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Induction of ATM/ATR pathway combined with V γ 2V δ 2 T cells enhance cytotoxicity of ovarian cancer cells

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Summary

Many ovarian cancer cells express stress-related molecule MICA/B on their surface that is recognized by V γ 2V δ 2 T cells through their NKG2D receptor, which is transmitted to downstream stress-signaling pathway. However, it is yet to be established how V γ 2V δ 2 T cells-mediated recognition of MICA/B signal is transmitted to downstream stress-related molecules. Identifying targeted molecules would be critical to develop a better therapy for ovarian cancer cells. It is well established that ATM/ATR signal transduction pathways, which is modulated by DNA damage, replication stress, and oxidative stress play central role in stress signaling pathway regulating cell cycle checkpoint and apoptosis. We investigated whether ATM/ATR and its down stream molecules affect V γ 2V δ 2 T cells-mediated cytotoxicity. Herein, we show that ATM/ATR pathway is modulated in ovarian cancer cells in presence of V γ 2V δ 2 T cells. Furthermore, downregulation of ATM pathway resulted downregulation of MICA, and reduced V γ 2V δ 2 T cells-mediated cytotoxicity. Alternately, stimulating ATM pathway enhanced expression of MICA, and sensitized

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Disclosure

Authors have no competing financial interests.

Author's contribution

Conceived and designed the experiments: JL, HD. Performed the experiments: JL, RA, SK, MD, MJ. Analyzed the data: RA, ML, AR, VP, CS, HD. Contributed reagents/materials/analysis tools: VP, CS. Wrote the paper: JL, AR, HD. All authors read and approved the final manuscript.

ovarian cancer cells for cytotoxic lysis by V γ 2V δ 2 T cells. We further show that combining currently approved chemotherapeutic drugs, which induced ATM signal transduction, along with V γ 2V δ 2 T cells enhanced cytotoxicity of resistant ovarian cancer cells. These findings indicate that ATM/ATR pathway plays an important role in tumor recognition, and drugs promoting ATM signaling pathway might be considered as a combination therapy together with V γ 2V δ 2 T cells for effectively treating resistant ovarian cancer cells.

Keywords

V γ 2V δ 2 T cells; ovarian cancer; cytotoxicity; ATM/ATR pathway; MICA; combination therapy

Introduction

Human innate immune system provides first line of defense against multiple viral or bacterial attacks, and provides critical surveillance against oncogenic development. Evidences show that individuals with primary immunodeficiency or induced immunosuppression during organ or cell transplantation showed higher risk for tumor development [1]. Alternately, impaired function of innate immune cells such as natural killer (NK) cells, subset of $\alpha\beta$ T cells, and $\gamma\delta$ T cells lead to increased susceptibility of the host for tumor growth [2, 3]. Among these innate immune cells, $\gamma\delta$ T cells (V γ 2V δ 2 subset) are of particular interest due to their dual role in the immune system for bridging the gap between the innate and adaptive immunity, and have been demonstrated critical anti-tumor activities [4–6]. Due to the robust metabolic activities in tumor cells, specific antigens such as MHC class-I chain-related molecules MICA/B were typically found to be highly expressed in most tumor cells compared to normal healthy cells [7]. These antigens were specifically recognized by the V γ 2V δ 2 T cells and were proposed to enhance the cytotoxic activity of V γ 2V δ 2 T cells towards variety of tumor cells [8–11]. The activating receptors, NKG2D, serves as one of the most important receptors present in V γ 2V δ 2 T cells and mediate the recognition process to eliminate the tumor cells. This NKG2D molecule not only interacts to the receptor MICA/B, but also interacts with molecules such as ULBPs [12].

Emerging T cell-based adaptive immunotherapy is under consideration for various cancers as a therapeutic regimen [13]. In the adoptive therapy, T cells are activated, expanded *ex vivo* and re injected into the patients with tumors [14, 15]. Adoptive T-cell therapy in renal cancer patients showed no adverse events, and 3 of 5 patients showed slower tumor progression. Patients documented positive response showed an increased number of V γ 2V δ 2 T cells in the peripheral blood and a strong *in vitro* response to phosphoantigen stimulation [14]. Various trials show promise for development of autologous V γ 2V δ 2 T cell therapies in eligible patients. However, for ovarian cancer, there is currently no effective immunotherapy. Interestingly, chemotherapeutic agents were shown to induce immunogenic tumor cell death, which is crucial for tumor eradication and long-term protection against relapse. Moreover, V γ 2V δ 2 T cells were recruited to the tumor bed after immunogenic chemotherapy and appear to be contributors to the efficacy of chemotherapy [16]. So, developing a combination therapy using chemotherapeutic reagent and V γ 2V δ 2 T cells will be a valuable option to be tested.

The V γ 2V δ 2 T cells induce cytotoxicity in many ovarian tumor cells via induction of apoptosis [17]. However, some of the ovarian tumor cells evade the apoptosis process and became resistant towards V γ 2V δ 2 T cells-mediated cytotoxicity. These resistant cell lines (such as A2780) showed slower proliferation compared to the sensitive cell line (such as OV4); interestingly, we found that the resistant cell line has reduced expression of MICA [17]. We proposed that the tumor cells may evade the V γ 2V δ 2 T cells cytotoxicity by down-regulating their MICA expression and at the same time enter into a dormancy stage, in which their proliferation were slowed down. In the current study, we further investigated the molecular mechanisms involved in the immune escape process. It has been shown that genotoxic stress or inhibitors of DNA-replication could up-regulate the expression of NKG2D ligand through activation of ATM (ataxia telangiectasia mutated) and ATR (ATM- and Rad3-related) protein kinase pathway in human fibroblast and in mouse tumor cell lines, which led to enhance cytotoxic lysis by NK cells [18, 19]. ATM and ATR are activated in response to DNA damage, oxidative stress, and replication stress resulting in apoptosis or cell cycle arrest. After activation ATM phosphorylates Chk2, and ATR phosphorylates Chk1 to start a cascade of downstream signaling events [20]. Activated Chk1 and Chk2 phosphorylate Cdc25 phosphatases, to inhibit their function, and the cells delay progression through the cell cycle [20]. After activation ATR and ATM also phosphorylates H2A variant H2AX at Ser-139 (γ H2AX) at the damage sites, or where chromosomes are fragmented by oxidative stress [21]. The γ H2AX has been used as a marker for DNA damage, oxidative stress, and replication stress. It was also shown that inhibition of ATM pathway by using synthetic inhibitor such as KU-55933 suppressed cell proliferation and induced apoptosis [22]. In this study, we examined whether the ATM and ATR protein kinases play a role in V γ 2V δ 2 T cells-mediated recognition of ovarian cancer cells. We found that treatment of ovarian cancer cells with V γ 2V δ 2 T cells results in down regulation of ATR and ATM signal transduction in resistant cells, but remain unchanged in sensitive cells. When we treated the cells with V γ 2V δ 2 T cells along with drugs activating ATM pathway, it resulted a significant increase in cytotoxicity of tumor cells. Thus, ATM-Chk2 signal transduction plays a critical role in regulating tumor survival in ovarian cancer upon V γ 2V δ 2 T cell treatment.

Materials and methods

Derivation of V γ 2V δ 2 T cells

Human peripheral blood was collected (30 ml) from adult healthy donors after obtaining the IRB approval from the Ohio State University Medical Center and obtaining written consents from donors. The ethic committee has also approved the procedure and records are saved in the laboratory. Freshly collected blood was processed to isolate peripheral blood mononuclear cells (PBMC) following the similar protocol published earlier [10, 11]. In brief, the peripheral blood was diluted twice with phosphate buffer saline (PBS, pH 7.4) and carefully layered over 10 ml of Ficoll-Paque Plus solution (GE Healthcare, Uppsala, Sweden). After 30 min of centrifugation in a swinging bucket rotor at 1400 rpm at room temp (24°C); the upper layer was aspirated out and the mononuclear cell layer (buffy coat) was collected. Buffy coat was washed three times with PBS to remove platelets. One million of PBMC in each well was stimulated with 10 μ M risedronate in a 24-well plate using 1 ml

RPMI 1640 supplemented with 10% fetal bovine serum (FBS, HyClone Lab Inc, Logan, UT), 2 mM glutamine, 1 nM β -mercapto ethanol, 1 nM HEPES, and 100 IU of penicillin and streptomycin at 37°C incubator. Recombinant IL-2, 0.5 nM (PeproTech Inc. Rocky Hill, NJ) was added to the culture on days 3 and 7. Cells were split after day 10 using the complete RPMI 1640 media supplemented with 0.5 nM rIL-2. Flowcytometric (FACS) analysis was performed (using a FACS Calibur machine, BD Biosciences, CA) at day 14, to evaluate phenotype of the expanded cell. FACS analysis data revealed that 99.8% of the expanded cells were CD3+, and 89.5% of them were V δ 2+. Cells were used between 15–19 days of initial culture for further experiments discussed below.

Ovarian cancer cells and chemicals

The ovarian cancer cells (A2780 and OV4; purchased from ATCC, VA, and used within six months of receipt) were cultured in 10 cm or 6 cm culture dishes in DMEM media supplemented with 10% FBS and antibiotics. Chemicals/drugs were used in this study includes H4073 (5 μ M, kind gift from Prof. Periannan Kuppusamy, Columbus, OH), Etoposide (500 μ M, MP Biomedical LLC, Santa Ana, CA), KU55933 (10 μ M, Selleck Chemicals, Houston, TX), Caffeine (5 mM, kind gift from Prof. Jeff Parvin, Columbus, OH), Neocarzinostatin, NCS (500 ng/ml, Cat# N9162, Sigma, St. Louis, MO, USA).

Protein analyses

Total protein analysis was performed using standard western blot (WB) technology. Half a million of tumor cells (A2780 or OV4) were pre-seeded in 6 cm Petri dish for 10 h before adding V γ 2V δ 2 T cells. Control plates were also plated at the same time to maintain equal cell numbers. We did not observe any significant change on cell numbers after 10 h of pre-seeding. Fresh medium (RPMI for A2780 and DMEM for OV4) containing 10% FBS were replaced to the culture plates. Two and half million V γ 2V δ 2 T cells were added to the tumor cells to make a ratio of C:T = 1:5 (where ever applicable). Protein was isolated after 4 and 24 h of addition of V γ 2V δ 2 T cells. Before isolation of total protein, V γ 2V δ 2 T cells were gently removed by washing with 1 \times PBS. Forty to sixty microgram of total protein from each sample was used for a single WB analysis. Various antibodies have been used for WB, such as ATM, phosphorylated (p) ATM, ATR, pATR, pCHK1, pCHK2, H2AX, γ H2AX to detect their level of expressions on tumor cells at various time points of co-culture with V γ 2V δ 2 T cells.

ATM silencing

ATM shRNA was obtained from Sigma Aldrich Inc (ATM shRNA plasmid from bacterial glycerol stock TRCN0000039949 (defined as #1) and TRCN0000039950 (defined as #2) and a irrelevant negative control shRNA plasmid). Transfection with various RNAs in A2780 cells were conducted using Lipofectamine™ 2000 transfection reagents (Invitrogen) according to the manufacturer's instruction and earliser published methods [23].

Cytotoxicity assays

The cytotoxicity assay was performed using the LIVE/DEAD® Cell-Mediated Cytotoxicity Kit (Invitrogen Inc) and repeated by 3–6 times for each condition. Protocol was followed as

suggested with little modification. Five hundred thousand of ovarian cancer cells (A2780) were pre-seeded on a 6 cm dish for 8 h. Cells were then stained with DiOC₁₈ (7.5 μM) for 3 h. After staining, DiOC₁₈ was washed by three times with 1xPBS, then, 1:10 ratio Vγ2Vδ2 T cells were added in combination of various treatment molecules including H4073, Etoposide, KU55933, Caffeine, and NCS. After 24 h, floating cells were collected and adhesion cells were collected by adding non-enzymatic cell dissociation buffer and mixed with corresponding floating cells. Collected cells were then stained with Propidium Iodide (PI 37.5 μM). After adding PI, cells were centrifuged for 5 min at 1500 r/min. Then cells were incubated at 37°C for 30 min. After staining with PI, the cells were evaluated immediately by flowcytometry for the cytotoxicity effect of Vγ2Vδ2 T cells. Upper-gated cells are PI stained dead cancer cells and lower gated cells are PI stain negative live cancer cells that are DiOC₁₈ positive.

MitoSox™Red staining

MitoSOX™ Red mitochondrial superoxide indicator was purchased from Invitrogen. The concentrations were maintained as 0.19 mg/ml to stain 500K cells. In brief, 500K A2780 tumor cells were pre-seeded in 6 cm petri-dish for 8 h in complete RPMI medium. After pre-seeding, medium was replaced with fresh complete RPMI. Tumor cells were co-cultured with Vγ2Vδ2 T cells at 1:10 ratio under different treatment conditions as indicated before including H4073, Etoposide, KU55933, Caffeine, and NCS. After 24 h cells were collected after washing with medium. Very few adherent cancer cells were washed away when we removed non-adherent Vγ2Vδ2 T cells from co-culture. After all cells were collected, staining reagent was added using concentration indicated before. After 10 min of staining, cells were washed one time with 1XPBS and processed immediately for flowcytometric analysis. Adherent cells were collected after adding non-enzymatic cell dissociation buffer (Sigma) and collected after spin down with PBS.

Flowcytometry

A2780 tumor cells were pre-seeded in 6 cm Petri dish for 8 h in complete RPMI medium. After pre-seeding, medium was replaced with fresh complete medium. Tumor cells were co-cultured with Vγ2Vδ2 T cells at 1:10 ratio under various treatment conditions as indicated before including H4073, Etoposide, KU55933, Caffeine, and NCS. After 24 h, all cells were collected after spinning down with existing medium. Adherent cells were collected after adding non-enzymatic cell dissociation buffer (Sigma) and collected after spin down with PBS. PE-conjugated MICA/B and PE-conjugated IgG antibodies were added to the different samples. After 45 min incubation in ice, cells were washed once with PBS and processed for flowcytometry.

Immunofluorescence staining

Immunofluorescence staining was performed to analyze protein expressions after the treatment with various relevant chemicals and drugs in presence or absence of Vγ2Vδ2 T cells. In brief, half a million of A2780 cancer cells (C) were pre-seeded in 6 cm Petri dish 10 h before adding Vγ2Vδ2 T (T) cells. Fresh RPMI medium containing 10% FBS were replaced to the culture plates. Vγ2Vδ2 T cells were added to the tumor cells to make a ratio of C:T = 1:10. Optimum concentration of chemical/drugs was added such as H4073,

Etoposide, KU55933, Caffeine or NCS before adding V γ 2V δ 2 T cells. Cells were stained after 24 h of co-culture. Briefly, cells were washed with 1XPBS for three times gently. Cells were fixed with 4% paraformaldehyde for 30 min and permeabilized with 0.1% triton-X-100 for 30 min. After blocking, cells were stained by various antibodies such as ATM, pATM, ATR, pATR, CHK1, pCHK1, CHK2, pCHK2, H2AX or γ H2AX to detect their level of expression on tumor cells.

Results

Differential expression of ATM/ATR signaling pathway molecules in ovarian cancer cells upon V γ 2V δ 2 T cells induction

Many epithelial cancer cells express stress-related molecules such as MICA/B on their surface and V γ 2V δ 2 T cells recognize those molecules through NKG2D receptor molecules expressed on them. However, it is yet to be investigated how V γ 2V δ 2 T cells-mediated recognition of MICA/B signal is transmitted to downstream stress-related molecules, and the molecular mechanism of this signal transduction pathway. Exploring this pathway would be critical to develop better therapies for ovarian cancer cells. Upon DNA damage, replication stress, and oxidative stress, ATM/ATR signal transduction pathway plays a central role in regulating cell cycle checkpoint and apoptosis [20]. To identify the stress-related downstream signaling pathways in response to V γ 2V δ 2 T cells, we have used OV4 (sensitive), and A2780 (resistant) ovarian cancer cell lines [17]. Downstream factors of ATM/ATR pathways and their activation by phosphorylation were analyzed using Western blot (Fig. 1). Western blot analysis revealed that there was no significant change in the total protein levels of ATM and ATR at both 4 h and 24 h time points, however pATM and pATR levels were enhanced at 24 h when co-cultured with V γ 2V δ 2 T cells in OV4 cells (Fig. 1, left panel). In contrast, H2AX is significantly phosphorylated in both 4 h and 24 h when co-cultured with V γ 2V δ 2 T cells, suggesting a general stress- and DNA damage-mediated phosphorylation of H2AX. Besides ATR and ATM, H2AX is also phosphorylated by DNA-pK. Therefore, there might be some contribution of DNA-pK in H2AX phosphorylation upon V γ 2V δ 2 T cell induction. A2780 resistant cells did not show any significant difference in total ATM and ATR levels when co-cultured with V γ 2V δ 2 T cells. However, the levels of pATM and pATR were downregulated when compared to total ATM and ATR, upon co-culture with V γ 2V δ 2 T cells, in both 4 h and 24 h time points (Fig. 1, right panel). Accordingly, ATM substrate Chk2 phosphorylation (pChk2) and ATR substrate Chk1 (pChk1) were also downregulated at 4 h and 24 h upon V γ 2V δ 2 T cell induction. This data showed that ATM-Chk2 and ATR-Chk1 signaling pathways are downregulated upon V γ 2V δ 2 T cells induction. We noticed that γ H2AX is substantially downregulated at 24 h, but this is not evident at 4 h, further indicating that H2AX phosphorylation is influenced by different kinases besides ATR and ATM. Based on these preliminary observations, we hypothesize that downregulation of ATR-Chk1 and ATM-Chk2 pathways might play a critical role in promoting the resistance of A2780 towards V γ 2V δ 2 T cells-mediated cytotoxicity.

Induction of ATM enhances V γ 2V δ 2 T cells-mediated cytotoxicity and specific inhibitors of ATM reduce cytotoxicity

It has been shown that genotoxic stress or inhibitors of DNA-replication could up-regulate the expression of NKG2D ligand through activation of ATM and ATR protein kinase pathways in human fibroblast and in mouse tumor cell lines, which led enhanced cytotoxic lysis by NK cells [18, 19]. The reduction of ATM activation upon V γ 2V δ 2 T cells induction in resistant ovarian cancer cells (A2780), suggest that somehow regulatory pathways involved in ATM activation are affected in this resistant cell line. Considering this possibility, we hypothesize that upregulation or induction of ATM would enhance V γ 2V δ 2 T cells-mediated cytotoxicity of these resistant cells. We next tested this hypothesis by doing following experiments. We selected three different chemicals or drugs (NCS, Etoposide, and H4073), known to influence ATM signaling pathway, and combined them with V γ 2V δ 2 T cells to determine the cytotoxicity in combination treatment. NCS is a macromolecular chromoprotein enediyne antibiotic, which leads to double-strand DNA cleavage [24]. Etoposide results in immediate ATM activation by inhibiting topoisomerase and thus DNA synthesis [25]. Studies implicated that etoposide could be used as a second-line of therapy both for platinum-resistant and platinum-sensitive ovarian cancer patients [26–28]. The H4073, a curcumin analog and food additive has demonstrated anti-tumor activity, but does not induce ATM [29]. We have optimized both the dose of molecules and V γ 2V δ 2 T cells concentration to demonstrate the specific effects on ovarian cancer cells. By using live/dead flowcytometric analysis study, we show that all three molecules have cytotoxic effect to the tumor cells and that the effect could be significantly enhanced by using application of V γ 2V δ 2 T cells (Fig. 2). The values (\pm SEM) for the level of cytotoxicity are as follows: A2780 = 3.45 \pm 0.74; A2780+T = 11.75 \pm 3.07; NCS = 9.63 \pm 0.74; NCS+T = 24.60 \pm 4.29; Etoposide = 7.50 \pm 0.18; Etoposide+T = 39.77 \pm 1.45; H4073 = 25.69 \pm 3.78; H4073+T = 36.72 \pm 8.12; Caffeine = 4.80 \pm 0.47; Caffeine+T = 7.64 \pm 0.34; KU3393 = 5.17 \pm 0.44; KU3393+T = 6.04 \pm 0.56. In the current experimental setting, the optimum dose of NCS and Etoposide were not able to induce remarkable lysis of tumor cells; however, when V γ 2V δ 2 T cells were added, the cytotoxicity effect was significantly boosted indicating synergistic effect of NCS and Etoposide with V γ 2V δ 2 T cells treatment. In contrast, H4073 was shown to be able to induce strong apoptosis to the A2780 cells; however, in combination with V γ 2V δ 2 T cells, no synergistic effect of apoptosis was observed. Our data support a possibility of the involvement of the ATM pathway in enhanced cytotoxicity of resistant ovarian cells. To further assess this possibility, we used two ATM inhibitors such as Caffeine (general inhibitor) and KU55933 (specific inhibitor) to abrogate or reduce ATM signaling in these ovarian cancer cells. Tumor cells were subjected to various treatments to optimize the synergistic effect of ATM on V γ 2V δ 2 T cell-mediated cytotoxicity in a live/dead flowcytometric assays (Fig. 3). Both caffeine and KU55933 were able to abolish the cytotoxic effect of V γ 2V δ 2 T cells. There is no significant difference in the inhibitory effect between caffeine and KU55933, indicating a possible high dependency of V γ 2V δ 2 T cells on the ATM pathway. In contrast, when ATM stimulator NCS was added, the cytotoxic effect of V γ 2V δ 2 T cells was boosted by two-fold. Interestingly, Caffeine or KU55933 (data not shown) alone was able to reduce the boost, indicating that ATM indeed play an important role in regulating the apoptotic response induced by V γ 2V δ 2 T cells in A2780 cells. However, there was no significant inhibitory effect of V γ 2V δ 2 T

cells-mediated cytotoxicity on tumor cells from caffeine in the presence of H4073, which does not induce ATM, confirming the caffeine and KU55933 effect was ATM specific.

Oxidative stress and ROS production in mitochondria of cancer cells upon induction of V γ 2V δ 2 T cells

It has been shown that ATM signaling pathway is activated upon certain levels of reactive oxygen species (ROS) induction [30]. Therefore, we next investigated whether ROS was the major contributor in the tumor cell lysis. ROS production was evaluated in tumor cells using MitoRed staining technique and flowcytometric evaluation in various treatment conditions. ATM inducers or ATM inhibitors alone could generate ROS production in the mitochondria of tumor cells. Even though V γ 2V δ 2 T cells were able to increase the ROS production compared with control tumor cells, addition of NCS or Etoposide didn't show any significant enhancement of ROS production (data not shown). Treating the cells with caffeine or KU55933 has showed slight decrease in ROS production compared to controls. However, H4073 significantly increased the ROS production in the presence of V γ 2V δ 2 T cells indicating that ROS production might play an important role in H4073 induced apoptosis (data not shown). However, we predict that in our experimental condition, overall ROS production doesn't seem to play an important role in the synergistic effect of V γ 2V δ 2 T cells and ATM inducers-induced cytolysis.

Downstream signaling molecules of ATM/ATR pathway involved in lysis of tumor cells in combination therapy

Immunocytochemical analysis was performed to better understand the modulation of the stress-related signaling molecules in tumor cells in the presence of ATM stimulatory chemicals (NCS and Etoposide) and V γ 2V δ 2 T cells after 24 h of co-culture (Fig. 4). As expected, NCS and Etoposide increased the expression of pATM and pATR, which leads to increased susceptibility towards tumor cells (Fig. 4AB). Additionally, the pChk2 and γ H2AX levels also showed increase in their levels upon combination treatment (Fig. 4DE). No significant change was observed on pCHK1 level, suggesting that ATR-Chk1 pathway might not be the predominant pathway activated when treated with Etoposide and NCS (Fig. 4C). We further confirmed our results by Western blot analysis. Our results also show that NCS, and Etoposide induced activation (phosphorylation) of ATM/ATR and their substrate Chk2/Chk1 respectively (Fig. 4F). Conversely, ATM inhibitors (caffeine, and KU55933) reduce expressions of the ATM/ATR and their substrate Chk2/Chk1 molecules (Fig. 4F).

ATM stimulator induces surface expression of MICA

To understand the contributory role of ATM signaling pathway, and its cross-talk with already known molecules activated by V γ 2V δ 2 T cells, next we examined the levels of MICA expression on ovarian cancer cells. We investigated whether the synergistic cytotoxicity by ATM inducer and V γ 2V δ 2 T cell treatment, is mediated by modulation of MICA expression. Therefore, we tested whether ATM inducer has any effect on MICA expression on the tumor cells. Flowcytometric analysis revealed that NCS and Etoposide, inducers of ATM, also enhanced surface expression of MICA in A2780 cells. As such these cells reduced surface expression of MICA in the presence of V γ 2V δ 2 T cells and become resistant to the cytotoxic lysis [17]. However, in the presence of ATM inducers these cells

could not reduce surface expression of MICA when V γ 2V δ 2 T cells were added; instead they increased the MICA expression (Fig. 5A). These data showed that ATM inducers directly promote MICA expression, which then enhance the cytotoxic effect in resistant tumor cells when ATM inducers and V γ 2V δ 2 T cells were combined. If this is true, then the ATM inhibitors should show an effect on MICA expression. In the absence of V γ 2V δ 2 T cells, inhibiting ATM by caffeine or KU55933 leads to down-regulation in surface expression of MICA on A2780 cells (Fig. 5B). However, stimulating ATM using NCS lead to an up-regulation of surface expression of MICA. These results indicated that activated ATM in A2780 cells leads to positive expression of MICA, which was able to boost the recognition of tumor cells by V γ 2V δ 2 T cells. This is consistent with finding in our previous published paper, where we showed that the MICA down-regulation played a critical role in A2780 cell's resistance towards V γ 2V δ 2 T cells cytotoxicity compared to OV4 cell line [17]. Molecules such as Etoposide were also able to promote MICA expression, which might follow similar mechanisms to NCS. When co-cultured with V γ 2V δ 2 T cells similar pattern was also observed in NCS and Etoposide (Fig. 5A). H4073 was able to slightly increase the expression of MICA compared to no drug treatment in the context of V γ 2V δ 2 T cells; even though the effect is much smaller compared to NCS and Etoposide, which may explain the little synergistic effect observed in H4073+ V γ 2V δ 2 T cells. This result emphasize that MICA regulation by ATM signaling pathway may play a critical role in the resistance of A2780 towards V γ 2V δ 2 T cells treatment, and stimulating ATM might be important in sensitizing A2780 cell line by up-regulating MICA expression.

To further confirm the direct involvement of ATM pathway in regulation of MICA molecule in ovarian cancer cells, we have knocked down ATM in A2780 cells using shRNA-mediated knock down approach. ATM knock down was evaluated by WB methods and found that one of the ATM shRNA (#2) is more effective in knocking down of ATM than the other (#1) (Fig. 5C). To investigate the effect of ATM knockdown on surface expression of MICA on A2780 cells, flowcytometric analysis was performed for MICA. We found that knock down of ATM by using ATM shRNA (#2) in A2780 cells also downregulated MICA expression in ovarian cancer cells (Fig. 5D).

Discussion

The cytotoxic T cells, particularly V γ 2V δ 2 T cells, are important for the consideration of immunotherapy due to the fact that they: 1) recognize transformed cells independent of antigen processing or presentation by classical MHC molecules, and 2) possess the strong anti-tumor effector functions. It has been shown that aminobisphosphonates, a class of drugs used as adjuvant cancer therapy for the treatment of malignant osteolytic bone disease, have effects of potentially activating the anti-tumor effector functions of human peripheral V γ 2V δ 2 T cells.

Despite the relative complexity of immunotherapy, such treatment may have an important role, in conjunction with other therapies in the treatment of cancers refractory to conventional treatments alone. Clinical studies have recently shown that adding immunotherapy to chemotherapy has survival benefits in comparison to chemotherapy alone [31]. Moreover, chemotherapeutic agents can sensitize tumors to immune cell-mediated

killing [32–34]. For instance, some can increase sensitivity of tumor cells *via* up-regulation of death receptors DR5 and Fas, ligands to TRAIL and FasL respectively [6]. Similarly, V γ 2V δ 2 T cells could be selectively activated by naturally occurring phosphoantigens, which are accumulated in remarkably higher amount in stressed cells, or synthetic drugs offers new avenues for the development of effective T cell-based immunotherapies. Currently, several protocols based on the *in vivo* activation of V γ 2V δ 2 T cells with phosphoantigens or aminobisphosphonates, or the adoptive transfer of *in vitro* expanded V γ 2V δ 2 T cells are in development for the treatment of several tumors [6].

Several mechanisms were identified in tumor cells by which they defend against V γ 2V δ 2 T cells, including inhibiting recruitment of tumor infiltrating lymphocytes, secreting immunosuppressive molecules, promoting immunosuppressive activities such as blocking dendritic cells maturation and promote regulatory T cells [35]. All these studies do not address, the molecular signaling pathways activated in tumor cells upon V γ 2V δ 2 T cells induction. We anticipate that identifying V γ 2V δ 2 T cells-mediated signaling pathways, and establishing the molecular mechanism of their function would help develop a more effective V γ 2V δ 2 T cell-mediated therapy. The V γ 2V δ 2 T cells induce oxidative stress, which upregulates NKG2D ligand MICA [36–38]. Furthermore, MICA upregulation transmits its signal through the phosphoinositide 3-kinase related kinases (PI3K) pathway [39]. The PI3K/AKT/mTOR pathway can become abnormally activated in many human tumors and thereby contributes to cell growth, cell proliferation, and angiogenesis [40–42]. We previously shown that ovarian tumor cell lysis due to V γ 2V δ 2 T cell induction were mediated via AKT and ERK pathway [17]. Thus, our results showed an oxidative stress-mediated regulation of PI3K/AKT/mTOR pathway [43]. Importantly, it has been shown that ATM is activated by phosphorylation upon oxidative stress, and results in DNA damage response [21, 44, 45]. Oxidative stress results in DNA single- and double-strand breaks, DNA-DNA and DNA-protein cross links, and base modifications, resulting in activation of ATM kinase and its downstream signaling pathway. This damage response then activates DNA repair, cell cycle checkpoint, changes in gene expression profiles, and apoptosis [21, 46]. Even though a link between NKG2D ligand activation and ATM damage response pathway has been implicated [47], whether ATM signal transduction is directly modulated upon V γ 2V δ 2 T cell induction has not been established.

Our results indicated that in sensitive ovarian tumor cells, ATM and ATR phosphorylation enhanced (Fig. 1, left panel). Interestingly, in resistant cells ATM-Chk2 pathway is downregulated upon the recognition of V γ 2V δ 2 T cells (Fig. 1, right panel), which resulted downregulation of MICA and subsequent reduction of cytotoxicity and cell death. Our observation of downregulation of ATM and its substrates upon V γ 2V δ 2 T cells induction is striking and led us to do the experiments using ATM enhancer and inhibitors. We observed that ATM-inducing drugs were able to stimulate ATM level, which promotes cytotoxicity. On the other hand, when we inhibited ATM signaling pathway by using ATM-specific inhibitors, it reduced the cytotoxic lysis of resistant cells after V γ 2V δ 2 T cells induction. Thus, both ATM enhancers and inhibitors showed that ATM-Chk2 signaling pathway plays a critical role in enhancing the susceptibility of tumor cells against V γ 2V δ 2 T cells. We investigated the mechanism further, and revealed the cross-talk between the MICA and

ATM pathway by examining the changes in the MICA expression level upon using ATM enhancers and inhibitors. We revealed that MICA expression is upregulated when ATM signaling pathway is upregulated and MICA expression is downregulated when ATM signaling is downregulated. These data support a direct cross-talk between ATM pathway and MICA regulation. At this point, we do not know how ATM influences MICA regulation, but it shows that ATM signal transductions are directly linked to MICA regulation and cytotoxicity to V γ 2V δ 2 T cells (Figs. 2, 3 & 5). This direct link between ATM and MICA is further confirmed by using ATM knock down experiments (Figs. 5). It is highly likely that ATM upregulation might influence the apoptosis pathway, as it has been shown that ATM can promote apoptosis [48–50]. It will be highly interesting to elucidate further signaling mechanisms involved in the process of sensitization, which will be greatly helpful to develop improved combination therapies for ovarian cancer.

Our results clearly demonstrate that inhibition of ATM pathway activation results in resistance to V γ 2V δ 2 T cell-mediated cell death. Therefore, enhancing ATM activation along with V γ 2V δ 2 T cell treatment would promote the cytotoxicity of resistant ovarian cancer cells. To our knowledge, this is the first report of direct cross-talk of ATM signaling and V γ 2V δ 2 T cell treatment. The molecular mechanism of the inhibition of ATM pathway upon V γ 2V δ 2 T cell-mediated induction is not clear yet, but it is obvious that ATM activation is affected by inhibition of its phosphorylation. Taken together, we propose that ovarian cancer cells inhibit ATM signaling pathway upon V γ 2V δ 2 T cell treatment, which in turn inhibit MICA surface expression, and reduce the cytotoxicity, resulting in resistance (Fig. 6). This also supports the mechanism that V γ 2V δ 2 T cells induce cytotoxicity through ATM signaling pathway. Based on our results, for the first time we tested a new combination therapy where drugs like Etoposide or NCS could be considered as a combination therapeutic regimens in conjunction with V γ 2V δ 2 T cell-mediated immunotherapy for the treatment of ovarian cancers. Moreover, if CD8+ $\alpha\beta$ T cells and NK cells function similar to the V γ 2V δ 2 T cells, then this combination therapy would be used to enhance cytotoxicity for these treatments. As resistance to chemotherapy is challenging in ovarian cancer and many other cancer types, we believe that this new therapeutic approach would highly benefit not only ovarian cancers, but also other cancer types.

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Highlights

- V γ 2V δ 2 T cells modulate ATM/ATR pathway in ovarian cancer cells
- Downregulation of ATM/ATR correlates with downregulation of MICA
- Upregulation of ATM/ATR correlates with enhanced MICA expression
- ATM/ATR upregulation mediates enhanced cytotoxicity by V γ 2V δ 2 T cells
- ATM promoting drug combined with V γ 2V δ 2 T cells for effective ovarian cancer lysis

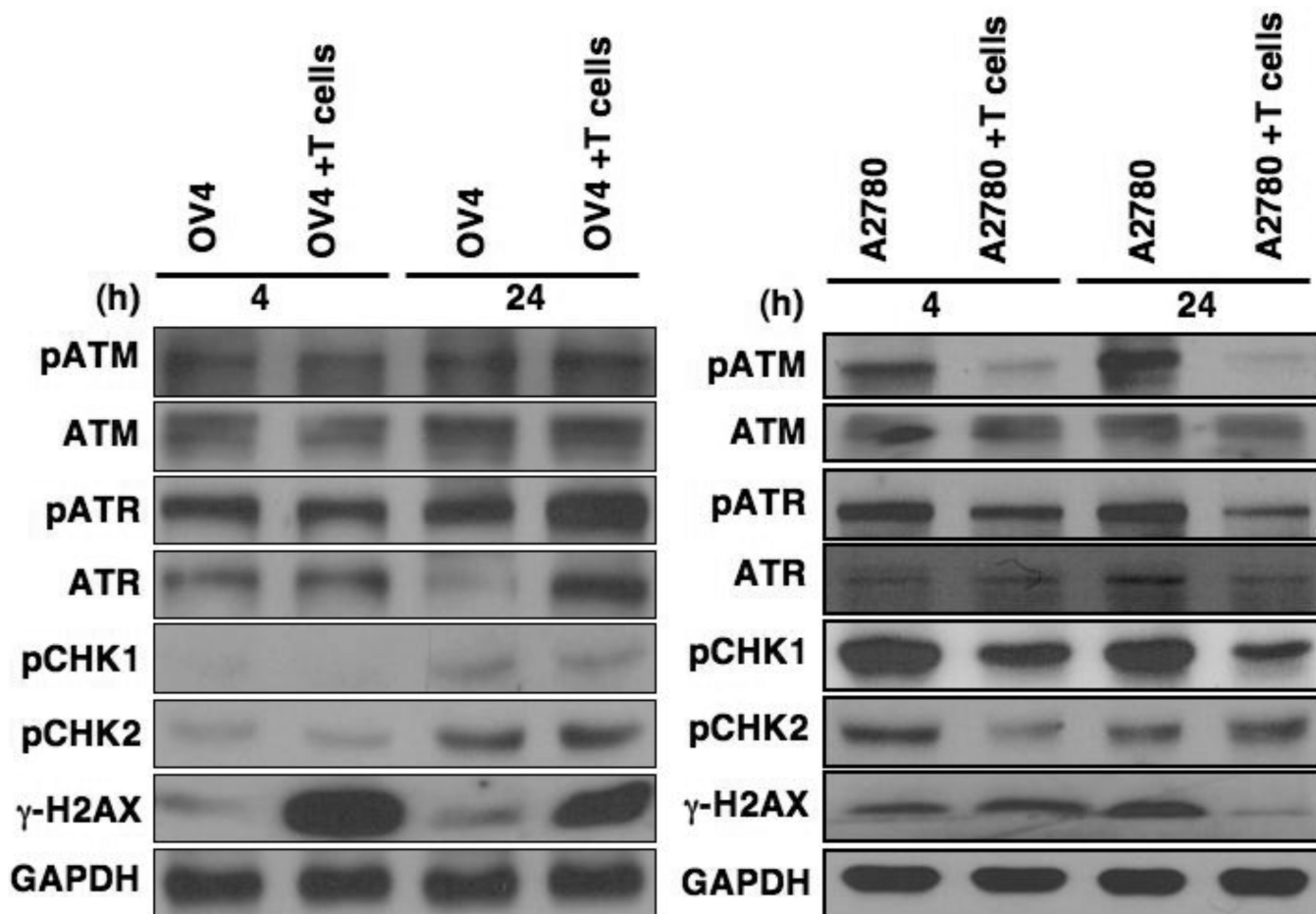


Figure 1. Levels of ATR and ATM signaling pathway molecules in ovarian cancer cells

A sensitive (OV4) and a resistant (A2780) ovarian cancer cells were co-cultured with ex-vivo-expanded V γ 2V δ 2 T cells at a ratio of Cancer: V γ 2V δ 2 T cells = 1:5, and the cancer cells were harvested at 4 h and 24 h time points to isolate total protein after gentle removal of V γ 2V δ 2 T cells. Total proteins were subjected to the Western blot analysis using relevant Abs as stated.

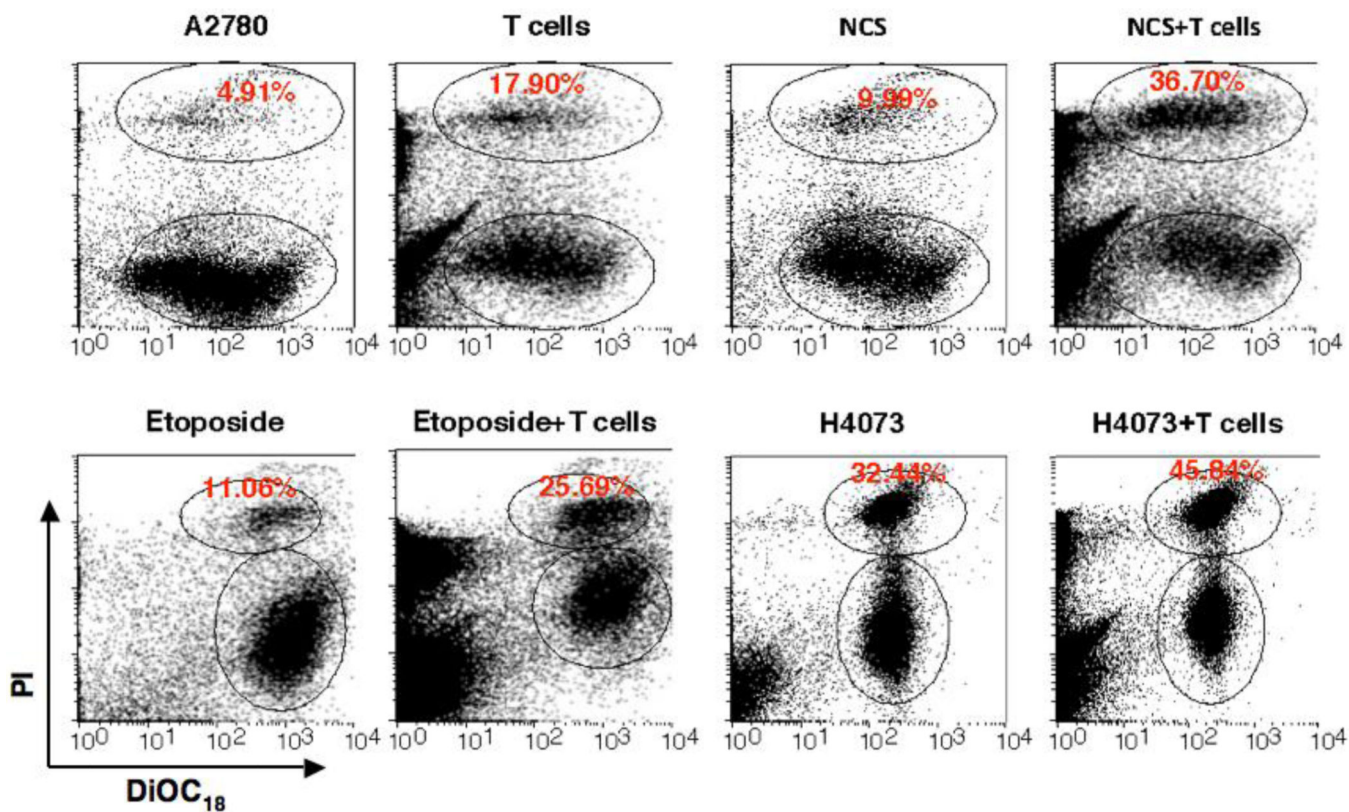


Figure 2. ATM-inducing molecules enhance V γ 2V δ 2 T cells-mediated cytotoxic lysis of ovarian cancer cells

Cytotoxicity of V γ 2V δ 2 T cells towards ovarian cancer cells was performed in presence of NCS, Etoposide, and H4073 molecules using a LIVE/DEAD cell-mediated viability/cytotoxicity kit and flowcytometric analysis following protocol mentioned in the Materials and Methods section. Upper-gated cells are PI stained dead cancer cells and lower gated cells are PI-stain negative live cancer cells that are DiOC₁₈ positive.

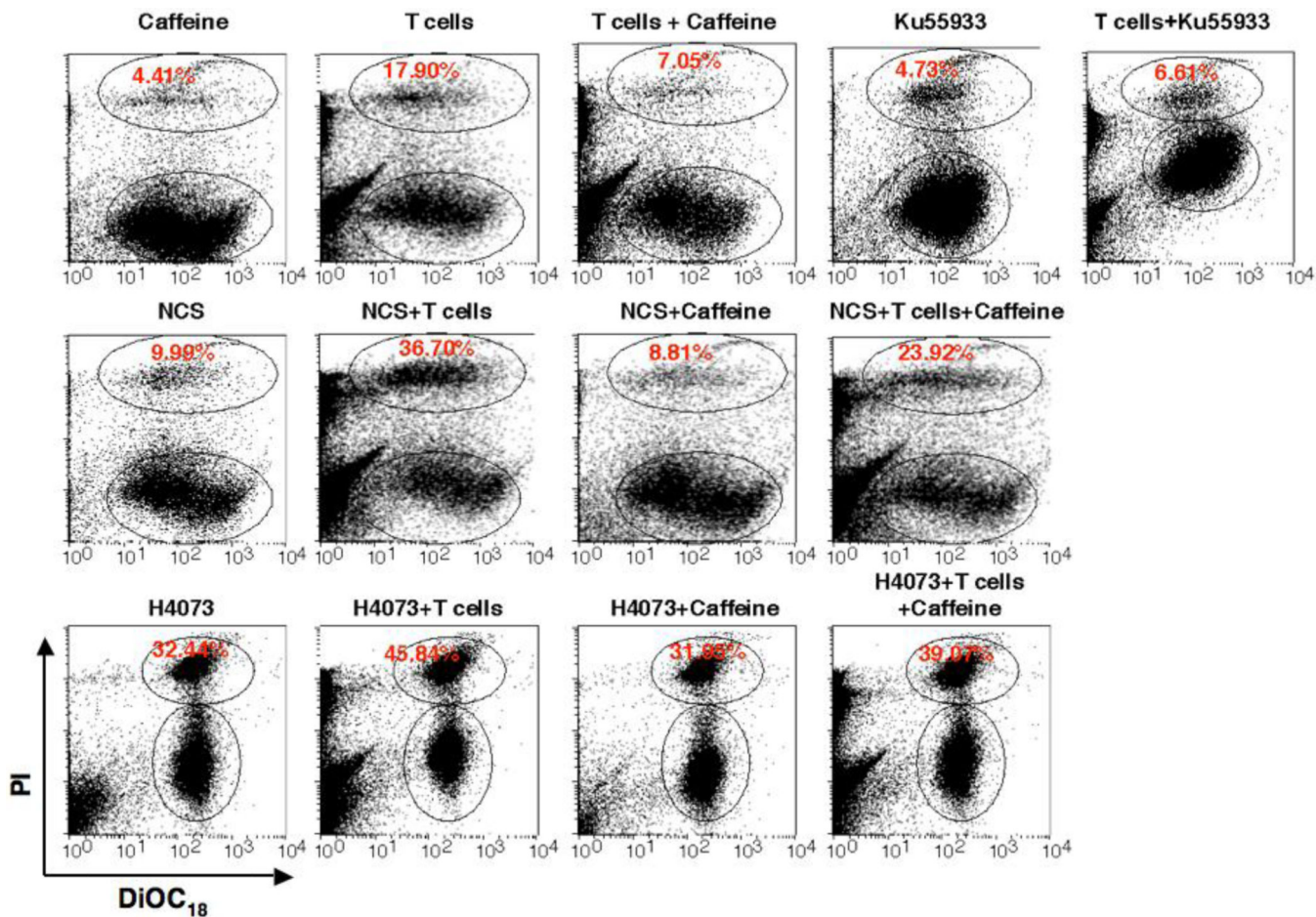


Figure 3. ATM inhibition reduces cytotoxic lysis of ovarian cancer cells by V γ 2V δ 2 T cells
 The V γ 2V δ 2 T cells-mediated cytotoxicity of ovarian cancer cells was evaluated in presence of NCS, Etoposide, and H4073 molecules along with ATM inhibitors. Flowcytometric analysis was performed to evaluate cytotoxicity using a LIVE/DEAD cell-mediated viability/cytotoxicity kit. Upper-gated cells are PI stained dead cancer cells and lower gated cells are PI-stain negative live cancer cells that are DiOC₁₈ positive.

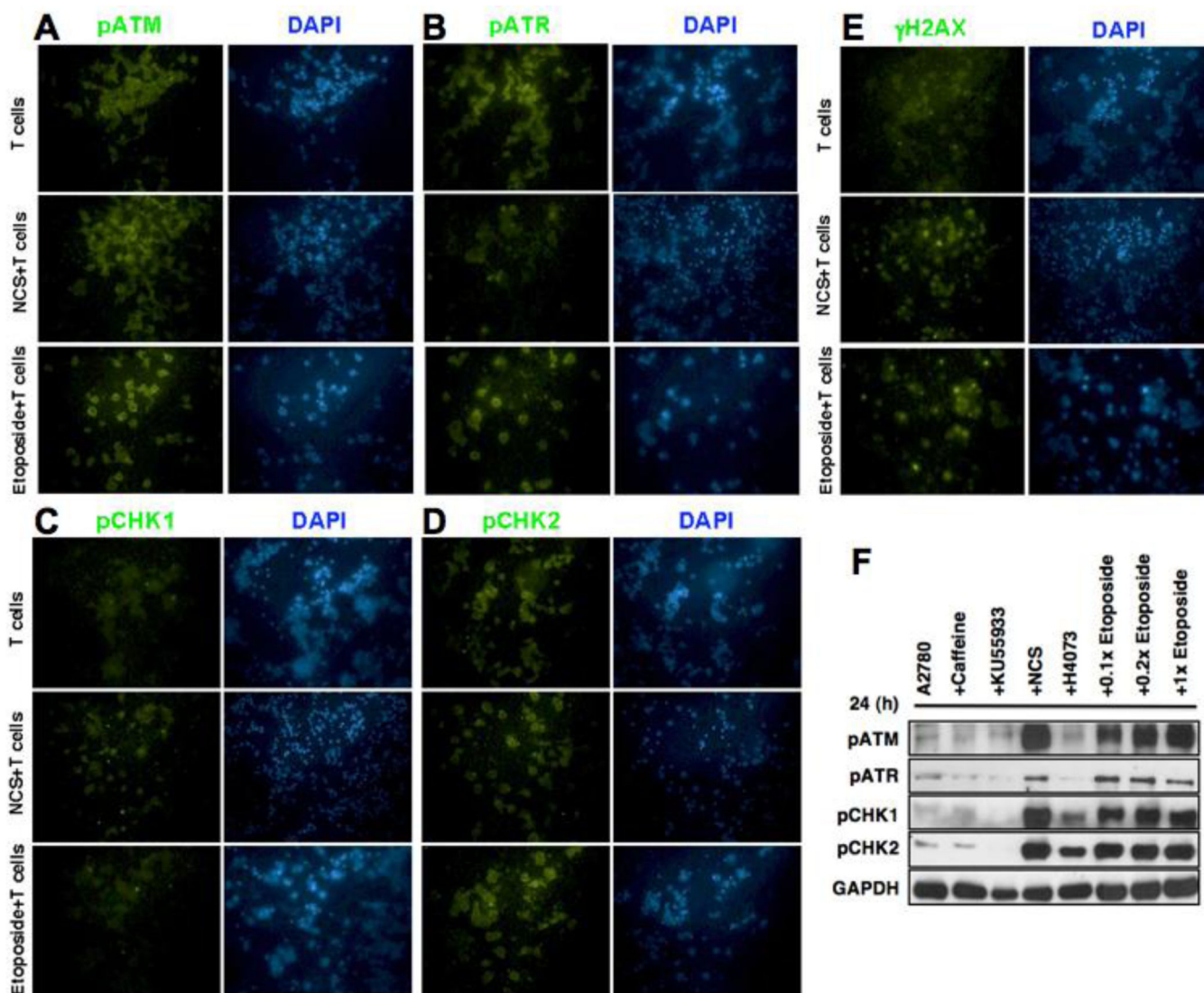


Figure 4. Expression of ATM/ATR pathway molecules in presence V γ 2V δ 2 T cells and ATM inducing factor

Ovarian cancer cells were co-cultured with V γ 2V δ 2 T cells for 24 h at 1:10 ratios along with ATM inducing factor (NCS), and various ATM/ATR pathway molecules were assessed using immunocytochemical techniques (A–E). F. Level of activation (phosphorylated form) in ATM/ATR and their substrate (Chk2/Chk1) molecules in A2780 cells with NCS, H4073, and Etoposide stimulus or with ATM-inhibitors (caffeine or Ku55933).

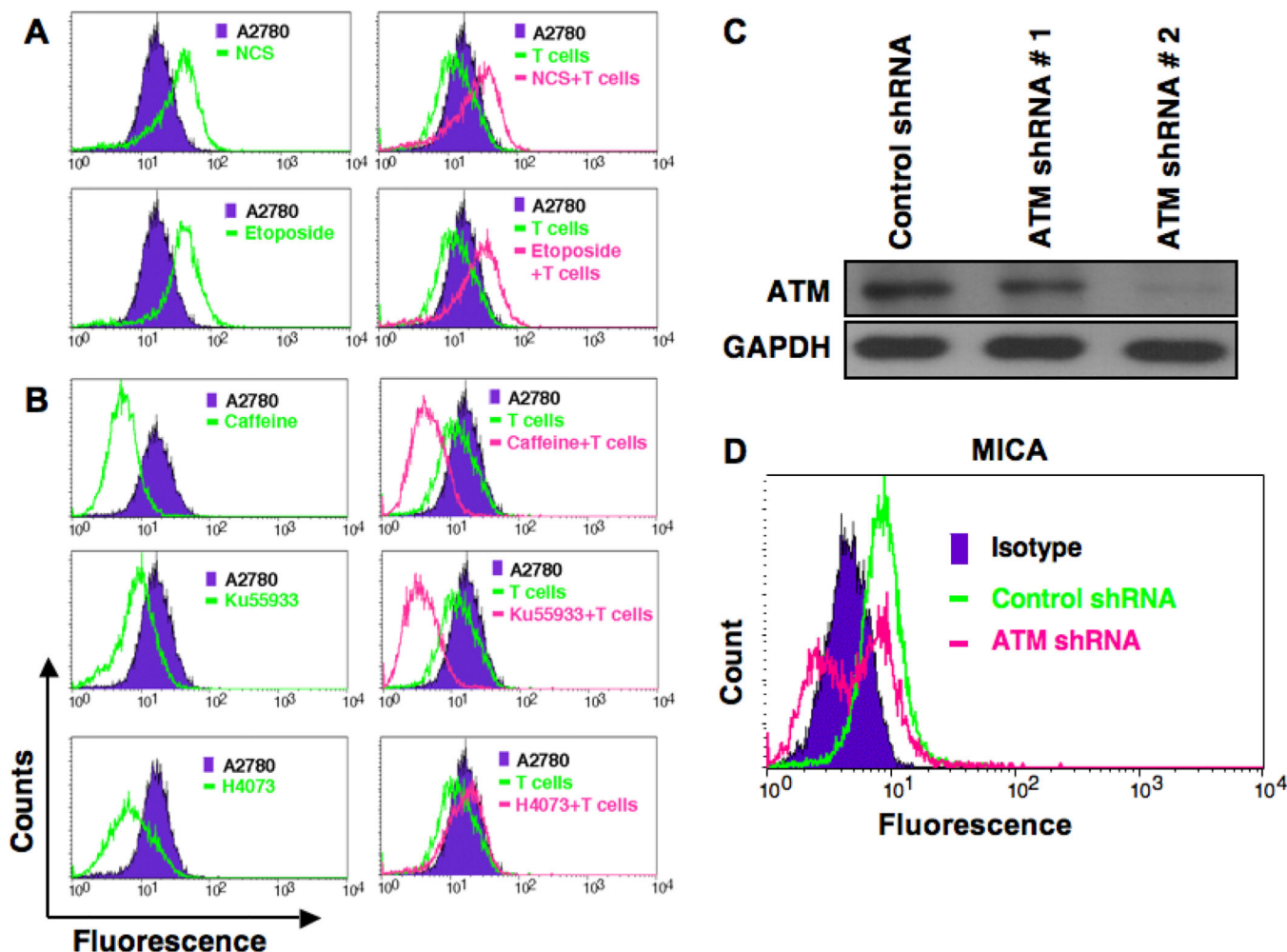


Figure 5. ATM-inducing molecule enhances surface expression of MICA in ovarian cancer cells and ATM-inhibitor reduces it

A. Ovarian cancer cells were co-cultured with V γ 2V δ 2 T cells for 24 h at 1:10 ratios in presence or absence of ATM-inducing factors (NCS or etoposide) and assessed for surface expression of MICA level using flowcytometry. Appropriate isotype Ab was used as a control. **B.** The surface expression of MICA molecule on ovarian cancer cells was also assessed by flowcytometry in presence or absence of V γ 2V δ 2 T cells and ATM-inhibiting factors (caffeine or Ku55933). **C.** ATM knock down in A2780 cells was evaluated by WB methods. Upper panel shows level of ATM and lower panel shows level of internal control GAPDH. **D.** Flowcytometric analysis for MICA. ATM knocked down cells was subjected to flowcytometric analysis for surface expression of MICA molecule.

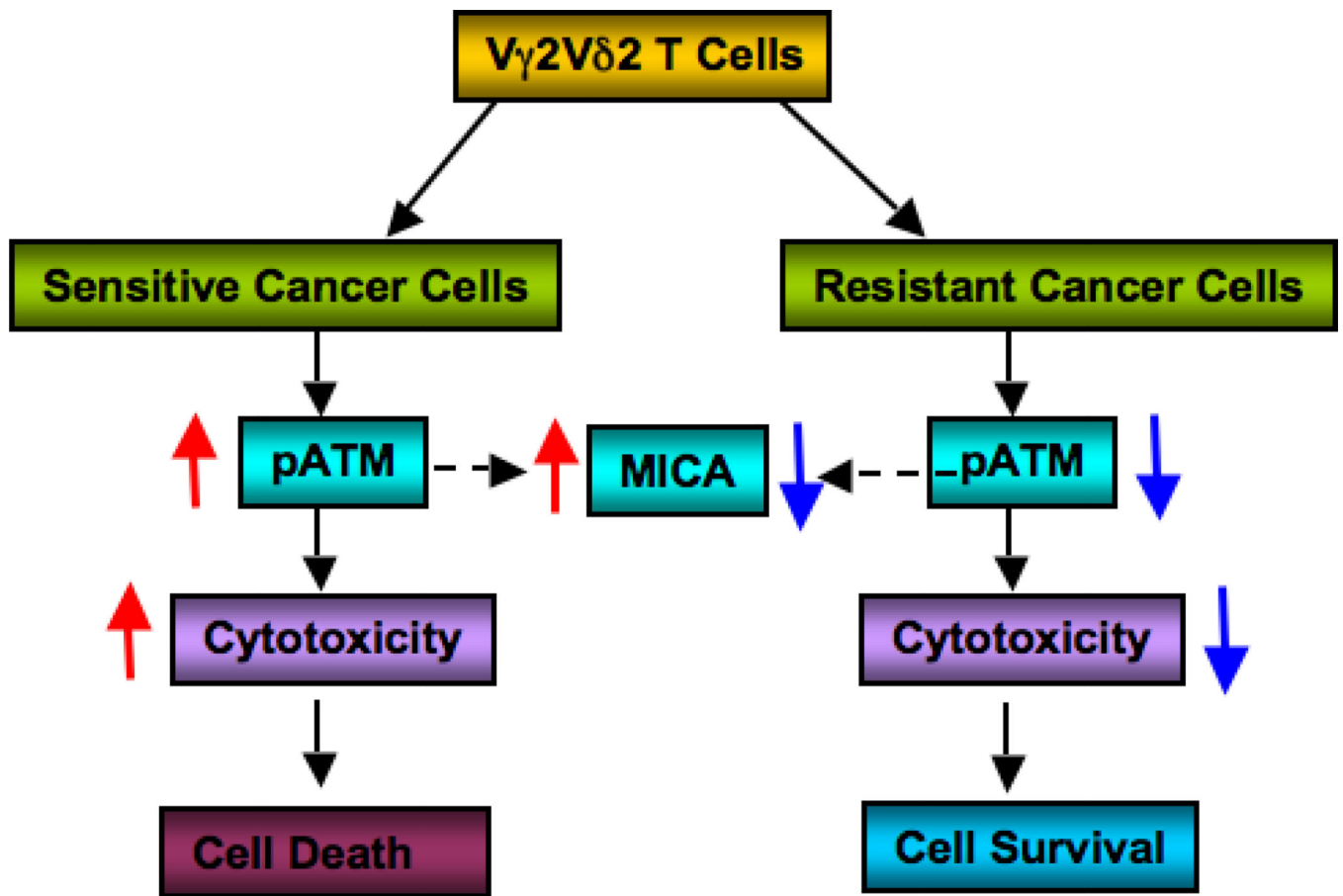


Figure 6. Interaction of V γ 2V δ 2 T cells and ovarian cancer cells
Schematics showing involvement of ATM pathway in V γ 2V δ 2 T cells-mediated cytotoxicity.