Honokiol-mediated Inhibition of PI3K/mTOR Pathway

A Potential Strategy to Overcome Immunoresistance in Glioma, Breast, and Prostate Carcinoma Without Impacting T Cell Function

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Summary: Inhibition of the phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway is an appealing method for decreasing the immunoresistance and augmenting T cell-mediated immunotherapy. A major impediment to this strategy is the impact of conventional PI3K/mTOR pathway inhibitors on T cell function. In particular, rapamycin, is a well-known immunosuppressant that can decrease the activity of the PI3K/mTOR pathway in tumor cells, but also has a profound inhibitory effect on T cells. Here we show that Honokiol, a natural dietary product isolated from an extract of seed cones from Magnolia grandiflora, can decrease PI3K/mTOR pathway-mediated immunoresistance of glioma, breast and prostate cancer cell lines, without affecting critical proinflammatory T cell functions. Specifically, we show that at doses sufficient to down-regulate levels of phospho-S6 and the negative immune regulator B7-H1 in tumor cells, Honokiol does not significantly impair T cell proliferation or proinflammatory cytokine production. In contrast to classic inhibitors, including LY294002, wortmannin, AKT inhibitor III and rapamycin, Honokiol specifically decreases the PI3K/mTOR pathway activity in tumor cells, but not in freshly stimulated T cells. Collectively, our data define a unique application for Honokiol and provide the impetus to more fully elucidate the mechanism by which T cells are resistant to the effects of this particular inhibitor. Honokiol is clinically available for human testing and may serve to augment T cell-mediated cancer immunotherapy.

Key Words: Honokiol, PI3 kinase, immunoresistance

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C ancer therapy is predicated upon specificity without toxicity. The most effective therapies have been able to exploit a unique attribute of the cancer cell with minimal impact on normal tissue. In this regard immunotherapy is very appealing as an approach to target unique tumor-associated antigens that are not expressed by healthy cells. Successful T cell-mediated immunotherapy requires expansion of both CD4 and CD8 positive tumor specific lymphocytes, target lysis, and subsequent development of

a memory T cell population that can serve to target cancer recurrence. As a result, several recent clinical trials have used antitumor vaccination strategies in an attempt to activate and expand tumor specific immune cells.[1–3] Many anticancer vaccination techniques have been described to evoke ex vivo T cell-mediated immunity, with documented peripheral immune responses that are tumor specific.[4,5] These vaccines include tumor specific peptides,[6] dendritic cell approaches,[7] and tumor derived heat shock proteins.[8] However, in the majority of clinical trials there has been no clear correlation between systemic immune responses and clinical efficacy. T cells in the periphery can be generated in response to vaccine, but they do not consistently kill their tumor target, suggesting that an intrinsic defect in T cell responses in the tumor environment.

The discordance between peripheral immune response and clinical efficacy highlights cancer immunoresistance as an emerging problem in immunotherapy. Many types of solid malignancies, including breast, prostate, and brain tumors have well characterized mechanisms of immunosuppression. Local and systemic inhibition of T cell activity can be due in part to factors secreted into the local milieu[9,10] or surface expression of proteins that prevent activation[11] or induce apoptosis[12] of effector cells. In some cases, immunoresistance is correlated with oncogenic pathway activation.[13] We recently uncovered a link between the phosphoinositide 3-kinase (PI3K)/mammalian target of rapamycin (mTOR) pathway and expression of the immunoresistant protein B7-H1 in glioma, breast, and prostate carcinoma cells.[14,15] Other mechanisms of PI3K/mTOR pathway-mediated cancer immunoresistance have been described,[16,17] suggesting that PI3K/mTOR inhibition may serve as a means to augment T cell-mediated immunotherapy. However, a major impediment to this strategy is the impact of conventional PI3K/mTOR pathway inhibitors on T cell function. For example, rapamycin (Rapa), a well known immunosuppressant can decrease the activity of the PI3K/mTOR pathway in tumor cells,[18–20] but also has a profound effect on T cells.[21] The ideal compound would inhibit the PI3K/mTOR in tumor targets without disrupting T cell function.

Here we show that Honokiol (HNK), a natural product isolated from an extract of Magnolia grandiflora seed cones, can decrease PI3K/mTOR-mediated immunoresistance in glioma, breast, and prostate cancer cells through PI3K/mTOR pathway inhibition, without significantly affecting proinflammatory T cell function. First characterized for its antimicrobial activity,[22] HNK has been shown to have antiangiogenic properties as well.[23] Specific antitumor properties include induction of caspase-dependent apoptosis in B cell chronic lymphocytic leukemia

cells,[24] and inhibition of the bone metastatic growth of human prostate cancer cells.[25] Most recently, work in breast cancer cells has shown that HNK can attenuate the PI3K/Akt/mTOR signaling by down-regulation of AKT phosphorylation, and up-regulation of PTEN expression.[26] In this report we show that HNK is effective in the prevention of PI3K/mTOR signaling pathway in glioma, breast, and prostate tumor cells, including decreased expression of B7-H1. Given the role of B7-H1 in the induction of T cell apoptosis, our findings indicate that PI3K/mTOR inhibition by HNK can enhance T cell specific tumor responses both through increased T cell survival as well as maintenance of proinflammatory T cell functions. Together these data support the clinical study of HNK as a means to augment T cell-mediated immunotherapy.

METHODS

Western Blot

Protein concentration of cellular extracts was measured using Protein Assay reagent (Bio-Rad) relative to bovine serum albumin standards. Protein (30 μg) was subjected to SDS-PAGE and electroblotted onto Immobilon-P membrane (Millipore). The membrane was blocked at 4°C overnight and incubated with mouse monoclonal antibody to α-tubulin, p70 S6K1 or phospho-S6 (T 389); rabbit monoclonal antibody to phospho-AKT (S473) (Abcam, Cambridge, MA); or goat polyclonal antibody to B7-H1 (Santa Cruz Biotechnology) after by incubation with isotype specific secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA). Bound protein was visualized using chemiluminescence Western blotting detection reagents (Amersham, Piscataway, NJ). Densitometric measurements were acquired and α-tubulin was used to verify equal loading.

Inhibitor Treatment

Tumor or T cells (1×10^6) were incubated for 48 hours with the following inhibitors: LY294002 (Ly294) (100 μM), wortmannin (WORT) (100 μM), AKT inhibitor III (50 μM), HNK (20 μM) or (100 μM). HNK was a gift from Dr Jack Arbiser at Emory University, all other inhibitors were obtained from Calbiochem, San Diego, CA). All inhibitors were reconstituted in dimethyl sulfoxide (vehicle). Where indicated, vehicle treatment refers to cells treated only with dimethyl sulfoxide for the same duration.

Lymphocyte/Tumor Cell Coculture

Peripheral blood lymphocytes (PBL) were isolated from whole blood by Ficoll centrifugation in accordance with the University of California, San Francisco Committee for Human Research approved protocol no. H47634-01655-34. Informed consent was obtained for collection of PBLs from patients with nonmalignant tumors. PBLs were enriched for CD3^{+} T cells using negative magnetic cell selection according to manufacturer's instructions (Stem-Cell Technologies, British Columbia). CD3^{+} cells were stimulated for 24 hours with 5 μg/mL plate bound antihuman CD3 (OKT3) and 2 μg/mL soluble antihuman CD28 (CD28.2) before coculture. Tumor cells (5×10^4) were plated in a 96-well round-bottom plate and allowed to adhere overnight, 5×10^4 enriched, and stimulated CD3^{+} cells were then added in a final volume of 200 μL.

Assay for Apoptosis

Cells were cocultured as described above for 4 hours and subsequently surface stained on ice with antihuman CD45 APC (BD Pharmingen, San Diego, CA) in phosphate buffered saline /2% bovine serum albumin for 20 minutes and washed for 3 times. Cells were then stained with Annexin V fluorescein isothiocyanate and 7-AAD (BD Pharmingen) for 10 minutes and fixed in 1% formaldehyde containing 5 μg/mL 7-AAD and analyzed by flow cytometry. As a positive control, cells were treated with 1 μM staurosporine (Sigma Aldrich, St Louis, MO), which served as a positive control for apoptosis.

Measure of T Cell Proliferation

CD3^{+} cells were enriched as described above and stained with 10 μM of carboxyfluorescein succinamidyl ester (CFSE) for 10 minutes at 37°C. Cells were quenched with 100% fetal bovine serum and then washed for 3 times in serum containing growth media (RPMI 1640). Cells were then CD3/CD28 stimulated for 72 hours, stained with CD45 APC, fixed in 2% paraformaldehyde and then analyzed by flow cytometry.

Intracellular Cytokine Staining

After the anti-CD3/CD28 stimulation in the presence of Brefeldin A (Sigma-Aldrich), CD3^{+} cells were surface stained with antihuman CD8 fluorescein isothiocyanate, antihuman CD4 PerCP (BD Pharmingen) and/or antihuman CD25 APC (eBioscience, San Diego, CA) on ice for 20 minutes and washed for 3 times. Cells were then fixed in 2% paraformaldehyde for 1 hour. CD3^{+} cells were then stained using antihuman interferon γ (IFNγ) APC or antihuman interleukin (IL)-17 phycoerythrin for the detection of intracellular cytokines (eBioscience) in saponin containing buffer (PermWash, BD Pharmingen). Cells were subsequently analyzed by flow cytometry.

Statistical Significance

Data for all the figures were collected from multiple independent experiments performed in triplicate. Representative experimental data is shown. Statistical significance was determined using a 2-tailed Student t test with $P < 0.05$. Error bars represent ± standard deviation.

RESULTS

We first aimed to demonstrate that HNK could inhibit the activation of the PI(3)K/mTOR pathway in PTEN deficient glioma cell lines. We found that in U87 and U251 glioma cell lines, treatment with HNK inhibited PI(3) kinase activation as demonstrated by a decrease in phospho AKT, phospho S6 kinase, and phospho S6 proteins (Fig. 1A). As expected, inhibition of this pathway was associated with a decrease in B7-H1 protein expression. Inhibition of this pathway after HNK treatment was similar to that observed after treatment with other well characterized inhibitors of the PI3K/mTOR pathway, Ly294, WORT, AKT inhibitor III and Rapa. We have previously demonstrated that a decrease in B7-H1 protein expression on tumor cells promotes the survival of activated T cells through inhibition of B7-H1-mediated apoptosis.[14] To determine if HNK treatment mediates a similar effect, we compared T cell survival after coculture with treated U87 and U251 cell lines before and after exposure to HNK (Fig. 1B). We found a significant decrease ($P < 0.01$) in the
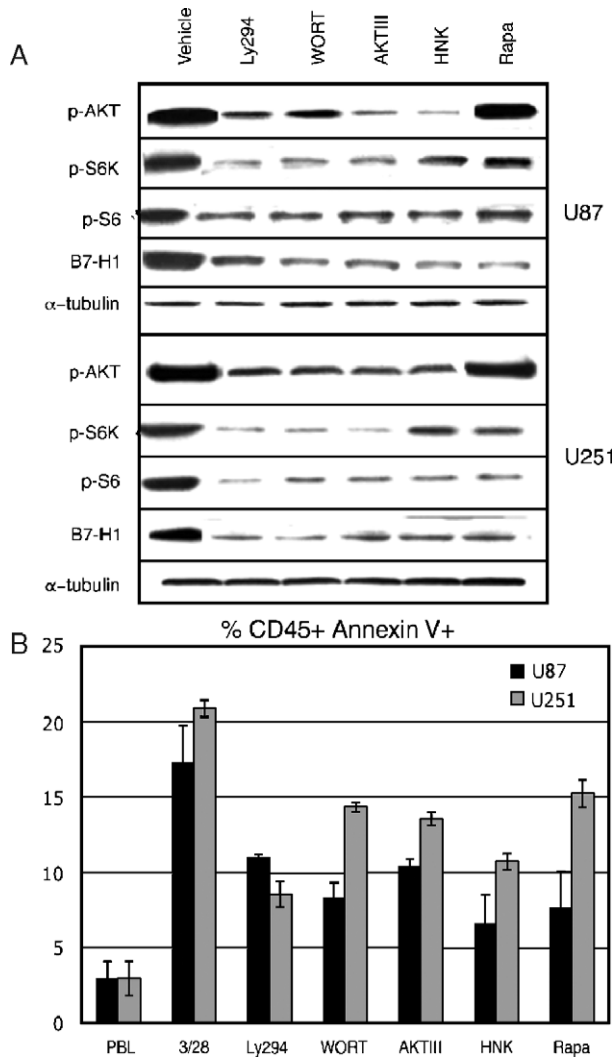
FIGURE 1. HNK inhibits phosphoinositide 3-kinase /mammalian target of rapamycin pathway activation, B7-H1 protein expression and T cell apoptosis in PTEN deficient glioma cells. A, PTEN deficient glioma cell lines U87 and U251 were treated for 72 hours with PI3K inhibitors LY294002 (100 μM), wortmannin (100 μM) , AKTIII inhibitor (50 μM), HNK (20 μM) or Rapamycin (100 μM) and whole cell lysates were analyzed by Western blot as described in Materials and Methods. B, CD3$^+$ lymphocytes (T cells) were stimulated with anti-CD3/anti-CD28 (3/28) or left unstimulated (NS) and cocultured with inhibitor treated-U87 or U251 cell lines for 4 hours. Cocultures were analyzed for T cell apoptosis using CD45/AnnexinV/7-AAD staining. Bars represent the average of triplicate samples from a representative experiment. Error bars represent ± SD. HNK indicates Honokiol.

To determine if this observation was a phenomenon restricted to PTEN deficient glioma cells, we next examined the effect of HNK treatment on PTEN deficient breast (Fig. 2A) and prostate cell lines (Fig. 2B). As observed in glioma cell lines, HNK treatment of BT549 and PC-3 cell lines significantly reduced the downstream phosphorylated components of the PI3K/mTOR pathway to levels similar
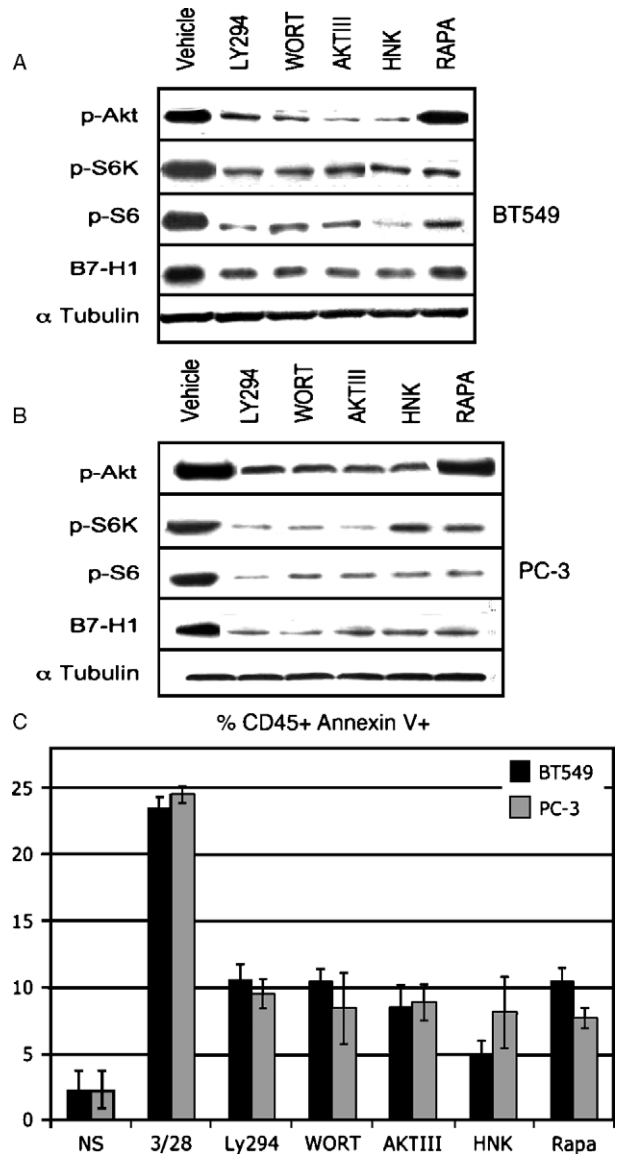

FIGURE 2. Honokiol inhibits PI3K/mammalian target of rapamycin pathway activation, B7-H1 protein expression (A, B) and T cell apoptosis (C) in PTEN deficient breast and prostate cells. PTEN deficient tumor cell lines BT549 (breast cancer, shown in A) or PC-3 (prostate cancer, shown in B) were treated for 72 hours with PI3K inhibitors described in Figure 1 and whole cell lysates were analyzed by Western blot for phospho-AKT, Phospho-S6K, phospho S6 and B7-H1. C, T cells were stimulated with anti-CD3/anti-CD28 (3/28) or left unstimulated (NS) and cocultured with inhibitor-treated BT549 or PC-3 cell lines. Cocultures were analyzed for T cell apoptosis using CD45/AnnexinV/7-AAD staining. Bars represent the average of triplicate samples from a representative experiment. Error bars represent˘ SD. PI3K indicates phosphoinositide 3-kinase.

percentage of T cells undergoing apoptosis when compared with cocultured cells treated with vehicle alone. The percentage of T cells undergoing apoptosis after coculture with U87 or U251 was reduced to levels comparable to those observed after treatment with any other PI3K/mTOR pathway inhibitor tested (average = 6.59% and 10.7%, respectively), suggesting that the decrease in PI3K/mTOR pathway activation observed by Western blot in Figure 1A has functional consequences on tumor cell-mediated induction of T cell apoptosis.

to those observed with treatment of other well-characterized PI3K inhibitors. After coculture with stimulated T cells, HNK-treated tumor cells also significantly reduced the induction of T cell apoptosis as measured by Annexin V staining to a similar degree as the other inhibitors tested (Fig. 2B). Interestingly, the decrease in T cell apoptosis after coculture with HNK-treated BT549 cells was significantly less ($P < 0.05$) than that was observed after the treatment with Ly294, WORT, AKT inhibitor III or Rapa, suggesting that HNK either promotes T cell survival, or it better inhibits the tumor cell-mediated T cell apoptosis.

One possible explanation for the reduction in T cell apoptosis after coculture with HNK-treated tumor cells is that the treatment is toxic to the tumor cells, reducing the number of tumor cells in coculture. To address this possibility, we treated tumor cells with HNK, Ly294, WORT, AKT inhibitor III or Rapa at doses sufficient to inhibit PI3K/mTOR signaling and assessed the amount of induced tumor cell death before coculture. We found that HNK was no more toxic to tumor cells than the other inhibitors (Fig. 3A), suggesting that preferential killing of tumor cells by HNK does not account for the reduction in T cell apoptosis observed in Figure 1. When inhibitor

concentrations were maintained throughout T cell-tumor cell coculture (Fig. 3B), we found that T cell apoptosis was decreased in HNK-treated tumor cells, whereas in the presence of Rapa, WORT, and AKT inhibitor III, T cell death was significantly increased. This may be due to the induction of T cell apoptosis as a result of inhibitor toxicity. Ly294 treatment also increases T cell apoptosis to a lesser degree, despite all of the inhibitors reducing tumor cell survival and B7-H1 expression on tumor cells (Fig. 1).
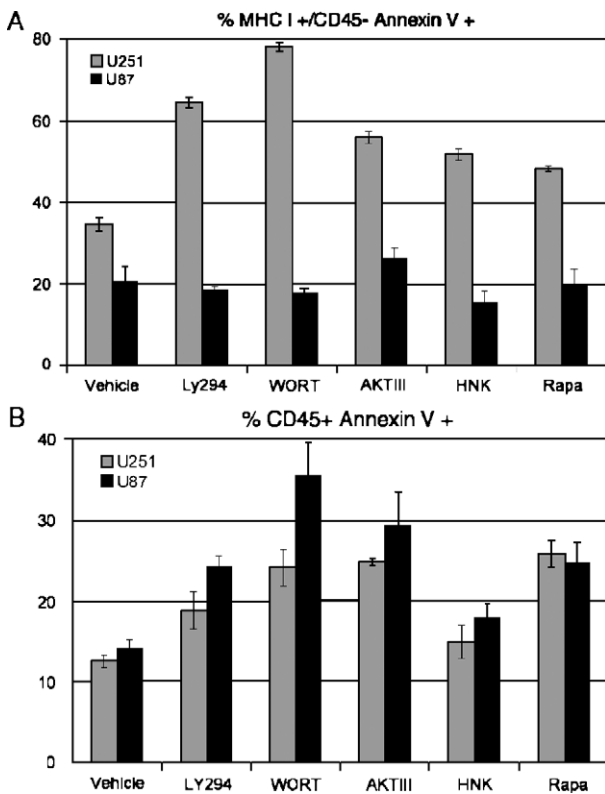

FIGURE 3. Honokiol treatment does not reduce T cell apoptosis by increased toxicity to tumor cells. A, PTEN deficient U87 and U251 tumor cell lines were treated with the following inhibitors for 72 hours: LY294002 (100 mM), wortmannin (100 mM), AKTIII inhibitor (50 mM), Honokiol (20 mM) or rapamycin (100 mM). Tumor cells were subsequently analyzed for apoptosis by flow cytometry using Annexin/7-AAD staining. B, Anti-CD3/CD28 stimulated enriched T cells were treated with inhibitors as in (A) and analyzed for apoptosis 72 hours later using Annexin/7-AAD staining. Bars represent the average of triplicate samples from a representative experiment. Error bars represent ± SD.
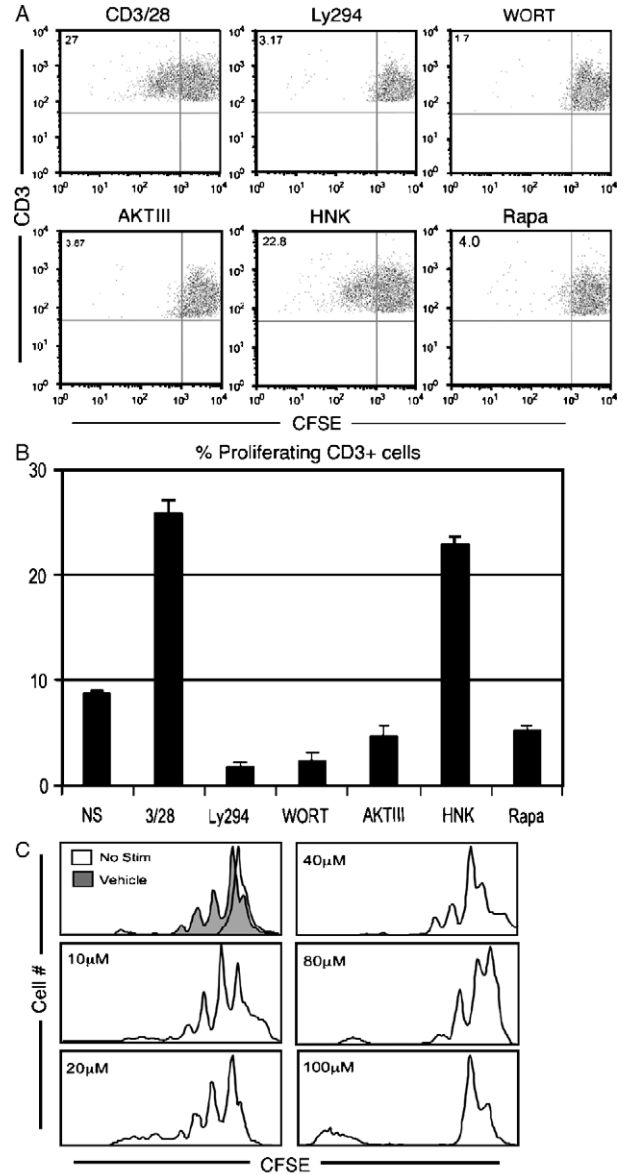

FIGURE 4. Honokiol-treated T cells proliferate in response to stimulation. A, Representative flow cytometry of T cells stained with CFSE and stimulated with CD3/CD28 in the presence of inhibitors. 72 hours later, cells were analyzed for dilution of CFSE. B, Average percentage of proliferating T cells after 72-hour CD3/CD28 stimulation. Percentages were determined using the gates drawn on the raw data shown in (A). Bars represent the average of triplicate samples from a representative experiment. Error bars represent ± SD. C, CFSE dilution by CD3+ T cells 72 hours after CD3/CD28 stimulation in the presence of increasing doses of Honokiol. CFSE indicates carboxyfluorescein succinimidyl ester.

Having demonstrated that HNK-mediated similar effects on tumor cell signaling and a reduction in tumor cell-mediated T cell apoptosis, we sought to determine the effects of HNK on activated T cell responses. We found that in contrast to other inhibitors, HNK supported proliferation of a high percentage of T cells after stimulation (Fig. 4). The slight reduction of proliferating T cells from 25.9% to 22.9% in response to vehicle treatment (Fig. 4B) was not statistically significant ($P = 0.58$), suggesting a minimal effect of HNK on T cell expansion. All other PI3K/mTOR inhibitors tested significantly reduced levels of proliferation to below that observed in unstimulated cells (8.83%), suggesting that baseline proliferation in patient lymphocytes is also affected by these inhibitors. Inclusion of inhibitors during the coculture with tumor cells did impair T cell proliferation (data not shown), suggesting that the observed effects are due to the anti-inflammatory properties of tumor cells and their effects on T cell proliferation, rather than the presence of HNK in the coculture. It has been reported, however, that HNK has anti-inflammatory properties in T cells, inhibiting the proliferation of the lipopolysaccharide responsive CTLL-2 cell line.[26] To determine if we could detect a similar inhibition of proliferation in primary T cells, we measured T cell proliferation by following the increasing doses of the HNK treatment. At doses sufficient to inhibit PI3K/mTOR signaling in tumor cells (20 µM), T cell proliferation is unaffected. However, at doses approaching 80 µM, T cell proliferation can be inhibited, suggesting that HNK does have the potential to act as an anti-inflammatory agent at relatively high doses.

After stimulation of T cells, several activation markers are up-regulated on the cell surface, including the IL-2
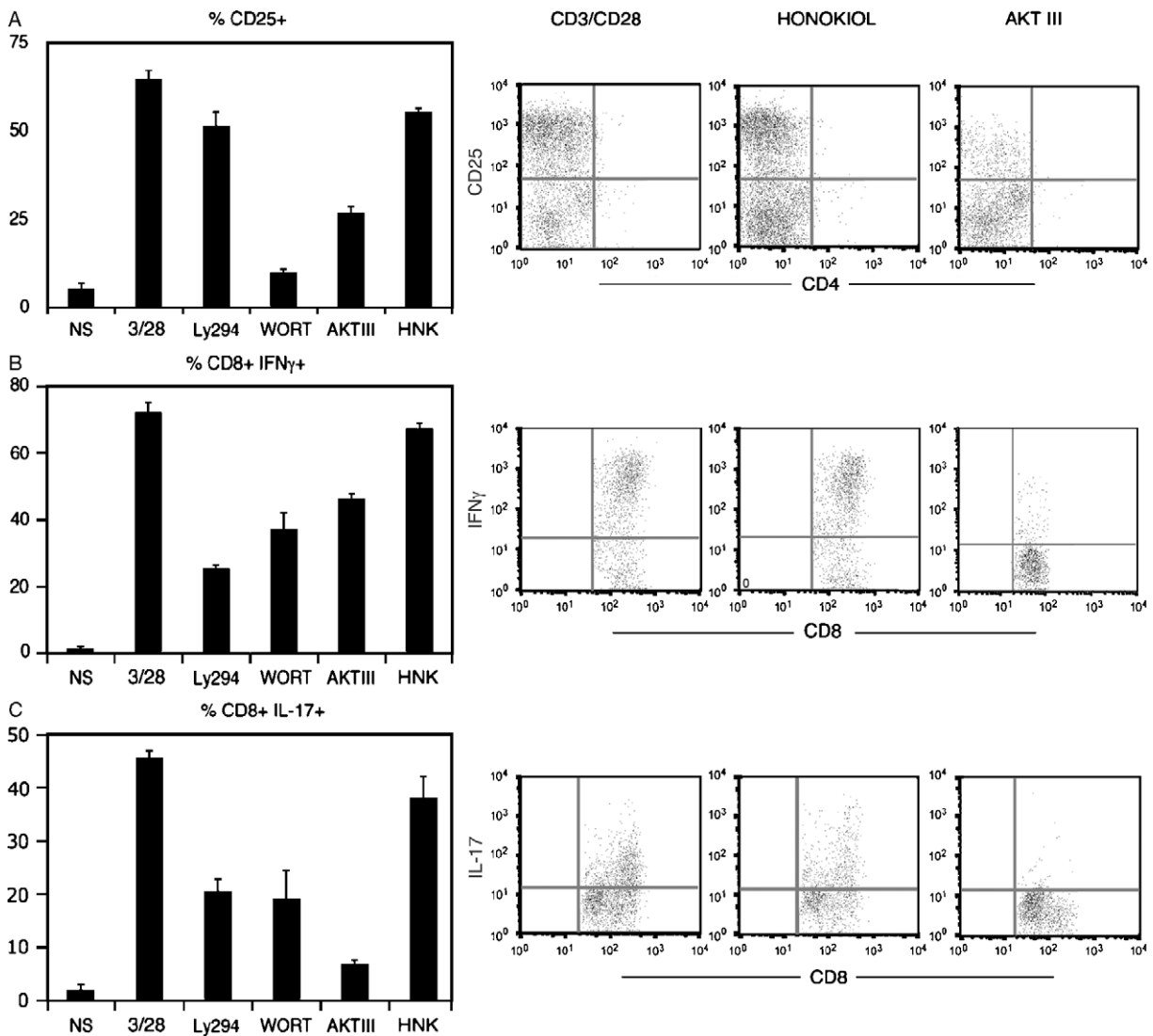

FIGURE 5. Honokiol-treated CD8+T cells produce proinflammatory cytokines in response to stimulation. A, An average percentage of CD8+ T cells expressing CD25 on the cell surface. T cells were stimulated for 72 hours as described in Materials and Methods and analyzed for surface expression of CD4 (x-axis) and CD25 (y-axis) by flow cytometry. B and C, T cells were stimulated for 72 hours and analyzed for CD8 (x-axis) expression and intracellular accumulation of (B) IFNγ (y-axis) or (C) IL-17 (y-axis). Bar graphs are representative of the average expression of triplicate samples. Percentages were determined using the gates drawn on raw flow cytometry data. Error bars represent +/− SD.

receptor, CD25. CD25 is an activation marker, but also functionally important in immune responses as activated T cell proliferation is dependent on IL-2 signaling through CD25. To determine if the difference in T cell proliferation observed after inhibitor treatment could be attributed to differential expression of CD25, we examined the surface expression on CD4 negative, CD8 positive, and T cells (Fig. 5A). Although HNK does significantly decrease the percentage of CD25 expressing cells ($P < 0.01$) relative to cells treated with vehicle only, we find that 55.5% of stimulated CD8 T cells express CD25, compared with only 9.9% after WORT treatment, and 27.0% after AKT inhibitor III treatment, which could explain the differences in T cell proliferation. Interestingly, Ly294 treatment and HNK treatment had similar effects on CD25 expression (51.5%), suggesting a CD25 independent mechanism for abrogation of T cell proliferation observed in Ly294-treated T cells.

Successful immunotherapy is dependent upon proinflammatory CD8 T cell responses characterized by either IFNγ or IL-17 production.[27,28] To determine if HNK treatment prevented proinflammatory cytokine production in response to stimulation, we next measured the effect on cytokine production by CD8 T cells. We found that HNK treatment did not significantly impair the ability of CD8 T cells to produce IFNγ (Fig. 5B) or IL-17 (Fig. 5C) when compared with T cells treated with vehicle alone. In contrast, all of the other inhibitors tested significantly reduced the percentage of proinflammatory cytokine producing cells. Importantly, the amount of cytokine produced on a per cell basis was similar to that observed in untreated cells after stimulation (Figs. 5B, C). T cell cytokine responses during tumor cell coculture were further decreased in the presence of inhibitor (data not shown), indicating that tumor cells with reduced PI3K/mTOR have an intrinsic ability to impair T cell proinflammatory responses. This suggests that HNK treatment would best function in conjunction with other treatments aimed to offset the anti-inflammatory tumor microenvironment. Together, these data indicate that HNK treatment at concentrations sufficient to down-regulate PI3K/mTOR pathway signaling, and B7-H1 expression, has a minimal effect on the proinflammatory response of adaptive immune effector cells.

We assessed the levels of phosphorylated S6, an endpoint of PI3K/mTOR signaling in T-cells after treatment with HNK to determine if the retention of proliferation and proinflammatory cytokine production occurs in spite of impaired PI3K/mTOR /S6 signaling in T cells. Whereas AKT inhibitor III treatment of T cells decrease the levels of phospho-S6 after stimulation, HNK treatment did not (Fig. 6A). However, HNK treatment of the PTEN deficient glioma cell line U87, did result in a 60% reduction of phospho-S6 levels (Fig. 6B). Because the activation of S6 is important for protein translation and activated T cell function, these data suggest that T cells may have a redundant mechanism to activate S6 despite the potent PI3K/mTOR pathway inhibition by HNK.

DISCUSSION

T cell-mediated cancer immunotherapy requires that a tumor specific CD8 T cell can identify and effectively target the cancer cell. Local and systemic immunoresistance is therefore a significant impediment to successful immunotherapy. Tumor cells have several mechanisms that facilitate immunoresistance and immunosuppression, including TGFβ secretion[29] or CTLA-4 expression.[30] We have previously linked the expression of B7-H1 protein to activation of the PI3K/mTOR pathway,[14,15] identifying this oncogenic pathway as a potential target for overcoming one known mechanism of immunoresistance in glioma, breast and prostate cancer cells, resulting in T cell apoptosis. Here we have shown that conventional inhibitors (Ly294, WORT, Rapa, and AKT inhibitor III) can reduce T cell apoptosis in coculture with PTEN deficient tumor cell lines, but that these inhibitors all have a significant impact on T cell proinflammatory function. Specifically, these inhibitors reduced T cell proliferation, decreased the percentage of CD8 T cells expressing interferon γ and IL-17, as well as decreased the percentage of T cells expressing the activation marker CD25. Accordingly, the strategy of augmenting T cell-mediated immunotherapy by inhibiting the PI3K/mTOR pathway using these conventional agents is likely to be minimally effective owing to the side effects on T cell function.

We first considered HNK as a possible candidate based on its proven ability to inhibit the PI3K/mTOR pathway in other cancers,[26] as well as its wide use as a herbal medicine, suggesting that it would be well tolerated as a clinical agent and unlikely to negatively impact the immune system. Here we show that HNK can inhibit B7-H1 expression in three types of PTEN deficient tumor cells as effectively as other conventional inhibitors. In doing so, HNK may also reduce the expression of other immunoresistant proteins that are the product of PTEN loss in these cells, by reducing cellular S6-mediated translation. In contrast to other conventional inhibitors, HNK has a minimal effect on the proliferation of T cells, expression of activation markers or proinflammatory cytokine production. The results of these experiments provide the impetus to more fully explore the clinical utility of HNK as an adjunct to active immunotherapy. Together with previously published work showing in vitro and in vivo inhibition of both tumor cell proliferation[25] and angiogenesis,[23] our data demonstrate a promising application for this natural PI3K/mTOR inhibitor that has previously not been described. The data presented here are also consistent with recently published studies using xenograft tumor models,[23,31]
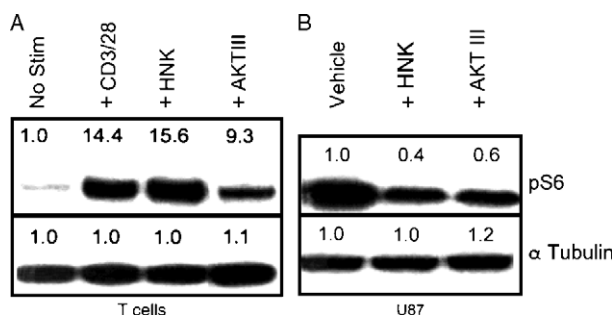

FIGURE 6. Honokiol treatment, but not AKT inhibitor III treatment allows the activation of phosphoinositide 3-kinase/ mammalian target of rapamycin pathway in T cells. (A) T cells or (B) U87 glioma cells were stimulated for 72 hours in the presence of Honokiol (20 μM) or AKT inhibitor III (50 μM) and analyzed for S6 phosphorylation by Western blot. Alpha tubulin was used as a loading control. Densitometry data provided a quantitative measure of protein expression levels.

suggesting a potential broad based application in patients with cancer.

Other published reports have indicated that HNK has anti-inflammatory effects, inhibiting the production of inflammatory cytokines,[32] scavenging free radicals produced during an ongoing immune response[33] in apparent contrast to our findings. However, the cytokines and responses evaluated in these studies are those produced during an innate immune response such as nitric oxide, IL-6, and TNFα, rather than those made during adaptive immune responses. One recent study suggests that HNK behaves instead as an anti-inflammatory agent in T cells.[26] Possible explanations for this discrepancy relate to the type of cells and stimulations used in the experiments. Here we describe the effects of low HNK doses on primary T cells stimulated with anti-CD3/CD28, whereas the previous work used the CTLL-2 cell line after lipopolysaccharide or Conconavalin A stimulation. After the treatment of T cells with increasing doses of HNK, we also find that this inhibitor can block T cell proliferation (Fig. 4), suggesting that in some conditions, HNK may be useful as an anti-inflammatory agent. Taken together with the data presented here, HNK may have the potential to inhibit the nonspecific tissue damage that innate cytokines and free radicals can cause and maintain T cell function and the specificity of adaptive immune therapy at doses sufficient to prevent PI3K/mTOR signaling.

The mechanism by which T cells are apparently resistant to the effects of HNK remains to be elucidated. Our data suggest that PI3K/mTOR pathway is unaffected in stimulated T cells exposed to HNK, in contrast to tumor cells exposed to the same dose over the same time course. The resistance may relate to redundancy in signaling pathways in T cells, oncogenic addiction in transformed cells, biochemical properties that distinguish permeability of T cells from tumor cells, or a combination of multiple factors. Regardless of the specific mechanism responsible for the discordance between glioma and lymphocytes, the biochemical and functional data in this report provide new insights into the potential clinical utility of HNK, within the context of immunotherapy for patients harboring PTEN deficient tumors.

Further studies are needed to extend these results into the clinical realm, including the use of preclinical animal models. Current studies are ongoing in our laboratory to evaluate the efficacy of HNK treatment on the growth of glioma cells in vivo. Longitudinal studies of these animals will reveal the ability of T cells to eliminate tumor cells treated with HNK, prolong animal survival and reduce tumor burden. In these studies, ex vivo analysis of T cells will also determine optimal doses and treatment regimens that promote T cell proinflammatory function whereas preventing PI3K/mTOR activation in tumor cells. We anticipate that higher doses will be required to achieve the results obtained in vitro owing to the complexity of the tumor microenvironment. However, the widespread dietary use of HNK suggests that this agent will be well tolerated at high doses. It has also been shown that a dosage of between 35 and 65 mg/kg 3 times a week is well tolerated in animal models and can induce tumor cell apoptosis in vivo, providing a median testable range for T cell function and toxicity in tumor bearing animals.[31]

In conclusion, we have identified a previously unknown feature of HNK that may offer a therapeutic advantage to patients being treated with T cell-based immunotherapy protocols. In vivo, HNK treatment may decrease tumor cell survival and PI3K/mTOR-mediated immunoresistance to adaptive immune responses. Taken with the current interest in T cell-mediated immune therapies for patients with many types of cancer, our data indicate that HNK may be best considered in combination with other therapies to maintain immune cell function during treatment.

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