level of phosphorylation of ATM

In initial studies to help define the mechanisms driving doxycycline-mediated increases in MICA/B surface expression, two cell lines were examined, one that increased MICA/B surface expression in response to both MMPi and doxycycline (UCI-101) and one that responded to doxycycline only (Ovarcar4). The overall levels of MICA/B in the cells were determined after different

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**Figure 1.** Doxycycline reduces shedding and enhances surface expression of MICA/B on tumor cells. (a) Doxycycline treatment reduces the shedding of soluble MICA (sMICA) or MICB (sMICB) from several cell lines. Doxycycline \( (1 \mu \text{g/ml}) \) or MMPi III \( (5 \mu \text{m}) \) were applied for 24 h and levels of soluble ligands in the media detected by ELISA after 24 h \( (* P < 0.05) \).

(b) Surface expression levels of MICA/B after different treatments. Surface expression of MICA/B was determined by flow cytometry (using an antibody that recognizes both forms) at 24 h after various treatments. The percentages of cells demonstrating positive staining relative to an isotype control are shown. Doxycycline and MMPi III treatment as before; HDACi were also applied for 24 h \( \text{(TSA was used at 0.375} \mu \text{M; VPA was used at 750} \mu \text{M; PXD101 was used at 0.625} \mu \text{M)} \) \( (* P < 0.05) \).

(c) Shedding of sMICA and sMICB was determined for cell lines after treatment with different HDACi (treatments and ELISA as before).
Doxycycline treatment enhances sensitivity of tumor cells to killing by CIK cells in vitro and in vivo

CIK (Cytokine Induced Killer) cells, like many NK and some T cells, target NKG2D ligands including MICA/B on tumor targets. The capability of these cells to destroy tumor cells with and without doxycycline treatment was therefore examined in a simple in vitro CTL assay. Initially it was necessary to ensure that doxycycline treatment alone did not mediate tumor cell killing. None of the tumor cells examined was destroyed directly by doxycycline treatment (Figure 3a). However, all of the cell lines tested displayed increased sensitivity to CIK cells after even low dose doxycycline treatment (1 μg ml⁻¹) (Figure 3b). This was apparently due to increased surface MICA/B expression as no increased killing was seen for SKOV3 cells that did not display enhanced MICA/B expression after doxycycline treatment.

In order to determine if the effects of doxycycline treatment could translate into enhanced anti-tumor effects in vivo a mouse tumor xenograft model was examined. UCI-101 cells were implanted subcutaneously into athymic nu/nu- mice and when palpable (50–100 mm³) doxycycline (100 μg) was added to the drinking water for 72 h prior to systemic treatment (tail vein) with 1 × 10⁷ human CIK cells pre-labeled with Cy5.5 for fluorescence imaging of cell trafficking (mice remained on doxycycline treatment for a further 14 days after treatment). It was seen (Figure 4a) that CIK cells displayed significantly enhanced trafficking or targeting of tumors in mice that had been pre-treated with doxycycline. This also translated into a significantly increased overall anti-tumor effect (Figure 4b). Doxycycline pre-treatment can therefore enhance sensitivity to systemic treatment with CIK cells. No treatment-associated toxicities were observed with or without doxycycline treatment.

Doxycycline enhances oncolytic vaccinia replication in tumor cells

We have previously described a novel therapeutic approach incorporating immune cells, such as CIK cells as carrier vehicles to deliver an oncolytic vaccinia virus to tumor targets after pre-infection of the CIK cells with the virus.⁶ The effect of doxycycline exposure on vaccinia replication was therefore determined for strain vvDD (vaccinia containing deletions in the viral growth factor and thymidine kinase genes) and expressing luciferase to quantify viral gene expression) (Figure 5).

Surprisingly doxycycline treatment increased viral replication in the majority of the tumor cell lines tested (8 of 12), this is of interest as several groups have described the incorporation of tetracycline responsive elements into vaccinia vectors, requiring doxycycline to induce or repress gene expression.⁶,³⁷ Several non-tumor cell types were also tested (MRC-5 and CIK cells), and...
Doxycycline treatment did not appear to enhance viral replication in these cells. Because doxycycline enhances viral replication in a tumor specific manner, and did not inhibit viral replication in any cells, it is likely that both oncolytic virus therapy and CIK-VV combination therapy would be enhanced by pre-treatment or combination treatment with the antibiotic.

Although there were no clear patterns between cells that responded to doxycycline with respect to enhanced MICA/B surface expression and viral replication, it was noted that of the 4 cell lines that did not show increased viral replication, two (SKOV3 and Ovcar4) also did not increase MICA/B expression in response to doxycycline, while a third (MCF-7) only increased expression by a small degree.

In order to examine the effects of doxycycline treatment on the oncolytic viral therapy in vivo, MDA-MB-231 cells were implanted into mice and treated systemically (tail vein) with $1 \times 10^7$ PFU of oncolytic vaccinia virus strain vvDD expressing luciferase. Mouse tumors reached 50–100 mm$^3$. Bioluminescence imaging was used to quantify viral gene expression from within the tumor at times after treatment for mice with and without additional doxycycline treatment. It was seen (Figure 6a) that doxycycline treatment significantly increased the level of viral gene expression from within the tumors of these animals. However, no such increase in viral gene expression was seen in non-tumor tissues, indicating that safety is not compromised with doxycycline treatment, instead an enhanced therapeutic index was achieved.

This increase in viral replication within the tumor also translated into a significantly increased anti-tumor effect in the same mouse model (Figure 6b). Doxycycline therefore has the capacity to enhance either CIK and oncolytic vaccinia therapies.

Doxycycline treatment significantly enhances CIK-VV therapy through action on multiple levels

The positive effect of doxycycline treatment on the action of both CIK cell and oncolytic vaccinia viral therapies used individually
sMICA/B, but also increased surface expression in cancer cells that increased surface expression of MICA/B in cells known to produce cleavage from the surface of cancer cells. 

The MMPi function of doxycycline was capable of blocking MICA/B/C2 treatment. Human CIK cells (hCIK) were pre-labeled with Cy5.5 and treated mice (CIK cell fluorescence was detected in the tumors of doxycycline fluorescence imaging 72 h after initial injection. Significantly more number of CIK cells in the tumor was determined by whole animal fluorescence imaging, however this needs further testing.

Figure 4. Doxycycline treatment enhances tumor targeting and therapeutic effect of CIK cells in mouse tumor models. (a) Athymic nu/nu- mice were implanted with UCI-101 tumor cells (1 x 10⁷ cells subcutaneously) and when tumors reached 50–100 mm³ treatments were begun. Some mice received doxycycline in their drinking water (100 μm) for a total of 17 days starting 3 days before CIK cell treatment. Human CIK cells (hCIK) were pre-labeled with Cy5.5 before 1 x 10⁷ cells were delivered via tail vein injection. The number of CIK cells in the tumor was determined by whole animal fluorescence imaging 72 h after initial injection. Significantly more CIK cell fluorescence was detected in the tumors of doxycycline treated mice (n = 8 per group; *P = 0.03). Sample images are shown. (b) Anti-tumor effects of CIK cells used as before with and without doxycycline combination. The tumor growth and response to therapy were followed in the same mice by caliper measurement. Doxycycline enhanced the anti-tumor effects of CIK cell therapy (*P < 0.05 for days 14–24).

MMPi function.²⁸ Our initial aim was therefore to examine whether the MMPi function of doxycycline was capable of blocking MICA/B cleavage from the surface of cancer cells. Surprisingly, doxycycline not only blocked cleavage and increased surface expression of MICA/B in cells known to produce sMICA/B, but also increased surface expression in cancer cells that did not shed the ligands, so negating any requirement for adding HDACi (to upregulate expression) in addition to MMPi (to block shedding). This was unexpected, and greatly increases the potential utility of doxycycline as an adjuvant to immune cell therapies. Initial investigation into the mechanism underlying this effect determined that doxycycline apparently increased both the overall level and surface trafficking of MICA/B. Although much is still unknown about the regulation of cell surface MICA/B levels, activation of ATM/ATR is the only recognized mechanism that enhances both transcription and cell surface trafficking. The level of phosphorylated ATM after doxycycline treatment was therefore examined, and an increase was found in many cell lines. However it should be noted that pATM levels increased in both cell lines that responded to doxycycline treatment and those that did not, indicating additional suppressive mechanisms might also be involved.

It is also unclear how doxycycline increases pATM levels, however there may be a link between the known activation of ATM as a result of increased DNA damage, the increased levels of DNA damage in tumor cells, and the known anti-apoptotic effects of tetracyclines.³³ Possibly inhibition of apoptosis may lead to increased accumulation of DNA damage, especially in tumor cells, and so increased levels of pATM and MICA/B surface expression, however this needs further testing. It was evident that doxycycline treatment in mouse tumor models led to enhanced CIK targeting and greater antitumor effects. We have previously described a highly promising combinatorial therapy in pre-clinical models involving the combination of CIK cells with an oncolytic vaccinia virus, such that the CIK cells can be pre-infected with virus and act as carrier vehicles, releasing the virus selectively in the tumor. The virus in turn acts as means for arming the CIK cells, and greatly increasing their capacity to destroy the tumor once they infiltrate. It is reasonable to expect that doxycycline would therefore also act to enhance the power of this CIK-VV combination therapy. However it was noted that doxycycline did not inhibit viral replication in the tumor cells. Surprisingly doxycycline treatment actually enhanced viral replication in the majority of tumor cells examined. This unexpected result has additional implications for the incorporation of tetracycline response elements into vaccinia strains to follow the effects of selective viral gene expression. Although, the mechanism is not understood, it appears that doxycycline effects on pATM levels, blocking of apoptosis or scavenging of reactive oxidative species is not the primary cause.

However, this effect of doxycycline on viral replication might be used to enhance the effects of oncolytic vaccinia therapies both used alone or especially in combination with CIK cell carrier vehicles. Indeed, doxycycline treatment was seen to significantly increase viral replication selectively in the tumor and overall therapeutic effect in mouse tumor models. Furthermore, the effects of doxycycline on the anti-tumor effects of CIK-VV therapy were even more dramatic with a highly significant therapeutic effect determined that doxycycline apparently increased both the therapeutic effect in mouse tumor models. Furthermore, the effects of doxycycline on the anti-tumor effects of CIK-VV therapy were even more dramatic with a highly significant therapeutic benefit. The current routine use of CIK cells in treatment of cancers in some regions, and the promising clinical data seen in recent Western trials mean that approaches that further increase their effectiveness and the range of cancers they can effectively treat would be highly beneficial. In addition, the recent exciting clinical data with oncolytic vaccinia virus raises the likelihood that these therapies may soon be approved for use in Western markets. Again, the observation that combining oncolytic vaccinia with a commonly used and approved agent such as doxycycline can safely enhance its therapeutic benefit would be important. Finally although CIK-VV combination therapies have only been examined in pre-clinical models to date, the combination has displayed improved therapeutic benefit over either CIK or VV used as single agents and clinical development is ongoing. The dramatically improved therapeutic effects of combining all these therapies with doxycycline make this an exciting potential future combination.

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Figure 4. Doxycycline treatment enhances tumor targeting and therapeutic effect of CIK cells in mouse tumor models. (a) Athymic nu/nu- mice were implanted with UCI-101 tumor cells (1 x 10⁷ cells subcutaneously) and when tumors reached 50–100 mm³ treatments were begun. Some mice received doxycycline in their drinking water (100 μm) for a total of 17 days starting 3 days before CIK cell treatment. Human CIK cells (hCIK) were pre-labeled with Cy5.5 before 1 x 10⁷ cells were delivered via tail vein injection. The number of CIK cells in the tumor was determined by whole animal fluorescence imaging 72 h after initial injection. Significantly more CIK cell fluorescence was detected in the tumors of doxycycline treated mice (n = 8 per group; *P = 0.03). Sample images are shown. (b) Anti-tumor effects of CIK cells used as before with and without doxycycline combination. The tumor growth and response to therapy were followed in the same mice by caliper measurement. Doxycycline enhanced the anti-tumor effects of CIK cell therapy (*P < 0.05 for days 14–24).

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approach. A variety of other immune cell based therapies also target NKG2D ligand expression on tumors and so these might also be enhanced by combination with doxycycline, while a number of other oncolytic virus strains are undergoing advanced clinical testing, and it would be of interest to know if they also benefit from doxycycline combination.

MATERIALS AND METHODS

Cell lines and reagents

Human ovarian cancer cell line UCI-101 and UCI-107 were a kind gift from Drs P DiSaia and A Menetta (University of California, San Diego). Ovcar4 and Ovcar8 were obtained from Dr T Hamilton (NCI-Frederick Cancer DCTD Tumor/Cell Line Repository, Frederick, MD, USA). Human ovarian cancer cell lines Skov3, cervical cancer cell line Hela, human colon cancer cell lines DLD1, HCT116 and HT29, human lung cancer cell line H596 and human normal lung fibroblast cell line MRC5 were obtained from American Type Culture Collection (ATCC), Manassas, VA, USA. All cells were cultured in recommended culture media supplemented with 5 or 10% fetal bovine serum and antibiotics.

Histone Dacetylase Inhibitors: TSA (Trichostatin A) and VPA (Valproic acid sodium salt) were purchased from Sigma-Aldrich (St Louis, MO, USA); Doxycycline was purchased from MP Biomedicals, Santa Ana, CA, USA; PXD101 (Belinostat) was obtained from Selleck Chemicals, Houston, TX, USA; The MMP inhibitor III was from Calbiochem (Merck, Darmstadt, Germany).

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Figure 5. Doxycycline enhances viral replication and gene expression after treatment of multiple tumor cell lines with oncolytic vaccinia strain vvDD. (a) Different tumor cell lines were infected (Multiplicity of Infection of 1.0 PFU/cell) with oncolytic vaccinia strain vvDD expressing luciferase, with and without the presence of 1 μg/ml doxycycline. Viral gene expression was determined at indicated times by bioluminescence imaging. Cell lines HeLa, HT29, OVCAR3, H596, OVCAR8, MDA-MB-231, DLD1 and HCT116 all displayed significantly increased gene expression at 48 h after infection, (P<0.05). (b) Viral plaque assays confirmed the increased gene expression correlated with viral replication for selected cell lines at 48 h. (c) The same gene expression assay was performed on the non-tumor (immortalized, non-transformed) cell line MRC5. (d) Viral replication was also determined by plaque assay in human CIK cells.
Flow cytometry

Cell surface MICA/MICB detection was with PE-conjugated anti-human MICA/MICB (eBioscience, San Diego, CA, USA). PE-conjugated mouse IgG1 isotype was used as control (eBioscience, San Diego, CA, USA). For cell apoptosis analysis FITC-conjugated Annexin-V and Propidium Iodide (PI) was used according to the manufacturer’s directions (Abcam Inc., Cambridge, MA, USA). Samples were analyzed using a BD Accuri C6 Flow Cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) and analysis on FlowJo (Treestar Inc, Ashland, OR, USA).

Cell immunofluorescence

Cells (5000 cells/chamber) were seeded and incubated overnight on 4-chambers chamber slides (Lab-Tek, Scott’s Valley, CA, USA). After incubation with indicated doses of Doxycycline for 24 h, cells were fixed with 2% paraformaldehyde and immunofluorescence was performed with mouse anti-human monoclonal to MICA+MICB (Abcam Inc., Cambridge, MA, USA), followed by the appropriate secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA) at a dilution of 1:600. Alexa Fluor 546-conjugated phalloidin was used at a 1:1000 dilution. To visualize nuclei, slides were incubated for 10 min in DRAQ-5 (Biostatus Limited, Shepshed, UK) diluted to 1:1000 in TBS (20 mM Tris-HCl, pH 7.5, 500 mM NaCl). Fluorescent images were collected using a Leica TCS SL Confocal microscope (Leica Microsystems, Bannockburn, IL, USA).

Western blot analysis

Protein extracts were isolated from 10⁶ treated cells using the mammalian cell lysis reagent containing protease inhibitor cocktail (Sigma-Aldrich, St Louis, MO, USA) following the manufacturer’s directions. Equal amounts of lysate protein were resolved on a 4–20% precast polyacrylamide gel (Bio-Rad Laboratories, Inc., Hercules, CA, USA) and were transferred to Immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). MICA/B, Ataxia telangiectasia mutated kinase (ATM), Phospho-ATM and beta-actin proteins were detected on Western blots using the mouse monoclonal to MICA+MICB (Abcam Inc., Cambridge, MA, USA), ATM (D2E2) Rabbit mAb, Phospho-ATM (Ser1981) (D6H9) Rabbit mAb (Cell

Figure 6. Doxycycline enhances in vivo effects of oncolytic vaccinia therapy alone, or in combination with CIK cell-mediated viral delivery. (a) Mice (athymic nu/nu- bearing subcutaneous MDA-MB 231 tumors of 50–100 mm³) were treated with oncolytic vaccinia strain WRTK- expressing luciferase (1 × 10⁷ PFU delivered via tail vein) with or without doxycycline (n = 7 per group). Subsequent viral gene expression was determined by bioluminescence imaging at the indicated times for a region of interest over the tumor (top) or the torso (bottom) of the mice (P < 0.05). (b) Therapeutic effect of the same treatments with tumor volume determined by caliper measurement (P < 0.05). (c) Mice as before (athymic nu/nu- with MDA MB 231 tumors) were treated with PBS, doxycycline, hCIK (1 × 10⁷) pre-mixed with vvDD (1 × 10⁷ PFU); or CIK/vvDD and doxycycline (n = 10 per group). CIK cells were labeled with cy5.5 prior to injection and fluorescence from within the tumor was determined after 72 h by whole animal fluorescence imaging (P = 0.027). (d) Bioluminescence imaging t the same time point determined viral gene expression from within the tumor (P = 0.0009). (e) Caliper measurements followed subsequent effect on tumor volume. Doxycycline alone had no effect on tumor growth, whereas CIK/vvDD showed significant benefit relative to control from day 60, and CIK/vvDD plus doxycycline was significantly better than any other treatment from day 44 onwards (P < 0.5). At the end of the experiment 2 of 8 mice in the CIK/vvDD group displayed complete responses and 6 of 8 in the CIK/vvDD plus doxycycline group.

Germany). Unless otherwise stated TSA was used at 0.375 μM; VPA was used at 750 μM; MMP inhibitor III was used at 5 μM; Doxycycline was used at concentrations range from 1 to 40 μg/ml in in vitro and 100 μg/ml in drinking water for in vivo work. The usual dose for treatment of infections in humans is 100 mg twice a day oral or IV (depending on the pathogen), meaning that the doses used here are below typical doses on a per kg basis.
Signaling Technology, Inc., Danvers, MA, USA), mouse monoclonal to β-actin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) antibodies, respectively. Blots were then incubated with appropriate peroxidase-conjugated secondary antibodies, and detected using the SuperSignal chemiluminescence system (Thermo Fisher Scientific, Rockville, MD, USA).

ELISA

ELISA measurement of soluble MICA and MICB levels in cell culture medium were determined by Human MICA Duoset ELISA Development kit and Human MICB Duoset ELISA Development kit (R&D Systems, Inc. Minneapolis, MN, USA). The procedures are in accordance with the protocols supplied with the kit.

Cellular cytotoxicity assay

Cell cytotoxicity was evaluated using a CytoTox 96 non-radioactive cytotoxicity assay kit (Promega, Madison, WI, USA) based on the measurement of lactate dehydrogenase (LDH) according to the manufacturer’s instructions. For CIK cell-mediated cytotoxicity assays, hCIK cells were prepared as stated before and were added to tumor cells using effector to target cell ratios of 50:1, 20:1, 10:1, and 1:1. After a 4 h incubation at 37 °C, culture medium was harvested for LDH production analysis, according to the manufacturer’s instructions.

Viral infection

Cells were treated as indicated for 24 h, then challenged with luciferase (Luc)-expressing oncolytic vaccinia virus strains WR,TK-, with a single deletion in the viral thymidine kinase gene (TK) and vP7D, with deletions in both the TK and vaccinia growth factor (VGF) genes, at a multiplicity of infection (MOI) of 1 plaque forming unit (pfu) per cell. At indicated time points post-infection, luciferin was added to each well [10 μM/106field of 30 mg/ml luciferin (Caliper Life Science, Perkin Elmer, Waltham, MA, USA)] and bioluminescence per well (photons/second) was measured on an IVIS 200 imaging system (Xenogen part of Perkin Elmer). In some experiments viral replication was determined by plaque assay on BSC-1 layer cells. All experiments were run in triplicate.

Mouse models

Athymic nu/nu-mice (female, 6–8 weeks) obtained from Taconic Corporation (Germantown, NY, USA) were used for xenograft studies. Mice received subcutaneous injection of 1.5 × 105 UC3-101 or MDA MB 231 tumor cells. Once palpable tumors (50–100 mm3) were formed, animals were regrouped and treatment was begun. Mice were treated with Doxycycline (100 mg/kg), twice daily, for 7 days. In some experiments viral replication was determined by plaque assay on BSC-1 layer cells. All experiments were run in triplicate.

Statistics

Student t-tests were used throughout, with statistical significance considered to be P<0.05.

CONFLICT OF INTEREST

Dr Thorne has a financial interest in Jennerex Biotherapeutics. The remaining authors declare no conflict of interest.

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