

Targeting AKT Signaling Sensitizes Cancer to Cellular Immunotherapy

Patricia S. Hähnel,¹ Sonja Thaler,² Edite Antunes,³ Christoph Huber,² Matthias Theobald,³ and Martin Schuler¹

¹Department of Medicine (Cancer Research), West German Cancer Center, University Hospital Essen, Essen, Germany; ²Department of Medicine III, Johannes Gutenberg University, Mainz, Germany; and ³Department of Hematology and Van Creveld Clinic, University Medical Center Utrecht, Utrecht, the Netherlands

Abstract

The promise of cancer immunotherapy is long-term disease control with high specificity and low toxicity. However, many cancers fail immune interventions, and secretion of immunosuppressive factors, defective antigen presentation, and expression of death ligands or serpins are regarded as main escape mechanisms. Here, we study whether deregulation of growth and survival factor signaling, which is encountered in most human cancers, provides another level of protection against immunologic tumor eradication. We show in two models that activated cell autonomous protein kinase B (PKB)/AKT signaling mediates resistance against tumor suppression by antigen-specific CTLs *in vitro* and adoptively transferred cellular immune effectors *in vivo*. PKB/AKT-dependent immunoresistance of established tumors is reversed by genetic suppression of endogenous Mcl-1, an antiapoptotic member of the Bcl-2 family. Mechanistically, deregulated PKB/AKT stabilizes Mcl-1 expression in a mammalian target of rapamycin (mTOR)-dependent pathway. Treatment with the mTOR inhibitor rapamycin effectively sensitizes established cancers to adoptive immunotherapy *in vivo*. In conclusion, cancer cell-intrinsic PKB/AKT signaling regulates the susceptibility to immune-mediated cytotoxicity. Combined targeting of signal transduction pathways may be critical for improvement of cancer immunotherapies. [Cancer Res 2008;68(10):3899–906]

Introduction

Malignantly transformed cells acquire multiple mutations to evade the cell-intrinsic tumor suppressor mechanisms. This leads to the development of a phenotype, which tolerates the stresses imposed by limited growth and survival factors or replicative DNA damage (1). Concomitantly, cancer cells become resistant to cytotoxic agents and radiation, thus resulting in treatment failure and relapse (2). Immunotherapy is one strategy to control such resistant cancer cells, which is currently explored in multiple preclinical and clinical trials. Once an immune response has been mounted, cancer-reactive lymphocytes should provide long-term disease control (3). However, many cancers fail immunologic therapies. This could be explained by insufficient immune

recognition of tumor cells, as well as by resistance to immune effector mechanisms.

To this end, we reasoned whether deregulated growth and survival factor signaling, which is common to most if not all human cancers, contributed to the immune escape phenotype. Amplification of growth factor receptors, activating mutations of receptor tyrosine kinases or mediators of downstream signaling, inactivation or loss of negative regulators, and chromosomal translocations producing constitutively active, cancer-specific kinases are frequently observed and are currently addressed by molecularly targeted therapies (4, 5). It is well established that deregulations in signal transduction pathways confer protection against cytotoxic anticancer therapies. However, it is presently unknown whether this may also translate into resistance against immune-mediated tumor suppression.

Protein kinase B (PKB)/AKT, a central regulator of growth and survival, acts at the converging point of several signal transduction pathways involved in oncogenic processes. Activated PKB/AKT signaling is found in a wide range of human cancers and has been implied as an indicator of prognosis. Downstream targets of PKB/AKT include regulators of protein synthesis, cell cycle progression, and apoptosis, which make it an attractive target for therapeutic interventions in cancer (6–9). Here, we study the role of PKB/AKT signaling in evasion from immune-mediated tumor suppression. Our results suggest that cancer cell-intrinsic PKB/AKT signaling confers protection against immune effectors and thus interferes with the efficacy of cancer immunotherapies and possibly immune surveillance.

Materials and Methods

Plasmids, antibodies, and reagents. A cDNA encoding AKT-ER^{tam} was generated by PCR using pcDNA3.AKT-ER^{tam} (provided by J. Downward) as template and cloned into the retroviral vector plasmid pQCxIP (Clontech) and pMxIG (provided by T. Kitamura). All inserts were verified by sequencing. Lentiviral vectors encoding short hairpin RNA (shRNA) from the MISSION TRC-Mm 1.0 library were purchased from Sigma; clone TRCN0000004691 was used for Mcl-1 suppression, and clone TRCN0000004693 served as negative control. The following primary antibodies were used: actin (C4, ICN); AKT1/2 (H-136), Bcl-2 (C2), Mcl-1 (all from Santa Cruz); Bcl-xL (54H6), PTEN, GSK3- β (all from Cell Signaling Technology); p70 S6 kinase, p-p70 S6 kinase (T389; R&D Systems); caspase-9 (MAB4609; Chemicon). All other phosphoepitope-specific antisera were purchased from Cell Signaling Technology. For the *in vitro* kinase assay (Cell Signaling Technology), AKT-ER^{tam} immunoprecipitated from fibrosarcoma extracts was incubated with a GSK3- β fusion peptide following the manufacturer's instruction. ABT-737 was generously provided by Abbott; rapamycin (sirolimus) was purchased from Wyeth; staurosporine, tamoxifen, and 4-hydroxytamoxifen were purchased from Sigma.

Cell lines and coculture experiments. HLA-A*0201 transgenic murine embryonic fibroblast (MEF) were generated from Cya2k^b mice (10) and

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Requests for reprints: Martin Schuler, Department of Medicine (Cancer Research), West German Cancer Center, University Hospital Essen, Hufelandstrasse 55, 45122 Essen, Germany. Phone: 49-201-723-2000; Fax: 49-201-723-5924; E-mail: martin.schuler@uk-essen.de.

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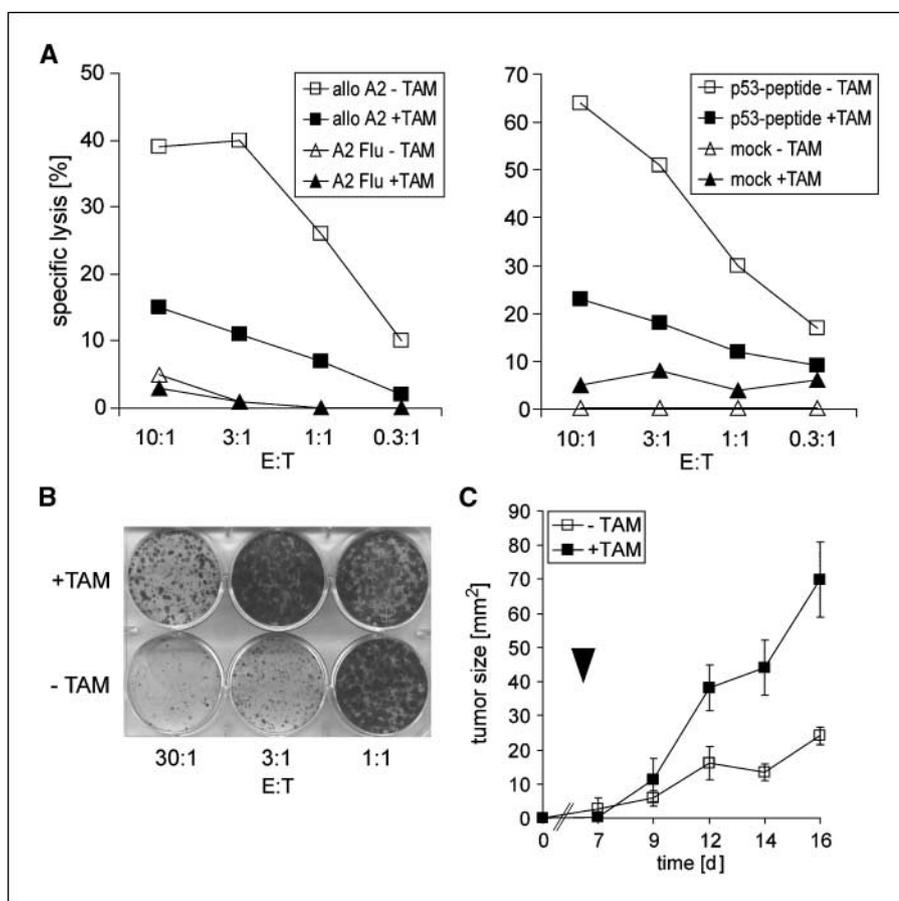


Figure 1. Withdrawal of PKB/AKT signaling sensitizes cancer cells to tumor suppression by cellular immune effectors. *A, left*, 5 h ^{51}Cr release assay of CyA2K^b-AKT-ER^{tam} MEFs coincubated with allo-A2K^b-reactive (allo A2; *boxes*) or HLA-A*0201/influenza matrix-reactive control CTL (A2 Flu; *triangles*); *right*, 5-h ^{51}Cr release assay of CyA2K^b-AKT-ER^{tam} MEFs loaded with the human p53 (264–272) peptide (*boxes*) or unloaded control cells (*triangles*) after coincubation with HLA-A*0201/human p53 (264–272)-specific CTL. Tamoxifen (*TAM*) was given (*closed symbols*) to activate conditional AKT-ER^{tam}. Mean values of duplicates of one of at least three independent experiments. *B*, proliferative survival of CyA2K^b-AKT-ER^{tam} MEFs coincubated with allo-A2K^b-reactive CTL either in the presence or absence of tamoxifen (representative photograph of at least four independent experiments). *C*, growth of CyA2K^b-AKT-ER^{tam} fibrosarcomas in NOD/SCID mice treated with tamoxifen (as *closed boxes*) for *in vivo* activation of PKB/AKT signaling or vehicle (*open boxes*). The arrowhead indicates the timepoint of adoptive transfer of unprimed C57BL/6-derived splenocytes. *Points*, mean of bidimensional tumor sizes of four mice per group; *bars*, SD.

retrovirally transduced as described previously (11). MEFs were maintained in DMEM supplemented with fetal bovine serum, L-glutamine, penicillin, and streptomycin (Invitrogen). Conditional AKT-ER^{tam} activation *in vitro* was achieved by 4-hydroxytamoxifen (100 nmol/L; Sigma). *P TEN*^{-/-} and *P TEN*^{+/+} HCT116 colorectal cancer cells (generously provided by T. Waldman) were maintained in supplemented McCoy's medium (12). Replication-defective lentiviral virions were generated by cotransfection of the respective plasmid with helper plasmids (a gift from I.M. Verma) in the 293T cell line. Allo A2, A2 Flu, and A2 p53 CTL have previously been described (13, 14). Cytotoxicity, apoptosis, and proliferation assays were carried out as published (11).

Adoptive splenocyte transfer model. Irradiated (150 rad) NOD/SCID mice received s.c. injections of 1×10^6 MEFs or 5×10^6 HCT116 cells. After the outgrowth of palpable tumors (between days 4 and 9), mice received single tail vein injections of unprimed splenocytes resuspended in saline (effector/target ratio, 50:1 for fibrosarcomas and 5:1 for HCT116 xenograft tumors, respectively) and an s.c. injection of 6×10^5 units interleukin 2 (Medicopharm) resuspended in saline and incomplete Freund's adjuvant. Tumor sizes were measured bidimensionally using a caliper. Daily i.p. injections of tamoxifen (1 mg; Sigma) resuspended in peanut oil (Sigma) were given for AKT-ER^{tam} activation *in vivo*. CD8a microbeads were used for CD8 depletion of splenocytes in a MACS system (Miltenyi Biotec); C57BL/6 Pfr1^{-/-} mice (15) were purchased from Charles River Laboratories. All animal studies were conducted in compliance with institutional guidelines and were approved by the regulatory authority.

Results

AKT signaling modulates susceptibility to CTL-mediated tumor suppression. For this study, we made use of a constitutively active AKT fused to a mutant murine estrogen receptor (AKT-

ER^{tam}). This system enables pharmacologic control of the activity of the fusion protein by tamoxifen (16). MEFs from HLA-A*0201 transgenic mice (10) back-crossed on an C57BL/6 background (CyA2K^b mice) were retrovirally transduced to express Myc and H-Ras. Such transformed MEFs were subsequently transduced to express physiologic levels of AKT-ER^{tam} generating CyA2K^b-AKT-ER^{tam} MEFs capable of conditional activation of PKB/AKT signaling within minutes after the addition of tamoxifen (Supplementary Fig. S1A). To study the inhibition of CTL-induced cytotoxicity by PKB/AKT, we devised CTL populations with defined specificities: Allo-A2K^b-reactive CD8-positive CTL (allo A2) lyse targets expressing the HLA-A*0201 antigen, HLA-A*0201/influenza matrix (58–66)-specific CTL (A2 Flu), and HLA-A*0201/p53 (264–272) CTL (A2 p53) lyse targets presenting the influenza matrix (58–66) epitope and the human p53 (264–272) epitope in the context of HLA-A*0201, respectively (13, 14). CyA2K^b-AKT-ER^{tam} MEFs cultured in the presence of tamoxifen exhibited marked resistance against CTL-induced cytotoxicity (Fig. 1A) and CTL-mediated inhibition of clonogenic survival (Fig. 1B). In turn, abrogation of AKT-ER^{tam} signaling by tamoxifen withdrawal sensitized cancer cells to CTL-mediated cytotoxicity (Fig. 1A) and tumor suppression *in vitro* (Fig. 1B). Control experiments confirmed that tamoxifen itself was inert in these systems.⁴

To study PKB/AKT-mediated resistance *in vivo*, HLA-A*0201 transgenic fibrosarcomas were established in NOD/SCID mice. Activation of AKT-ER^{tam} by daily administration of tamoxifen did

⁴ P.S. Hähnel and M. Schuller, unpublished observation.

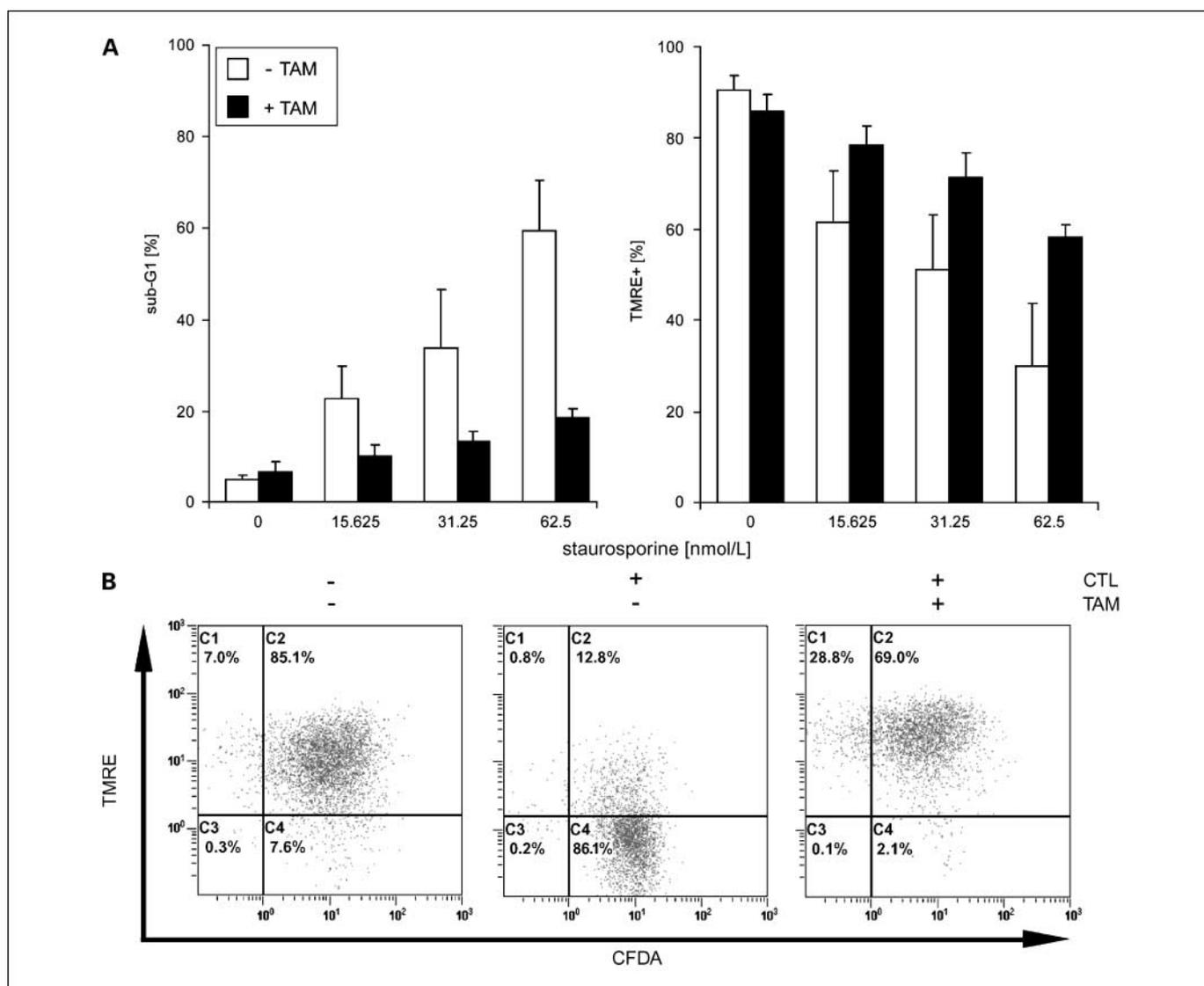


Figure 2. Conditional activation of PKB/AKT signaling protects cancer cells against apoptotic mitochondrial damage. *A*, CyA2K^b-AKT-ER^{tam} MEFs were treated with staurosporine in the presence (*closed bars*) or absence (*open bars*) of tamoxifen. After 24 h, the fraction of apoptotic cells with subgenomic DNA content (*left*) or viable cells with maintained mitochondrial transmembrane potential $\Delta\Psi_m$ (*right*) was determined by staining with propidium iodide or the fluorescent mitochondrial dye TMRE (Molecular Probes), respectively, and flow cytometry. *Columns*, mean of three independent experiments; *bars*, SD. *B*, flow cytometric measurement of the $\Delta\Psi_m$ (indicated by TMRE positivity) of tamoxifen-treated or vehicle-treated CyA2K^b-AKT-ER^{tam} MEFs cocultured with allo A2 CTL. For separation of the two cell populations, MEFs were loaded with the green fluorescent dye CFDA (Molecular Probes). Note the loss of TMRE staining indicative of dissipation of $\Delta\Psi_m$ in MEFs devoid of tamoxifen-induced PKB/AKT signaling.

not alter the growth rate of these tumors (Supplementary Fig. S1B). To model adoptive cellular immunotherapy, mice with established fibrosarcomas received single i.v. injections of unprimed splenocytes derived from C57BL/6 mice, followed by one s.c. dosing of interleukin-2. Despite the fact that a strong allogeneic immune response against HLA-A*0201 transgenic tumor cells was evoked, splenocyte transfer failed to inhibit the growth of fibrosarcomas in tamoxifen-treated mice. In contrast, tumor growth in vehicle-treated mice with inactive AKT-ER^{tam} was significantly suppressed (Fig. 1C). Hence, the inactivation of cell-intrinsic PKB/AKT signaling sensitized cancer cells toward cytotoxicity by antigen-specific CTL *in vitro* and tumor suppression by cellular immune effectors *in vivo*.

The model of adoptive splenocyte transfer for treatment of established fibrosarcomas was chosen to recapitulate the graft-

versus-tumor effect (GvT) mediated by allogeneic hematopoietic stem cell transplantation (ASCT) or donor lymphocyte infusion, adoptive immunotherapies widely used in clinical practice (17, 18). Therapeutic efficacy of these modalities relies at least in part on CTL targeting minor histocompatibility antigens and/or alloantigens presented by cancer cells. To characterize the effector population in this model, we studied splenocyte preparations depleted of the CD8-positive fraction, splenocytes from perforin-deficient C57BL/6 mice (Pfr1^{-/-}; ref. 15), or splenocytes derived from HLA-A*0201 transgenic CyA2K^b mice (10). Either depletion of the CD8-positive population or perforin deficiency greatly reduced the tumor-suppressive capacity of transplanted splenocytes (Supplementary Fig. S2A and B). Also, splenocytes from CyA2K^b mice (10) were tolerant to HLA-A*0201 transgenic fibrosarcomas (Supplementary Fig. S2C). In conclusion, the bulk

tumor-suppressive activity of adoptively transferred splenocytes was mediated by perforin-dependent cytotoxicity of CD8-positive cells, which target HLA-A*0201-presented antigens and/or HLA-A*0201 itself. This was consistent with previous findings in a similar model, in which alloreactive CTL lysed targets primarily via granule-dependent mechanisms (11).

AKT mediates immunoresistance by preventing apoptosis. The activation of PKB/AKT can be monitored by specific phosphoepitopes (6). To confirm that tamoxifen treatment indeed activated AKT-ER^{tam} *in vivo*, we prepared extracts from fibrosarcomas explanted from mice treated with tamoxifen or vehicle. Phosphorylation of AKT-ER^{tam} at residues corresponding to threonine-308 and serine-473 of wild-type AKT was only detectable in tumors explanted from tamoxifen-treated mice, and phosphorylation of glycogen synthase kinase 3- β (GSK3- β), a downstream target of PKB/AKT, was strongly increased (Supplementary Fig. S3A). Furthermore, the *in vitro* kinase activity of AKT-ER^{tam} immunoprecipitated from fibrosarcoma extracts was strongly enhanced by tamoxifen (Supplementary Fig. S3B). These results

showed that the activity of AKT-ER^{tam} in established fibrosarcomas was effectively regulated by tamoxifen *in vivo*.

PKB/AKT signaling has pleiotropic effects on cancer cells (9, 19). As activation of AKT-ER^{tam} failed to accelerate fibrosarcoma growth (Supplementary Fig. S1B), we reasoned whether AKT-ER^{tam} conferred immunoresistance by preventing apoptosis. Indeed, CyA2K^b-AKT-ER^{tam} MEFs cultured in the presence of tamoxifen were protected against caspase activation and apoptosis induced by staurosporine (Fig. 2A and Supplementary Fig. S1C), which engages the same mitochondrial pathway of apoptosis in MEFs as alloreactive CTL (11). Accordingly, active AKT-ER^{tam} prevented dissipation of the mitochondrial transmembrane potential $\Delta\Psi_m$ by staurosporine (Fig. 2A) or allo A2 CTL (Fig. 2B). In contrast, AKT-ER^{tam} provided no further protection to MEFs expressing Bcl-xL, which inhibits apoptotic mitochondrial outer membrane (MOM) permeabilization (Supplementary Fig. S3C). Collectively, these findings suggest that AKT-ER^{tam} interferes with apoptotic signal transduction at or upstream of MOM permeabilization.

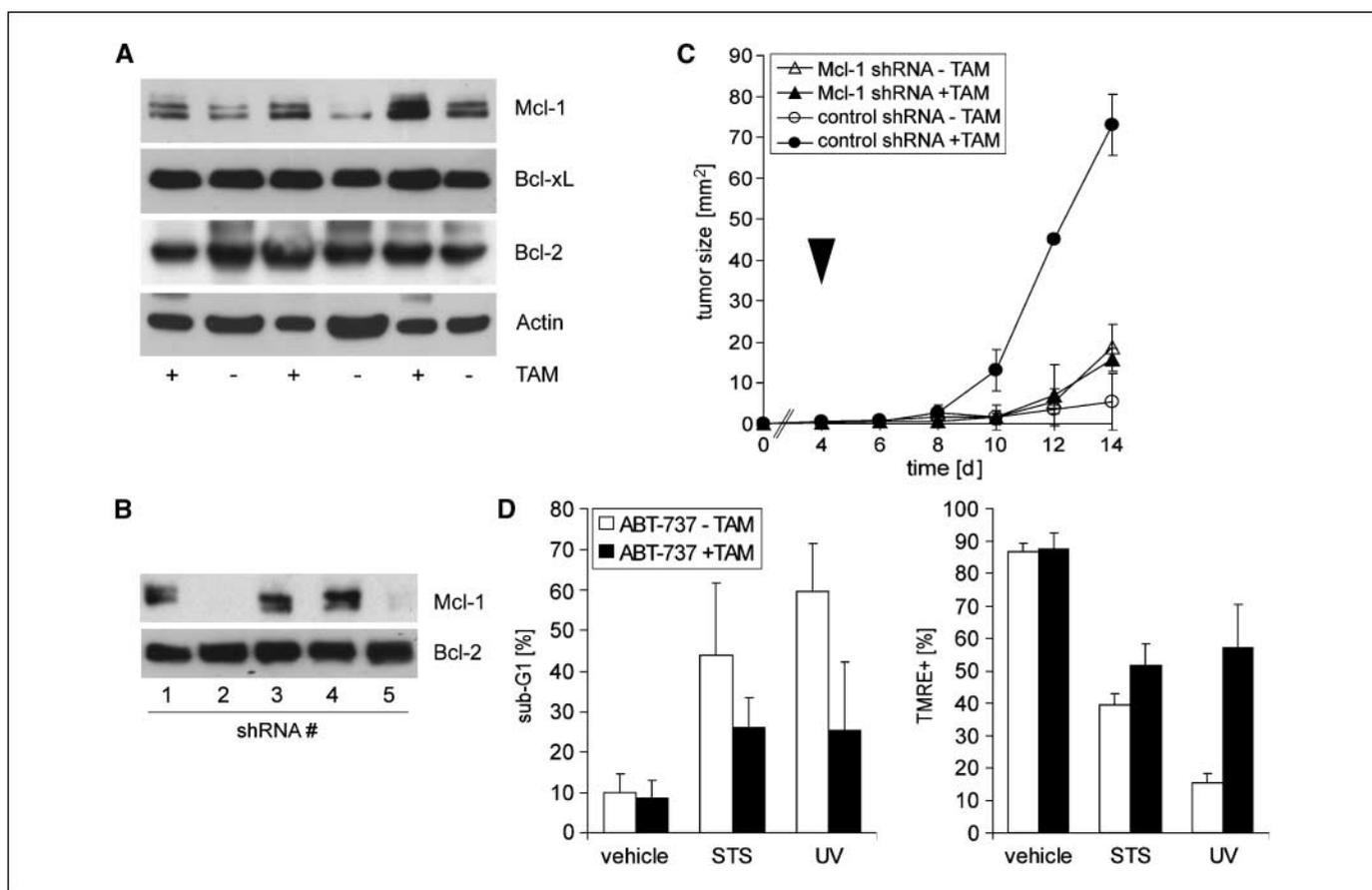
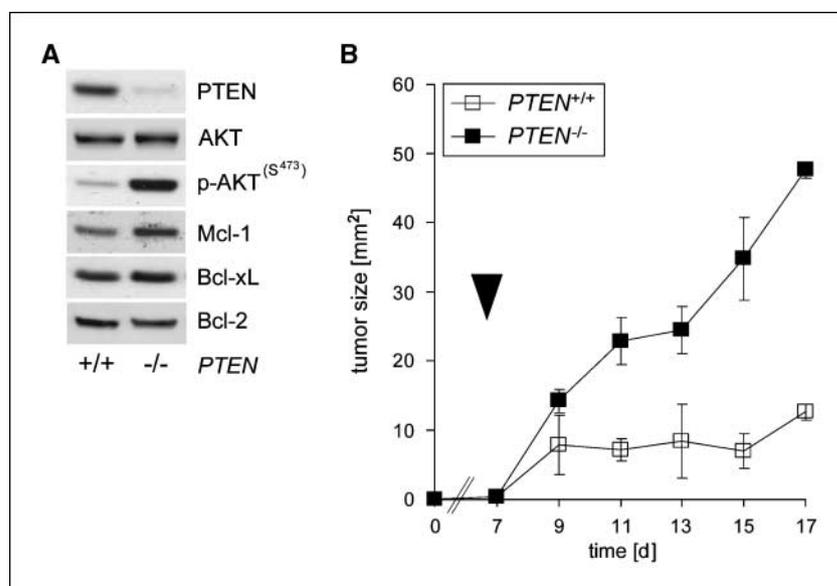


Figure 3. PKB/AKT regulates Mcl-1 to confer resistance to apoptosis and immune-mediated tumor suppression. *A*, immunoblot analysis of extracts prepared from six CyA2K^b-AKT-ER^{tam} fibrosarcomas explanted from mice treated with tamoxifen (+) or vehicle (-). Note the up-regulation of Mcl-1 in the presence of tamoxifen-induced PKB/AKT signaling. *B*, immunoblot analysis of CyA2K^b-AKT-ER^{tam} MEF populations expressing five different shRNA targeting vectors for murine Mcl-1. Population 2 was used for further studies; population 3 subsequently served as negative control. *C*, growth of CyA2K^b-AKT-ER^{tam} fibrosarcomas with Mcl-1 knockdown (Mcl-1 shRNA; triangles) or controls (control shRNA; circles) in NOD/SCID mice treated with tamoxifen (closed symbols) for *in vivo* activation of PKB/AKT signaling or vehicle (open symbols). The arrowhead indicates the timepoint of adoptive transfer of unprimed C57BL/6-derived splenocytes. Note the loss of protection against splenocyte-mediated tumor suppression by PKB/AKT in the absence of endogenous Mcl-1 expression (closed triangles). Points, mean of bidimensional tumor sizes of 10 mice per group; bars, SD. *D*, CyA2K^b-AKT-ER^{tam} MEFs were treated with staurosporine (31.25 nmol/L) or UVC radiation (50 mJ/cm²) in combination with ABT-737 (12.5 μ mol/L). PKB/AKT signaling was induced by tamoxifen (closed bars). After 24 h, the fraction of apoptotic cells with subgenomic DNA content (left) or viable cells with maintained $\Delta\Psi_m$ (right) was determined by flow cytometry after staining with propidium iodide or TMRE, respectively. Columns, mean of three independent experiments; bars, SD.

Figure 4. Endogenously activated PKB/AKT protects cancer cells against CTL-mediated cytotoxicity by up-regulation of Mcl-1. **A**, immunoblot analysis of extracts prepared from $PTEN^{+/+}$ and $PTEN^{-/-}$ HCT116 colorectal cancer xenograft tumors. Note the up-regulation of Mcl-1 in the $PTEN^{-/-}$ tumor. The weak PTEN signal in the $PTEN^{-/-}$ sample results from contaminating murine stromal cells. **B**, growth of $PTEN^{+/+}$ (open boxes) and $PTEN^{-/-}$ HCT116 colorectal cancer xenografts (closed boxes) in NOD/SCID mice. The arrowhead indicates the timepoint of adoptive transfer of unprimed C57BL/6-derived splenocytes. Points, mean of bidimensional tumor sizes of five mice per group; bars, SD.



AKT regulates Mcl-1 to confer resistance to apoptosis and immune-mediated tumor suppression. The integrity of MOM is guarded by antiapoptotic members of the Bcl-2 family, such as Bcl-xL, Mcl-1, or Bcl-2 (20, 21). Examining these proteins in CyA2K^b-AKT-ER^{tam} fibrosarcoma extracts, we observed an up-regulation of Mcl-1 in tumors from tamoxifen-treated mice (Fig. 3A). To investigate whether AKT-ER^{tam}-mediated resistance indeed required Mcl-1, we devised shRNA technology to stably suppress endogenous Mcl-1 in CyA2K^b-AKT-ER^{tam} MEFs (Fig. 3B). The knockdown of Mcl-1 had no effect on the growth of CyA2K^b-AKT-ER^{tam} fibrosarcomas *in vivo* (Supplementary Fig. S4A). However, when endogenous Mcl-1 was suppressed, the activation of AKT-ER^{tam} by tamoxifen failed to protect fibrosarcomas against tumor suppression by transplanted splenocytes (Fig. 3C). These findings showed that PKB/AKT conferred cancer immunoresistance by Mcl-1-dependent inhibition of apoptosis. To corroborate that PKB/AKT-mediated resistance indeed depended on antiapoptotic Mcl-1, we made use of ABT-737, a pharmacologic compound which effectively sensitizes cancer cells to apoptotic caspase activation via the mitochondrial pathway (22) unless high levels of Mcl-1 are expressed (23–25). Whereas ABT-737 at low micromolar concentrations sensitized CyA2K^b MEFs expressing Bcl-xL or a control vector to apoptosis (Supplementary Fig. S3D), ABT-737 failed to overcome PKB/AKT-mediated apoptosis resistance in CyA2K^b-AKT-ER^{tam} MEFs (Fig. 3D). This observation lent further support to a role of Mcl-1, but not Bcl-2 or Bcl-xL, in PKB/AKT-mediated protection of cancer cells.

PKB/AKT is a downstream effector of phosphatidylinositol 3-kinase (PI3K), which itself is antagonized by the tumor suppressor *PTEN*. To assess whether human cancer cells with endogenously activated PKB/AKT were also protected against CTL-mediated cytotoxicity, we devised the HLA-A*0201-positive colorectal cancer cell line HCT116. Targeted deletion of the *PTEN* gene resulted in constitutive activation of PKB/AKT signaling in HCT116 cells (12), as evidenced by phosphorylation of the PKB/AKT target GSK3- β and PKB/AKT itself (Fig. 4A). After s.c. injection into NOD/SCID mice, *PTEN*-deficient ($PTEN^{-/-}$) and *PTEN*-proficient ($PTEN^{+/+}$) HCT116 cells formed xenograft tumors growing at comparable rates (Supplementary Fig. S4B). However,

$PTEN^{-/-}$ HCT116 cells exhibited resistance to allo A2 CTL-mediated cytotoxicity *in vitro*, as well as to tumor suppression by adoptively transferred murine splenocytes *in vivo* (Fig. 4B). Thus, deregulated endogenous PKB/AKT signaling is even capable to protect human cancer cells against a strong xenogeneic immune response. Examining the expression of antiapoptotic members of the Bcl-2 family in $PTEN^{-/-}$ HCT116 xenograft tumor extracts, we confirmed increased Mcl-1 levels, whereas Bcl-xL and Bcl-2 were equally expressed in the presence or absence of endogenous *PTEN* (Fig. 4A). This was in line with our observation in CyA2K^b-AKT-ER^{tam} fibrosarcomas (Fig. 3A).

Immune escape by up-regulation of Mcl-1 requires continued AKT signaling. To further characterize the mechanism of PKB/AKT-mediated up-regulation of Mcl-1, we analyzed quantitative RNA expression of CyA2K^b-AKT-ER^{tam} MEFs after the addition of tamoxifen. No effect of AKT-ER^{tam} signaling on Mcl-1, Bcl-xL, or Bcl-2 RNA expression was observed (Supplementary Fig. S5A), suggesting posttranscriptional regulation of Mcl-1 by PKB/AKT. Several mechanisms of posttranslational regulation of Mcl-1 have been described. Its proteasomal degradation is controlled by the E3 ligase Mule/ARF-BP1 (26). In addition, Mcl-1 protein stability is influenced by the BH3-protein Noxa (23) and the PKB/AKT target GSK3- β (27). Examining Bcl-2 family proteins in CyA2K^b-AKT-ER^{tam} MEFs treated with tamoxifen or vehicle, we found that active AKT-ER^{tam} enhanced the stability of Mcl-1, but not Bcl-xL or Bcl-2 (Fig. 5A). Thus, PKB/AKT signaling specifically increased Mcl-1 expression by a rapid, posttranscriptional mechanism.

Accordingly, abrogation of PKB/AKT signaling could be an effective therapeutic strategy to sensitize cancers to cellular immunotherapies. First, we tested this hypothesis by kinetic studies of tamoxifen withdrawal in CyA2K^b-AKT-ER^{tam} fibrosarcoma-bearing NOD/SCID mice receiving adoptive cellular immunotherapy. This model allowed us to define an essential window of cancer cell protection by PKB/AKT signaling during the first 4 days after splenocyte transfer (Fig. 5B).

Rapamycin prevents AKT-mediated up-regulation of Mcl-1 and overcomes immunoresistance *in vivo*. Rapamycin is an inhibitor of the mammalian target of rapamycin (mTOR) kinase, which acts downstream of PKB/AKT to promote RNA synthesis

and translation (8). Inhibition of mTOR by rapamycin was shown to sensitize cancer cells to cytotoxic therapies (28), and several rapamycin analogues are in clinical development for cancer treatment (7). Although rapamycin is clinically applied as an immunosuppressive agent, it was found that growth and survival of CD8-positive T cells can be promoted in the presence of rapamycin (29). Against this background, we reasoned whether rapamycin could overcome PKB/AKT-mediated and Mcl-1-dependent immunoresistance. Rapamycin treatment effectively suppressed the phosphorylation of the mTOR target p70 S6 kinase *in vivo* (Fig. 6A and C), but had no effect on the growth rate of CyA2K^b-AKT-ER^{tam} fibrosarcomas or HCT116 xenografts (Supplementary Fig. S4B and C). Whereas rapamycin did not interfere with tamoxifen-induced activation of AKT-ER^{tam} and subsequent phosphorylation of GSK3- β (Fig. 6A), it suppressed the AKT-ER^{tam}-mediated up-regulation of Mcl-1 (Fig. 6A). Similar effects were seen after rapamycin treatment of NOD/SCID mice bearing *PTEN*^{+/+} and *PTEN*^{-/-} HCT116 xenograft tumors (Fig. 6C and Supplementary Fig. S5B). Importantly, rapamycin abrogated the PKB/AKT-dependent protection of fibrosarcomas and HCT116 xenografts against tumor suppression by adoptively transferred splenocytes (Fig. 6B and D). Thus, pharmacologic inhibition of mTOR in the presence of cell-

autonomous PKB/AKT signaling decreased the expression of antiapoptotic Mcl-1 and consecutively sensitized established cancers toward immune-mediated tumor suppression.

Discussion

Cancer cell-intrinsic resistance mechanisms play a major role in determining the susceptibility to cytotoxic chemotherapy and radiotherapy. Efflux pumps, such as MDR-1, are frequently induced under selection with anticancer agents, lowering intracellular drug concentrations below a threshold required for induction of apoptosis (30). Furthermore, functional inactivation of tumor suppressor mechanisms, which occurs during oncogenic transformation and tumor progression, may also confer resistance to DNA damage-induced cell cycle arrest and cell death (1, 2). Hence, alternative therapeutic approaches are required to eradicate such resistant cancers. Antigen-specific cellular immunotherapy is such a modality, which could provide long-term control of cancer cells while sparing nonmalignant tissues. Accordingly, numerous studies are under way to explore this possibility to the benefit of patients and to define conditions which are suited to specifically break immunologic tolerance to autologous cancers. One particularly effective modality might be the adoptive transfer of cellular immune effectors which exhibits impressive clinical activity in the setting of ASCT for patients with high-risk leukemias (17). However, cancer cells may even find ways to escape the GvT in the allogeneic setting, and failure from ASCT due to leukemic relapse is more frequently encountered in heavily pretreated patients transplanted advanced disease stages (17, 31).

Against this background, we reasoned whether cell autonomous mechanisms conferring resistance to cytotoxic therapies might also affect the susceptibility of cancer to cellular immunotherapies. To this end, we have studied PKB/AKT, a central regulator in several signal transduction pathways involved in cellular proliferation, metabolic processes, and survival. Enhanced PKB/AKT signaling in cancer frequently results from inactivation of the tumor suppressor PTEN, which negatively regulates PI3K upstream of PKB/AKT. In addition, multiple deregulations of growth and survival factor signal transduction pathways in cancer impinge on PKB/AKT (8, 19). It is well established that PKB/AKT can mediate resistance against growth arrest and apoptosis as induced by anticancer drugs or withdrawal of survival factors. Accordingly, pharmacologic inhibitors of PKB/AKT signaling are clinically developed for cancer therapy in combination with cytotoxic drugs and targeted agents (7). Our present results suggest that resistance to antigen-specific cellular immunotherapies should be added to the list of PKB/AKT activities in cancer.

These findings have several implications. First, we show that PKB/AKT signaling confers resistance to cytotoxicity and tumor suppression by antigen-specific cellular immune effectors *in vitro* and *in vivo*. As PKB/AKT is activated in a high proportion of human cancers, this mechanism may broadly interfere with the efficacy of cancer immunotherapies. In support, established murine fibrosarcomas or human colorectal cancer xenograft tumors were successfully sensitized to adoptive cellular immunotherapy through the conditional shutdown of PKB/AKT signaling or treatment with rapamycin. Secondly, we show that PKB/AKT-dependent immunoresistance primarily depends on the inhibition of apoptotic MOM permeabilization. While PKB/AKT affects several apoptosis regulators, posttranscriptional stabilization of Mcl-1 proved critical in both models. This identifies Mcl-1 as

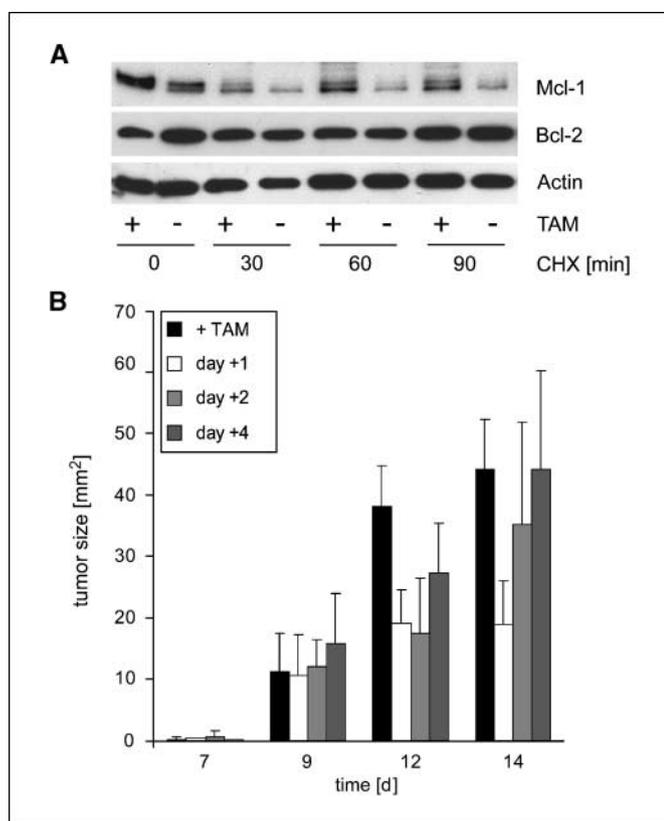
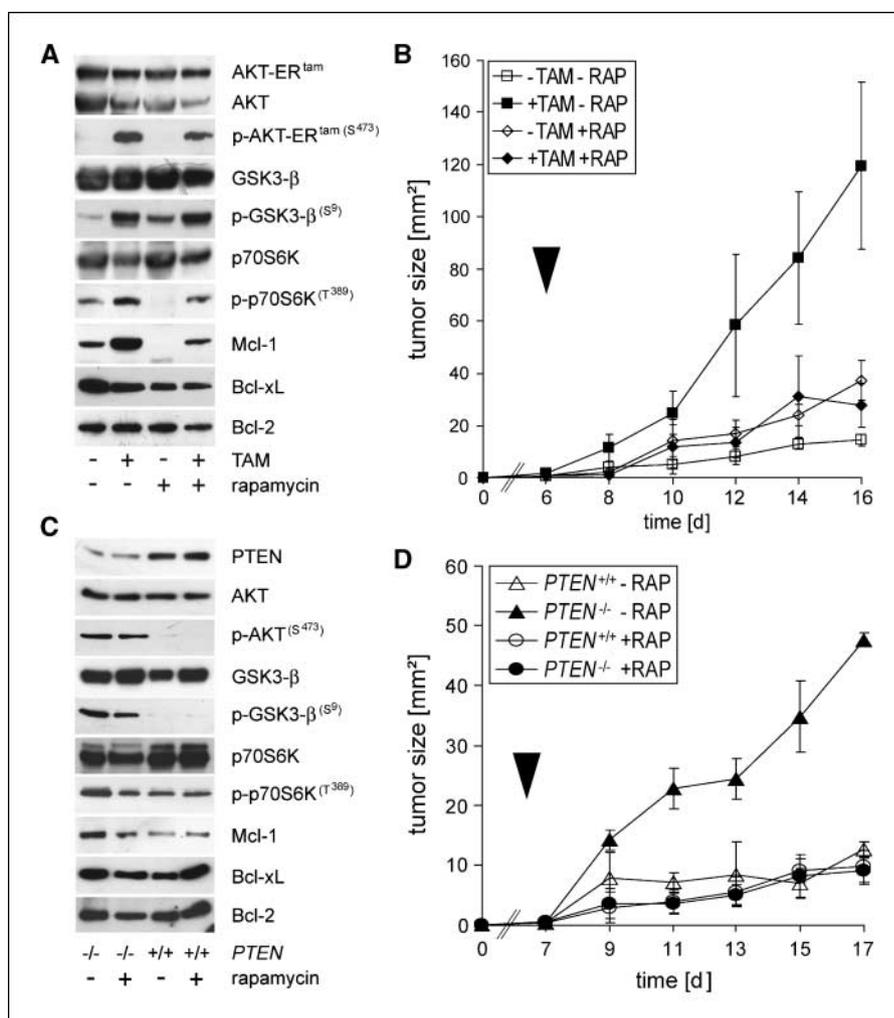


Figure 5. Conditional activation of PKB/AKT signaling up-regulates Mcl-1 by a posttranscriptional mechanism. *A*, immunoblot analysis of extracts prepared from serum-starved CyA2K^b-AKT-ER^{tam} MEFs cultured in the presence (+) or absence (-) of tamoxifen at various time points after the addition of the translation inhibitor cycloheximide (CHX; 200 μ g/mL; Sigma). Conditional PKB/AKT signaling prolongs the protein stability of endogenous Mcl-1. *B*, tumor sizes of CyA2K^b-AKT-ER^{tam} fibrosarcomas in NOD/SCID mice in relation to the time point of withdrawal of PKB/AKT signaling. Columns, mean of four mice per group; bars, SD. Tamoxifen was either given for the whole experiment (black columns) or until 1 d (white columns), 2 d (light grey columns), or 4 d (dark grey columns) after the adoptive transfer of unprimed C57BL/6-derived splenocytes.

Figure 6. Rapamycin prevents PKB/AKT-induced up-regulation of Mcl-1 to sensitize established cancers to immune-mediated tumor suppression.

A. immunoblot analysis of extracts obtained from CyA2K^b-AKT-ER^{tam} fibrosarcomas explanted from mice treated with combinations of tamoxifen, rapamycin (4 mg/kg), and/or vehicle. Note the reduction of PKB/AKT-induced up-regulation of Mcl-1 and phosphorylation of the mTOR target p70 S6 kinase in the presence of rapamycin.
B. growth of CyA2K^b-AKT-ER^{tam} fibrosarcomas in NOD/SCID mice treated with combinations of tamoxifen (closed symbols), rapamycin (+RAP, diamonds), and/or vehicle for *in vivo* activation of PKB/AKT signaling and suppression of mTOR. The arrowhead indicates the timepoint of adoptive transfer of unprimed C57BL/6-derived splenocytes. Note the loss of protection against splenocyte-mediated tumor suppression by PKB/AKT in the presence of rapamycin (closed diamonds). Points, mean of bidimensional tumor sizes of five mice per group; bars, SD.
C. immunoblot analysis of extracts obtained from PTEN^{+/+} and PTEN^{-/-} HCT116 colorectal cancer xenograft tumors explanted from mice treated with rapamycin (4 mg/kg) or vehicle. Note a decrease in Mcl-1 levels and phosphorylation of the mTOR target p70 S6 kinase in the presence of rapamycin.
D. growth of PTEN^{+/+} (open symbols) and PTEN^{-/-} HCT116 colorectal cancer xenografts (closed symbols) in NOD/SCID mice treated with rapamycin (+RAP; circles) or vehicle (-RAP; triangles) for *in vivo* suppression of mTOR. The arrowhead indicates the timepoint of adoptive transfer of unprimed C57BL/6-derived splenocytes. Note the loss of protection against splenocyte-mediated tumor suppression of PTEN^{-/-} tumors in mice treated with rapamycin (closed circles). Points, mean of bidimensional tumor sizes of five mice per group; bars, SD.



gatekeeper of the cellular response to immune-mediated cytotoxicity. Thirdly, down-regulation of Mcl-1 by shRNA targeting or rapamycin sensitized established cancers to cellular immunotherapy. Hence, PKB/AKT, Mcl-1, and mTOR are promising targets for improving efficacy of immunotherapies. Currently, allogeneic cellular immunotherapies generally require concomitant immunosuppression for prevention of Graft-versus-Host disease (17, 18). Based on our findings, the incorporation of rapamycin into these regimens warrants further exploration. From a broader perspective, we have shown that signal transduction events in target cells may be critical determinators of susceptibility to immune-mediated cytotoxicity. Future studies of cancer immunotherapy might greatly benefit from adding such inhibitors of signal transduction, which do not interfere with the activation of immune effectors.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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