ATM-Mediated DNA Damage Signals Mediate Immune Escape through Integrin-αvβ3–Dependent Mechanisms

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Abstract

Although recent evidences have been unveiling the critical role of tumor microenvironments in tumor progression and metastasis, it remains unclear how resistance to various anticancer modalities is linked with the modulation of tumor microenvironments. We identified a novel mechanism whereby constitutively activated DNA damage signals in anticancer therapy-resistant tumor cells suppress antitumor immunity in an integrin-αvβ3-dependent manner. Integrin-αvβ3 was upregulated on various therapy-resistant tumor cells through chronic activation of ATM/chk2-and NF-κB-mediated pathways. The inhibition of tumor-specific integrin-αvβ3 improved therapeutic efficacies of anticancer drugs by stimulating endogenous host immune systems. As a mechanism of action, tumor-specific integrin-αvβ3 targets dendritic cells to facilitate phagocytosis of live resistant tumor cells, leading to impaired cross-priming of antigen-specific T lymphocytes. Our findings clarified the detrimental effects of DNA damage signals in chemosensitivity and antitumor immunity, and targeting integrin-αvβ3 has a major implication for treating patient refractory to current anticancer regimens.

Introduction

Although various intrinsic mechanisms, such as genetic alterations, histone demethylase-mediated chromatin modifications, activation of multidrug resistance transporters (1-3) and enrichment of cancer stem/initiating cells (4), may determine responses to anticancer therapeutics, accumulating evidence has clarified the role of tumor microenvironments in the regulation of therapeutic outcomes (5,6). Conversely, tumor cells may modify the biologic properties of stromal cells, endothelial cells, and host immunity in local microenvironments, resulting in further tumor progression and a worse prognosis (7, 8). Therefore, the acquisition of anticancer drug resistance may render tumor cells with the ability to modulate their microenvironments in a paracrine fashion, further enhancing survival signals and the progression of tumors.

The cytotoxic effects of chemotherapy rely mainly on the cellular stress responses through the rapid activation of DNA damage signals. DNA damage signals are also activated at early stages of tumorigenesis and should circumvent tumor progression in part through the induction of oncogene-induced senescence (9-12). On the other hands, DNA damage signals in tumors are linked with activation of innate immune pathways, represented by the NKG2D ligand expression on innate lymphocytes and the pro-inflammatory cytokine secretion from senescent cells (13, 14). However, it remains unclear whether DNA damage pathways modify

tumorigenicity and antitumor immunity in the process of therapeutic interventions.

Integrin-αvβ3 and αvβ5 are expressed on tumor cells, endothelial cells and stromal cells, implying broad activities on tumor microenvironments (15). Integrin-αvβ3 plays a critical role in triggering invasive and metastatic activities through the coordinated activation of multiple oncogenic signals and thus contributes to the resistance to specific molecular target therapies (15-17). Integrin-αvβ3 is also critically involved in angiogenesis in cooperation with several growth factors such as VEGF (18). In addition, integrin-αvβ3 on myeloid cells regulates tissue inflammation and autoimmunity by regulating helper T cell differentiation and cytokine profiles (19-21). Due to its critical involvement in inflammation and cancer, specific inhibitors targeting integrin-αvβ3 have been developed for treatment of cancer at advanced stage, and they have demonstrated significant clinical effects (22, 23). Therefore, there is a critical need to address the optimal conditions and therapeutic options in which integrin-αvβ3 inhibitors should be used to treat malignant disorders.

Here we present the first evidence that DNA damage signals are responsible for triggering integrin-αvβ3 upregulation on drug-resistant tumor cells. As machineries by which integrin-αvβ3 promotes tumorigenicity, the resistant tumor cells triggers the impairment of DC immunogenicity and antitumor immunity due to the integrin-αvβ3-mediated recognition of live tumor cells. Our findings reveal a unique role for integrin-αvβ3 in linking drug resistance with

immune evasion and implicate integrin-αvβ3 as a novel therapeutic target in cancer patients.

Materials and Methods

Mice and tumor cells

ATM-deficient mice were backcrossed at least nine generations onto the C57BL/6 strain and housed under specific pathogen-free conditions. Genotypes were confirmed by PCR, and the experiments were conducted as described previously (24, 25). C57BL/6 and NOD-SCID animals were purchased from SCL and Charles River, respectively. All experiments were conducted under a protocol approved by the animal care committees of Hokkaido University.

The tumor cells (MC38, HCT116 for colorectal carcinoma cells; MCF-7 for breast carcinoma cells; A375 and B16 for melanoma cells: NCI-H1975, PC3 for NSCLC cells: Hep3B for hepatoma cells) were obtained from the American Tissue Culture Collection (ATCC). K029 melanoma cells were established from the metastatic lesion of advanced patients as previously described (26). The therapy-resistant variants were established by the exposure of increasing concentrations of therapeutic agents (50~100 times higher than maximal toxic concentrations).

Human samples

The clinical protocols for this study project were approved by the committees in Institutional Review Board of Hokkaido University Hospital (Approval number: 10-0114). Pleural effusion

cells were obtained from patients with stage IV non small cell lung carcinomas after written informed consents had been obtained. The cells were isolated by Ficoll Hypaque density centrifugation, and further purified as $EpCAM^+$ epithelial tumor cells from pleural effusion and $CD45^+$ leukocytes from pleural effusion.

Evaluation of integrin-* $\alpha v \beta 3$ *expression

The cell lines and primary tumor infiltrates obtained from patients with advanced cancer were analyzed by flow cytometry using mAbs against human integrin-αvβ3 (clone: LM690: Millipore). In primary cells, the epithelial tumor cells were fractionated as EpCAM-positive populations. All clinical protocols received approval from Institutional Review Board of Hokkaido University Hospital (Approval number: 10-0114).

Immunoblotting

A375 melanoma cells or those resistant to PLX-4720 were stimulated with 50nM PLX-4720, and the cell lysates were then subjected to Western blotting with Abs specific for anti-phospho-chk2 (Thr165), phospho-ATM (Ser138) (Cell Signaling Technologies) and integrin-αvβ3 (Millipore). Beta-actin was used as a loading control to check the integrity of each sample.

siRNA transduction

The design, preparation, and transduction of siRNA vectors were performed as described previously[50]. In brief, expression plasmids containing human chk-2 siRNA, integrin-β3 as well as ATM siRNA was obtained from Thermo-Scientific, and transfected into tumor cells according to the manufacturer's instructions. The gene knockdown efficacy was assessed by protein immunoblot analysis, and proved to be more than 95 %.

Detection of apoptosis

Tumor cells were exposed to γ-irradiation (50 Gy) and the induction of apoptosis was quantified by flow cytometry with annexin-V/ propidium iodide staining according to the manufacturer's instructions (BD Bioscience).

In vivo antitumor activities of BRAF inhibitor and anti-integrin-αv mAb

For *in vivo* tumor experiments, C57BL/6 or NOD-SCID mice were challenged subcutaneously in the flank with B16 melanoma cells transfected with V600E BRAF (B16.V600E) or those resistant to PLX-4270 ($1x10^5$) on day 0. For the therapy model, mice were injected with 250ug of mouse anti-integrin-αv mAb (RMV-7) twice a week and 250mg/kg of PLX-4270 (Merck-Calbiochem) three times a week. Tumor growth was measured every five days. In some

instances, intratumoral administration of BMDCs (1 x 10^6 / mouse) was performed with PLX-4270 and anti-integrin-αv mAb to examine the effect on antitumor responses.

Immune phenotypic assay

The frequency and phenotype of T lymphocytes in tumor draining lymph nodes (TDLs) were analyzed by flow cytometry using anti-CD4, anti-CD8, anti-CD44 and anti-CD62L Ab (BD-Bioscience). For intracellular staining, TDLs were dispersed and pretreated with anti-CD3 and anti-CD28 agonistic Ab (BD-Bioscience) for 24 h. The cells were then treated with brefeldin-A (Sigma-Aldrich), stained with anti-CD4 or anti-CD8, fixed, permeabilized with Cytofix/Cytoperm buffer, and stained again with PE-conjugated Abs for Foxp3, IFNγ, IL-17. In some cases, IFNγ, IL-10 and IL-12 were quantified by ELISA (BD Bioscience) using supernatant obtained from cultured TDL or BMDCs. The frequency of each immune cell population was determined by flow cytometry.

Antigen-specific $CD8^+$ responses against H-$2D^b$-restricted epitopes derived from gp100 were determined by incubating lymphocytes for 72 hours with $1x10^5$ irradiated B16 cells and measuring the frequency of gp100-specific CTLs by flow cytometry after staining with H-$2D^b$-gp100 tetramer (MBL International).

Phagocytosis assays

B16 melanoma cells or those resistant to PLX-4270 or dacarbazine were untreated or exposed to γ-irradiation (50 Gy) to trigger apoptosis. The live or apoptotic cells were labeled with PKH26 red fluorescence dye (Sigma-Aldrich), as described[36]. BMDCs were generated from bone marrow cells by culturing for seven days in the presence of conditioned media (days 0, 2, 4, 6) from CHO cells secreting GM-CSF. BMDCs were cocultured with the labeled live or apoptotic cells for 4h and evaluated for phagocytosis efficiency by flow cytometry. In some experiments, the tumor cells were pretreated with anti–integrin-αv mAb (30 ug/ml) for 30 min before the co-culture to evaluate the contribution of integrin-αvβ3 to tumor cell engulfment.

Cross-priming assays

BMDCs were co-cultured with untreated or irradiated B16-OVA cells (1:10 ratio) that were labeled with PKH26 in 12-well round-bottom plates for 4 h and phagocytosis was determined with flow cytometry. $CD11c^+$ cells were isolated by magnetic cell sorting (Miltenyi Biotec), and cocultured with naïve $CD8^+$ T cells from the spleens of OT-I mice. Intracellular IFNγ expression in T cells was then determined by flow cytometry. In some experiments, the tumor cells were pretreated with anti–integrin-αv mAb (30 ug/ml) for 30 min before the co-culture to evaluate the contribution of integrin-αvβ3 to the cross-priming of OVA-specific CTLs.

Immunofluorescence microscopy

MoDCs were generated from $CD14^+$monocytes isolated from peripheral blood leukocytes with GM-CSF and IL-4 (20ug/mL, Peprotech). The MoDCs (1×10^4) were stained with an Alexa-488 conjugated mAb for CD11c (Biolegend), while Jurkat cells transfected with control or integrin-αvβ3 expression vectors ($1x10^5$) were stained with PKH-26. Live or apoptotic tumor cells were co-cultured with MoDCs for 8 hours on glass slides. The samples were then washed three times, fixed with 4% paraformaldehyde at -20^o for 5 minutes, and visualized using a TE2000-U inverted fluorescence microscope (Olympus).

Statistics

The differences between two groups were determined with the Student's *t* test or the two-sample *t* test with Welch's correction. The differences among three or more groups were determined with a one-way ANOVA. The *P* values less than 0.05 are considered as statistically significant.

Results

Expression of integrin-αvβ3 on chemoresistant tumor cell variants

Although it has been established that integrin-αvβ3 plays a critical role in tumorigenesis through multiple oncogenic signaling pathways (15), it remains unknown whether it affects tumorigenic activities during the course of anticancer therapies. Thus, we generated stable drug-resistant variants of tumor cells of various origins through prolonged exposure to increasing concentrations of anticancer drugs and examined the expression levels of integrin-αvβ3 on the tumor cells. The drug resistant phenotypes were confirmed with decreased caspase-3 activities in resistant variant of tumor cells (Supplemental Fig 1). Integrin-αvβ3 was expressed at much higher levels on various tumor cells with resistance to cytotoxic and molecular targeting agents than on parental cell lines. This is represented by K029 melanoma cells resistant to BRAF kinase inhibitor PLX-4720 and HCT116 colon carcinoma cells resistant to epithelial growth factor receptor (EGFR) mAb (Cetuximab) (Fig 1A and 1B). Furthermore, we found that integrin-αvβ3 expression was highly expressed on tumor cells obtained from patients with non small cell lung cancer (NSCLC) who received multiple rounds of chemotherapy but did not respond clinically (TX) compared to those before initial chemotherapy (Fig 1C). Thus, integrin-αvβ3 induction in tumor cells seems to be correlated with resistance to anticancer drugs.

DNA damage signals are critical for integrin-αvβ3 induction in therapy-resistant tumors

To further examine the functional significance of integrin-αvβ3 on tumor chemoresistance, we focused on melanoma cells resistant to anticancer drugs because integrin-αvβ3 has been associated with progression in patients with melanoma (27, 28). In addition, BRAF kinase inhibitors have emerged as a new option against advanced melanomas in the clinic (29), but therapeutic responses were mostly transient and closely associated with the emergence of drug resistance (30, 31). Although recent evidence has unveiled the intrinsic alterations of oncogenic signals in BRAF inhibitor-resistant melanomas, BRAF inhibition also manipulates host immune responses, underscoring their role in the regulation of tumor microenvironments (32). Thus, it is critical to address the additional mechanisms and its biological consequences in which melanoma cells acquire resistance to therapeutic regimens including BRAF inhibitors.

Various cellular stresses, including UV, γ-irradiation and cytotoxic drugs, trigger DNA damage responses through coordinated interplay of the ATM-chk2 and ATR-chk1 pathways, which may regulate the therapeutic antitumor responses induced by chemotherapy and radiotherapy (10, 11). To our surprise, the ATM-chk2 activation triggered by BRAF inhibition was significantly stronger in resistant A375 cells (A375-BRAFIR) than sensitive A375 tumor cells. Furthermore, A375-BRAFIR cells but not their sensitive counterparts expressed the active form of ATM/chk2

under steady state (Fig 2A). The ATM activities in sensitive A375 cells were maximized 12h after PLX-4720 stimulation or increased in a dose-dependent manner, but activation levels remained constant in A375-BRAFIR cells at all concentrations and time course of the treatment (Supplemental Fig 2B). Several siRNA-mediated targeting of ATM genes suppressed integrin-αvβ3 expression in various therapy-resistant but not naïve melanoma cells (A375, K008, B16) and colon cancer cells (MC38) (Fig 2B and Supplemental Fig 3A), suggesting that constitutive activation of ATM-chk2-mediated DNA damage pathways is required for integrin-αvβ3 upregulation. The mRNA levels of integrin-αv and β3 were higher in A375-BRAFIR than A375 at steady state, and treatment with PLX-4720 increased the expression levels in sensitive A375 cells although less than A375-BRAFIR cells. Furthermore, treatment with ATM inhibitor significantly depressed integrin-αv and β3 expression in B16-BRAFIR (Supplemental Fig 4). These results indicated that ATM regulates integrin-αvβ3 expression at transcriptional levels. In stark contrast, the knockdown of ATR or p53 did not repress integrin-αvβ3 in A375-BRAFIR cells, indicating the specific role of an ATM-dependent, p53-independent cascade in integrin-αvβ3 expression (Fig 2C). Furthermore, integrin-αvβ3 expression in A375-BRAFIR cells was mostly depressed with NF-κB inhibitor BAY11-7082 at similar extent to ATM inhibitor KU55933, consistent with previous findings that genotoxic stress-mediated activation of ATM was linked with the NF-κB signaling cascade, which serves as a transcriptional activator of

integrin-αv (33) (Fig 2D).

To further define the role of DNA damage pathways in modulating integrin-αvβ3 expression, peripheral blood leukocytes from ATM-knockout or wild-type mice were exposed to γ-irradiation, and integrin-αv expression was evaluated on apoptotic (annexin-V+) and surviving (annexin-V-) populations, in which the proportion of apoptotic cells was similar in ATM-deficient and wild-type cells (Fig. 2E). Integrin-αv expression was upregulated on surviving leukocytes from wild-type but not ATM-deficient mice, but this induction was abrogated in apoptotic leukocytes from both wild-type and ATM-knockout mice (Fig 2E). These results indicate that the ATM-dependent DNA damage pathway was necessary for triggering integrin-αvβ3 without oncogenic activation or transformation. We next evaluated the involvement of DNA damage signals in the *in vivo* antitumor activities of BRAF kinase inhibitor. For this purposes, we utilized B16 melanoma stably transfected with mutant V600E BRAF (B16-V600E) because murine melanoma cells including B16 rarely bear V600E BRAF activating mutations. We confirmed that B16-V600E conferred sensitivities to BRAF kinase inhibitors (Supplemental Fig 1). B16-V600E cells were further stimulated with PLX-4270 to create their drug-resistant variant (B16-BRAFIR). The concurrent administration of anti–integrin-αv mAb triggered apoptotic cell death in B16-BRAFIR cells but less than in B16-V600E treated with PLX-4720 alone. The specific ATM inhibitor (KU53955)

markedly decreased apoptosis in B16-BRAFIR treated by PLX-4720 with or without anti-integrin-αv mAb (Supplemental Fig 5). These results demonstrated that inhibition of tumor-derived integrin-αvβ3 conferred anticancer agents with the ability to kill chemoresistant tumor cells. Furthermore, PLX-4720 exhibited little antitumor effect *in vivo* on B16-BRAFIR, but concurrent administration of neutralizing anti-integrin-αv mAb with PLX-4720 significantly reduced the tumor burden in C57BL/6 mice (Fig 2F). However, PLX-4720 was sufficient to suppress ATM-deficient B16-BRAFIR tumor growth without integrin-αv mAb treatment. In either case, integrin-αv mAb alone was insufficient to trigger antitumor activities against B16-BRAFIR (Fig 2F). The *in vivo* resistance to anticancer agent and its reversal by anti-integrin-αv mAb or ATM inhibition was also confirmed in MC38 colon cancer cells resistant to the chemotherapeutic drug CPT-11 (MC38-CPT11R) (Fig 2G), in which integrin-αvβ3 was upregulated in an ATM-dependent manner (Fig 2B). Together, these results demonstrate that integrin-αvβ3 is important for triggering anticancer drug resistance in tumor cells *via* the ATM-chk2-mediated pathway.

Therapy-resistant tumors suppress host antitumor immunity in an integrin-αvβ3-dependent manner

The activation of DNA damage signals causes proinflammatory cytokine secretion from

senescent tumor cells (14, 34) and activates innate immune signals through induction of the stress-related molecules such as NKG2D ligands (13). Therefore, we evaluated the role of integrin-αvβ3 triggered by DNA damage signals in host antitumor immune responses. For this purpose, B16-BRAFIR were injected subcutaneously into C57BL/6 mice or immunodeficient NOD-SCID mice, and treatments with PLX-4720 and anti-integrin-αv mAb were given to each mouse with or without T cell depletion using anti-CD4- and CD8-specific Abs. The antitumor effect mediated by PLX-4720 and anti-integrin-αv mAb was significantly reduced by T cell depletion in wild-type C57BL/6 wild type, but not in NOD-SCID mice (Fig 3A). Consistent with a key role for adaptive immunity in the generation of long-lived and specific protective immunity, mice that completely eradicated B16-V600E tumors by the PLX-4720 and anti-integrin-αv mAb rejected a subsequent lethal challenge with B16 melanoma but not unrelated MC38 colon carcinoma, suggesting that the integrin-αvβ3 blockade contributed to the induction of immunological memory responses (Supplemental Fig 6).

To further define whether ATM regulation of tumor integrin-αvβ3 is responsible for growing tumors in an T cell-dependent manner, C57Bl/6 mice were challenged with B16-BRAFIR transfected with siRNA vectors specific for integrin-β3 or ATM, and PLX-4720 was administered to each group with or without T cell depletion. The tumor suppressive activities of PLX-4720 were potent against B16-BRAFIR transfected with integrin-β3 or ATM siRNAs, and

we confirmed that these siRNAs efficiently suppress integrin-αvβ3 expression (Supplemental Fig 3B). However, T cell depletion largely abrogated the antitumor effects mediated by integrin-β3 or ATM knockdown (Fig 3B and Fig 3C). These results further validate that ATM-mediated regulation of tumor integrin-αvβ3 is responsible for impeding antitumor responses of PLX-4720 by T cell-mediated mechanisms.

We next evaluated whether integrin-αvβ3 affected host immune phenotypes in tumor microenvironments. Anti-integrin-αv mAb therapy decreased the percentage of $CD4^+$ cells expressing IL-10 and Foxp3 (Fig 4A) and increased the frequencies of IL-17^+ and IFN-γ^+IL-17^+ $CD4^+$ T cells, which have been recently identified as key mediators of antitumor activities (35) (Fig 4B). The treatments also enriched the frequencies of total and activated $CD8^+$ T cells ($CD44^{hi}CD62L^{low}$) in tumor-draining lymph nodes (TDL) (Fig 4C). These activated phenotypes were not observed in splenocytes or non-tumor-draining lymphocytes in the treatment with PLX-4720 and RMV-7, indicating that tumor microenvironments trigger immune tolerance in an integrin-αvβ3-dependent manner (data not shown). The treatment with PLX-4720 resulted in high levels of IL-12 and IFN-γ, but showed little capacity to produce IL-10, in TDL isolated from established B16-BRAFIR cells transfected with the ATM or integrin-β3 siRNA (Fig 4D).In contrast, PLX-4270 or anti-integrin-αv mAb alone had no effect on IFN-γ secretion and $Foxp3^+$ Treg cell frequencies in TDL when PLX-4270-sensitive B16-V600E tumors were

targeted (Supplemental Fig 7). These results demonstrate that integrin-αvβ3 plays a critical role in suppressing antitumor immunity against therapy-resistant tumors.

Integrin-αvβ3 on therapy-resistant tumor cells targets dendritic cells to induce immune tolerance

Since integrin-αvβ3 elicits multiple effects on host immune functions (19-21), we next examined the effect of integrin-αvβ3 on the functions of dendritic cells, which serve as strong antitumor adjuvants (36). We found that murine bone marrow-derived dendritic cells (BMDCs) manifested a substantial uptake of live therapy-resistant B16-BRAFIR cells, which was mostly abrogated by the integrin-αvβ3 blockade, but the uptake of apoptotic B16-BRAFIR cells was not affected by the integrin-αvβ3 (Fig 5A). Immunofluorescence microscopy confirmed the uptake of live B16-BRAFIR cells by BMDCs, whereas the naïve B16-V600E cells were not recognized (Supplemental Fig 8). Human monocyte-derived dendritic cells (MoDCs) also have the ability to engulf live Jurkat cells transfected with integrin-αvβ3, but not control plasmid (Fig 5B and 5C). Moreover, the integrin-β3 knockdown of tumor cells, but not MoDCs, by specific siRNA resulted in impaired DC engulfment of viable integrin-αvβ3-expressing Jurkat cells (Supplemental Fig 9), suggesting that the tumor-derived integrin-αvβ3 was responsible for triggering DC uptake of viable tumor cells. The live epithelial tumor cells isolated from pleural

effusions of NSCLC patients were also engulfed by MoDCs from the same donor in an integrin-αvβ3-dependent manner, whereas apoptotic cells were recognized by MoDCs irrespective of the integrin-αvβ3 blockade (Fig 5D). Collectively, these results demonstrate that tumor-derived integrin-αvβ3 facilitates DC engulfment of viable tumor cells.

Antigen-presenting cells exploit multiple receptors and secreted proteins to affect the clearance of dying cells (37-39). Since RGD sequence in several ligands, such as MFG-E8 and osteopontin (OPN), serves as a target recognized by integrin-αvβ3, we examined the role of RGD motifs in DC recognition of live resistant tumor cells. The inhibition of RGD recognition with cyclic RGD peptide, but not control RGE peptide, partially reduced the DC uptake of live integrin-αvβ3-expressing Jurkat cells (Supplemental Fig 10). These results demonstrated that integrin-αvβ3 acts on the ligands bearing RGD sequence to promote live tumor cell engulfment. However, the addition of various neutralizing Abs, such as anti-MFG-E8 Ab and anti-OPN mAb, had little inhibitory effects on the live tumor cell phagocytosis (Supplemental Fig. 11).

The uptake of apoptotic cells by phagocytes has been known to influence various immune functions, such as T helper cell polarization, antigen processing, presentation and the generation of antigen-specific CTL (40). Thus, we next examined the immunogenic consequences of DC engulfment of live therapy-resistant tumor cells. The recognition of live A375-BRAFIR cells, but not apoptotic one, resulted in a marked down-regulation of CD11c (Supplemental Fig 12),

and reduced levels of IL-6 and IL-12p40 in MoDCs, which were restored by the integrin-αvβ3 blockade (Fig 5E). Furthermore, MoDCs loaded with live integrin-αvβ3-expressing Jurkat cells induced tolerogenic Foxp3 ^{high} and IFNγ / IL-17 ^{low} $CD4^+$ T cells, whereas those loaded with control Jurkat cells generated $Foxp3^{low}$ IFNγ / IL-17 ^{high} populations in $CD4^+$ T cells (Supplemental Figure 13).

As DCs serve as sentinels to cross-present immunogenic antigens for T cell activation by capturing dying tumor cells, we examined the impact of live tumor cells on cross-presentation of immunogenic targets by DCs. To do so, we utilized B16-V600E engineered to express OVA (B16-V600E.OVA) and C57BL/6 mice harboring a transgenic TCR specific for an MHC class I-restricted OVA (OT-I). The uptake of apoptotic B16-V600E.OVA cells, irrespective of the anti-integrin-αv blockade, enhanced DC stimulation of OT-I transgenic $CD8^+$ T cells. In contrast, the uptake of live B16-V600E.OVA cells resistant to PLX-4720 (B16-V600E.OVA-BRAFIR) resulted in a reduced activation of OVA-specific $CD8^+$ T cells, which was restored by the blockade of integrin-αbβ3 (Fig 5F). Collectively, these findings suggest that therapy-resistant tumor cells compromise antitumor CTL responses by facilitating the engulfment of live tumor cells in an integrin-αvβ3-dependent manner.

Integrin-αvβ3 on therapy-resistant tumors is responsible for impeding the antitumor immunogenicity of DC vaccines

Although DCs have been considered to be a potent adjuvant for triggering antitumor immunity, the clinical effects of dendritic cell-based vaccination remain dismal (36). To elucidate whether the integrin-αvβ3-mediated recognition of live tumor cell may impede antitumor immunogenicity of DCs, immature BMDCs loaded with viable live or apoptotic B16-BRAFIR cells were used as vaccine adjuvant against established B16-BRAFIR tumors in combination with the BRAF kinase inhibitor PLX-4720. The treatment with PLX-4720 was ineffective against established B16-BRAFIR tumors in combination with DCs loaded with live B16-BRAFIR cells, whereas the vaccination of DCs loaded with apoptotic B16-BRAFIR augmented substantial antitumor effects on PLX-4720. In contrast, the concurrent administration of RMV7 with PLX-4720 considerably diminished tumor burdens of B16-BRAFIR even with DCs loaded with live B16-BRAFIR cells (Figure 6A). Moreover, PLX-4720 alone was sufficiently reduced B16-BRAFIR tumor burden when DCs loaded with either live or apoptotic integrin-β3-deficient B16-BRAFIR tumor cells were vaccinated two days before tumor challenge (Fig. 6B)

Collectively, these findings validate the importance of integrin-αvβ3 in restraining protective immunity through compromised antitumor immunogenicity of DCs.

Discussion

Recent evidence has unveiled the molecular events leading to anticancer drug resistance. Genetic and epigenetic modifications rendered tumor cells with the abilities to activate alternative oncogenic pathways, trigger chromatin modulation, or acquire functions associated with stem cell activities (2-4, 27-29). These events represent key steps in protecting the surviving tumor populations from complete eradication by lethal insults. In addition to direct cytotoxic and proapoptotic effects, anticancer agents also regulate tumor microenvironments, leading to conditions that determine the balance between tumor-promoting and -suppressing responses (5, 6). For example, subsets of cytotoxic agents activate antitumor immune responses through induction of multiple types of innate systems and cytokine-mediated senescence (18, 41-43). In contrast, chemotherapy also creates conditions for tumor survival in part through paracrine secretion of IL-6 and TIMP-1 from endothelial cells in a DNA damage-dependent manner (5). From these perspectives, the interplay between tumor cells and their environments should be critical in determining the mode of host immunity in the settings of therapeutic interventions.

Here we provide the first evidence that therapy-resistant tumors adopt a novel strategy to suppress antitumor immunosurveillance by triggering integrin-αvβ3 on tumor cells. The chronic

activation of DNA damage signals mainly mediated by the ATM-chk2 pathway is responsible for upregulating integrin-αvβ3 expression on tumor cells. The tumor-derived integrin-αvβ3 rendered DCs with ability to engulf live tumor cells. The live cell phagocytosis resulted in reduced immunogenicity and cross-priming of DCs, leading to impairment of tumor-specific adaptive responses. The blockade of integrin-αvβ3 either with specific antibodies or tumor-specific knockdown promoted efficient tumor recognition by the host immune system, thus overcoming the resistant niche created by therapy-resistant tumors. Altogether, these findings demonstrate that the ATM-integrin-αvβ3 axis confers drug-resistant tumor cells with the ability to circumvent endogenous immunosurveillance at tumor microenvironments (Fig 6C). The significance and pathological relevance for anticancer drug-resistant tumor cells to evade from antitumor immunosurveillance remain unclear in the present study, but we speculate that these cells have evolved to protect themselves from multiple defense mechanisms against tumorigenesis, including endogenous immune systems. Thus, these multifaceted properties of therapy-resistant tumor cells may contribute to further progression and worse prognosis, although further elucidation is required to prove this hypothesis.

DNA damage checkpoint machineries serve as barriers to tumorigenesis mainly by p53-dependent mechanisms (9-12). Here, we delineate some unexpected aspects of DNA damage signals on anticancer therapy resistance: they suppress antitumor immunity by inducing

integrin-αvβ3 on tumor cells in the setting of a chemoresistant niche. Thus, although DNA damage signals are generally linked with p53-dependent regulation to trigger senescence and apoptosis, intrinsic and environmental regulation of therapeutic responses may change the genetic profiles of tumor cells to evade “classical” DNA damage pathways. Indeed, we found that unique genotoxic stress-induced signals are required for triggering integrin-αvβ3 on therapy-resistant tumor cells by ATM-dependent, but p53-independent mechanisms (Fig. 2D). Intriguingly, previous reports unveiled novel pathways in which ATM stimulates NF-κB signaling cascade in response to genotoxic stimuli such as cytotoxic drugs and irradiation (30). Furthermore, these ATM-NF-κB pathways could mediate cell survival independently of p53-mediated effector mechanisms (44, 45). Since NF-κB has been established as a critical sentinel linking inflammation with carcinogenic process, it will be of great interest to determine whether ATM-dependent DNA damage responses activated by tumor cells may be associated with the activation of key carcinogenic inflammatory pathways at chemoresistant niche, further promoting tumorigenic and metastatic potential of therapy-resistant tumors.

Another novel mechanism clarified in this study is that integrin-αvβ3 on therapy-resistant tumors mediates immune tolerance by facilitating the DC engulfment of live tumor cells. The DC uptake of live tumor cells is mediated by ligands bearing RGD-motif, although MFG-E8 and OPN had little role in this process. These results demonstrate that tumor-derived integrin-

αvβ3 uses a distinct system to recognize live tumors from those used to recognize apoptotic cells, although further studies are needed to identify the specific ligand for the integrin-αvβ3. Although live microbes are sensed by DCs through recognition by specialized phagocytic receptors (46), there has been no literature indicating that DCs have the capacity to engulf viable transformed cells. In this regard, integrin-αvβ3 induction on therapy-resistant tumors serves as a novel strategy for compromising DC immunogenicity and antitumor immunosurveillance. Whether live tumor cells, like dying cells, are digested and processed via phagolysosomal pathways in DCs remains obscure, although we did not observe digested forms of chemoresistant tumor cells in DCs in long-term in vitro culture or infiltrating into tumors in vivo (unpublished observation). Further studies should address the intracellular dynamics in DCs whereby tumor cells interrupt their cross-priming machineries.

In summary, we identified integrin-αvβ3 as an indispensable factor for creating immune evasion systems in a chemoresistant niche. Although recent clinical development of integrin-αv inhibitors produced significant benefits against advanced gliomas (22), it is critical to clarify the optimal conditions in which particular types of anticancer drugs should be combined with integrin-αvβ3 blockade (23). As subsets of cytotoxic drugs may be classified as inducers of "immunogenic cell death" (41, 42), it is possible that the therapeutic options for boosting host immunity, including chemotherapy, radiotherapy, and/or immunotherapy, may maximize the

antitumor activities of integrin-αvβ3 inhibitors in patients with advanced stages of malignancies.

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Figure legends

Figure 1.

High expression of integrin-αvβ3 in tumor cells that acquired resistance to anticancer drugs. (A) The cell surface expression of integrin-αvβ3 (green line) was examined in K029 melanoma cells with acquired BRAF-kinase inhibitor resistance (K029-BRAFIR), and HCT116 colon carcinoma cells and HCT116 with acquired anti-EGFR mAb resistance (HCT116-EGFRIR). The staining with isotype-matched Ig was overlaid as filled histograms. (B) The percentages of integrin-αvβ3 positive cells were evaluated by flow cytometry in various tumor cell lines and their drug resistant variants: K029-CDDPR; cisplatin-resistant variant of K029, A375-BRAFIR; BRAF inhibitor-resistant variant of A375 human melanoma cells, MCF7-HER2IR; Her2 inhibitor (Herceptin)-resistant variant of MCF7 human breast cancer cells, Hep3B-SorafenibR; multi-kinase inhibitor sorafenib-resistant variant of Hep3B human hepatoma cells, PC3; EGFR kinase inhibitor (gefitinib)-sensitive human lung cancer cells, NCI-H1975; gefitinib-resistant human lung cancer cells, B16-BRAFIR; BRAF inhibitor resistant variant of murine B16.V600E melanoma cells; MC38-EGFRIR or MC38-CPT11R; EGFR inhibitor (AG494) or CPT-11-resistant variant of MC38 murine colon cancer cells. * $p<0.05$. (C) The $EpCAM^+$ epithelial tumors isolated from patients with non-small cell lung carcinomas who received multiple cycles

of chemotherapy (TX) or not (non-TX) were evaluated for integrin-αv expression by flow cytometry. Similar results were observed in three experiments.

Figure 2.

DNA damage signals induce integrin-αvβ3 expression in therapy-resistant cells. (A) Immunoblots for phospho-chk2 (Thr168), chk2 and β-actin on lysates from A375 melanoma cells or those resistant to PLX-4720 (A375-BRAFIR), after exposure to PLX-4720 for 1 h. (B) Various melanoma cells or their therapy-resistant counterparts (A375-BRAFIR, K008-DTICR, B16-BRAFIR), as well as MC38 colon cancer cells or those resistant to CPT-11 (MC38-CPT11R) were infected with scrambled siRNA (scr) or siRNA specific for chk2 or ATM, and integrin-αvβ3 expression was examined by immunoblot (left) or flow cytometry (right). (C) A375-BRAFIR were infected with siRNA specific for scramble sequences (scr), ATM, ATR or p53 and examined for integrin-αvβ3 expression by flow cytometry. (D) A375-BRAFIR was treated with inhibitors for MEK1 (U0126), PI3K (Ly294002), p38 (SB203580), NF-κB (BAY11-7082) or ATM (KU55933) for 24 h, and the integrin-αvβ3 expression was examined by flow cytometry. (E) Peripheral blood leukocytes were isolated from ATM knockout (ATM KO) or wild-type (WT) mice, and exposed to γ-irradiation (10Gy). The cells were subdivided into annexin-V positive (+) or negative (-) populations, and subjected to staining with integrin-

αvβ3 mAb (filled histograms) or isotype control IgG (white histograms) in each group. Representative data (left) or those from three independent experiments (right) are presented. * $p<0.05$ (F, G) PLX-4720-resistant variant of B16 bearing a BRAF V600E mutation (B16-BRAFIR) (F) or MC38-CPT11R (G) were stably transfected with lentivirus encoding siRNAs for ATM gene (ATM siRNA) or not (control), and inoculated subcutaneously into wild-type C57BL/6 mice (n=4 per group). The established tumors were treated with PLX-4720 (F), CPT-11 (G) and/or anti-integrin-αv neutralizing antibody (RMV7) on days 10, 12, 14 and 16 after tumor challenge, and tumor growth was measured on the indicated days. Similar results were obtained in at least three experiments. *: $p<0.05$, ns: not significant.

Figure 3.

Integrin-αvβ3 on therapy-resistant cells suppresses T cell-mediated antitumor immunity. (A) C57BL/6 (WT) or NOD-SCID mice harboring established B16-BRAFIR melanomas (25 mm^2) (n=4 per group) were treated with systemic PLX-4270 and RMV-7 and tumor diameters measured on the indicated days. In some mice, T cell depletion was performed by depletion Abs for anti-CD4 and anti-CD8 three days before tumor challenge. (B, C) B16-BRAFIR melanomas were infected with siRNA lentivirus vectors specific for integrin-β3 (integrin-β3 siRNA), ATM (ATM siRNA) or scrambled control genes (control siRNA), and inoculated into C57Bl/5 mice

(n=4 per group) along with PLX-4720 with or without pretreatment with CD4- and CD8-depletion Abs. Results are representative of two independent experiments. *; $p<0.05$, ns; not significant.

Figure 4.

Integrin-αvβ3 impede host immune responses at tumor microenvironments. (A, B) Tumor-draining lymph nodes (TDLs) were harvested from mice bearing B16-BRAFIR tumors 5 d after the indicated treatment. $CD4^+$ T cells from the TDLs were gated as $CD3^+CD4^+$ cells, and assayed for (A) IL-10 and Foxp3 and (B) IFN-γ and IL-17 by flow cytometry (numbers in quadrants are percentages). The means ± SEM from six mice per group are shown in the adjacent bar graph. (C) $CD8^+$ T cells from the TDL were also gated as $CD3^+CD8^+$ cells, and assayed for $CD8^+$ T cell frequency and CD44 and CD62L expression by flow cytometry. Representative staining patterns are presented. (D) The mice bearing established B16.V600E or B16-BRAFIR tumors transfected with control, ATM or integrin-β3 siRNA lentivirus vectors were treated with PLX-4720. Draining lymph node cells were assayed for the production of IL-12p40, IL-10 and IFN-γ in the culture supernatants. Each experiment was independently performed four times with similar results. * $p<0.05$

Figure 5.

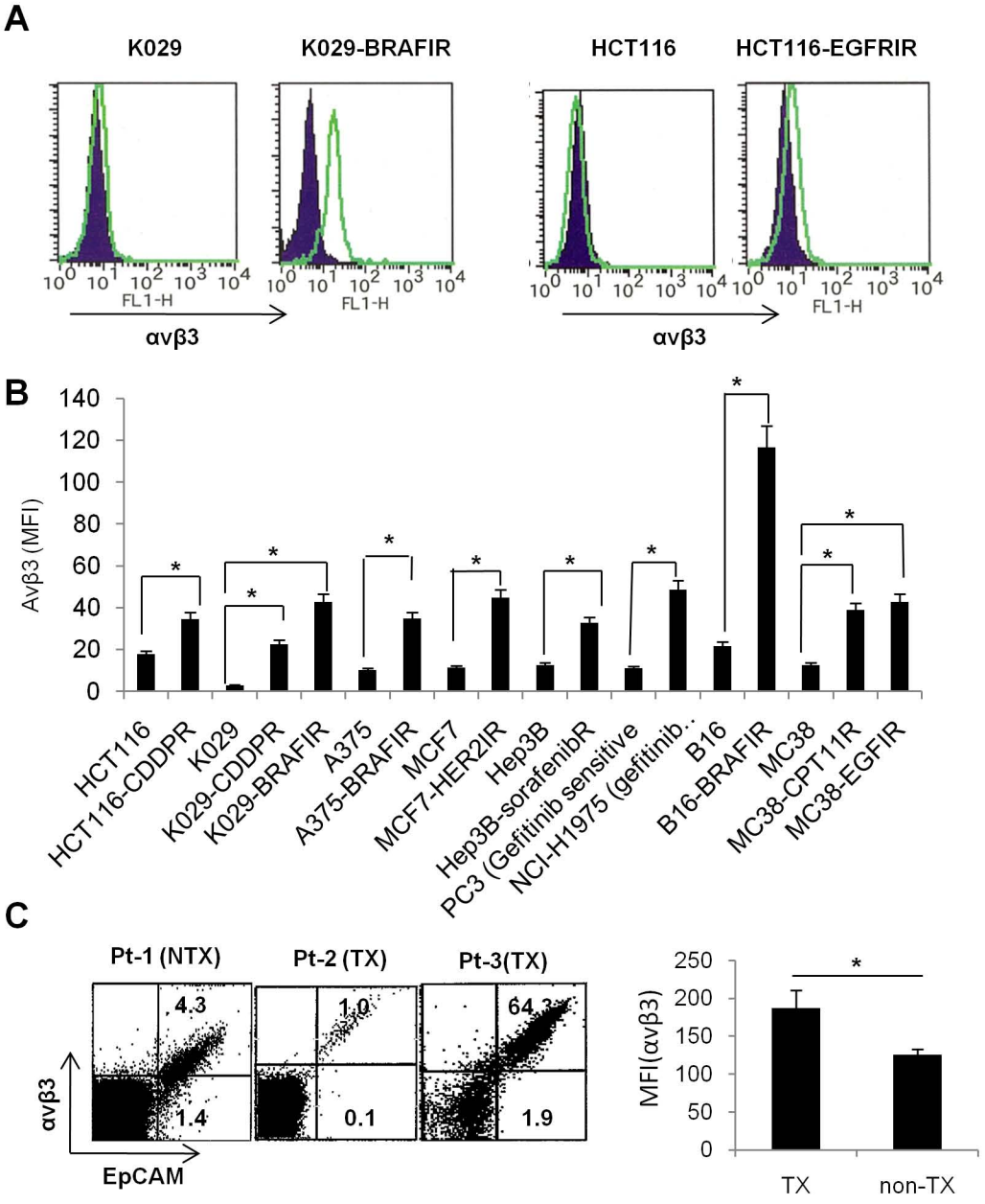
Integrin-αvβ3 regulates the dendritic cell phagocytosis of live therapy-resistant tumor cells. (A) PKH26-labeled B16-BRAFIR cells were untreated or exposed to high-dose γ-irradiation (50 Gy) to induce apoptotic cell death. The untreated live or irradiated apoptotic cells were incubated with BMDCs in the presence of isotype-matched control IgG -or RMV-7 for 4 h. The uptake of B16-BRAFIR cells by CD11c+BMDCs was quantified by flow cytometry. Representative dot plots (above) and a graph of data from three experiments (below) are shown. * $p<0.05$ (B, C) Live Jurkat lymphoma cells transfected with control or integrin-αvβ3 plasmid were labeled with PKH26, and added to alexa488-labeled CD11c+MoDCs from 2 donors (MoDC-1 and MoDC-2) for 4 h. The uptake of Jurkat cells by CD11c+DCs was quantified by flow cytometry (B) or visualized by fluorescence microscopy (Magnification, x200) (C). The percentages of cell uptake (left) are shown as a PKH-$26^{+}$$CD11c^{+}$ fraction within the total CD11c population. The engulfment of tumor cells was visualized by immunofluorescence microscopy. (D) The $EpCAM^{+}$ epithelial tumor cells isolated from pleural effusion of a NSCLC patient were untreated (Live) or exposed to γ-irradiation to induce apoptosis (Apoptosis). The live or apoptotic cells were labeled with PKH26, and added to MoDCs of the same donors in the presence of isotype-matched control Ig or anti-integrin-αvβ3 (LM609) for 4 h. Uptake of the cells was evaluated by flow cytometry. * $p<0.05$ (E) MoDCs were loaded with apoptotic or live

A375-BRAFIR melanomas (A375R) in the presence of control Ab or LM609. IL-6 and IL-12 in culture supernatant were quantified by ELISA. (F) BMDCs loaded with PKH26-labeled B16-V600E.OVA cells or those resistant to PLX-4720 (B16.OVA-BRAFIR) were co-cultured with OVA-specific TCR- transgenic $CD8^+$ T cells in the presence of isotype-matched control IgG or RMV-7, and IFNγ production in TCR-β5.1-positive cells was evaluated by flow cytometry. Similar results were obtained from three experiments, and the means ± SEM are shown. *; $p<$ 0.05, np; not significant.

Figure 6

(A). Integrin-αvβ3 regulates the antitumor effects of DC vaccines. B16-BRAFIR cells were injected subcutaneously into C57Bl/6 mice (n=4 per group) in a mixture with BMDCs loaded with live or apoptotic B16-BRAFIR cells. Mice were also treated with PLX-4720 and RMV7 on days 3, 6, 9, 12 and 15. Tumor growth was measured on the indicated days. (B). BMDCs were loaded with B16-BRAFIR melanomas infected with siRNA vectors specific for integrin-β3(integrin-β3 siRNA) or scrambled control genes (control siRNA), and used as vaccines along with PLX-4720 against established B16-BRAFIR tumors in C57Bl/6 mice (n=4 per group). Results are representative of two independent experiments. *; $p<0.05$, ns; not significant compared to unloaded DC vaccines. (C). A model for integrin-αvβ3-mediated immune

suppression whereby ATM induction of αvβ3on chemoresistant tumors facilitate phagocytosis of live tumor cells, leading to impaired antigen-processing and priming of specific antitumor CTL.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K029
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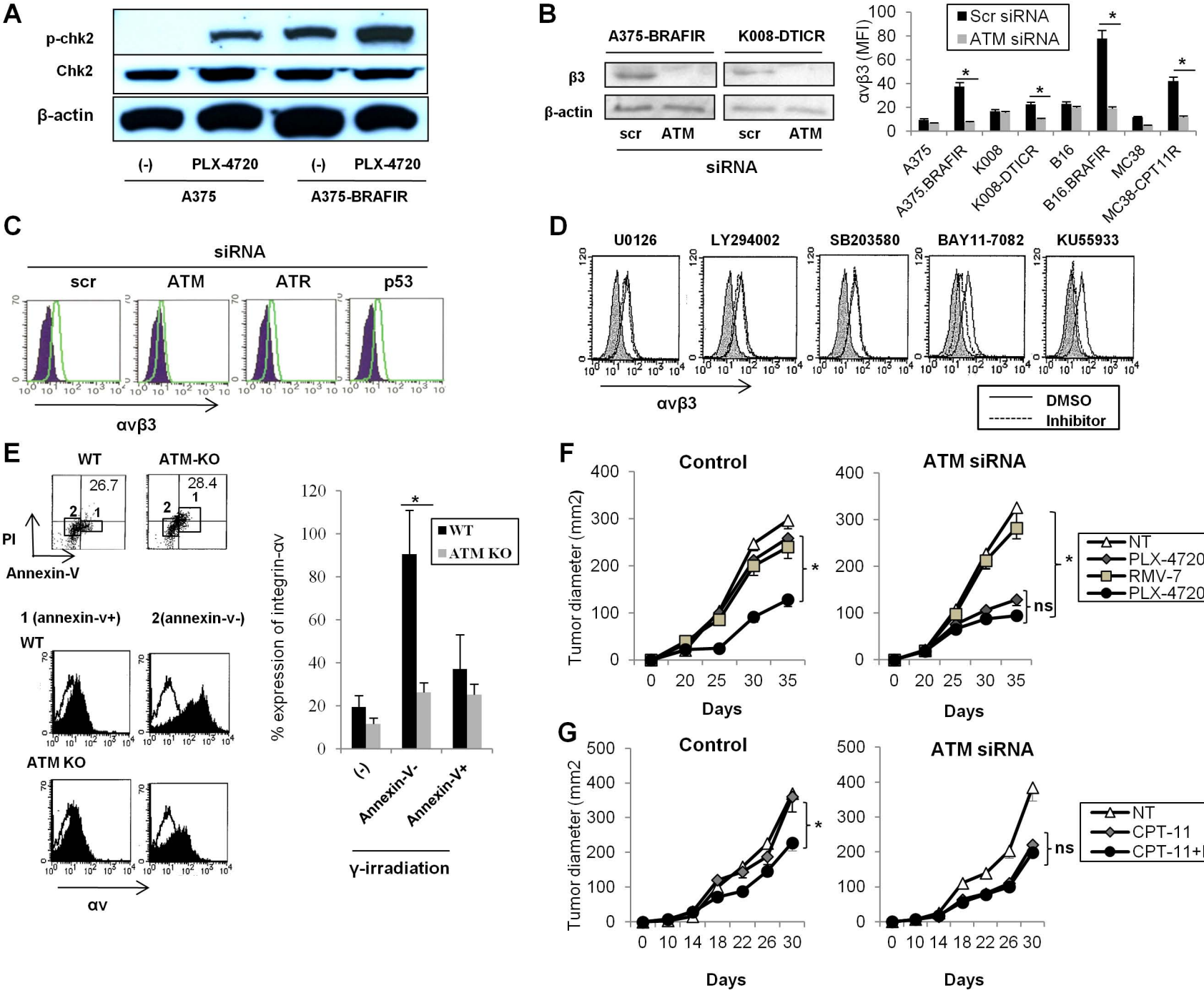
HCT116
HCT116-EGFRIR
FL1-H
αvβ3
B
Avβ3 (MFI)
HCT116
HCT116-CDDPR
K029
K029-CDDPR
K029-BRAFIR
A375
A375-BRAFIR
MCF7
MCF7-HER2IR
Hep3B
Hep3B-sorafenibR
PC3 (Gefitinib sensitive
NCI-H1975 (gefitinib..
B16
B16-BRAFIR
MC38
MC38-CPT11R
MC38-EGFIR
C
Pt-1 (NTX)
Pt-2 (TX)
Pt-3(TX)
4.3
1.4
1.0
0.1
64.3
1.9
αvβ3
EpCAM
MFI(αvβ3)
TX
non-TX

Figure 1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Chk2
β-actin
(-)
PLX-4720
(-)
PLX-4720
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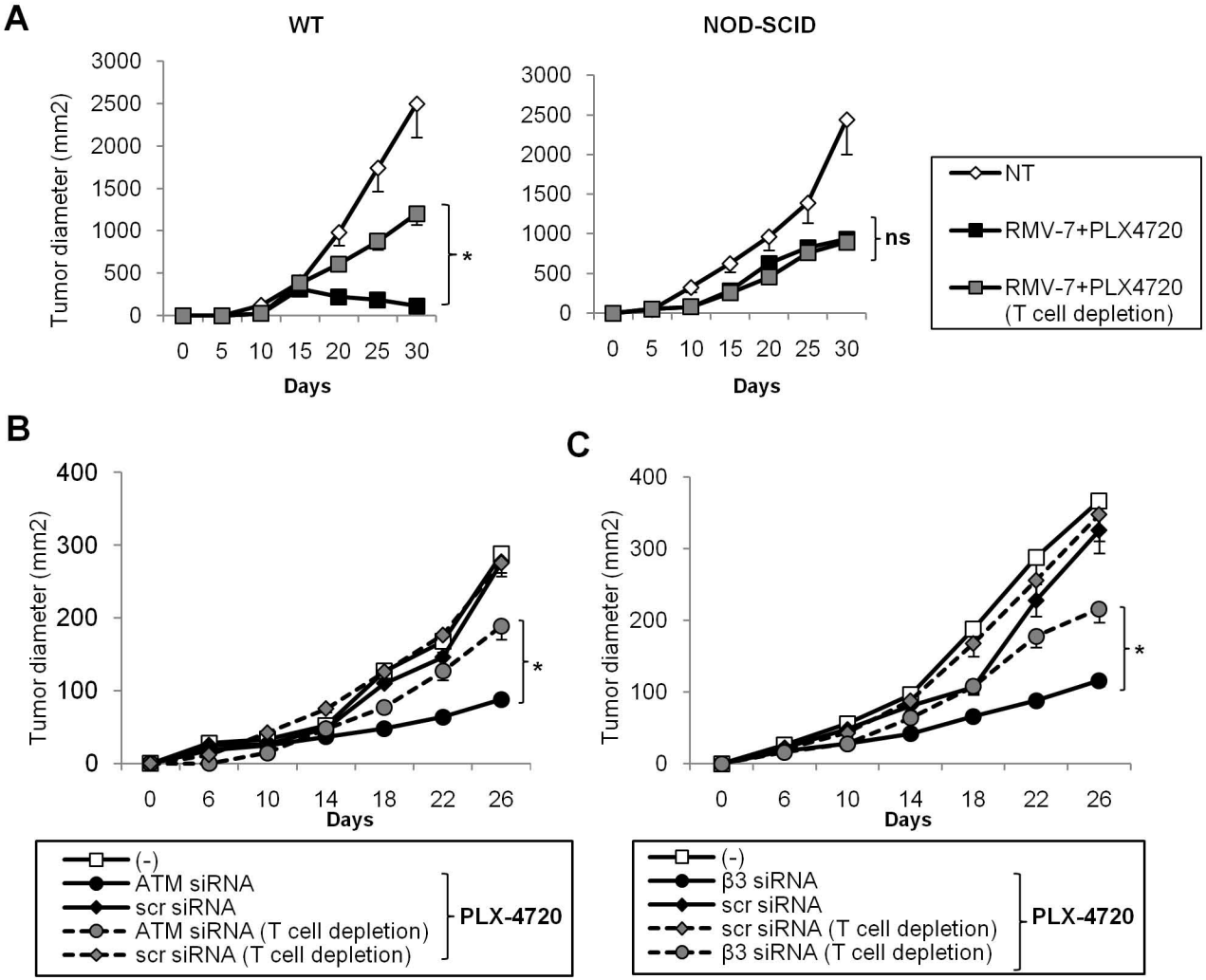
A375-BRAFIR
B
A375-BRAFIR
K008-DTICR
β3
β-actin
scr
ATM
scr
ATM
siRNA
Scr siRNA
ATM siRNA
αvβ3 (MFI)
A375
A375.BRAFIR
K008
K008-DTICR
B16
B16.BRAFIR
MC38
MC38-CPT11R
C
siRNA
scr
ATM
ATR
p53
αvβ3
D
U0126
LY294002
SB203580
BAY11-7082
KU55933
αvβ3
DMSO
Inhibitor
E
WT
ATM-KO
26.7
28.4
PI
Annexin-V
1 (annexin-v+)
2(annexin-v-)
WT
ATM KO
αv
WT
ATM KO
% expression of integrin-αv
(-)
Annexin-V-
Annexin-V+
γ-irradiation
F
Control
ATM siRNA
Tumor diameter (mm2)
Days
NT
PLX-4720
RMV-7
PLX-4720+RMV7
ns
G
Control
ATM siRNA
Tumor diameter (mm2
Days
NT
CPT-11
CPT-11+RMV-7
ns

Figure 2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WT
NOD-SCID
Tumor diameter (mm2)
Days
*
ns
NT
RMV-7+PLX4720
RMV-7+PLX4720
(T cell depletion)
B
C
(-)
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scr siRNA
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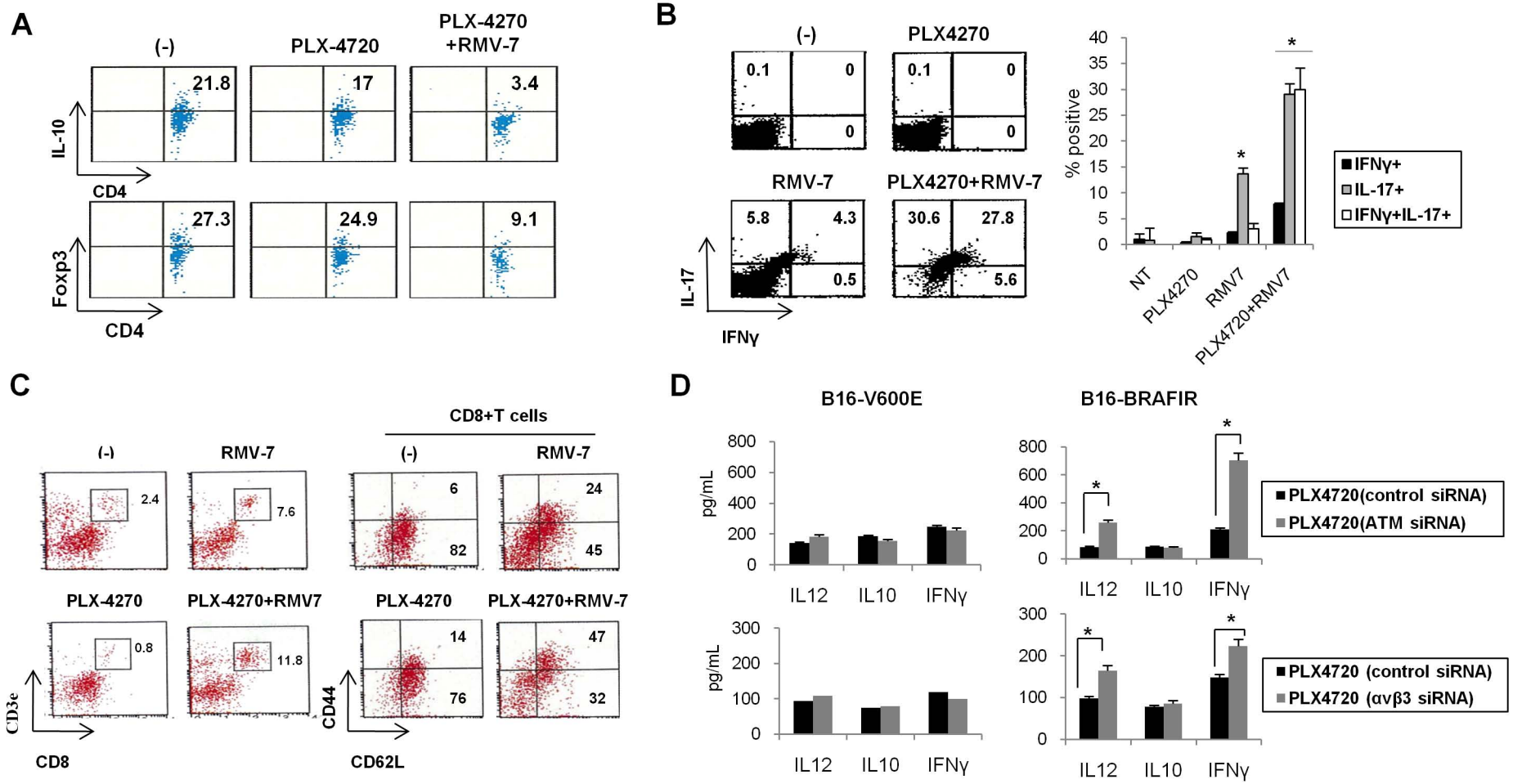
scr siRNA (T cell depletion)
PLX-4720
β3 siRNA
β3 siRNA (T cell depletion)

Figure 3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PLX-4720
PLX-4270 +RMV-7
21.8
17
3.4
IL-10
CD4
27.3
24.9
9.1
Foxp3
CD4
B
(-)
PLX4270
0.1
0
0.1
0
0
0
RMV-7
PLX4270+RMV-7
5.8
4.3
30.6
27.8
0.5
5.6
IL-17
IFNγ
% positive
40
35
30
25
20
15
10
5
0
*
*
NT
PLX4270
RMV7
PLX4720+RMV7
IFNγ+
IL-17+
IFNγ+IL-17+
C
CD8+T cells
(-)
RMV-7
(-)
RMV-7
2.4
7.6
6
24
82
45
PLX-4270
PLX-4270+RMV7
PLX-4270
PLX-4270+RMV-7
0.8
11.8
14
47
76
32
CD3e
CD8
CD44
CD62L
D
B16-V600E
B16-BRAFIR
pg/mL
800
600
400
200
0
IL12
IL10
IFNγ
*
*
*
PLX4720(control siRNA)
PLX4720(ATM siRNA)
pg/mL
300
200
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IL10
IFNγ
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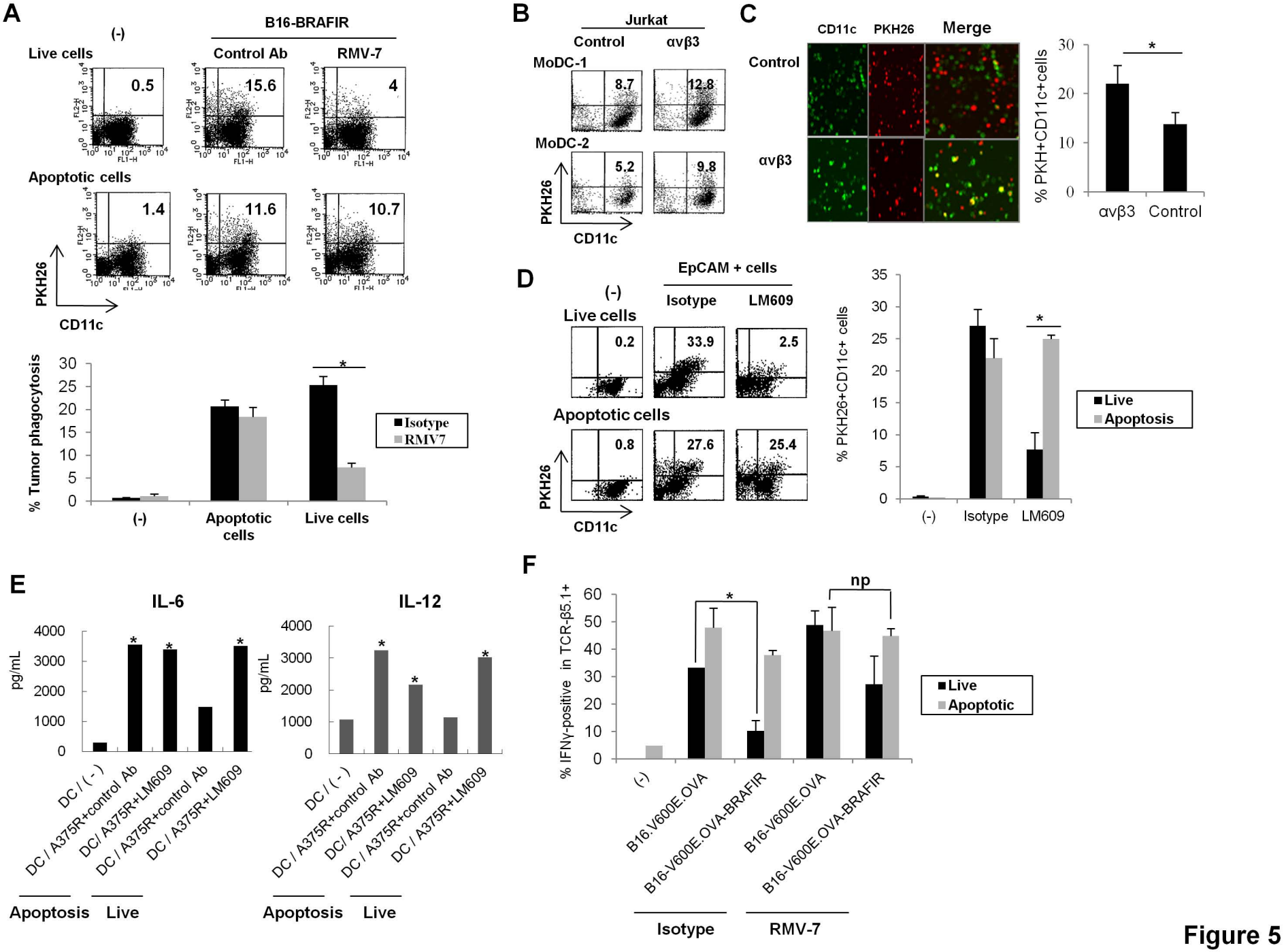
*
*
PLX4720 (control siRNA)
PLX4720 (αvβ3 siRNA)

Figure 4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(-)
B16-BRAFIR
Live cells
Control Ab
RMV-7
0.5
15.6
4
Apoptotic cells
1.4
11.6
10.7
PKH26
CD11c
% Tumor phagocytosis
Isotype
RMV7
(-)
Apoptotic cells
Live cells
B
Jurkat
Control
αvβ3
MoDC-1
8.7
12.8
MoDC-2
5.2
9.8
PKH26
CD11c
C
CD11c
PKH26
Merge
Control
αvβ3
% PKH+CD11c+cells
αvβ3
Control
D
EpCAM + cells
(-)
Isotype
LM609
Live cells
0.2
33.9
2.5
Apoptotic cells
0.8
27.6
25.4
PKH26
CD11c
% PKH26+CD11c+ cells
Live
Apoptosis
(-)
Isotype
LM609
E
IL-6
IL-12
pg/mL
DC / (-)
DC / A375R+control Ab
DC/ A375R+LM609
DC / A375R+control Ab
DC / A375R+LM609
Apoptosis
Live
F
% IFNγ-positive in TCR-β5.1+
np
Live
Apoptotic
(-)
B16.V600E.OVA
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B16-V600E.OVA
B16-V600E.OVA-BRAFIR
Isotype
RMV-7

Figure 5

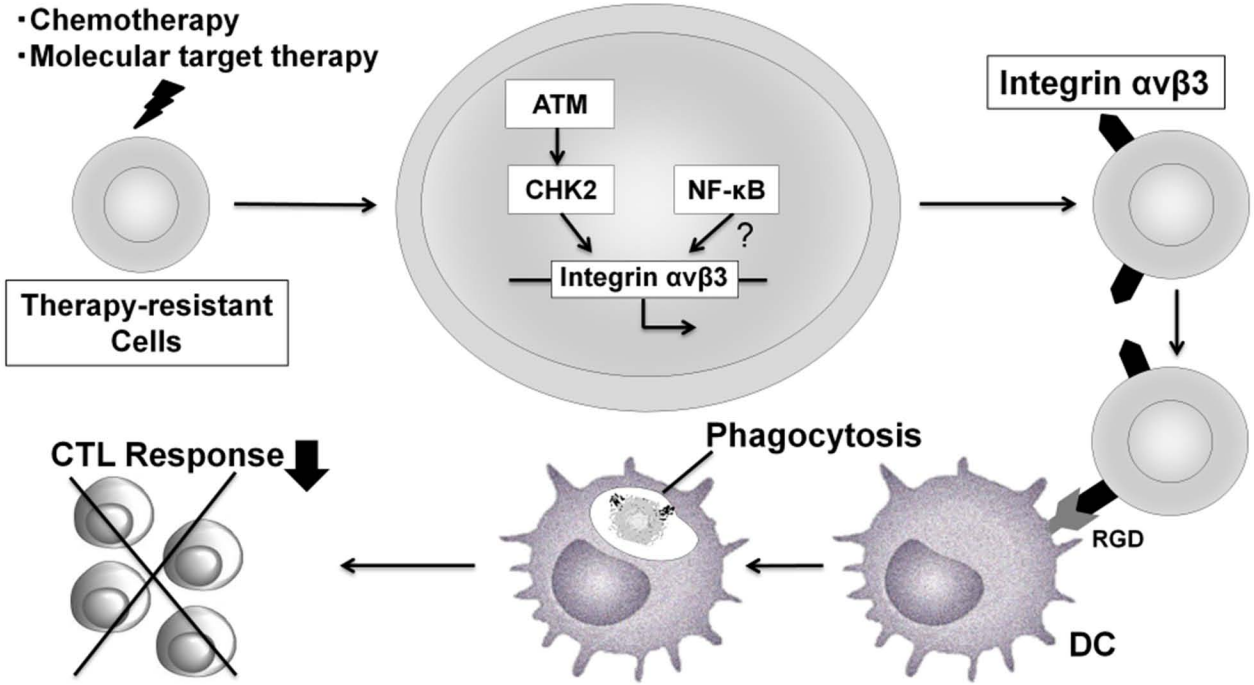
・Chemotherapy
・Molecular target therapy
Therapy-resistant Cells
ATM
CHK2
NF-κB
?
Integrin αvβ3
Integrin αvβ3
RGD
DC
Phagocytosis
CTL Response

Figure 6