Autophagy induced by conventional chemotherapy mediates tumor cell sensitivity to immunotherapy

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Abstract

Autophagy attenuates the efficacy of conventional chemotherapy but its effects on immunotherapy have been little studied. Here we report that chemotherapy renders tumor cells more susceptible to lysis by cytotoxic T cells (CTL) in vivo. Moreover, bystander tumor cells that did not express antigen were killed by CTL. This effect was mediated by transient but dramatic upregulation of the mannose-6-phosphate receptor (MPR) on the tumor cell surface. Antitumor effects of combined treatment related to the kinetics of MPR upregulation and abrogation of this event abolished the combined effect of immunotherapy and chemotherapy. MPR accumulation on the tumor cell surface during chemotherapy was observed in different mouse tumor models and in patients with multiple myeloma. Notably, this effect was the result of redistribution of the receptor caused by chemotherapy-inducible autophagy. Together, our findings reveal one molecular mechanism through which the antitumor effects of conventional cancer chemotherapy and immunotherapy are realized.
Introduction

Therapeutic cancer vaccines and adoptive T-cell transfer are attractive options for the treatment of different types of cancer. In recent years, it has become apparent that cancer immune therapy may provide only limited clinical benefits and that its combination with targeted or cytotoxic chemotherapeutic treatment will be necessary to achieve substantial clinical benefit. However, the use of conventional cancer chemotherapy in combination with immunotherapy was previously not deemed appropriate due to potent immunosuppressive effects usually associated with chemotherapy. This paradigm was challenged in recent years by the serendipitous observations made in a number of phase I/II clinical trials in which high rates of objective clinical responses were observed when cancer vaccines were combined with chemotherapy (1-5). These observations were made using various cancer vaccines and different chemotherapeutic regimens in patients with diverse types of cancer (6). The mechanisms of the potential beneficial effect of combined immuno-chemo therapy remains unknown.

In animal tumor models it has been shown that conventional chemotherapy and radiation therapy can induce immune responses against antigens generated in dying tumor cells (7, 8). Single injections of chemotherapeutic drugs may induce an antitumor immune response by directly causing immunogenic cell death (9). When used in non-cytotoxic doses, drugs like paclitaxel (TAX), doxorubicin (DOX), mitomycin C, and methotrexate increased antigen presentation by antigen presenting cells (10). Dendritic cells (DCs), treated with vinblastine, underwent maturation and exhibited better ability to induce CD8 T-cell responses compared to untreated DCs (11). Chemotherapy has also been shown to render cancer cells more susceptible to killing by CTLs. 5-fluorouracil, CPT-11, and cisplatin (CIS) were all shown to increase the sensitivity of the SW480 colon cancer cell line to killing by T cells (12). Cytotoxic drugs can
modulate systemic immune suppression or expand antigen-specific T-cells via cytoreduction (rev. in (13)). However, the paradox is that the chemotherapeutic agents used in those studies are known to suppress immunity in cancer patients during treatment, which includes multiple repeated doses of drugs (14). There is ample evidence that chemotherapeutic agents can ablate T-cell function and blunt anti-tumor immune responses (15). Previous studies indicated that conventional doses of chemotherapy did not support the development and maintenance of antitumor immune responses (16). Even in patients who benefited from combined treatment, chemotherapy inhibited antigen-specific T cells generated by previously administered cancer vaccines (2). Moreover combination of chemotherapy with high dose cytokines have failed to improve survival of patients with metastatic melanoma (17).

These observations suggested that chemotherapy can potentiate immunity if used in either single dose or in low non-cytotoxic doses. However, the critical question is- Can immunotherapy be used in combination with conventional chemotherapy in patients with advanced stage cancer?

We recently demonstrated that chemotherapy makes tumor cells permeable to granzyme B (GrzB) released by activated CTLs in a perforin independent manner (18). We suggested that this effect in vitro could be mediated via up-regulation of the cation-independent mannose-6-phosphate (MPR) receptor on the surface of tumor cells. MPR (also termed insulin-like growth factor 2 receptor) is a multifunctional protein with high-affinity binding to insulin growth factor 2 (IGF-2). Within the cell, MPRs are found in the trans-Golgi network (TGN), endosomes, and are also present within the plasma membrane. On the membrane, these receptors bind to their ligands and the whole complex is packaged in transport intermediates consisting of clathrin-coated vesicles (CCVs) and the AP-1 assembly complex. CCVs mediate the sorting of MPRs from TGN for transportation to endosomal compartments. Within early endosomes, ligand
dissociates from the receptor due to the low pH (19). The receptors are recycled back to TGN by the complex containing Golgi-localized, γ-ear containing ADP-ribosylation factor binding proteins (GGAs) (20). MPR function is associated with endocytosis and degradation (21). MPR was previously implicated in GrzB delivery to cells, bypassing perforin (22). Although under normal conditions MPR play a minor role in CTL mediated killing (23-25), this receptor is considered to be an important factor that, together with perforin, mediates GrzB entry into the cell (24, 26).

In this study we investigated the role of MPR during combined chemotherapy and immune therapy of cancer in vivo.
Materials and Methods

Mice and tumor models. Animal protocols were approved by the University of South Florida Animal Care and Use Committee. Female C57BL/6J (B6, H-2^b), BALB/c (H-2^d), and Pmel mice were purchased from The Jackson Laboratory (Bar Harbor, ME) or Charles River Laboratories (Wilmington, MA). Murine lymphoblastoma cell line, EL4 and B16-F10 melanoma, mammary carcinoma 4T1 lines were purchased from ATCC after 2009. B16-F10Kb- cells were isolated as an escape mutant from vaccinated mice (27). Small cell lung cancer cell line 86M1 and human multiple myeloma cell lines H929, U266 and 8226 were purchased from ATCC after 2008. The cells were maintained in RPMI 1640 media containing 10% FBS. B16-F10-luc-G5 mouse melanoma cell line that expresses the firefly luciferase was obtained from Xenogen (Alameda, Calif, USA) and maintained with 200µg/ml Zeocin (Invitrogen). Tumor cells were treated with 12.5 nM TAX, 25 ng/ml DOX and 25 ng/ml CIS for 18 hr prior to use as targets in various assays.

Patients samples. Patients with a histologically confirmed diagnosis of multiple myeloma (MM) were enrolled during 2002-2006 to clinical protocol MCC12733 approved by the University of South Florida institutional review board. Patients were treated for three days with melphalan (50 mg/m^2/d IV) and escalating doses of topotecan (starting at 10 mg/m^2/d IV). Bone marrow (BM) samples were collected before and immediately after three days of high-dose chemotherapy. Cell suspensions from BM aspirates were cytospun on slides and kept frozen in liquid nitrogen. Paired samples from 10 patients treated under this protocol were available for the analysis in our study.

Immunohistochemistry for MPR. Protocol is provided in supplemental materials.
Transfection of cell lines with MPR shRNA, ATG5siRNA, or ATG5shRNA vector. B16F10 and B16F10 kb- cell lines were stably transfected with either a control plasmid shRNA or MPR or Atg5 shRNA vector incorporating the puromycin resistance gene for selection (Mission®, Sigma-Aldrich) using Geneporter® 2 kit (Genlantis). B16F10 cells were transfected with ATG5siRNA using the Nucleofector Kit C (Lonza; Program X-05 on Amaxa) and 300nM of atg5 siRNAs or with 300nM of scrambled siRNA. The cells were re-suspended in DMEM medium and rested for 48h before treatment with TAX.

Detection of MPR and GrzB in cell lines. Cell lines were treated with 12.5nM TAX for 16h and labeled for MPR and GrzB as described earlier (18). A portion of the cells was fixed with 2% paraformaldehyde for 20 min at room temperature prior to staining. Dead cells were discriminated from the live population by either DAPI stain or Live/Dead Fixable Dead cell stain kit (Invitrogen).

Treatment protocol for B16F10 control shRNA/MPR shRNA tumor models. C57BL/6 mice were inoculated with 0.5 x 10^6 tumor cells/mouse on Day 0. Spleen cells from Pmel mice were activated with 0.1µg/ml of gp-100 peptide in vitro for 72h and T cells were purified using nylon wool. On day 11, mice were injected i.v. with 5 x 10^6 of Pmel T cells. On day 14, TAX was administered i.p. at a dose of 12.5mg/kg.

Luciferase murine model for bystander assay. Detailed protocol is provided in supplemental materials. For adoptive transfer using endogenous, vaccine-generated T cells, B16 tumor-bearing mice received 5x10^6 Trp2_180-specific CD8^+T cells generated in B6 mice immunized with Trp2_180TriVax (27).

Quantitative real-time polymerase chain reaction (qRT-PCR) and Western blotting. Protocol is provided in supplementary methods.
Confocal imaging. U266 MM cells were treated with 25ng/ml DOX for 18h, cytospun and fixed with 4% paraformaldehyde for 30 min in humid chamber. The cells were blocked with 10% human serum for 30 min and then labeled with primary MPR antibody, followed by goat anti rabbit Alexa 555 (Invitrogen). For inhibition of autophagy, the cells were treated with 1mM 3 methyladenine (3MA) for 4h prior to treatment with DOX overnight and then stained as described above. The cells were imaged with a Leica TCS SP5 laser scanning confocal microscope through a 63X/1.40NA Plan Apochromat oil immersion objective lens (Leica Microsystems, Germany). 405nm and 555nm diode lasers lines were applied to excite the samples. An acousto-optical beam splitter (AOBS) were used to collect peak emission photons sequentially to minimize crosstalk between fluorochromes. Protocol is provided in supplemental methods.

Statistical analysis. Statistical analysis was performed using a 2-tailed Student t-test and GraphPad Prism 5 software (GraphPad Software Inc.), with significance determined at $P < 0.05$. Analysis of tumor growth curves was performed, using a two-way Anova test with a Bonferroni posttest.
Results

The antitumor effect of combined chemo-immuno therapy is linked to a transient induction of MPR

We tested the effects of chemotherapy on the expression of MPR in vivo using several mouse (B16F10 melanoma, 4T1 mammary carcinoma) and human (multiple myeloma (MM) 8226, H929, U266) tumor cell lines. Consistent with the results of our previous study (18), different chemotherapeutic agents like TAX, DOX, CIS caused substantial and similar up-regulation of MPR expression in vitro in all tested mouse and human tumor cell lines (Fig. 1A).

To determine the effects of chemotherapy on the level of the receptor in vivo, tumors were established in either syngenic mice (C57BL/6 for B6F10, BALB/c for 4T1) or SCID/NOD or Nude mice (human tumors). When tumors reached 1 cm in diameter mice were treated with doses of appropriate chemotherapy (TAX for B16F10 and 4T1 tumors; DOX for MM tumors, CIS for lung carcinoma) that resulted in a substantial inhibition of tumor progression within 7-10 days after administration (data not shown). In all tumor models significant (p<0.01) up-regulation of MPR was detected 48 hr after the injection of chemotherapeutic drugs. It remained elevated for another 24 hr and then returned to the pre-treatment level within 5 days after injection (Fig. 1B,C and Fig. S1).

We then asked whether the kinetics of MPR upregulation was relevant for the antitumor effects of combination therapy. Mice with established B6F10 melanoma were treated with TAX alone, adoptive transfer of in vitro activated Pmel-1 CTLs that recognize gp100-derived epitope on B16F10 cells, or with the combination of these two therapies. Administration of either T cells or TAX alone substantially delayed tumor growth, which however, resumed one week after the treatment (Fig. 1D). When TAX was combined with T-cell therapy, a significant (p<0.05)
potentiating effect was observed. However, this effect was seen only if TAX was administered two days after T cells. When TAX was injected 5 days prior to T-cell administration, no increased antitumor effect was observed (Fig. 1D). Thus chemotherapy induced transient up-regulation of MPR on tumor cells, which was directly associated with antitumor effect of combined chemo-immunotherapy.

**The role of MPR in the antitumor effect of combination chemo-immuno therapy**

We generated a B16F10 tumor cell line with stable expression of MPR shRNA. In contrast to cells transfected with control shRNA, cells transfected with MPR shRNA did not up-regulate MPR in response to TAX treatment in vitro (Fig. 2A) or in vivo (Fig. 2B). TAX did not increase granzyme B (GrzB) penetration into cells expressing MPR shRNA (Fig. 2C). Treatment of mice bearing B16F10 tumors expressing control shRNA with the combination of TAX and Pmel-1 CTL demonstrated antitumor activity that was significantly (p<0.05) higher than each therapy alone. This effect was not seen in mice bearing tumors expressing MPR shRNA (Fig. 2D).

We tested the direct role of MPR in chemotherapy-inducible penetration of recombinant GrzB to the tumor cells in vitro. As expected, treatment of control shRNA B16F10 with GrzB during 6 hr did not result in induction of apoptosis. However, pretreatment of these cells with TAX made them sensitive to the subsequent treatment with GrzB. This effect was completely abrogated in MPR shRNA B16F10 cells (Fig. 2E).

To test this hypothesis in vivo we used wild-type (H2Kb) B16F10 cells expressing luciferase (B16-Luc) and B16F10 H2Kb− cells. B16-H2Kb− cells were transfected with control or MPR shRNA. As effector cells we used CTLs that recognize a Trp2180-188-derived peptide epitope expressed on B16F10 cells. Since this epitope is recognized in the context of H2Kb, these CTLs do not kill B16-H2Kb− cells (data not shown). In this experimental system we measured the
tumor size of both B16-Luc and B16-H2Kb⁻ tumors with calipers and independently monitored the growth of B16-Luc tumor using in vivo imaging.

First, we used control shRNA B16-H2Kb⁻ cells. B16-Luc and B16-H2Kb⁻ cells demonstrated similar growth rates after s.c. injection into C57BL/6 mice (data not shown). To test the effect of therapy, B16-Luc and B16-H2Kb⁻ tumors were established in the opposite flanks of the same mouse. When tumors became palpable, mice were split into four groups and treated with either TAX or CTLs alone, no treatment or with the combination. TAX, at the selected dose, had a modest antitumor effect against both tumors, whereas Trp2 CTLs had antitumor effects only against wild-type B16-Luc cells (Fig. 3A) but not against B16-H2Kb⁻ cells (Fig. 3B). Significant (p<0.05) potentiation of the antitumor effects of combined TAX and CTLs was observed only in the flank with B16-Luc tumor (Fig. 3A) but not with B16-H2Kb⁻ tumors (Fig. 3B).

In the other sets of experiments B16-Luc and B16-H2Kb⁻ cells were mixed together at a 1:1 ratio and injected s.c. into mice. Mice with established tumors were then treated with TAX and Trp2 CTLs either separately or in combination. Combining TAX and CTLs resulted in a significantly (p<0.05) greater antitumor effect than each of them separately. This was seen by monitoring tumor size with calipers (Fig. 3C) and by in vivo imaging (Fig. 3D and Fig. S2). Similar experiments were performed by mixing B16-Luc and B16-H2Kb⁻ cells transfected with MPR shRNA. In contrast to described above results, potentiation of the antitumor effect of combination therapy was not detected when B16-Luc cells were mixed with B16-H2Kb⁻cells transfected with control shRNA (Fig. 3E). This effect was most likely due to the outgrowth of B16-H2Kb⁻ cells since combination of TAX and CTLs had potent antitumor activity against B16-Luc cells when these cells were monitored by in vivo imaging (Fig. 3F and Fig. S2). These results indicate that chemotherapy and immunotherapy combined in vivo resulted in bystander
killing of tumor cells without the need for antigen recognition and that this effect was mediated by MPR.

**Mechanism of MPR up-regulation by chemotherapy**

We then asked how chemotherapy could regulate MPR on tumor cells. We tested the possible regulation of MPR synthesis or degradation by chemotherapy agents. We did not observe the effect of TAX, CIS, or DOX on total amount of MPR protein by using Western blot (**Fig. 4A**) or flow cytometry after fixation and permeabilization of the cells (**Fig. S3**). No effect of chemotherapy drugs on *Mpr* expression was found (**Fig. 4B**).

We asked whether MPR accumulation is limited to the cell membrane. In three different tumor cell lines treated with TAX or DOX, chemotherapy induced substantial accumulation of MPR only in a membrane fraction (**Fig. 4C**). To verify these findings we evaluated MPR expression in human MM U266 cells treated with DOX using confocal microscopy. Chemotherapy caused redistribution of MPR from a primarily cytoplasmic to a predominantly membrane localization of MPR (**Fig. 4D**). Similar results were obtained with mouse B16F10 cells treated with TAX (**Fig. S4**).

To determine whether similar effects could be observed in cancer patients, we used archived BM samples obtained from patients with MM treated on phase I trial of high-dose melphalan and topotecan followed by peripheral blood stem cell rescue. BM cells were collected from these patients before and after 3 days of high-dose chemotherapy. The type of immunoglobulin secreted by MM for each patient has been determined; therefore we could use appropriate kappa or lambda chain-specific antibodies to detect MM cells. In addition, cells were stained with MPR-specific antibody. We assessed the proportion of MM cells with predominantly membrane localization of MPR (**Fig. 5A**). Prior to treatment only 20% of the MM cells had such a
characteristic, whereas after 3 days of high-dose chemotherapy this proportion increased to more than 50% of all MM cells ($p=0.02$) (Fig. 5B).

**Autophagy as a mechanism of MPR accumulation on the cell membrane**

How can chemotherapy induce accumulation of MPR on the cell surface? Autophagy is a common effect of chemotherapy on tumor or endothelial cells (28-31). It is a relatively rapid process and often associated with tumor cell survival from chemotherapy. The mechanism of accumulation of autophagosomes in tumor cells depends on type of chemotherapy used. We tested the possible role of autophagy in MPR up-regulation. As expected, treatment of tumor cells with TAX, CIS, or DOX caused rapid induction of autophagy as determined by the appearance of autophagy-specific LC3 punctae in treated cells (Fig. 6A). For pharmacological inhibition of autophagy we used 3-methyladenin (3MA), established specific inhibitor of autophagy. 3MA abrogated upregulation of MPR on the surface of U266 MM cells treated with DOX (Fig. 6B) or B16F10 cells treated with TAX (Fig. 6C). 3MA also blocked the DOX-inducible redistribution of MPR to the cell membrane (Fig. 6D). The cytotoxic activity of Pmel-1 CTLs against B16F10 target cells treated with or without chemotherapy was tested in a standard chromium release assay. Pretreatment of tumor cells with TAX dramatically increased Pmel-1 mediated killing of target cells. In contrast, this effect was completely abrogated in the presence of 3MA (Fig. 6E).

To further evaluate the role of autophagy in MPR upregulation, we down-regulated expression of *atg5*, the gene critically important for induction of autophagosomes, using siRNA with two different siRNA (data not shown). Transfection of B16F10 cells with *atg5*siRNA completely abrogated the TAX-inducible increase in MPR presence on the cell surface (Fig. 7A). Transfection of *atg5*siRNA to U266 MM cells abrogated DOX inducible up-regulation of MPR.
(Fig. 7B). It also completely abrogated GrzB penetration to these cells (Fig. 7C). To verify these findings independently we established melanoma B16F10 cell line with stable expression of atg5 shRNA. These cells have substantially lower level of Atg5 than B16F10 cells transfected with control shRNA (Fig. 7D). Down-regulation of Atg5 abrogated TAX-inducible up-regulation of MPR on the surface of the tumor cells (Fig. 7E) and increased killing of tumor cells by specific CTLs (Fig. 7F).

To assess a direct role of autophagy on regulation of MPR expression on the cell surface we used rapamycin, a known inducer of autophagy (32). Autophagy (determined by LC3 punctae) was detected in U266 MM (Fig. 7G) and B16F10 (Fig. 7H) cells treated with rapamycin at concentration of 100 nM. At this and higher doses, rapamycin caused a substantial increase in MPR levels on the tumor cell surface (Fig. 7 I,J).
Discussion

In this study we tried to determine why the combination of chemotherapy with adoptive T-cell transfer resulted in an enhanced antitumor effect. This study was based on previous serendipitous findings obtained in a number of clinical trials demonstrating an unusually high rate of objective clinical responses observed in patients treated with conventional chemotherapy after failing cancer vaccines (6). These effects were obtained with different chemotherapeutic drugs and different types of cancer vaccines suggesting that there was some common mechanism involved. Moreover, in all those cases, conventional doses of chemotherapy were used, which are known to inhibit immune responses. Therefore, although the ability of chemotherapy to induce immunogeneic tumor cell death has been described (33, 34), this mechanism was unlikely involved in this case. Recently, we demonstrated in experiments in vitro a possible role of MPR in this process. Whether this mechanism is indeed operational in vivo remained unclear. More importantly, the role of this process in mediating the antitumor effect of combination therapy as well as the mechanism of MPR up-regulation on tumor cells was unknown. To address these questions we used different tumor models and chemotherapeutic drugs with different mechanism of action. The doses of the drugs were selected to impact on tumor progression. Most of the experiments with combined treatment were performed with TAX. Previous studies have shown that at selected doses, repeated injections of TAX are immune suppressive (35).

As immune therapy we used the adoptive transfer of antigen-specific T cells, a promising new method of treatment that has demonstrated antitumor effects in mice and patients (36-38). We found that chemotherapy caused dramatic, albeit only transient up-regulation of MPR on tumor cells in vivo. This effect was seen in every tumor model tested and with all drugs used. Importantly, the effect of combined treatment was seen only when chemotherapy was given
within a window of time during which increased level of MPR was observed on tumor cells. After the MPR level was normalized, addition of adoptive T-cell transfer did not induce any further therapeutic benefit. This result suggested that the effect of combination therapy depended on up-regulation of MPR. To test this hypothesis directly we inhibited MPR expression on tumor cells using shRNA and found that the antitumor effect of combination chemo-immunotherapy was abrogated in mice bearing MPR deficient tumor cells.

We suggested that if MPR did indeed mediate the antitumor effect of CTLs in combination with chemotherapy, then tumor cells not expressing specific antigens, and thus not recognized by CTLs would not be sensitive to the effect of combination therapy. Our data have shown that no antitumor effect of T-cell transfer was seen in mice bearing tumors lacking one of MHC class I allele (H2Kb), which binds the specific peptide (Trp2180-188) that is recognized by CTLs. Not surprisingly, in that tumor, combination therapy did not provide any additional benefit over the effect of chemotherapy alone. However, when wild type and H2Kb− tumor cells were mixed together the effect of combined chemo-immunotherapy was prominent. Apparently, activated CTLs were able to kill not only wild-type tumor cells that expressed specific antigen but also those that could not be recognized by CTLs (H2Kb− cells). This effect was abrogated when MPR deficient H2Kb− tumor cells were mixed with wild-type tumor cells. These data strongly support the concept that in combination with chemotherapy, activated CTLs can release GrzB, which is able to bind MPR on neighboring tumor cells regardless of whether those cells express MHC class I or not.

The other main question we addressed in this study was how chemotherapy can up-regulate MPR expression on the surface of tumor cells. Our data indicate that chemotherapy did not induce MPR synthesis and did not inhibit its degradation. Instead, we observed redistribution of MPR
within the cells. Normally more than 90% of total MPR is localized inside the cells in different compartments (endosomes and TGN) (39). After chemotherapy, large amount of MPR was localized on the cell membrane. This might account for stronger IHC staining of tumor tissues after treatment with chemotherapy (MPR localized on cell surface is more accessible for antibodies and thus is better stained than MPR localized in intracellular compartments) and could explain the increased uptake of GrzB by tumor cells after interaction with CTLs observed in our previous study (18).

What could cause redistribution of the MPR in the cell? In recent years, increasing evidence has accumulated regarding the importance of autophagy in cancer treatment (40-42). Autophagy is a reversible process that can contribute both to tumor cell death and survival. This catabolic pathway is initiated by the formation of a phagophore around cytoplasmic organelles and/or some portion of the cytosol. The enclosed material is sequestered in a vacuole lined by two membranes called the autophagosome. Autophagosomes then undergo fusion with either endosomes or lysosomes. The mechanism of autophagosome formation depends on the type of chemotherapeutic drug. In our study, TAX, DOX and CIS caused rapid induction of autophagy in tumor cells, which was associated with up-regulation of MPR. Blockade of autophagy with either its inhibitor 3MA or by down-regulating \textit{atg5} resulted in abrogation of chemotherapy induced up-regulation of MPR on the tumor cell surface. How exactly this may happen is not entirely clear. We propose that MPR is re-directed to autophagosomes either as part of clathrin coated vesicles, or as part of fusion of autophagosomes with endosomes. In both cases, low pH in autophagosomes results in release of the MPR cargo followed by shuttle of the receptor to the surface. The detailed mechanism of this effect needs to be clarified.
Recently Michaud et al demonstrated that autophagy was dispensable for chemotherapy-induced cell death but required for its immunogenicity. In response to chemotherapy, autophagy-competent, but not autophagy-deficient cancers attracted dendritic cells and T lymphocytes into the tumor bed. This effect was mediated via ATP (43). The issue of the relationship between autophagy and tumor immunity requires further elucidation since there is evidence that autophagy can limit immune-mediated cytotoxicity (44) and that autophagy-deficient tumors may be rare (45).

Our study presents a novel concept relating to the interaction between CTLs and tumor cells undergoing autophagy that can be clinically exploited not only in the setting of chemotherapy but also radiation therapy, as well as other treatments that cause autophagy of tumor cells. Our data demonstrate that combining chemotherapy and immunotherapy as a front line therapy in patients with advanced cancer has a strong rationale. However, it needs to be carefully timed and may be monitored using MPR expression.

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References


Figure Legends

Figure 1. Chemotherapy causes transient up-regulation of MPR in tumors. A. Up-regulation of MPR in vitro on the surface of indicated tumor cells after overnight treatment with 12.5 nM TAX (B16F10 and 4T1) or 25 ng/ml DOX (8226, H929, U266). Typical examples of 3-5 performed experiments are shown. Staining with isotype IgG showed less than 101 fluorescence intensity (not shown). B. Effect of chemotherapy on MPR expression in tumors in vivo. 4T1 and B16F10 tumor-bearing mice were treated with 12.5 mg/kg TAX and U266 tumor-bearing SCID/NOD mice were treated with 25 mg/kg DOX. Tumors were excised at indicated times after chemotherapy and stained for MPR. Bar = 100 µm. C. Mean±SD of cumulative results are shown. Quantitation was performed using image algorithm as described in Methods. Each group included 3-4 mice. * - statistically significant differences from untreated mice (p<0.05). D. Effect of combination therapy depends on time of the treatment. B16F10 tumors were established in C57BL/6 mice. On day 11 mice received 5x10⁶ activated pmel T cells (T cells and T cells + TAX group) or TAX (12.5mg/kg) (TAX+T cells group). Mice from T cells +TAX groups received TAX on day 14 and mice from TAX+T cells group received T cells on day 16. Each group included 5 mice, mean±SD are shown.

Figure 2. MPR is responsible for the antitumor effect of combined chemo-immunotherapy. B16F10 cells were transfected with control shRNA or MPR shRNA. A. Effect of overnight treatment with 12.5 ng/ml TAX on expression of MPR on tumor cells surface; B. Effect of TAX (12.5 mg/kg) treatment of B16F10 tumor-bearing mice on expression of MPR in tumors; C. GrzB penetration into tumor cells after overnight treatment of B16F10 cells with TAX. D. Treatment of mice with adoptive T-cell transfer (T cells), TAX, or combination as described in Fig. 1F. Each group included 5 mice. Mean±SD are shown. E. Down-regulation of MPR
abrogated GrzB mediated killing of tumor cells pre-treated with TAX. Control shRNA and MPR shRNA B16F10 cells were treated with vehicle alone (Unt) or 12.5 ng/ml TAX overnight followed by 6 hr treatment with 40 nM recombinant mouse GrzB. The percentage of Annexin-V positive cells from 3 performed experiments is shown.

**Figure 3. MPR mediates a bystander effect of combination therapy.** A,B. B16-Luc (H2Kb⁺) tumor cells (A) were established in the left flank and B16-H2Kb⁻ in the right flank of the same mice (B). Mice were treated with Trp2-specific CD8⁺ T cells on days 10 and 16 followed by vaccinations with specific peptide on days 11 and 17. TAX group and combination therapy group (T cells + TAX) received TAX (12.5mg/kg) on days 13 and 22. Each group included 4-5 mice. Mean ± SD are shown. C-F. B16-luc tumor cells were mixed at 1:1 ratio (1.5x10⁵ cells each) with either H2Kb⁻ cells transfected with control shRNA (C,D) or H2Kb⁻ cells transfected with MPR shRNA (E,F) and injected s.c. Mice were treated as described above. Tumor size was monitored using calipers (C, E) and by *in vivo* imaging (D,F). Each group included 4-5 mice. Mean ± SD are shown.

**Figure 4. The mechanism of MPR up-regulation in tumor cells after chemotherapy.** A,B. 4T1 or B16F10 tumor cells were treated overnight with TAX and U266 with DOX. The amount of total protein was evaluated by Western blotting (A) and mpr mRNA by qRT-PCR (B). C. Membrane and cytoplasmic fractions of tumor cells treated with TAX or DOX were isolated and MPR was detected by Western blotting. D. U266 were treated overnight with DOX, fixed and stained with MPR specific antibody and analyzed by confocal microscopy. Bar = 25 µm.

**Figure 5. Effect of chemotherapy on MPR expression in patients with MM.** Paired samples of BM aspirates from patients with MM collected before and after 3 days of chemotherapy stained with the indicated kappa or lambda antibodies (depending on the types produced by MM
cells) and MPR antibody. **A.** Typical example of staining. Bar = 50 µm. **B.** The proportion of cells with membrane MPR staining. Cumulative results from 10 samples is shown.

**Figure 6. The link between autophagy and MPR expression in tumor cells.** **A.** Formation of LC3 punctae in tumor cells by overnight treatment with different drugs. Staining with LC3 specific antibody (red). Bar = 25 µm. **B-D** MPR up-regulation in B16F10 cell induced by TAX (B) or in U266 cells induced by DOX (C,D) was abrogated by inhibitor of autophagy 3MA. Staining of cells was evaluated by flow cytometry (B,C) and by confocal microscopy (D). **A-D** - Each experiment was performed at least three times. **E.** Autophagy inhibitor 3MA abrogated combined cytotoxic effect of TAX (12.5 ng/ml, overnight treatment) and CTLs (activated Pmel T cells) against B16F10 tumor cells. Standard $^{51}$Cr-release assay was performed in duplicates. Results of two experiments are shown (Mean ± SD).

**Figure 7. Autophagy causes up-regulation of MPR in tumor cells.** **A.** Down-regulation of \textit{atg}5 in B16F10 cells using siRNA abrogated TAX-inducible up-regulation of MPR. **B.** Down-regulation of \textit{atg}5 in U266 cells using siRNA abrogated DOX-inducible up-regulation of MPR. **C.** Down-regulation of \textit{atg}5 in U266 cells using siRNA abrogated DOX-inducible uptake of GrzB. **A-C** - Typical examples are shown. Each experiment was performed at least three times. **D.** Down-regulation of Atg5 in B16F10 cell line with stable transfection of \textit{atg}5 shRNA. **E.** Effect of TAX on up-regulation of MPR on B16F10 tumor cells transfected with control or \textit{atg}5 shRNA. **F.** Down-regulation of Atg5 in tumor cells abrogated TAX-inducible up-regulation of tumor-cell killing by CTLs in 4-hr chromium release assay. Target:effector ratio 1:12.5 is shown. Experiment was performed in triplicates. **G-J** - U266 (G,I) or B16F10 (H,J) cells were treated overnight with 100 nM rapamycin. Autophagy was evaluated by LC3 staining (bars = 25 µm)
(G,H) and MPR expression by flow cytometry (I,J). Two experiments with the same results were performed.
Figure 1
Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
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