Stat3 Inhibition Augments the Immunogenicity of B-cell Lymphoma Cells, Leading to Effective Antitumor Immunity


Introduction

Previous studies in murine models of B-cell lymphoma indicate that generation of effective anti-lymphoma immunity requires (i) conversion of bone marrow–derived antigen-presenting cells (APC) from a noninflammatory (or tolerogenic) status into inflammatory APCs that trigger effective T-cell responses (1, 2) and (ii) augmentation of the APC function of the malignant B cells (3). Therapeutic strategies endowed with the ability of fulfilling both requirements might not only lead to successful eradication of B-cell tumors but also to a long-lasting immunity, and the latter is a desirable effect for certain B-cell malignancies characterized by their high tendency to relapse.

Mantle cell lymphoma (MCL) is the prototype of a B-cell malignancy in which relapse is the major challenge to overcome. In spite of a good initial response to first-line treatment with chemotherapy plus monoclonal antibodies, almost all patients with MCL will eventually relapse, becoming less responsive to further lines of treatment and ultimately will succumb to their disease (4, 5). Given these sobering characteristics, MCL has one of the worst prognoses among all B-cell non–Hodgkin lymphomas (NHL; ref. 6). As such, novel non–cross-resistant treatment modalities capable of improving the response rate and more importantly able of sustaining these responses are greatly needed for patients with MCL.

Several lines of evidence point to manipulation of the immune system as an enticing non–cross-resistant therapeutic strategy for MCL. The demonstration that immune cells are able to kill chemotherapy-resistant tumor cells (7, 8) together with the findings that T-cell responses can be elicited in vaccinated patients with MCL (9), and the encouraging responses observed in patients with relapsed/refractory MCL treated with immunomodulatory drugs (10, 11) suggest that harnessing the immune system and, in particular, eliciting its exquisite specificity and long-lasting protection might lead to sustained immune responses in MCL (12).

Given the above rationale, a significant effort has been devoted to identify molecular target(s) capable of influencing inflammatory pathways in APCs as well as in malignant B cells. Stats are cytoplasmic transcription factors that are key mediators of cytokine and growth factor signaling pathways (13). One of the members of the Stat family, Stat3, has emerged as a negative regulator of inflammatory responses in a variety of immune cells (14–16). For instance, we have previously shown that pharmacologic or genetic disruption of Stat3 in APCs resulted in diminished production of the anti-inflammatory cytokine IL-10, enhanced expression of costimulatory molecules, and increased release of proinflammatory mediators leading to augmentation of the function of these cells to effectively prime T cells and restore the responsiveness of anergic CD4+ T cells (17). These observations prompted us to ask whether targeting Stat3 in malignant B cells might also influence the immunogenicity and inflammatory status of malignant B cells and anti-lymphoma immunity in vivo. Our findings therefore indicate that Stat3 inhibition may represent a therapeutic strategy to overcome tolerance to tumor antigens and elicit a strong immunity against MCL and other B-cell malignancies. Cancer Res; 72(17); 4440–8. ©2012 AACR.
these cells and whether such an effect might unleash effective antitumor immune responses in a murine model of MCL.

Materials and Methods

Mice

Six-week-old male BALB/c (H-2b) and C57BL/6 (H-2b) mice were obtained from the NIH (Frederick, MD). Male BALB/c severe combined immunodeficient (SCID) or C57BL/6 SCID mice, aged 6 weeks, were purchased from Jackson Laboratories. T-cell receptor (TCR) transgenic cell lines expressing an αβ T-cell receptor specific for amino acids 110 to 120 from influenza hemagglutinin presented by I-Ed were a gift by H. von Boehmer (18). TCR transgenic mice (OT-II) expressing an αβ TCR-specific for peptide 323–339 from ovalbumin (OVA) presented by MHC class II and I-Ab (19) were provided by Dr. W. Heath (The Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). All experiments involving the use of mice were carried out in accordance with protocols approved by the Animal Care and Use Committees of the University of South Florida College Of Medicine (Tampa, FL).

Tumor cells

Mature A20 lymphoma cells (H-2k) and human JEKO MCL cells were obtained from American Type Culture Collection. A20 lymphoma cells expressing HA (Hemagglutinin influenza) as a model tumor antigen were selected and grown in vitro as previously described (20). FC-muMCL1 cell line (H-2k) was derived from a tumor-explanted, 1-year-old Bcl-1 transgenic mouse injected with pristane intraperitoneally (i.p.; ref. 21). Cells were cultured in RPMI-1640 medium supplemented with 15% FBS. All cell lines were passaged at least every other day and washed twice in RPMI-1640. Cells were cultured for 3 hours at 37°C, 5% CO2, and nonadherent cells were collected for further experiments.

Reagents

LPS (Escherichia coli 055:B5, L-2880) was purchased from Sigma-Aldrich. CPA-7 was provided by S. Sebti (H. Lee Moffitt Cancer Center, Tampa, FL). CPA-7 was first reconstituted in dimethyl sulfoxide (DMSO) and then further diluted in culture medium for in vitro or in HBSS for in vivo use.

Transfection of tumor cells

A20 B cells were transfected with either a dominant-negative variant of Stat3, Stat3β (22, 23), or Stat3c, a mutant form of Stat3 that is constitutively activated without tyrosine phosphorylation (24). Transfections were conducted via electroporation according to the manufacturer’s instructions (Bio-Rad). Briefly, A20 B cells were harvested and washed with cold PBS, then resuspended at the concentration of 1 × 10^7/0.3 mL in PBS and transferred into an electroporation cuvette. Then, 15 μg of either GFP, Stat3β GFP DNA, or PBS was added and cells were subjected to a high-voltage electrical pulse of defined magnitude and length as per manufacturer’s instructions. A similar procedure was followed to transfect A20 cells with a Stat3c expression vector or control vector. Inhibition of Stat3 in JEKO human MCL was accomplished with siRNA specific for Stat3 using Amaxa Nucleofector methodology per manufacturer’s protocol (Dharmacon).

Isolation of malignant B cells in vivo

Mice were sacrificed and tumor nodules were carefully dissected from their livers. Tumors were gently mashed in tissue culture plates and cells were transferred to a conical tube and washed twice in RPMI-1640. Cells were cultured for 3 hours at 37°C, 5% CO2, and nonadherent cells were collected for further experiments.

Immunoblotting

Whole-cell lysates were prepared using modified radioprecipitation assay (RIPA) lysis buffer. Fifty micrograms of protein was subjected to SDS-PAGE and transferred onto polyvinylidene difluoride (Millipore) membranes and incubated overnight with primary antibodies, followed by horseradish peroxidase–conjugated secondary antibodies (Pierce) and finally, proteins were visualized with a Chemiluminescent Detection Kit (Pierce). Primary antibodies against phospho-Stat3 (Tyr705), phospho-AKT, and phospho-p42/44 MAPK were purchased from Cell Signaling Technology. Anti-Stat3 and anti-AKT antibodies were purchased from Santa Cruz Biotechnology and anti-glyceraldehyde-3-phosphate dehydrogenase was purchased from Sigma (Sigma-Aldrich).

In vitro and in vivo pharmacologic inhibition of Stat3

CPA-7 is a platinum-containing compound that disrupts Stat3 DNA-binding activity, but not Stat5 or Stat1 in malignant cells (25). For in vitro studies, FC-muMCL1 cells were treated with CPA-7 alone (30–1,000 nmol/L) or in combination with LPS (2 μg/mL), and their ability to present cognate peptide to antigen-specific CD4^+ T cells was determined as described under In vitro antigen presentation studies. For in vivo studies, FC-muMCL1 or A20 tumor–bearing mice were given CPA-7 i.v. at the dose of 5 mg/kg every 3 days as previously described (26).

In vivo generation of anergized CD4^+ T cells

Briefly, 2.5 × 10^6 CD4^+ transgenic T cells specific for an MHC class II epitope of HA were injected i.v. into A20HA lymphoma–bearing mice. Twenty-one days after T-cell transfer, animals were sacrificed and anergized T cells were reisolated from their spleens as previously described (20). Cytokine production by reisolated clonotypic CD4^+ T cells in response to HA peptide 110–120 presented by A20 B cells was determined as described under antigen presentation studies.

For induction of antigen-specific T-cell tolerance in H-2^b tumor–bearing mice, a similar experimental approach was used, the only difference being that 1 × 10^6 anti-OVA CD4^+ transgenic T cells (OT-II) were transferred into animals bearing an ovalbumin-expressing tumor (B16/OVA). Fourteen days after T-cell transfer, animals were sacrificed and anergized OT-II cells were reisolated from their spleens (17). Cytokine production by OT-II cells in response to OVA peptide 253–329.
presented by FC-muMCL1 cells was determined as described below.

**In vitro antigen presentation studies**

A20 or FC-muMCL1 cells (1 x 10^6 per well) were cultured with 5 x 10^5 purified naive or tolerized antigen-specific CD4^+ T cells in the presence or absence of cognate peptide (either synthetic HA peptide p110-120 SFERFEIFPEK for studies with A20 B cells or OVA peptide 223-239 SQAVHAAHAEINEAGR for studies with FC-muMCL1 cells; American Peptide Company) After 48 hours, supernatants were collected and assayed for IL-2 and IFN-γ production by ELISA (R&D Systems). Values for T cells cultured in media alone were routinely less than 10% of the values for antigen-stimulated T cells.

**Flow cytometric analysis**

FC-muMCL1 cells were stained with phycoerythrin (PE) anti-CD5 (53-7.3; BD Bioscience), PE-Cy7 anti-CD19 (1D3; BD Bioscience), and fluorescein isothiocyanate anti-cyclin D1 (DCS-6, Millipore) antibodies. The expression of B7.1, B7.2, CD40, and MHC class I and II in FC-muMCL1 and A20 B cells was determined using specific antibodies (16-10A1, GL1, IC10, 2B4, 2B1, Stat3c). Fifty thousand gated events were collected on a FACSCalibur (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Statistical analysis**

A 2-way ANOVA was used to evaluate the magnitudes of cytokine production by clonotypic T cells. Differences in survival were assessed with the log-rank test.

**Results**

**Genetic manipulation of Stat3 signaling in malignant B cells influences antigen-specific T-cell responses in vitro**

First, we asked whether genetic manipulation of Stat3 in A20 lymphoma B cells could influence their intrinsic antigen-presenting capabilities and the responsiveness of antigen-specific CD4^+ T cells. To inhibit Stat3 in A20 cells, we used a dominant-negative variant of Stat3, Stat3β (22). A20 cells were transfected with Stat3β (A20-Stat3β) and then cultured in vitro with syngeneic naive CD4^+ T cells specific for an MHC class II restricted epitope of HA in the presence or absence of cognate HA-peptide. As shown in Fig. 1 (left), clonotypic T cells encountering HA-peptide on A20-Stat3β cells displayed a significantly enhanced production of IL-2 (Fig. 1A) and IFN-γ (Fig. 1B), relative to those T cells encountering cognate peptide on either nontransfected (None), mock-transfected (Mock), or GFP-transfected (GFP) A20 B cells.

In previous studies, we have shown that adoptive transfer of naive anti-HA transgenic CD4^+ T cells into mice bearing A20 B cell lymphoma, expressing HA as a model tumor antigen (A20HA), resulted in the induction of antigen-specific CD4^+ T-cell tolerance. In this system, reisolated T cells from lymphoma-bearing mice were found to be anergic by their failure to be primed in vitro as well as by their diminished IL-2 and IFN-γ production in response to in vitro restimulation with cognate HA-peptide (20). However, as shown in Fig. 1 (right), in vitro incubation of these same tumor-anergized T cells with A20-Stat3β lymphoma cells resulted in restoration of T-cell responsiveness to cognate HA-antigen. Indeed, presentation of HA-peptide by A20-Stat3β triggered IL-2 (Fig. 1C) and IFN-γ (Fig. 1D) production by tolerant CD4^+ T cells (Fig. 1D). In sharp contrast, anergic T cells encountering HA antigen on nontransfected, mock-transfected, or GFP-transfected A20 B cells remained unresponsive.

Given the above findings, we next asked whether an opposite effect would be observed when Stat3 is overexpressed in malignant B cells. A20 cells were therefore transfected with Stat3c, a mutant form of Stat3 that is constitutively activated without tyrosine phosphorylation (24). Unlike naive anti-HA CD4^+ T cells that produce IL-2 and IFN-γ in response to cognate antigen presented by control A20 B cells (Fig. 2A and B, non-transfected or PC-DNA transfected), CD4^+ T cells cultured with A20-Stat3c cells were rendered unresponsive given their minimal production of IL-2 (Fig. 2A and B) and IFN-γ (Fig. 2B and D) by ELISA. Values represented mean ± SE of triplicate cultures and are representative of 3 independent experiments (*, P < 0.05 statistically significant for the difference in cytokine production).

**Pharmacologic inhibition of Stat3 in murine B-cell lymphomas augments APC function**

Given that inhibition of Stat3 in malignant B cells induces better activation of antigen-specific CD4^+ T cells and restores the responsiveness of tolerized T cells, we asked next whether this positive effect was due to the augmentation of the APC...
function of malignant B cells. Therefore, the expression of MHC and costimulatory molecules was determined in A20 B cells treated in vitro with CPA-7, a platinum-containing Stat3-specific inhibitor (25). As shown in Fig. 3, CPA-7–treated A20 B cells displayed elevated expression of the costimulatory molecules B7.1, B7.2, and CD40. No significant changes in the expression of MHC class I or II molecules were observed among untreated or CPA-7–treated malignant B cells (data not shown). However, the expression of MHC class I and II were increased in A20 cells treated with CPA-7 plus LPS in comparison with cells treated with LPS alone (data not shown).

Recently, Smith and colleagues have developed a murine model of MCL by injecting pristane i.p. into 1-year-old Bcl-1 transgenic mice (Eμ-cyclin D1). In these animals, the pattern of disease consists of diffuse adenopathy, splenomegaly, bone marrow infiltration as well as lung, kidney, and periportal hepatic infiltration. Analysis of tumor explants revealed malignant B cells that coexpress cyclin D1, CD20, and CD5, but lack expression of CD23, findings reminiscent of human MCL (21). A cell line, FC-muMCL1, has been derived from one of these lymphoma explants and phenotypic analysis confirmed that they express cyclin D1, CD19, and CD5 (Fig. 4A). Furthermore, all C57BL/6 mice challenged with 5 × 10^6 FC-muMCL1 given either subcutaneously (black circle) or i.p. (open circle) developed tumors and showed decreased survival (Fig. 4B). In mice challenged subcutaneously, tumor nodules developed by day 21. Intraperitoneal injection of MCL cells resulted in the development of ascites by day 30 and at necropsy we found enlarged mesenteric and retroperitoneal lymph nodes as well as tumor nodules in the peritoneum and small bowel (data not shown).

Next, we determined the in vitro effects of CPA-7 upon FC-muMCL1 cells. First, CPA-7 did not affect the viability or proliferation of malignant B cells (Supplementary Fig. S1). Second, we assessed whether FC-muMCL1 cells can present cognate antigen to antigen-specific CD4^+ T cells and if so, whether this intrinsic APC function can be enhanced by CPA-7. Given the background of FC-muMCL1 cells (H-2^b), we assessed their ability to present ovalbumin peptide to transgenic CD4^+ T cells expressing a β2TCR specific for OVA peptide 323–339 (19). First, anti-OVA CD4^+ T cells encountering cognate antigen on untreated MCL cells (ovalbumin peptide) or in LPS-treated MCL cells (LPS+OVA) produced IL-2 (Fig. 4C, left) and IFN-γ (Fig. 4C, right) indicative of the ability of murine MCL cells to present antigen to CD4^+ T cells in vitro. This intrinsic APC function was enhanced following exposure of MCL cells to LPS in the presence of increasing concentrations of Stat3 inhibitor. Indeed, CPA-7–treated MCL cells triggered an increased production of IL-2 and IFN-γ by CD4^+ T cells (Fig. 4C; CPA-7+LPS). Importantly, anergized anti-OVA CD4^+ T cells cultured in vitro with CPA-7–treated FC-muMCL1 cells regained their ability to produce IFN-γ in response to cognate ovalbumin peptide (Fig. 4D; CPA-7+LPS). In contrast, anergized T cells encountering ovalbumin peptide on untreated MCL cells (OVA) or in cells treated with LPS+OVA peptide (in the absence of CPA-7) remained unresponsive (Fig. 4D). Finally, reminiscent of our observations in CPA-7–treated A20 B cells, FC-muMCL cells treated with CPA-7 also displayed enhanced expression of the costimulatory molecules B7.1 and B7.2 relative to untreated cells (Fig. 4E). However, no changes in the expression of CD40 was observed in CPA-7–treated cells relative to controls (data not shown). Taken together, treatment of murine MCL cells with CPA-7 augments their immunogenicity resulting in enhanced activation of naive CD4^+ T cells.

Figure 2. Increased Stat3 activity in malignant B cells inhibits antigen-specific CD4^+ T-cell responses. A20 B cells were transiently transfected with either pcDNA3 empty vector or Stat3c expression vector. Then, 1 × 10^5 transfected or control cells were incubated with 5 × 10^6 naive anti-HA CD4^+ T cells in the presence or absence of 12.5 μg of HA peptide. After 48 hours, supernatants were collected and assayed for IL-2 (A) and IFN-γ (B) production by ELISA. Values represent mean ± SE of triplicate samples. P < 0.05 statistically significant for the difference in cytokine production.

Figure 3. Enhanced expression of costimulatory molecules on malignant B cells treated with CPA-7. Expression of B7.1, B7.2, and CD40 on A20 B cells treated or not with CPA-7 (15 μmol) for 15 hours was assessed by flow cytometry. Fifty thousand gated events were collected on a FACSCalibur and analyzed using FlowJo software. Gray histogram shows isotype control. Shown are representative of 3 experiments with similar results.
In vivo inhibition of Stat3 delays progression of murine B-cell lymphomas

Next, we determined whether CPA-7 inhibits Stat3 signaling in malignant B cells in vivo. Previous studies have shown that CPA-7 induces in vivo antitumor responses when used at the dose of 5 mg/kg given i.v. every 3 days (16). We therefore injected 5 × 10⁶ FC-muMCL1 cells i.p. into C57BL/6 mice and 21 days later, animals were treated (or not) with 5 mg/kg of CPA-7. After 5 mg/kg of CPA-7 given i.v. on days +21, +24, and +27. Two days later (day +29), animals were sacrificed and tumor nodules were dissected from their livers. As shown in Fig. 5A, a decrease in phospho-Stat3 expression was observed in malignant B cells isolated from MCL-bearing mice treated with CPA-7. No such effect was observed in untreated tumor-bearing mice or animals treated with vehicle control. Of note, less tumor burden was observed in CPA-7–treated mice. These results prompted us to determine the in vivo anti-MCL effect of CPA-7 in a larger group of animals. C57BL/6 mice were challenged with 5 × 10⁶ FC-muMCL1 cells given subcutaneously. Half of the mice received vehicle control and the other half received CPA-7 (5 mg/kg/i.v. every 3 days, starting on day +5 after tumor challenge). Unlike untreated MCL-bearing mice, which rapidly developed tumors (Fig. 5B, solid line), mice treated with CPA-7 had a significant delay in MCL tumor growth (Fig. 5B, dashed line). Of note, analysis of immune cells infiltrating MCL lymphoma nodules revealed no differences in intratumoral T-cell recruitment (Supplementary Fig. S2). Similarly, BALB/c mice challenged with 1 × 10⁶ A20 lymphoma cells subcutaneously and then treated with CPA-7 (same dose and frequency as in the MCL model) rejected this B-cell tumor (Fig. 5B, solid line). No such rejection was observed in A20 B cell lymphoma-bearing mice given vehicle control (Fig. 5C, dashed line). Therefore, in vivo treatment of lymphoma-bearing mice with CPA-7 resulted in decreased Stat3 phosphorylation in malignant B cells and a strong anti-lymphoma effect.
The in vivo antitumor effect of CPA-7 requires an intact adaptive immune system

Previous studies have shown that disruption of Stat3 in malignant cells, including MCL cells, resulted in the induction of apoptosis (27, 28). As such, the in vivo antitumor effect observed in CPA-7–treated lymphoma-bearing mice (Fig. 5) could be a reflection of a direct effect of this drug upon tumor cells themselves rather than immune effects triggered by Stat3 inhibition. To address this question, C57BL/6 SCID mice (Fig. 6A) or BALB/c-SCID mice (Fig. 6B) were challenged with 5 × 10⁶ FC-muMCL1 cells or 1 × 10⁶ A20 lymphoma cells given subcutaneously, respectively. Half the mice in each group were treated with CPA-7 (5 mg/kg every 3 days, starting on day +5 after tumor challenge) and the other half received vehicle control. Unlike immunocompetent lymphoma-bearing mice treated with CPA-7 in which a strong antitumor effect was clearly shown (Fig. 5B and C), such an antitumor effect was not observed in immunodeficient animals treated with CPA-7. Indeed, no difference in the kinetics of tumor growth was observed among untreated or CPA-7–treated lymphoma-bearing SCID mice (Fig. 6A and B, MCL and A20, respectively). To rule out the possibility that CPA-7 might not be affecting its tumor target in SCID mice, we determined the expression of p-Stat3 in malignant B cells isolated from tumor nodules of untreated and CPA-7–treated SCID mice. As shown in Fig. 6C, in vivo treatment with CPA-7 resulted in decreased p-Stat3 expression, an effect that was more pronounced in A20 lymphoma cells. These results indicate that the antitumor effect of CPA-7 requires an intact adaptive immune system and points to the immunologic rather than the nonimmunologic.
In the in vivo immune response against B-cell lymphomas, it is likely that both malignant cells themselves as well as bone marrow–derived APCs present tumor antigens to antigen-specific CD4+ T cells. B-cell lymphomas are the transformed counterparts of cells endowed with antigen-presenting capabilities. Normal B lymphocytes have long been known to interact with CD4+ T cells during physiologic immune responses in a process that involves presentation of peptide MHC class II complexes, along with costimulatory signals to antigen-specific T cells (31, 32). Like normal B cells, malignant B cells also express MHC class I and II molecules and low but inducible levels of adhesion and costimulatory molecules (1, 33, 34). In spite of these intrinsic properties, it is quite paradoxical that B-cell malignancies fail to be eliminated in the very same compartment lymph nodes where tumor antigen–specific T-cell responses are initiated.

Several factors might account for the failure of malignant B cells to properly activate T cells in vivo. First, their expression of MHC molecules, costimulatory molecules, and/or adhesion molecules that participate in T-cell priming might not be sufficient to trigger full T-cell activation. This “state” of partial T-cell activation, in the absence of additional signals capable of sustaining this initial response and/or in the presence of dominant suppressive mechanisms, might be followed instead by a state of T-cell anergy (1, 35). Among the active suppressive mechanisms, the ability of malignant B cells to induce T-cell immunologic synapse dysfunction (36) together with the ability of bone marrow–derived APCs to create a tolerogenic environment that favors T-cell anergy over T-cell activation (37–39) have gained particular attention. It is plausible therefore that the combination of these mechanisms would be conducive to T-cell unresponsiveness, a barrier that needs to be overcome if effective immunity against B-cell tumors is to be generated.

In the past several years, a number of therapeutic approaches have sought to improve the antigen-presenting capabilities of malignant B cells by either genetically modifying these cells to enforce the expression of adhesion and costimulatory molecules as well as proinflammatory cytokines (40–43), or by repairing a dysfunctional T-cell immunologic synapse with immunomodulatory drugs such as lenalidomide (36). Other approaches have focused instead on the induction of inflammatory APCs as a strategy to improve cross-presentation of tumor antigens to antigen-specific T cells (3, 44). Although each of these approaches induced productive immune responses, the duration and magnitude of these effects were transient and not strong enough to fully eradicate systemic lymphoma. A potential explanation for their limited success is that they have targeted either the malignant B cell or the APC, but not both, and as such, they were unable to fully overcome tolerogenic mechanisms in cancer. Therefore, from a therapeutic perspective, it would be desirable to find novel approaches with the dual ability of enhancing the antigen-presenting function of malignant B cells and inducing inflammatory APCs displaying enhanced cross-presentation of tumor antigens to antigen-specific T cells.

Inhibition of Stat3 signaling represents a novel strategy given its known ability to influence the inflammatory status of the APC (17, 29, 45) and, as shown here, to augment the APC

### Discussion

In this study, we have shown that genetic or pharmacologic disruption of Stat3 in malignant B cells increased their immunogenicity leading to augmentation of antigen-specific CD4+ T-cell function and restoration of responsiveness of tolerated T cells. These findings expand the previously known proinflammatory effects of Stat3 inhibition upon other immune cells such as bone marrow–derived APCs (17, 29, 30). This unique property of Stat3 inhibition to influence the inflammatory status of both the malignant B cell as well as the APC points to pharmacologic inhibition of this signaling pathway as an appealing strategy to overcome tolerance to tumor antigens and elicit a strong antitumor immunity.

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Figure 7. CPA-7 specifically inhibits Stat3 phosphorylation in human MCL cells. JEKO cells were treated or not treated with CPA-7 (30 µmol) for 24 hours (left). In parallel, cells were transiently transfected with Stat3-specific siRNA (Stat3 siRNA) or nontargeting control (Control). Then, cells were harvested and protein extracts were obtained and subjected to Western blot analysis using antibodies against p-Stat3, Stat3, p-MAPK, MAPK, p-Akt, and Akt. Shown is a representative experiment of 2 with similar results.

antitumor effects of this Stat3 inhibitor, as playing a dominant role in its in vivo anti-lymphoma activity.

**CPA-7 inhibits Stat3 in human MCL**

Next, we determined whether CPA-7 would also inhibit Stat3 in human MCL cells. As shown in Fig. 7, p-Stat3 was diminished in JEKO cells treated in vitro with CPA-7. This inhibition was specific as other signaling pathways such as phospho-MAPK or phospho-AKT were not affected in MCL cells treated with even higher doses of CPA-7 (30 µmol). The selectivity of this agent is further highlighted by the demonstration that the phenotype displayed by CPA-7–treated JEKO cells is recapitulated in cells in which Stat3 was knocked down using specific Stat3 siRNA (Fig. 7, right). Of note, in vitro culture of CPA-7–treated human JEKO cells with allogeneic human peripheral blood mononuclear cells also resulted in enhanced IL-2 and IFN-γ production by T cells as determined by ELISPOT assay (data not shown).

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antitumor effects of this Stat3 inhibitor, as playing a dominant role in its in vivo anti-lymphoma activity.

**CPA-7 inhibits Stat3 in human MCL**

Next, we determined whether CPA-7 would also inhibit Stat3 in human MCL cells. As shown in Fig. 7, p-Stat3 was diminished in JEKO cells treated in vitro with CPA-7. This inhibition was specific as other signaling pathways such as phospho-MAPK or phospho-AKT were not affected in MCL cells treated with even higher doses of CPA-7 (30 µmol). The selectivity of this agent is further highlighted by the demonstration that the phenotype displayed by CPA-7–treated JEKO cells is recapitulated in cells in which Stat3 was knocked down using specific Stat3 siRNA (Fig. 7, right). Of note, in vitro culture of CPA-7–treated human JEKO cells with allogeneic human peripheral blood mononuclear cells also resulted in enhanced IL-2 and IFN-γ production by T cells as determined by ELISPOT assay (data not shown).

### Discussion

In this study, we have shown that genetic or pharmacologic disruption of Stat3 in malignant B cells increased their immunogenicity leading to augmentation of antigen-specific CD4+ T-cell function and restoration of responsiveness of tolerated T cells. These findings expand the previously known proinflammatory effects of Stat3 inhibition upon other immune cells such as bone marrow–derived APCs (17, 29, 30). This unique property of Stat3 inhibition to influence the inflammatory status of both the malignant B cell as well as the APC points to pharmacologic inhibition of this signaling pathway as an appealing strategy to overcome tolerance to tumor antigens and elicit a strong antitumor immunity.
function of malignant B cells. Indeed, treatment of malignant B cells with CPA-7 rendered these cells better activators of antigen-specific CD4+ T cells and capable of restoring the responsiveness of tolerant T cells isolated from lymphoma-bearing mice. In addition to these in vitro effects, treatment of MCL-bearing mice with CPA-7 decreased Stat3 phosphorylation in tumor cells and resulted in protective immunity. Of note, the lack of antitumor activity in immunodeficient mice treated with CPA-7 points to the effects of Stat3 inhibition upon immune cells as being essential for effective lymphoma eradication in vivo. It should be mentioned that our in vivo results in SCID mice are at odds with the results reported by Wang and colleagues who treated SCID mice bearing human SP53 or Grant 519 MCL cells with Atiprimod, a compound known to inhibit Stat3 (27). Unlike our study, SCID mice treated with Atiprimod had significantly less tumor burden compared with control mice, pointing to a direct antitumor effect of this compound upon MCL cells. Several differences between their study and ours might explain these seemingly divergent outcomes. First, unlike CPA-7, which mainly inhibits Stat3 without affecting other signaling pathways in human MCL (Fig. 7), Atiprimod is a less selective inhibitor of Stat3 as it also activates c-jun-NH2-kinase and inhibits NF-kB, an important survival pathway that is constitutively activated in MCL. It is plausible, therefore, that the antitumor effect observed in their study could be related to the targeting of pathway(s) other than Stat3. Second, in our in vivo studies, mice were challenged with murine MCL cells, whereas in their study, SCID mice were challenged with human MCL cells. Third, there were also differences in the dose and frequency of in vivo administration of CPA-7 and Atiprimod (CPA-7: 5 mg/kg/i.v. every 3 days, starting on day +5 after tumor challenge vs. Atiprimod: 25 mg/kg/i.p. daily for 6 days starting 3–4 weeks after tumor challenge).

Our observation that Stat3 inhibition is an effective strategy in a murine model of MCL provides the framework for its future combination with agents able to repair defective T-cell immunologic synapse, such as lenalidomide, or as adjuvants to lymphoma vaccines. Furthermore, our findings, together with the demonstration that Stat3 is constitutively activated in human MCL cells (46, 47), provide the basis for evaluating the clinical efficacy of Stat3 inhibition in human MCL. Of note, inhibition of Stat3 in tumor cells displaying aberrant activation of this pathway has been shown to result in “inflammatory death”, a process associated with release of proinflammatory mediators that could amplify ongoing anti-tumor immune responses also triggered by the effects of Stat3 inhibition upon APCs and other immune cells (26, 48). This proinflammatory environment generated by Stat3 inhibition is further enhanced by the inability of malignant B cells and immune cells to produce IL-10 in the absence of intact Stat3 signaling (15, 17). Such a lack of production of IL-10 has the dual advantage of not only diminishing the generation of an immunosuppressive environment but also depriving malignant B cells of an important survival factor (49, 50).

Taken together, the dual effects of Stat3 inhibition upon both the malignant B cells as well as immune cells triggers a positive loop of proinflammatory events that likely generates an activating rather than a tolerogenic environment in the lymph nodes, which might be ultimately conducive to effective antilymphoma immunity. Such a unique property of Stat3 inhibition makes this approach suitable for future evaluation in human MCL and other B-cell malignancies, either alone or in combination with lenalidomide, or as an adjuvant to therapeutic vaccines.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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References


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