**5-aza-2'-deoxycytidine/valproate combination induces CTL response against mesothelioma**

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**Running title:** Mesothelioma epigenetic treatment

**ABSTRACT**

Malignant pleural mesothelioma (MPM) is an aggressive tumor with limited response to conventional therapy. The aim of this study was to evaluate the anticancer effect of a DNA methyltransferase inhibitor, 5-aza-2'-deoxycytidine (5-azaCdR), and two histone deacetylase inhibitors, valproic acid (VPA) and suberoylanilide hydroxamic acid (SAHA).

Human mesothelioma cells were treated with each epigenetic drug, either alone or in combinations. The cytotoxic effects on treated cells, and the expression of specific tumor antigens, were evaluated. The recognition of treated cells by a specific CD8+ T-cell clone was also measured. Additionally, the effect of combined treatments was tested in a murine model of mesothelioma.

We showed that VPA and SAHA synergized with 5-azaCdR to kill MPM cells and to induce tumor antigen expression in the remaining living tumor cells. As a consequence, tumor cells expressing these antigens were recognized and lysed by specific CD8+ cytotoxic T-cells. *In vivo*, treatment with 5-azaCdR/VPA inhibited tumor growth and promoted lymphocyte infiltration and an immune response against tumor cells.

Appropriate epigenetic drug combinations, in addition to inducing mesothelioma cell death, also affect the immunogenic status of these cells. This property could be exploited in clinical investigations to develop MPM treatments combining chemotherapeutic and immunotherapeutic approaches.

**INTRODUCTION**

Malignant pleural mesothelioma (MPM) is an aggressive tumor of the pleura, usually associated with chronic asbestos exposure (1). Worldwide, the incidence of MPM is increasing and is expected to peak around the year 2020 (2). MPM treatments include chemotherapy, radiotherapy and surgery, but are of limited efficacy, urging the development of new therapeutic strategies. Numerous preclinical and clinical studies have proved that hypomethylating drugs such as 5-aza-2'-deoxycytidine (5-azaCdR, Decitabine), and histone deacetylase inhibitors (HDACi) such as valproic acid (VPA, Depakine) and suberoylanilide hydroxamic acid (SAHA, Vorinostat), have a potent anti-cancer activity and promising therapeutic potential (3-11).

The DNA methyltransferase inhibitor (DNMTi) Decitabine, allows the expression of silenced genes through the demethylation of CpG islands. This drug has recently demonstrated clinical efficacy in the treatment of hematopoietic malignancies (12). HDACi aim to block chromatin compaction due to histone deacetylation, thereby increasing gene expression (13). Among HDACi, Depakine and Vorinostat are currently being evaluated alone, or in combination, as cytotoxic agents for MPM treatment (8, 9, 14). In these MPM clinical studies, and for many others cancer types, Decitabine and HDACi are being evaluated for their ability to slow down tumor progression by inducing cell cycle arrest, differentiation and/or apoptosis.

In addition, these drugs may also have an impact on the immune response. As spontaneous regression has been observed in mesothelioma patients with infiltration of tumor-specific lymphocytes (15), immunotherapy strategies for the treatment of MPM should be considered (16). However, candidate antigens that can be targeted on MPM are poorly defined. One way to by-pass this lack of identified antigens for MPM is to force tumor cells to express tumor-specific associated antigens (TAA). With the aim to favor TAA expression, it has been shown

that hypomethylating drugs and/or HDACi, in addition to restoring a normal epigenetic status in cancer cells, can also induce tumor antigen expression (17-21).

The aim of our investigation was to demonstrate the combined effect of the epigenetic drugs, DNMTi and HDACi, both on cell toxicity and on the new expression of TAA that could activate a cytotoxic T-cell response. We, thus, investigated the toxicity of 5-azaCdR, VPA and SAHA, alone and in sequential combinations, on human MPM cell lines. The effects of these drugs on the expression of several Cancer Testis Antigens (CTA) (such as *NY-ESO-1* [New-York esophageal cancer], *MAGE-A1* and *–A3* [melanoma-associated antigens]), which are of increasing interest as immunotherapeutic targets, were also analyzed, as well as recognition of treated cells by cytotoxic lymphocytes (CTL). Our study showed that VPA and SAHA trigger MPM cell death and synergize with 5-azaCdR to induce *NY-ESO-1* expression in the remaining living cells, which become sensitive to lysis mediated by NY-ESO-1-specific cytotoxic T-lymphocytes. Additionally, using a mouse model of mesothelioma, we report *in-vivo* tumor regression associated with lymphocyte infiltrates after sequential 5-azaCdR and VPA treatment. These infiltrates contained CD8a+ lymphocytes able to secrete IFNγ in response to tumor cells.

## MATERIALS AND METHODS

### Cell culture

Human epithelioid mesothelioma cell lines were established from pleural effusions in our laboratory (22). The human melanoma cell line (M117) was provided by Dr. N.Labarrière (INSERM, U892, Nantes). Cells were cultured in RPMI-1640 medium supplemented with 10% heat-inactivated FCS, 2mM L-glutamine, 100IU/ml penicillin and 0.1mg/ml streptomycin (complete medium). Human mesothelial cells were isolated from normal pleura

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by enzymatic disaggregation and cultured in complete medium supplemented with hydrocortisone (0.4µg/ml), epidermal growth factor (10ng/ml), insulin (0.5µg/ml) and non-essential amino-acids. 5azaCdR, VPA and cell culture reagents were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France). SAHA was provided by Dr. P.Bertrand (CNRS UMR 6514, University of Poitiers). After drug treatments, cell viability was assessed by TO-PRO-3-iodide labeling (Invitrogen, Cergy-Pontoise, France) and flow cytometry analysis.

The HLA-A*0201-restricted, NY-ESO-1(157-165)-specific CD8+ T-cell clone, M117.32H, was obtained from tumor-infiltrating lymphocytes of melanoma patient 117 and cultured as described by Fonteneau *et al* (23).

**RNA extraction and real-time RT-PCR**

Total RNA was extracted with the RNeasy Mini kit (Qiagen, Courtaboeuf, France) according to the manufacturer's protocol. One microgram of total RNA was reverse-transcribed using Moloney-Murine Leukemia Virus Reverse Transcriptase (Invitrogen). PCR reactions were carried out using 10X QuantiTect Primer Assays (Qiagen) and RT$^2$ Real-Time SYBR-Green/ROX PCR mastermix (Tebu-bio, Le-Perray-en-Yvelines, France), according to the manufacturer's instructions.

**Activation of HLA-A*0201-restricted, NY-ESO-1-specific CD8+ T lymphocytes**

Tumor cells were co-cultured with NY-ESO-1-specific CD8+ T lymphocytes in complete medium containing 10µg/ml brefeldin-A (Sigma-Aldrich) for 5hrs at 37°C and washed. Cells were stained with APC-conjugated mouse anti-human CD8 and PE-conjugated mouse anti-human IFNγ mAb (BD Biosciences, Le Pont de Claix, France) using methods described by Jung *et al* (19). CD8 and IFNγ expression were analyzed by flow cytometry.

**Cytotoxicity assay**

Tumor cells were incubated with $Na_2^{51}CrO_4$ (PerkinElmer, Boston, USA) for 1hr at 37°C and either pulsed, or not, with 10µM NY-ESO-1(157-165) peptide for 1hr at 4°C. Cells were subsequently co-cultured with HLA-A*0201-restricted NY-ESO-1-specific CD8+ T-cells for 4hrs at 37°C, in triplicate. Each supernatant was collected and added to scintillation liquid cocktail (OptiPhase "Supermix", PerkinElmer, Boston, USA) before liquid scintillation counting. The percentage of specific lysis was calculated as follows: 100x(experimental release–spontaneous release)/(maximum release–spontaneous release). The spontaneous release of $^{51}Cr$ was determined from targeT-cells cultured alone. The maximum release of $^{51}Cr$ was obtained from targeT-cells which were lysed in medium containing 1% Triton.

**Animal experiments**

The experiments reported here were carried out in compliance with the guidelines of the European Union for the care and use of animals in research protocols. Five million AK7 murine mesothelioma cells per mouse (24) were administered intraperitoneously to 4 groups of C57BL/6 mice (Elevage Janvier, Le Genest-St-Isle, 53, France) (day 0). Group 1 did not receive any further treatment. Groups 2 and 4 were given 3 successive i.p. injections of 5-azaCdR (4mg/kg) at days 7, 9 and 14. VPA (5mM) was added to the drinking water of mice from Group 3 and 4 from day 10 to 20. All Animals were necropsied on day 22.

**Histopathology and immunohistochemistry**

Tumors and tissues were fixed in 4% PBS/formaldehyde, embedded in paraffin, cut to 5µm sections and stained with hematoxylin-eosin-saffron.
Immunohistochemistry was performed on tumor slices (paraffin-embedded) using standard techniques. The primary antibody, anti-Foxp3 (Abcam, Paris, France), was used at a dilution

of 1/100. N-Histofine Simple Stain Mouse MAX Peroxidase (Nichirei Biosciences, Tokyo, Japan) was used as the detection reagent.

**IFNγ ELISPOT assay**

Spleens, as well as AK7 tumors, were pooled for each group of mice (control and 5-azaCdr/VPA) and were dissociated using the gentleMACS Dissociator (Miltenyi Biotech, Paris, France), according to the manufacturer's optimized protocols. Cell suspensions were 70μm-filtered, aseptically, and red blood cells were lysed with BD Pharm Lyse solution (BD Biosciences). Living cells were labeled with anti-CD8a (Ly-2) MicroBeads (Miltenyi Biotech). CD8a+ cells were positively-sorted using MACS columns and a MACS separator (Miltenyi Biotech). CD8a+ splenocytes ($5x10^5$ cells/well) and CD8a+ tumor-infiltrating lymphocytes ($1x10^5$ cells/well) were cultured in complete medium supplemented with 50μM 2-mercaptoethanol (Merck, Nottingham, UK) at 37°C in a 96-well ELISPOT plate for 36hrs. The stimulator cells were AK7 cells ($5x10^4$ cells/well), treated or not, beforehand, with 0.5μM 5-azaCdR (72hrs) and 5mM VPA (48hrs). The ELISPOT assay was performed in triplicate according to the manufacturer's instructions (BD Biosciences). Spots were counted automatically using an ELISPOT plate reader (Autoimmun Diagnostika GmbH, Strassberg, Germany).

**Statistical analysis**

Statistical significance was calculated using GraphPad PRISM. Comparisons between groups were made using the Mann-Whitney U-test or Kruskal-Wallis ANOVA followed by Dunn's post test (multiple comparisons). A two-tailed $P$-value <0.05 was considered as significant.

**RESULTS**

**Toxic and proliferative effects of 5-azaCdR, VPA and SAHA on MPM cells**

Carcinogenesis results from a combination of genetic abnormalities and epigenetic modifications leading to dysregulation of genes involved in cell cycle control and apoptosis. Modifications of chromatin depend on the activities of enzymes such as histone acetyltransferases, histone deacetylases and DNA methyltransferases, which are considered to be good targets for cancer therapy. Thus, we first studied the toxic effects of the HDAC inhibitors, VPA and SAHA, and the hypomethylating drug 5-azaCdR, on MPM tumor cells, using TO-PRO-3 iodide staining and flow cytometry. These drugs were used at concentrations known to be compatible with their biological activity. After 48hrs, VPA or SAHA triggered MPM tumor cell death with an $IC_{50}$ of 10mM and 10µM, respectively (Figure 1). This toxic effect was linked to increased MPM cell apoptosis, as indicated by Annexin-V staining (data not shown). On the contrary, 5-azaCdR showed no toxicity and no obvious effect on MPM cell proliferation.

**Effects of 5-azaCdR, VPA and SAHA on TAA expression**

MPM displays multiple epigenetic abnormalities that lead to the silencing of tumor suppressor genes (25). Such observations suggest that MPM may be a good target for epigenetic drugs that would favor the expression of new, tumor-associated genes. Thus, we studied the impact of 5-azaCdR, VPA and SAHA on the expression of three TAA (*NY-ESO-1*, *MAGE-A1* and *MAGE-A3)*. Using RT-qPCR, we showed that *NY-ESO-1* mRNA was undetectable in all non-treated MPM cell lines and that *MAGE-A1* and *-A3* mRNA were also undetectable in half of the tested cell lines. After treatment, 5-azaCdR induced or up-regulated the expression of the three antigens tested in a dose-dependent manner (Figure 2). A concentration of 0.5µM 5-

azaCdR was sufficient to achieve optimal induction levels. In contrast, VPA or SAHA alone had no effect on mRNA expression of the TAA tested (data not shown).

**VPA and SAHA enhance the expression of TAA in MPM tumor cells pre-treated with 5-azaCdR**

Many studies have evaluated the effects of combined treatment of cancer cells with a hypomethylating agent (especially 5-aza-2'-deoxycytidine) and a histone deacetylase inhibitor (26, 27). Since these drug-types affect two cooperative epigenetic mechanisms, we sought to determine the best drug combination schedules. Thus, in order to improve treatment efficiency, we first assessed whether VPA treatment before or after 5-azaCdR treatment would be more efficient. Two different schedules of treatment with 5-azaCdR and VPA were tested. MPM cell lines were treated with 0.5μM 5-azaCdR followed by 5mM VPA, or with 5mM VPA followed by 0.5μM 5-azaCdR (Figure 3A). Analysis of *NY-ESO-1* mRNA expression by RT-qPCR showed an induction after both of these sequential treatments. However, treatment with 5-azaCdR/VPA, as compared VPA/5-azaCdR, was associated with much higher *NY-ESO-1* mRNA expression levels in all MPM cell lines tested. The optimal schedule of sequential 5-azaCdR/VPA treatment was investigated (Figure 3B). The best inductions of *NY-ESO-1* expression were observed after sequential treatments with 0.5μM 5-azaCdR (48hrs)/5mM VPA (48hrs) and 0.5μM 5-azaCdR (72hrs)/5mM VPA (48hrs).

Genetic abnormalities and epigenetic modifications are characteristics of chromatin alteration that lead to carcinogenesis. Thus, epigenetic drug inhibitors should not have an effect on normal cells. The effects of the most effective combination, 5-azaCdR/VPA, on *NY-ESO-1* expression in normal mesothelial cells (NMC) were evaluated. NMC primary cultures were obtained from pleural biopsies of patients undergoing cardiac surgery. NMC cultures were positive for normal mesothelium markers: calretinin and cytokeratin-5 and -6 (data not shown). *NY-ESO-1* mRNA was not detected in NMC, either before or after treatment with 5-

azaCdR/VPA (Figure 3C). Conversely, *NY-ESO-1* was induced in MPM-treated cells, but to a lower level than that observed in the melanoma cell line, M117. Similar results were obtained for *MAGE-A1* and *–A3* (data not shown).

In another set of experiments, we compared the effects of the combinations, 5-azaCdR/VPA or 5-azaCdR/SAHA, on TAA expression and cell proliferation. MPM cells were treated with 0.5μM 5-azaCdR and with either 5mM VPA or 2.5μM SAHA (Figure 4). No impact of 5-azaCdR on cell proliferation was observed after treatment. Conversely, the combination of 5-azaCdR with VPA or with SAHA (used at doses below their $IC_{50}$) led to a 50% inhibition of cell proliferation (40% when the HDACi was used alone). However, as previously noted with HDAC inhibitors alone, no complete toxicity was observed with combination treatments. Furthermore, 5-azaCdR/VPA treatment was associated with higher TAA mRNA levels than treatment with 5-azaCdR alone or the 5-azaCdR/SAHA combination. We, thus, focused on the combination of 5-azaCdR/VPA for subsequent experiments.

**HLA-A*0201-restricted NY-ESO-1-specific CD8+ T lymphocytes respond to all HLA-A*0201+, 5-azaCdR/VPA-treated MPM cell lines**

NY-ESO-1 is considered as a very valuable target for T-cell based immunotherapy [28]. Thus, we evaluated whether the *NY-ESO-1* expression observed in 5-azaCdR/VPA-treated, HLA-A*0201+ MPM cells was able to promote their recognition by an NY-ESO-1(157-165)/HLA-A*0201-specific CD8+ T-cell clone. This T-cell clone, M117.32H, was obtained from limiting-dilution cultures of tumor infiltrating lymphocytes from a melanoma patient, and recognized the autologous tumor cell, M117 (Dr N. Labarrière, unpublished observation). Furthermore, this clone exhibited 100% staining with PE-conjugated NY-ESO-1(157-165)/HLA-A*0201 and no staining with HLA-A*0201 tetramer loaded with irrelevant peptide (data not shown).

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In a first set of experiments, IFNγ production by the CD8+ T-cell clone, in response to an MPM cell line treated, or not, with 5-azaCdR/VPA, was tested (Figure 5A). Untreated and treated tumor cells were washed and then co-cultured with the NY-ESO-1-specific T-cell clone. No T-cell clone response was observed with tumor cells which were not treated with 5-azaCdR/VPA. IFNγ production by the clone was observed by intracytoplasmic staining only in response to 5-azaCdR/VPA-treated cells. In some conditions, IFNγ was added with VPA to the culture medium, in order to increase the expression of HLA class I molecules at the surface of MPM cells (data not shown) and, therefore, their recognition by CD8+ T-cells. As expected, a slightly better activation of the clone was observed (e.g., up to 32.3% cells versus 26.2% for meso96) when tumor cells were pre-treated with exogenous IFNγ.

The level of activation of the clone in response to 5-azaCdR/VPA-treated MPM cell lines and its level of recognition of the control autologous M117 melanoma cell line were then compared. All 5-azaCdR/VPA-treated, HLA-A2+ MPM cell lines were recognized by the clone, but at levels lower than the level of recognition of M117 (Figure 5B). As a control, it was also demonstrated that the HLA-A*0201[neg] MPM cell line was not recognized, as it did not express the NY-ESO-1(157-165)-presenting HLA-A*0201 molecule. Interestingly, IFNγ T-cell production in response to all treated HLA-A*0201+ cell lines confirmed that 5-azaCdR/VPA-treated cell lines express enough NY-ESO-1 protein to efficiently process and present the peptide on their surface and to induce their recognition by an NY-ESO-1-specific CD8+ T-cell clone. Finally, all the clone responses were partially inhibited (56.6% ±7.7) by the presence of a blocking anti-HLA-A*0201 monoclonal antibody (clone BB7.2) in the T-cell clone/MPM cell line co-cultures.

In addition, we tested whether IFNγ T-cell production in response to 0.5μM 5-azaCdR (72hrs)/5mM VPA (48hrs)-treated HLA-A*0201+ MPM tumor cells was accompanied by tumor cell lysis by the NY-ESO-1-specific CD8+ T-cells. The cytotoxicity observed, as

revealed by $^{51}$Cr release, was undetectable in untreated MPM cells (Figure 5C). As expected, MPM cells were lysed when they were pre-treated with 10μM NY-ESO-1(157-165) peptide or when they were treated with 5-azaCdR/VPA. Overall, the clone's cytotoxic activity was well-correlated with its IFNγ production, suggesting that 5-azaCdR/VPA treatment of MPM tumor cells induces sufficient NY-ESO-1 expression to cause substantial recognition and lysis of tumor cells by NY-ESO-1-specific CD8+ T-cells.

**VPA, in conjunction with 5-azaCdR, inhibits tumor growth in a murine model of mesothelioma**

In order to assess the *in-vivo* efficiency of the combination of 5-azaCdR and VPA, we used C57BL/6 mice engrafted with AK7 cells. In pilot studies, we showed that these cells, similarly to human MPM cells, expressed TAA equivalent to human CTA, such as the tumor rejection antigen P1A, upon 5-azaCdR/VPA treatment (data not shown). We also determined that intraperitoneal (i.p.) injection of $5\times10^6$ AK7 cells represented the best tumorigenic dose. The administration schedule of 5-azaCdR was based on those described by Guo *et al* (29), who found that i.p. injections of 5-azaCdR (1mg/kg body weight) twice daily for 5 consecutive days were associated with mild toxicity and an induction of P1A. In our experiments, mice received only three injections of 5-azaCdR, but at a higher dose (4mg/kg), and without obviously-increased toxicity. In correspondence with our *in-vitro* findings, VPA (5mM) was delivered to mice after the first two injections of 5-azaCdR. The mice were weighed daily, with no differences noted between the groups. Upon completion of the treatment, mice from the treated and untreated groups were killed and tumor tissues were collected. All the results are summarized in Table 1 and representative illustrations for each group are shown in Figure 6. In mice from Group 1 (control, N=10), the omentum, invaded by AK7 cells, constituted a hard, white-colored tissue which was easily isolated from the

brown/yellowish soft pancreatic tissue. The tumor mass varied greatly between untreated animals (m=261.7mg ±43.5), and the presence of lymphocytes was rare. In animals from Group 2 (N=6), who received only 5-azaCdR, the tumor mass was slightly reduced (m=199.2mg ±32.5) and some areas showed accumulation of non-organized lymphocytes. With VPA alone (Group 3, N=6), the tumor mass was even more reduced (m=135mg ±18.7) and important foci of lymphocytes were observed (containing several regulatory T-cells labeled with Foxp3). Finally, mice receiving the combination of 5-azaCdR/VPA (N=6) showed the most important reduction in tumor mass (m=99.8mg ±22.1, p<0.05) and a greater number of lymphocyte aggregates. However, in contrast to Group 3, tumor cells were excluded from lymphocyte foci, which contained rare Foxp3+ cells localized at the periphery. In a second series of experiments, we determined if 5-azaCdR/VPA treatment induces a specific T-cell response against AK7 cells. We isolated CD8a+ T-cells from the spleens of mice having received, or not, the combined treatment (N=5, both). We then stimulated these CD8a+-purified T-cells with AK7 tumor cells (Figure 7A). As determined by ELISPOT assay, the number of IFNγ-secreting cells was greater in spleens from 5-azaCdR/VPA-treated mice: $80\pm6$ spots/$5x10^5$ cells compared to $18\pm4$ spots/$5x10^5$ cells for untreated mice, suggesting the induction of a tumor cell-specific immune response by the treatment. Furthermore, this response was mainly directed towards treatment-induced tumor antigens. Indeed, the IFNγ response doubled with CD8a+ T-cells from the spleen of treated mice stimulated by AK7 cells which were previously-treated with 0.5μM 5-azaCdR for 72hrs and 5mM VPA for 48hrs ($154\pm9$ spots/$5x10^5$ cells), as compared with untreated AK7 cells ($80\pm6$ spots/$5x10^5$ cells). As control, no spot was observed in wells containing medium alone and $12\pm4$ spots/$5x10^5$ cells were observed in wells containing only T-cells. Most importantly, we succeeded in isolating CD8a+ lymphocytes from the tumors of mice having received the 5-azaCdR/VPA treatment, but not from untreated animals. As for the experiment performed

with splenocytes, we observed an increase of the number of T-cells producing IFNγ in response to treated AK7 cells (28±2 spots/$10^5$ cells), as compared with untreated AK7 (10±2 spots/$10^5$ cells) (Figure 7B) and medium alone (4±2 spots/$10^5$ cells).


## DISCUSSION

Hypomethylating drugs and HDACi are used clinically for their ability to promote growth arrest, differentiation and apoptosis of tumor cells (13, 30). Decitabine (5-azaCdR), an inhibitor of DNA methyltransferase enzymes, is the most-commonly-used hypomethylating drug (31). Depakine (VPA) and Vorinostat (SAHA) are two HDACi currently being evaluated for MPM treatment (8, 9, 14). The histone acetylation mechanism involves two enzyme families: histone acetyltransferases (HATs) and histone deacetylases (HDACs). HATs transfer acetyl groups to amino-terminal lysine residues in histones, whereas HDACs catalyze the removal of acetyl groups, leading to chromatin condensation and transcriptional repression (32, 33).

Apart from their intrinsic toxic properties, our work demonstrated that epigenetic drugs used alone, or in combination, could have an impact on the expression of tumor-associated antigens and, therefore, could improve the recognition of tumor cells by cytotoxic T-lymphocytes. We focused our attention on CTA, which are of growing interest as immunotherapeutic targets because of their *in-vivo* immunogenic properties, their expression among tumors of different types, and their absence in normal tissues (28, 34).

In agreement with a previous observation made by Sigalotti *et al* (19), we showed that 5-azaCdR used alone can induce or increase the expression of *NY-ESO-1*, *MAGE-A1* and *MAGE-A3* in MPM cells of epithelioid subtype at doses that are not cytotoxic. In contrast to 5-azaCdR, its close analog 5-azacytidine (Vidaza), which is preferentially incorporated into RNA but not DNA, was not efficient in inducing the expression of the CTA analyzed (data

not shown). Conversely, VPA or SAHA alone displayed relatively high toxicity on these cells but did not affect CTA expression. However, we showed that these two drugs, used at doses below optimal toxicity, enhanced CTA expression in MPM cells pre-treated with 5-azaCdR. In addition, the overall toxicity of the combined treatment was slightly increased, when compared with the HDACi used alone. In proliferating cells, pre-treatment with 5-azaCdR induces demethylation of the CpG islands located in the promoters of silenced genes such as CTA, as reported for NY-ESO-1 in a glioma cell model (35), and, as a consequence, allows their expression. Thus, the subsequent use of VPA or SAHA, which can directly inhibit HDAC activity and leave the chromatin in an open conformation, enhances the expression of CTA induced by 5-azaCdR, through a better accessibility of transcription factors to DNA. Importantly, normal mesothelial cells similarly-treated did not express CTA such as NY-ESO-1, suggesting that such treatment did not affect the immunogenic status of normal, non-proliferating cells.

Furthermore, we observed that all HLA-A*0201+ MPM cell lines treated with 5-azaCdR/VPA (or 5-azaCdR/SAHA) can be recognized by a CD8+ T-cell clone, HLA-A*0201-restricted specific to NY-ESO-1(157-165). Hence, *de-novo* synthesized NY-ESO-1 antigen is functionally processed and presented on the tumor cell surface through MHC class I molecules. Consequently, we have shown that, in addition to the cytotoxicity induced by the HDACi, the sequential 5-azaCdR/VPA treatment induces a specific lysis of MPM cell lines by NY-ESO-1-specific CD8+ T-cells.

Finally, using a murine model of mesothelioma, we demonstrated that such sequential association of treatment with 5-azaCdR and VPA could inhibit tumor progression by approximately 60% after 3 weeks. This effect is due to both the toxicity of the treatment itself (indicated by active caspase-3 staining within the tumors of treated mice (data not shown)) and also to the activation of an anti-tumor immune response. Indeed, even though AK7 cells

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are known to secrete large amount of transforming growth factor β (TGFβ) and to promote tolerance towards the tumor (36), we saw large lymphocyte infiltrates within the tumors of treated mice but, only rarely, Foxp3+ cells. Furthermore, we showed that pools of CD8a+ T cells isolated from the tumors (and also from the spleens) of treated, compared with untreated, mice, contained more T-cells able to recognize AK7 cells. As IFNγ has opposite immunomodulatory effects to TGFβ, this cytokine may play an important role in the anti-tumor response observed after 5-azazCdR/VPA treatment.

Although various clinical trials have already shown anti-tumor activity of 5-azaCdR, SAHA or VPA alone in leukemias (3, 6, 7, 11), very few data are available concerning the activity of these drugs in patients with MPM (4). Schrump *et al.* evaluated the feasibility and toxicity of continuous infusion of 5-azaCdR alone for 72hrs in 35 patients with lung and esophageal cancers or MPM, in a phase I clinical trial. The authors determined that the maximum tolerated dose of 5-azaCdR was 60 to 75mg/m$^2$ and, interestingly, that prolonged infusion induced CTA gene expression (*NY-ESO-1* and *MAGE-3*) in 36% of patients.

SAHA is the first HDACi approved for the treatment of cancer, in particular cutaneous T-cell lymphoma (5). Its safety is being evaluated in numerous phase I clinical trials for the treatment of solid tumors. Collected safety data showed that the use of SAHA in combination therapies, most commonly at 400mg daily for 14 days, is generally well-tolerated, and preliminary evidence of anti-cancer activity has been observed across a range of malignancies (11). Of interest, in a phase I study, 2 out of 13 patients with advanced MPM who had progressed after first-line chemotherapy demonstrated a partial clinical response after oral administration of SAHA for several cycles of therapy (9).

Among HDACi, VPA is known to be less active than hydroxamic acids, such as SAHA, but its pharmacokinetic properties and bioavailability favor its use as a clinical treatment. For instance, VPA has a longer half-life than SAHA (16-17hrs and 19-47min, respectively) and its

widespread use as an anti-epileptic drug over several decades has proven that its side-effects are acceptable in the long term. VPA is currently being tested as an anti-cancer agent, either alone, or in combination with other agents, mostly in hematological diseases (7). In a phase I/II study, 53 patients with advanced leukemia were treated with a fixed dose of 5-azaCdR (15mg/m²/day i.v. for 10 days) administered concomitantly with 50mg/kg/day VPA, given orally for 10 days (37). This combined 5-azaCdR/VPA treatment was shown to be safe and active, and was also associated with significant DNA hypomethylation, induction of histone acetylation, and gene reactivation. Clinical studies with VPA alone, or in combination with other agents, have also been performed on solid tumors (38). For example, in a phase I study, patients with advanced solid tumors were treated with VPA by intravenous infusion: in agreement with the observations in leukemia patients, doses up to 60mg/kg/day for 5 consecutive days were well tolerated and showed detectable biological activity, i.e. accumulation of hyperacetylated H3 and H4 histones in cell lysates of peripheral blood lymphocytes (39). In the context of MPM, it has been shown recently that VPA improves the pro-apoptotic effect of pemetrexed/cisplatin, the usual first-line treatment regimen for this tumor, in tumor cells from patients' biopsies and in a mouse model of MPM (8). The clinical benefit of VPA plus doxorubicin was recently evaluated in a phase II trial (see protocol 01062 at http://www.elcwp.org). There are currently very few data available concerning the use of 5-azaCdR and VPA combinations (sequential or concomitant) for the treatment of solid malignancies. Only one clinical trial (phase I) for the treatment of patients with non-small cell lung cancer is ongoing (see protocol NCT00084981 at http://www.clinicaltrials.gov). Of note, in all these studies patients received 5-azaCdR, SAHA or VPA by intravenous infusion or orally at doses below those used here. However, other administration protocols can be proposed, such as infusion into the pleural cavity in direct contact with MPM tumor cells. This route of injection, which has been used successfully in clinical trials with interleukin-2

(40), may allow the use of higher doses of drugs that are incompatible with systemic administration and may also diminish side-effects in other organs.

Altogether, these clinical trials have allowed the measurement of 5-azaCdR, SAHA and VPA toxicities but, considering our results presented here, the impact of these molecules on the immune response towards cancer cells should be evaluated further. Our investigations have demonstrated that sequential 5-azaCdR/VPA or 5-azaCdR/SAHA treatments represent potential new strategies to induce MPM cell death and to modulate the immunogenicity of remaining, drug-resistant tumor cells. This work opens up new opportunities for the development of additional MPM treatment strategies which use chemotherapeutic drug combinations, such as 5-azaCdR/VPA or 5-azaCdR/SAHA, to induce MPM cell apoptosis, and neo-expression of CTA, such as NY-ESO-1, in the remaining, apoptosis-resistant tumor cells. These latter cells then become targets for a cytotoxic and TAA-specific immune response. Furthermore, this strategy may bypass the development of antigen-specific tolerance mechanisms that occur when CTA are expressed by cancer cells during tumor progression. The association of such chemotherapeutic with immunotherapeutic treatments should be evaluated in the future.

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**TABLES**

**Table 1: Staging of omental invasion by the tumor, and of lymphocyte infiltration.**

|  | Tumor mass (mg) | AK7 tumor/omental tissue [#] | Lymphocyte aggregates [$] |
|---|---|---|---|
| **Control (vehicle)** | 261.7 ± 43.5 | ++++ / - | 0 |
| **5-azaCdR** | 199.2 ± 32.5 | +++ / + | < 1 |
| **VPA** | 135 ± 18.7 | ++ / ++ | 3 |
| **5-azaCdR/VPA** | 99.8 ± 22.1 * | ++ / ++ | 6 |

[#] Degree of omental tissue invasion by AK7 tumor cells: ++++ / - = omentum totally occupied by tumor cells, no residual fat cells, +++ / + = residual fat cells in the central part of the omentum, ++ / ++ = equivalent amounts of AK7 and fat cells in the omentum.

[$] Quantification of lymphoid cell infiltrates: Average number of lymphocyte aggregates per section of tumor in the whole group of mice. An aggregate was defined as a dense, organized accumulation of lymphoid cells covering an area $> 225 \times 300 \mu m^2$.

* $P < 0.05$

**FIGURE LEGENDS**

**Figure 1: Effect of 5-azaCdR, VPA and SAHA on MPM tumor cell viability and proliferation.** MPM cell lines were treated with various doses of 5-azaCdR (A), VPA (B) or SAHA (C) for 48hrs. Cell number was measured by counting living cells at the end of the treatments. Cell viability was measured by TOPRO-3 incorporation and flow cytometry. Viability and growth are expressed as percentages (mean with SEM of the 4 tested cell lines).
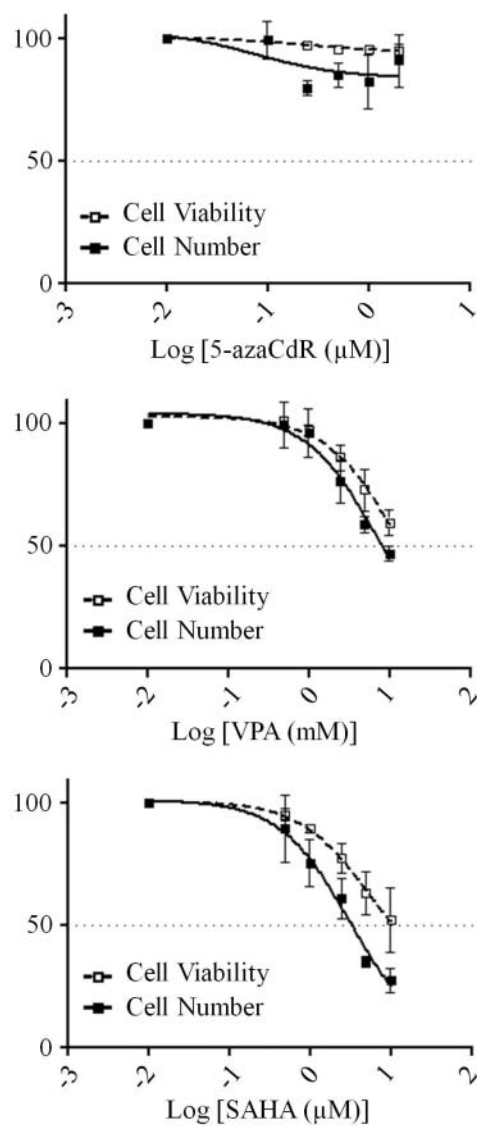
Figure 1

**Figure 2: Effect of 5-azaCdR on CTA expression.** (A) MPM cell lines (N=4) were treated with various doses of 5-azaCdR for 48hrs. SAHA and VPA alone had no effect (data not shown). *NY-ESO-1*, *MAGE-A1* and *MAGE-A3* gene expression was measured by real-time RT-PCR. For each gene, results were normalized to the endogenous reference gene, *RPLPO* (x1000). Results are represented as means with SEM. Statistical significance was calculated using Kruskal-Wallis ANOVA followed by Dunn's post test (* *P*<0.05; ** *P*<0.01: *** *P*<0.001).
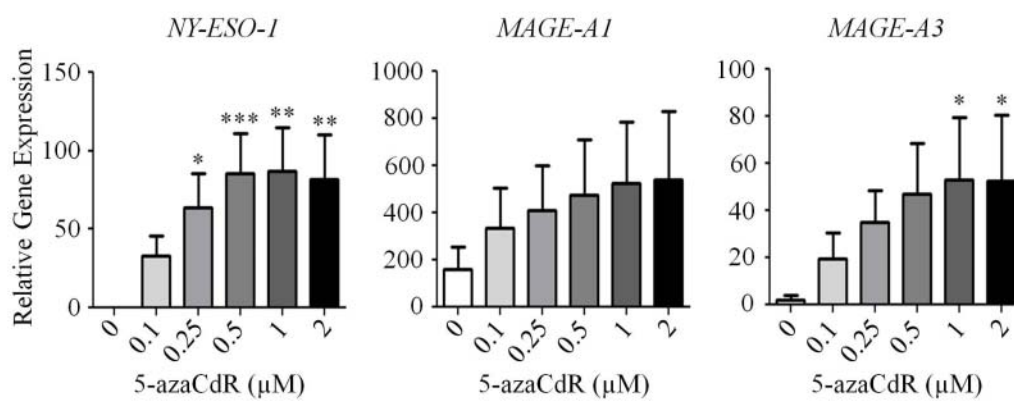
Figure 2

**<u>Figure 3</u>: Impact of different schedules of 5-azaCdR and VPA administration on NY-ESO-1 expression in MPM cells and normal mesothelial cells.** (A) *NY-ESO-1* expression

was assessed by RT-qPCR in different MPM cell lines (N=5) treated sequentially with 0.5μM 5-azaCdR for 48hrs and 5mM VPA for 24hrs, or with 5mM VPA for 24hrs and 0.5μM 5-azaCdR for the next 48hrs. (B) *NY-ESO-1* expression was assessed by RT-qPCR in 2 MPM cell lines treated with 0.5μM 5-azaCdR and 5mM VPA, alone or in combination, with different time schedules. (C) Normal mesothelial cells in primary cultures (N=2) were treated sequentially with 0.5μM 5-azaCdR for 72hrs and 5mM VPA for 48hrs. *NY-ESO-1* expression in NMC was compared to that obtained in 5 MPM cell lines, and the melanoma cell line (M117) as a positive control. *NY-ESO-1* expression was normalized to the endogenous reference gene, *RPLPO* (x1000). Data are represented as means ±SEM. Statistical significance was calculated using Kruskal-Wallis ANOVA followed by Dunn's post test (A) or Mann-Whitney test (C) (** $P$<0.01).
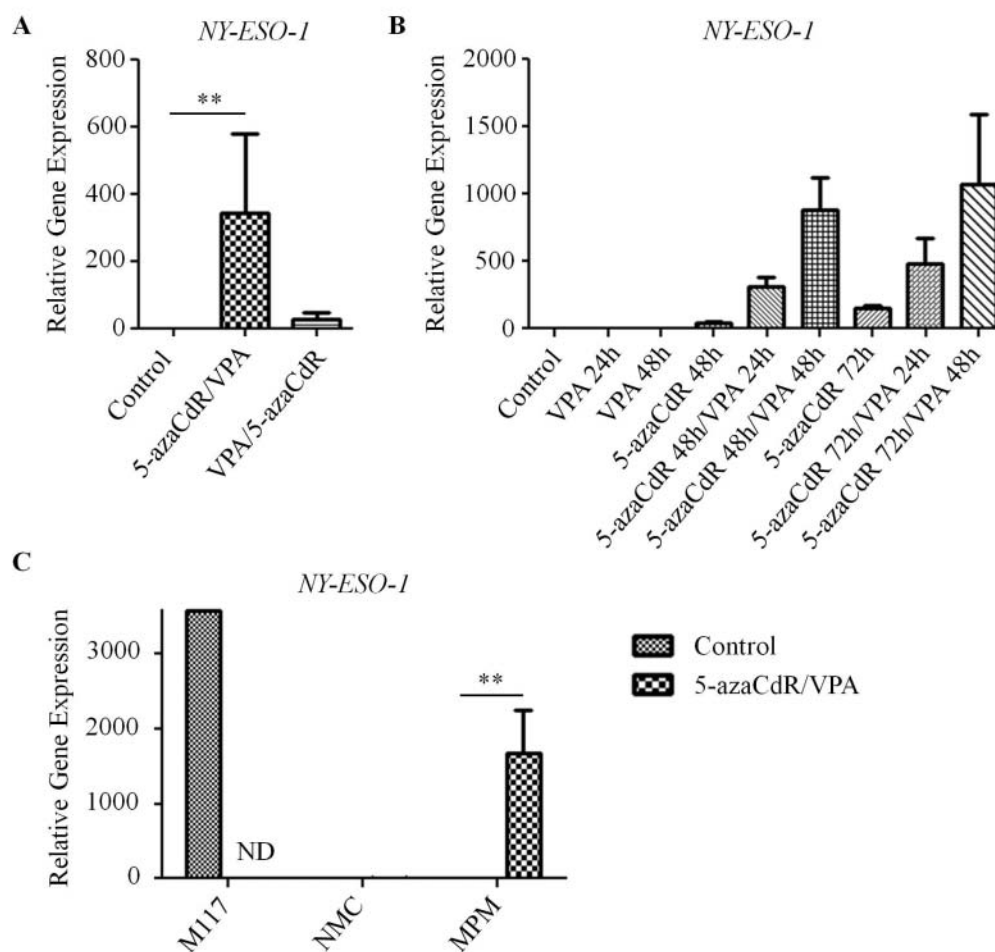
**Figure 4: Effects of the combinations 5-azaCdR/VPA and 5-azaCdR/SAHA on cell proliferation and CTA expression.** MPM cell lines were treated sequentially with 0.5 µM 5-

azaCdR for 72hrs and 5mM VPA or 2.5µM SAHA for 48hrs. (A) The numbers of living cells were measured at the end of the treatments. Each curve represents the growth mean (with SEM) of the 6 tested cell lines. (B) *NY-ESO-1*, *MAGE-A1* and *MAGE-A3* gene expression were measured by real-time RT-PCR. For each gene, results were normalized to the endogenous reference gene, *RPLPO* (x1000). Results are represented as means with SEM. Statistical significance was calculated using 2-way ANOVA (A) or Kruskal-Wallis ANOVA followed by Dunn's post test (B) (* $P<0.05$; ** $P<0.01$: *** $P<0.001$).
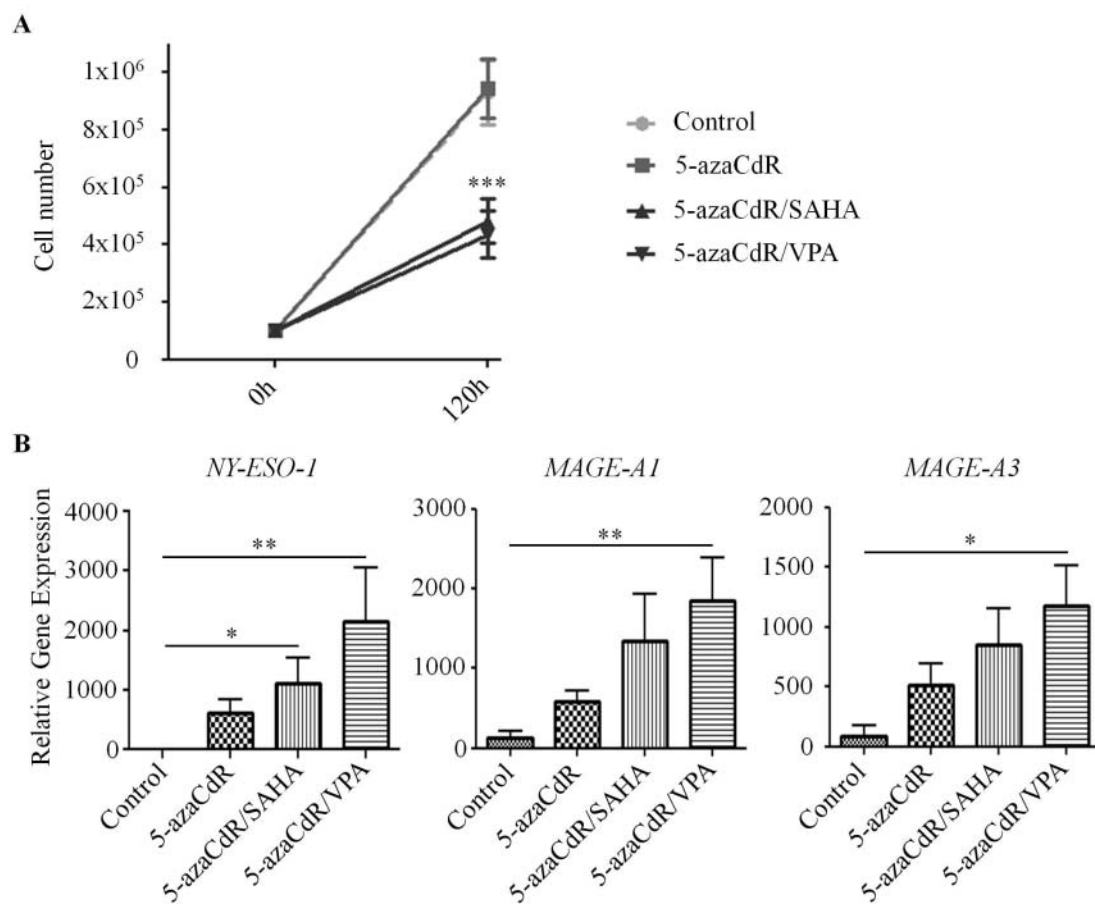
Figure 4

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**Figure 5**: **Recognition of 5-azaCdR/VPA-treated, HLA-A\*0201+ MPM cell lines by a HLA-A\*0201/NY-ESO-1(157-165)-specific CD8+ T-cell clone.** (A) IFNγ production by the NY-ESO-1-specific CD8+ T-cell clone in response to meso96 treated or not with 0.5µM 5-azaCdR (72hrs)/5mM VPA (48hrs) ± 500IU/ml IFNγ (48hrs). IFNγ production was measured by intracytoplasmic staining of IFNγ and surface staining of CD8 followed by flow cytometry analysis. (B) IFNγ production by the NY-ESO-1-specific CD8+ T-cell clone in response to the autologous melanoma cell line (M117), a HLA-A\*0201neg MPM cell line (meso13) and four HLA-A\*0201+ MPM cell lines (meso34, meso62, meso96 and meso122), treated or not with 0.5µM 5-azaCdR (72hrs)/5mM VPA (48hrs) ± IFNγ (48hrs). (C) Cytotoxic activity of the NY-ESO-1-specific CD8+ T-cell clone in response to the autologous melanoma cell line (M117) and an HLA-A\*0201+ MPM cell line (meso96), treated or not ( = control) with 0.5µM 5-azaCdR (72hrs)/5mM VPA (48hrs) and pulsed or not with 10µM NY-ESO-1(157-165) peptide. Effector (HLA-A\*0201-restricted, NY-ESO-1-specific CD8+ T-cells):target (tumoral cells) (E:T) ratios 5.000:1.000; 10.000:1.000 and 20.000:1.000 were used. Cytotoxic activity was measured by $^{51}$Cr release. Experiments were performed in triplicate for each data point.
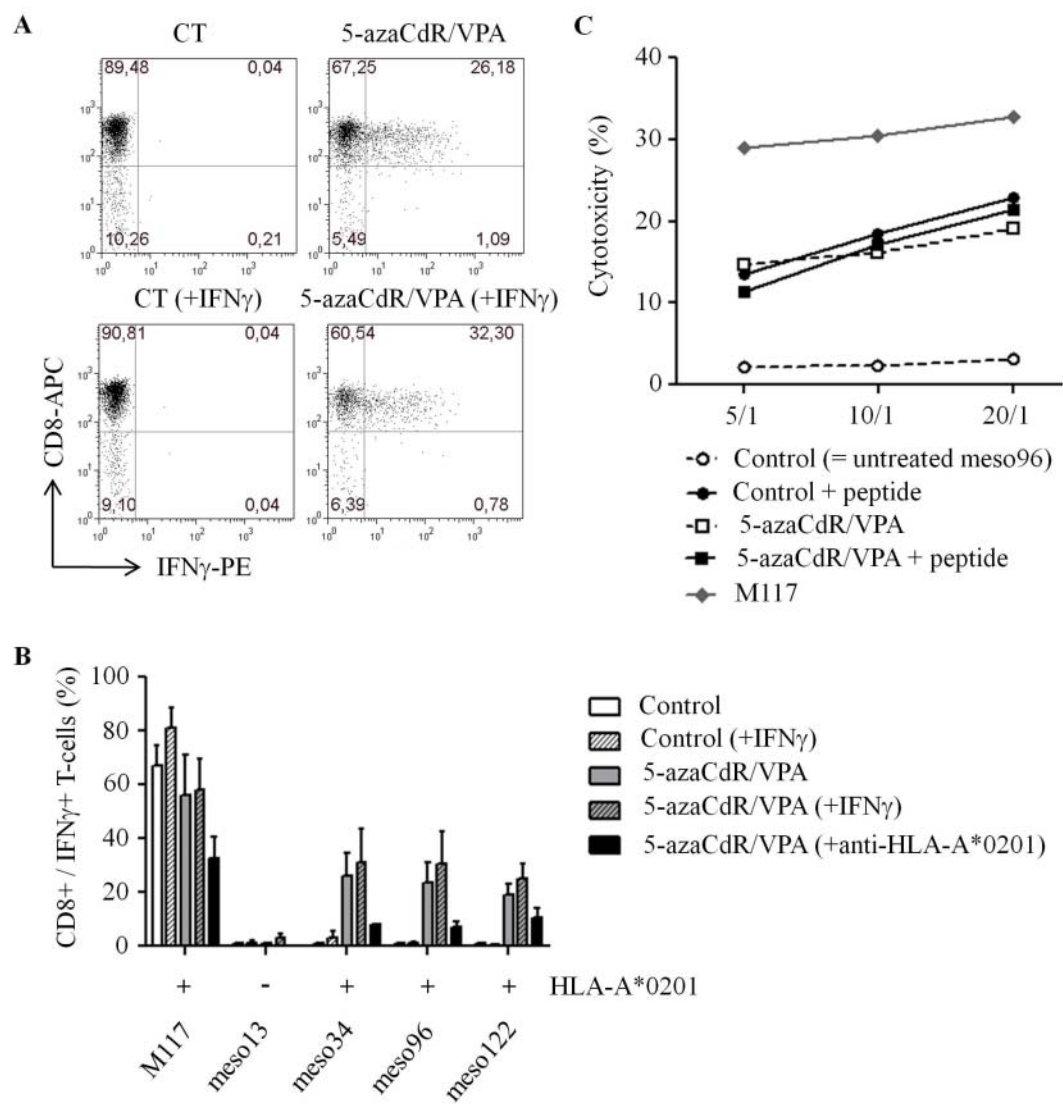
Figure 5

**Figure 6: Histopathological examination of AK7 tumors implanted in mice.** $5\times10^6$ AK7 murine mesothelioma cells were injected intraperitoneously into C57BL/6 mice. Mice from Group 1 (N=10) had no treatment. Mice from Groups 2 (N=6) and 4 (N=6) received 3 injections of 5-azaCdR (4mg/kg). VPA (5mM) was added to the drinking water of mice from Groups 3 (N=6) and 4. Top: Tumor sections stained with hematoxylin-eosin-saffron. Bottom: Immunohistochemical staining of tumor sections from mice treated with VPA alone or in combination with 5-azaCdR, using anti-Foxp3 antibodies. Foxp3+ cells appear with a brownish nuclear staining.
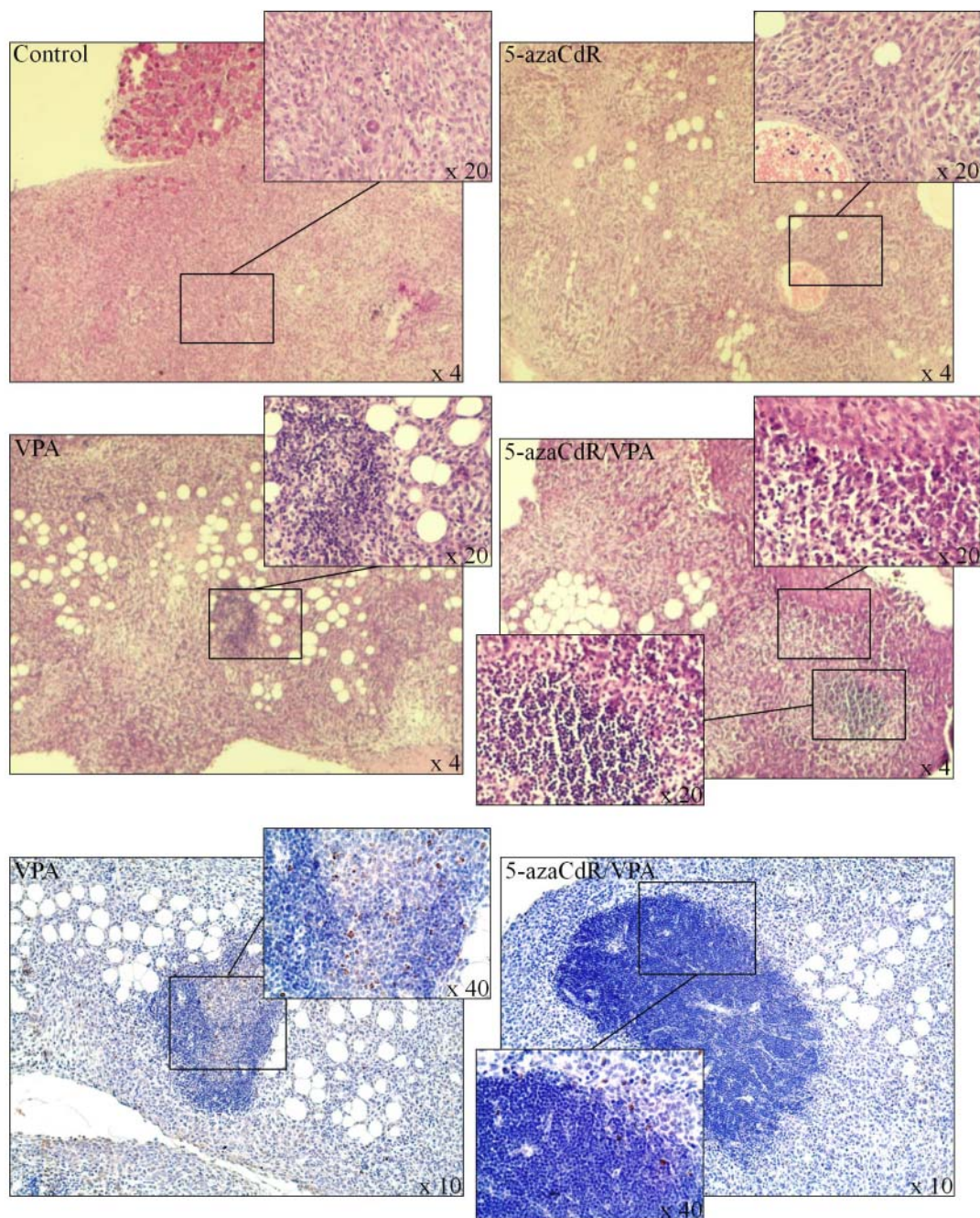
Figure 6

**Figure 7: T-cell anti-tumor response *in vivo* after 5-azaCdR/VPA treatment**. CD8a+ T lymphocytes from the spleen (A) and CD8a+ tumor-infiltrating T lymphocytes (B) from C57BL/6 mice bearing tumors (treated or not with 5-azaCdR/VPA) were stimulated for 36hrs with untreated AK7 cells or 5-azaCdR/VPA-treated AK7 cells. Secretion of IFNγ by CD8a+ cells was measured using an ELISPOT assay. The mean number of spots ±SEM in triplicate wells was calculated and expressed as number of spots per $5x10^5$, or $1x10^5$, CD8a+ cells. Significant differences in response were determined using Kruskal-Wallis ANOVA followed by Dunn's post test (A) or Mann-Whitney test (B) (* $P<0.05$; ** $P<0.01$: *** $P<0.001$). A representative well for each condition is displayed under the corresponding bar of the histogram.
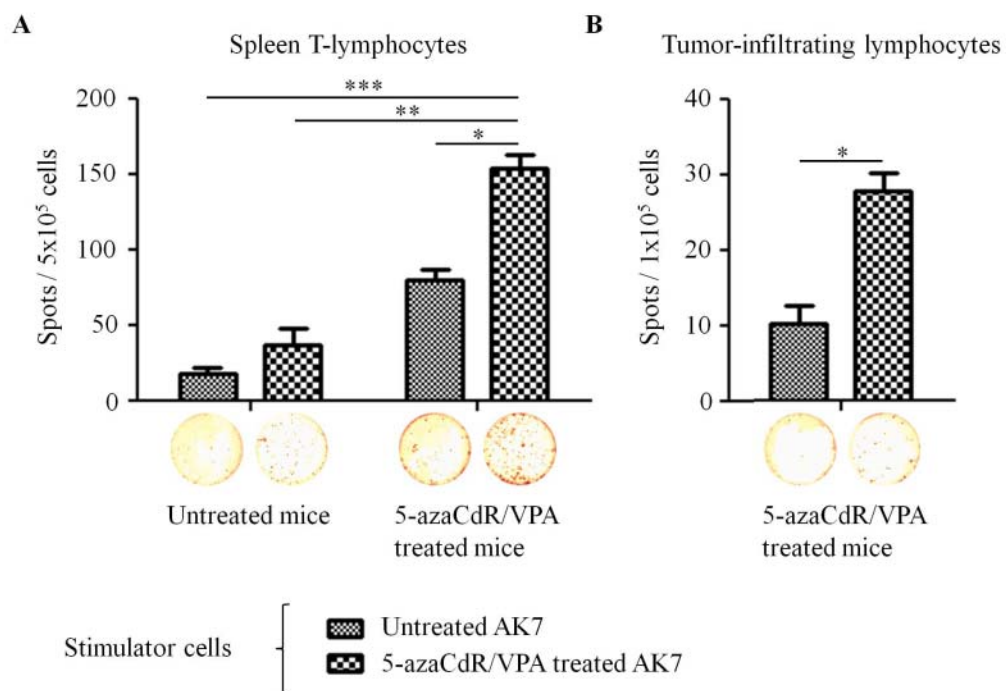
**A** Spleen T-lymphocytes

**B** Tumor-infiltrating lymphocytes

Stimulator cells
- Untreated AK7
- 5-azaCdR/VPA treated AK7

Figure 7