STAT3 INHIBITION AUGMENTS THE IMMUNOGENICITY OF B-CELL LYMPHOMA CELLS LEADING TO EFFECTIVE ANTITUMOR IMMUNITY

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ABSTRACT

Mantle cell lymphoma (MCL) is an aggressive and incurable subtype of B-cell Non-Hodgkin’s lymphomas. Although patients often respond initially to first-line treatment with chemotherapy plus monoclonal antibodies, relapse and decreased response to further lines of treatment eventually occurs. Harnessing the immune system to elicit its exquisite specificity and long-lasting protection might provide sustained MCL immunity that could potentially eradicate residual malignant cells responsible for disease relapse. Here we show that genetic or pharmacologic disruption of Stat3 in malignant B-cells augments their immunogenicity leading to better activation of antigen-specific CD4+ T-cells and restoration of responsiveness of tolerized T-cells. In addition, treatment of MCL-bearing mice with a specific Stat3 inhibitor resulted in decreased Stat3 phosphorylation in 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.
**Introduction**

Previous studies in murine models of B-cell lymphoma indicate that generation of effective anti-lymphoma immunity requires: (1) Conversion of bone marrow (BM)-derived antigen presenting cells (APCs) from a non-inflammatory (or tolerogenic) status into inflammatory APCs that trigger effective T-cell responses (1,2) and, (2) Augmentation of the antigen-presenting cell function of the malignant B-cells (3). Therapeutic strategies endowed with the ability of fulfilling both requirements might lead not only to successful eradication of B-cell tumors but also to a long-lasting immunity, the latter 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’s lymphomas (NHL) (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 MCL patients.

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 MCL patients (9) and, the encouraging responses observed in patients with relapsed/refractory MCL treated with immunomodulatory drugs (IMIDs) (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. Signal transducer and activators of transcription (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 demonstrated that pharmacologic or genetic disruption of Stat3 in APCs resulted in diminished production of the anti-inflammatory cytokine IL-10, enhanced expression of co-stimulatory molecules, and increased release of pro-inflammatory 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 these cells and whether such an effect might unleash effective antitumor immune responses in a murine model of MCL.
Materials and Methods

Mice. 6 weeks-old male BALB/c (H-2\textsuperscript{d}) and C57BL/6 (H-2\textsuperscript{b}) mice were obtained from the NIH (Frederick, Maryland). Male BALB/c SCID or C57BL/6 SCID mice, aged 6 weeks, were purchased from Jackson Laboratories (Bar Harbor, ME). TCR transgenic mice expressing an $\alpha\beta$ T-cell receptor specific for amino acids 110-120 from influenza hemagglutinin presented by I-E\textsuperscript{d} were a gift of H. von Boehmer (18). TCR transgenic mice (OT-II) expressing an $\alpha\beta$ TCR specific for peptide 323-339 from Ovalbumin (OVA) presented by MHC class II, I-A\textsuperscript{b} (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 performed in accordance with protocols approved by the Animal Care and Use Committees of the University of South Florida College Of Medicine.

Tumor Cells. Murine A20 lymphoma cells (H-2\textsuperscript{d}) and human JEKO MCL cells were obtained from ATCC (Rockville, MD). A20 lymphoma cells expressing HA (Hemagglutinin influenza) as a model tumor antigen were selected and grown \textit{in vitro} as previously described (20). FC-muMCL1 cell line (H-2\textsuperscript{b}) was derived from a tumor explanted one year-old Bcl-1 transgenic mice injected with pristane intraperitoneally (21). Cells were cultured in RPMI 1640 medium supplemented with 15% FBS. All cell lines were evaluated by flow cytometry for characterization before experimentation. For \textit{in vivo} experiments, cells were washed three times in sterile HBSS and then $1\times10^6$ A20 or $5\times10^6$ FC-muMCL1 cells were injected either subcutaneously (sc) or intraperitoneally (ip) into BALB/c or C57BL/6 mice respectively.
**Reagents.** LPS (Escherichia coli 055:B5, L-2880) was purchased from Sigma-Aldrich (St. Louis, MO). CPA-7 was provided by Dr. Said Sebti (Moffitt Cancer Center, Tampa, FL). CPA-7 was first reconstituted in 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 a mutant form of Stat3 that is constitutively activate without tyrosine phosphorylation, Stat3c (24). Transfections were performed via electroporation according to the manufacturer’s instructions (Bio-Rad, Hercules, CA). Briefly, A20 B-cells were harvested and washed with cold PBS then resuspended at the concentration of 1x10⁷/0.3 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, Lafayette, CO).

**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 non-adherent cells were collected for further experiments.
**Immunoblotting.** Whole-cell lysates were prepared using modified RIPA lysis buffer. 50μg of protein was subjected to SDS-PAGE and transferred onto PVDF (Millipore, Billerica, MA) membranes and incubated overnight with primary antibodies, followed by HRP-conjugated secondary antibodies (Pierce, Rockford, IL) and finally, proteins were visualized with a Chemiluminescent Detection kit (Pierce, Rockford, IL). Primary antibodies against phospho-Stat3 (Tyr705), phospho-AKT, and phospho-p42/44 MAPK were purchased from Cell Signaling Technology (Cambridge, MA). Anti-Stat3 and anti-AKT antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and anti-GAPDH was purchased from Sigma (Sigma-Aldrich, St. Louis, MO).

**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 nor Stat1 in malignant cells (25). For *in vitro* studies, FC-muMCL1 cells were treated with CPA-7 alone (30 to 1000nM) or in combination with LPS (2μg/ml) and their ability to present cognate peptide to antigen-specific CD4+ T-cells was determined as described under antigen presentation studies. For *in vivo* studies, FC-muMCL1 or A20 tumor bearing mice were given CPA-7 intravenously 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 x10^6 CD4+ transgenic T-cells specific for an MHC class II epitope of influenza hemagglutin (HA) were injected intravenously (iv) into A20HA lymphoma bearing mice. Twenty-one days after T-cell transfer, animals were sacrificed and anergized T-cells were re-isolated from their spleens as previously described (20). Cytokine production by
re-isolated 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 utilized, the only difference being that 1x10\(^6\) anti-OVA CD4\(^+\) transgenic T cells (OT-II) were transferred into animals bearing an OVA-expressing tumor (B16OVA). Fourteen days after T-cell transfer, animals were sacrificed and anergized OT-II cells were re-isolated from their spleens (17). Cytokine production by OT-II cells in response to OVA-peptide\(^{323-339}\) presented by FC-muMCL1 cells was determined as described under antigen-presentation studies.

**In vitro antigen-presentation studies.** A20 or FC-muMCL1 cells (1x10\(^5\)/well) were cultured with 5x10\(^4\) purified naïve or tolerized antigen-specific CD4\(^+\) T cells in the presence or not of cognate peptide (either synthetic HA peptide\(^{110-120}\) SFERFEIFPKE for studies with A20 B-cells or OVA peptide\(^{323-339}\) ISQAVHAAHAEINEAGR for studies with FC-muMCL1 cells, American Peptide Company, Sunnyvale, CA) After 48 hours, supernatants were collected and assayed for IL-2 and IFN-\(\gamma\) production by ELISA (R&D Systems, Minneapolis, MN). 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 PE anti-CD5 (53-7.3, BD Bioscience), PE-Cy7 anti-CD19 (1D3, BD Bioscience) and FITC anti-Cyclin D1 (DCS-6, Millipore) antibodies. The expression of B7.1, B7.2, CD40, MHC class I and II in FC-muMCL1 and A20 B-cells was determined using
specific antibodies (16-10A1, GL1, 1C10, 28-14-8, M5/114.15.2, eBiosience, San Diego, CA). Fifty-thousand gated events were collected on a FACSCALIBUR (BD Biosciences) and analyzed using FlowJo software (Tree Star).

**Statistical Analysis.** A 2-way analysis of variance (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 naïve CD4⁺ T-cells specific for an MHC class II restricted epitope of influenza hemagglutinin (HA) in the presence -or not- of cognate HA-peptide. As shown in Fig. 1 (left panel), 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 non-transfected (None), mock-transfected (Mock) or GFP-transfected (GFP) A20 B-cells.

In previous studies we have demonstrated that adoptive transfer of naïve 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, re-isolated T-cells from lymphoma bearing mice were found to be anergic by their failure to be primed \textit{in vivo} as well as by their diminished IL-2 and IFN-γ production in response to \textit{in vitro} re-stimulation with cognate HA-peptide (20). However, as shown in Fig. 1 (right panel), \textit{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 production by tolerant CD4⁺ T-cells (Fig. 1D). In sharp contrast, anergic T-cells encountering HA-antigen on non-transfected, 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 over-expressed in malignant B-cells. A20 cells were therefore transfected with Stat3c, a mutant form of Stat3 that is constitutively activate without tyrosine phosphorylation (24). Unlike naïve 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-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, Stat3c) and inability to produce IFN-γ in response to cognate antigen (Fig. 2B, Stat3c). Taken together, genetic manipulation of Stat3 in A20 B-cells determines the functional outcome of antigen-specific T-cells and points to Stat3 inhibition as an enticing approach to overcome T-cell anergy.
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 Figure 3, CPA-7-treated A20 B-cells displayed elevated expression of the co-stimulatory 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 collaborators have developed a murine model of MCL by injecting pristane intraperitoneally (ip) into one 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 co-express 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 $5 \times 10^6$ FC-muMCL1 given either sc (black circle) or ip (open circle) developed tumors and demonstrated decreased survival (Fig. 4B). In mice challenged sc, tumor nodules developed by day 21. Intra-peritoneal 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 (Supplemental Figure 1). 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 (OVA) peptide to transgenic CD4$^+$ T-cells expressing a $\alpha\beta$ TCR specific for OVA-peptide$_{323-339}$ (19). First, anti-OVA CD4$^+$ T-cells encountering cognate antigen on untreated MCL cells (OVA 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 OVA-peptide (Fig. 4D, CPA-7+LPS). In contrast, anergized T-cells encountering OVA-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 naïve CD4+ T-cells and restoration of responsiveness of tolerized CD4+ T-cells.

**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 iv every 3 days (16). We therefore injected 5x10^6 FC-muMCL1 cells ip into C57BL/6 mice and twenty-one days later, animals were treated (or not) with 5 mg/kg of CPA-7 given iv 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 Figure 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 \textit{in vivo} anti-MCL effect of CPA-7 in a larger group of animals. C57BL/6 mice were challenged with $5 \times 10^6$ FC-muMCL1 cells given sc. Half of the mice received vehicle control and the other half received CPA-7 (5mg/kg/iv every three 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 (Supplemental Figure 2). Similarly, BALB/c mice challenged with $1 \times 10^6$ A20 lymphoma cells sc and then treated with CPA-7 (same dose and frequency as in the MCL model) rejected this B-cell tumor (Fig. 5C, dashed line). No such rejection was observed in A20 B-cell lymphoma bearing mice given vehicle control (Fig. 5C, solid line). Therefore, \textit{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.

\textbf{The \textit{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 \textit{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 5x10^6 FC-muMCL1 cells or 1x10^6 A20 lymphoma cells given sc, respectively. Then, half the mice in each group were treated with CPA-7 (5mg/kg every three 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 demonstrated (Fig. 5B-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: MCL, Fig. 6B: A20). 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 Figure 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 immunological rather than the non-immunological 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 Figure 7, p-Stat3 was diminished in JEKO cells treated in vitro with
CPA-7. This inhibition was specific since other signaling pathways such as phospho-MAPK or phospho-AKT were not affected in MCL cells treated with even higher doses of CPA-7 (30μM). 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 (Figure 7, right panel). 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 tolerized T-cells. These findings expand the previously known pro-inflammatory effects of Stat3 inhibition upon other immune cells such as BM-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.

In the in vivo immune response against B-cell lymphomas, it is likely that both malignant cells themselves as well as BM-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 physiological immune responses in a process that involves presentation of peptide-MHC class II complexes, along with co-stimulatory signals to antigen specific T-cells (31,32). Like normal B-cells, malignant B-cells also express major histocompatibility complex (MHC) class I and II molecules and low but inducible levels of adhesion and co-stimulatory 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 \textit{in vivo}. First, their expression of MHC molecules, co-stimulatory 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 BM-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 pro-inflammatory cytokines (40-43), or by repairing a dysfunctional T-cell immunological 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 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 Atipromid 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 inhibit Stat3 without affecting other signaling pathways in human MCL (Figure 7), Atipromid is a less selective inhibitor of Stat3 since it also activates JNK and inhibits NF-κB, 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, while 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 Atipromid (CPA-7: 5mg/kg/iv every three days, starting on day +5
after tumor challenge versus Atipromid: 25 mg/kg/ip daily x 6 days starting three to 4 weeks after tumor challenge).

Our demonstration 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 immunological synapse, such as lenalidomide, or as adjuvants to lymphoma vaccines. Furthermore, our findings together with the demonstration that Stat3 is constitutively activate in human MCL cells (46,47) provides 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 pro-inflammatory mediators that could amplify ongoing antitumor immune responses also triggered by the effects of Stat3 inhibition upon APCs and other immune cells (26,48). This pro-inflammatory 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 pro-inflammatory events that likely generates an activating rather than a tolerogenic environment in the lymph nodes, which might be ultimately conducive to effective anti-lymphoma 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, in combination with lenalidomide, or as an adjuvant to therapeutic vaccines.

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References


**Figure Legends**

**Figure 1. Disruption of Stat3 in malignant B-cells augments antigen-specific CD4\(^+\) T-cell responses.** A20 B-cells were untransfected (None), mock transfected or transiently transfected with GFP or Stat3β GFP expression vector. Then, 1x10\(^6\) transfected or control cells were incubated with either 5x10\(^4\) naïve (left panel) or tolerized anti-HA CD4\(^+\) T-cells isolated from the spleen of A20HA-bearing mice (right panel), in the presence of 12.5 \(\mu\)g of HA peptide\textsubscript{110-120} SFERFEIFPKE. After 48 hours, supernatants were collected and assayed for IL-2 (A, C) and IFN-γ (B, D) by ELISA. Values represent mean ± S.E of triplicate cultures and are representative of three independent experiments (*p<0.05 statistically significant for the difference in cytokine production).

**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, 1x10\(^6\) transfected or control cells were incubated with 5x10\(^4\) naïve anti-HA CD4\(^+\) T-cells in the presence or not of 12.5 \(\mu\)g 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 ± S.E of triplicate cultures and are representative of three independent experiments (*p<0.05 statistically significant for the difference in cytokine production).
Figure 3. Enhanced expression of co-stimulatory 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 (15μM) for 15 hours was assessed by flow cytometry. Fifty-thousand gated events were collected on a FACSCALIBUR and analyzed using FlowJo software. Gray histogram: Isotype control. Shown are representative of three experiments with similar results.

Figure 4. CPA-7 augments the antigen presenting function of FC-muMCL1 cells. (A) Expression of Cyclin D1, CD19 and CD5 by FC-muMCL1 cells (open histogram) was assessed by flow cytometry. Gray histogram: Isotype control. (B) In vivo growth of FC-muMCL1 tumors was determined in C57BL/6 mice injected either sc or ip with 5x10^6 FC-muMCL1 cells. Five mice were included in each group and inspected three times a week for the development of tumor. Shown is a representative experiment of two with similar results. (C-D). To ascertain antigen-presenting function of MCL cells, FC-muMCL1 cells (1x10^5 cells/well) were treated with LPS (2 μg/ml), LPS + increasing concentrations of CPA-7, or left untreated (Media) for 24 hours. Then, cells were washed and plated with either 5x10^4 naïve or anergized anti-OVA CD4+ T-cells isolated from tumor bearing mice in presence of 3μg/ml of OVA peptide323-339. Forty-eight hours later, supernatants were collected and the production of IL-2 and IFN-γ by naïve T-cells (C) and the production of IFN-γ by anergized T-cells (D) were determined by ELISA. (E) Expression of B7.1 and B7.2 by FC-muMCL1 cells treated -or not- with CPA-7 (15μM) for 15 hours was assessed by flow cytometry. Gray
histogram: Isotype control. Shown is a representative experiment of three independent experiments with similar results (*$p<0.05$ statistically significant for the difference in cytokine production).

**Figure 5.** *In vivo* treatment with CPA-7 results in decreased Stat3 phosphorylation in malignant B-cells and an anti-lymphoma effect. (A) $5\times10^6$ FC-muMCL1 cells were injected ip into C57BL/6 mice. Twenty-one days later animals were treated or not with 5 mg/kg of CPA-7 given iv every three days (days +21, +24 and +27). On day +29 animals were sacrificed and tumor nodules were carefully dissected from their livers. Malignant B-cells were then isolated and the expression of phopho-Stat3 was determined by western blot using an anti-p-Stat3 (Tyr705) antibody. Shown is a representative of two experiments with similar results. (B) C57BL/6 mice (n=10) were challenged with $5\times10^6$ FC-muMCL1 cells given sc in the right leg. Half the mice were then treated with CPA-7 (5mg/kg) given iv every three days, starting on day +5 after tumor challenge (dashed line). The other half of the mice received vehicle control (solid line). Tumor volumes were calculated (LengthxWidthxWidthX1/2) from measurements as indicated. Two independent experiments were performed with similar results. (C) BALB/c mice (n=10) were challenged with $1\times10^6$ A20 lymphoma cells given sc in the right leg. Half the mice were treated with CPA-7 (dash line), and the other half with vehicle control (solid line) as indicated in (B). Animals were monitored for the development of tumor and tumor volumes.
measured on days indicated. Two independent experiments were performed with similar results ($p<0.01$).

**Figure 6. Similar kinetics of lymphoma growth in CPA-7 treated SCID mice.**

(A) C57BL/6 SCID mice ($n=10$) were challenged with $5 \times 10^6$ Fc-muMCL1 cells given sc. Then mice were treated with CPA-7 (dash line) (5mg/kg iv given every three days, starting on day +5 after tumor challenge) or vehicle control (solid line). (B) BALB/c SCID mice ($n=10$) were challenged with $1 \times 10^6$ A20 cells given sc. Mice were treated with CPA-7 (dash line) or vehicle control (solid line) as indicated in A. Tumors were measured at the indicated times. (C) C57BL/6 SCID and BALB/c SCID mice were treated as indicated in (A) and (B). Mice were sacrificed 19 days after tumor challenge and tumor nodules were collected. Malignant B-cells were then isolated and the expression of phopho-Stat3 was determined by western blot using an anti-p-Stat3 (Tyr705) antibody. Two independent experiments were performed with similar results.

**Figure 7. CPA-7 specifically inhibits Stat3 phosphorylation in human MCL cells.** JEKO cells were treated or not with CPA-7 (30μM) for 24 hours (left panel). In parallel, cells were transiently transfected with Stat3-specific siRNA (Stat3 siRNA) or non-targeting control (Control). Then, cells were harvested and protein extracts were obtained and subjected to western blot using antibodies against p-Stat3, Stat3, p-MAPK, MAPK, p-Akt and Akt. Shown is a representative experiment of two with similar results.
Naïve T-Cells

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Tolerant T-Cells

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A: IL-2 (pg/ml) vs. Treatments

- None
- PC-DNA
- Stat3C

B: IFN-γ (pg/ml) vs. Treatments

- None
- PC-DNA
- Stat3C

For both panels, treatments showed significant differences:
- A: p < 0.005
- B: p < 0.01
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STAT3 INHIBITION AUGMENTS THE IMMUNOGENICITY OF B-CELL LYMPHOMA CELLS LEADING TO EFFECTIVE ANTITUMOR IMMUNITY

Fengdong Cheng, Hongwei Wang, Pedro Horna, et al.

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