

CANCER IMMUNOLOGY

Dual enhancement of T and NK cell function by pulsatile inhibition of SHIP1 improves antitumor immunity and survival

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The success of immunotherapy in some cancer patients has revealed the profound capacity for cytotoxic lymphocytes to eradicate malignancies. Various immunotherapies work by blocking key checkpoint proteins that suppress immune cell activity. The phosphatase SHIP1 (SH2-containing inositol polyphosphate 5-phosphatase) limits signaling from receptors that activate natural killer (NK) cells and T cells. However, unexpectedly, genetic ablation studies have shown that the effector functions of SHIP1-deficient NK and T cells are compromised *in vivo*. Because chronic activation of immune cells renders them less responsive to activating signals (a host mechanism to avoid autoimmunity), we hypothesized that the failure of SHIP1 inhibition to induce antitumor immunity in those studies was caused by the permanence of genetic ablation. Accordingly, we found that reversible and pulsatile inhibition of SHIP1 with 3- α -aminocholestane (3AC; “SHIPi”) increased the antitumor response of NK and CD8⁺ T cells *in vitro* and *in vivo*. Transient SHIP1 inhibition in mouse models of lymphoma and colon cancer improved the median and long-term tumor-free survival rates. Adoptive transfer assays showed evidence of immunological memory to the tumor in hematolymphoid cells from SHIPi-treated, long-term surviving mice. The findings suggest that a pulsatile regimen of SHIP1 inhibition might be an effective immunotherapy in some cancer patients.

INTRODUCTION

Host defense against malignancy is mediated, in part, by T cell- and natural killer (NK) cell-mediated killing of malignantly transformed cells (1–3). However, compensatory mechanisms, called T cell exhaustion and NK cell disarming, induce a state of “hyporesponsiveness” among chronically activated T and NK cells to prevent misdirected targeting of healthy cells and autoimmune disease (4–7). Increased survival among cancer patients treated with immune checkpoint inhibitors has revealed the potential for T cells to mediate immune control of malignancy (8). This approach lifts self-tolerance limitations imposed on the T cell compartment by tumors via antibody blockade of the T cell inhibitory receptor CTLA4 (cytotoxic T-lymphocyte antigen 4) or PD-1 (programmed cell death-1), or the PD-1 ligand PD-L1, and has enabled the emergence of potent antitumor CD8⁺ T cell responses (9, 10). This has resulted in the clearance of widely metastatic disease and consequently long-term survival in a small subset of patients (11). Several groups have also begun to target NK cells with immunomodulatory strategies in ongoing clinical trials (12).

The SH2-containing inositol polyphosphate 5-phosphatase (SHIP1) catalyzes the hydrolysis of the phosphatidylinositol 3-kinase (PI3K) product PI(3,4,5)P₃ to form PI(3,4)P₂ (13, 14). Numerous biochemical and *in vitro* studies have suggested that SHIP1 can limit T cell receptor

(TCR) signaling in T cells and inhibit NK cell responses (15–20). However, *SHIP1*^{−/−} mice do not have hyperresponsive T or NK cells *in vivo*; rather, mice with T cell-specific deletion of *SHIP1* have profound T cell death at mucosal sites, defective effector T cell differentiation, and increased numbers of regulatory T cells (19, 21, 22). Additionally, despite increased signaling downstream of activating receptors, NK cells from mice with NK cell-specific deletion of *SHIP1* are also hyporesponsive *in vivo* (23).

Given the numerous reports that SHIP1 can limit TCR signaling and NK cell activation *in vitro*, we hypothesized that loss of SHIP1 signaling might initially result in increased T and NK cell activity, but that hyporesponsiveness is eventually induced among these cells to preserve self-tolerance when inhibition or loss of SHIP1 signaling is sustained. The recent discovery of small-molecule SHIP1 inhibitors (SHIPi) has enabled testing of this hypothesis through the reversible and pulsatile inhibition of SHIP1 signaling *in vivo* (24–28). We proposed that intermittent, short-term inhibition of SHIP1 would promote increased responses by these two lymphocyte lineages, and that intermittent dosing would prevent the induction of hyporesponsiveness caused by chronic activation. We show here that SHIPi increased both NK and T cell responsiveness and reduced the growth of hematological and solid tumors in mice. Additionally, after lymphoma challenge, SHIPi-treated mice had prolonged and, in some cases, long-term tumor-free survival with evidence of immunological memory to the tumor.

RESULTS

Short-term inhibition of SHIP1 *in vivo* promotes increased responsiveness of T and NK cells

SHIP1 is recruited directly to the TCR and to the scaffolding protein Dok1 that limits T cell activation by the TCR (15–19). SHIP1 also opposes activation of the PI3K-AKT-mTOR signaling pathway in NK cells (23, 29). Accordingly, mice with extended duration of SHIP1 deficiency

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either from genetic ablation or from long-term treatment with SHIP1 inhibitors have increased activation of the PI3K signaling pathway. However, NK cells from these mice have decreased capacity to perform effector functions (23, 24, 29). We hypothesized that SHIP1 deficiency might initially result in increased PI3K signaling with resultant hyper-responsiveness, but that continuous PI3K activation for an extended duration induces the hyporesponsive phenotype described previously. To test this hypothesis, we used the small-molecule 3- α -aminocholestane (3AC; herein called SHIPi) to selectively inhibit SHIP1 signaling. Specifically, we treated host mice for two consecutive days with either SHIPi or its vehicle, isolated splenocytes, and then used intracellular flow cytometry to measure NK cell interferon- γ (IFN- γ) production after cross-linking each of three major NK cell-activating receptors *ex vivo*. NK cells from SHIPi-treated mice had significantly greater IFN- γ production after stimulation of each activating receptor than those from vehicle-treated mice (Fig. 1, A and B). Consistent with SHIP1's proximal role in limiting receptor signaling, NK cells stimulated with phorbol 12-myristate 13-acetate (PMA) and ionomycin showed no difference between SHIPi-treated and vehicle-treated controls. This indicates that SHIP1 plays a role in limiting NK cell receptor responsiveness and that short-term inhibition of SHIP1 function results in NK cell hyperresponsiveness, which is not sufficient to disarm the NK cell compartment.

To determine whether SHIPi is also capable of activating the other major type of cytotoxic lymphocyte, we examined the responsiveness of CD8⁺ T cells using this same short-term regimen of SHIPi treatment. As described above, T cells from SHIPi-treated mice also exhibited increased responsiveness compared to those from vehicle-treated controls (Fig. 1, C and D). SHIPi treatment significantly increased not only the percentage of activated CD8⁺ T cells *in vivo* as determined by the emergence of CD69 expression but also the surface abundance of CD69 among CD8⁺ T cells (Fig. 1, C and D). Splenic CD8⁺ T cells harvested from SHIPi-treated mice had enhanced cytolytic capacity with increased abundance of LAMP-1 (CD107a), a marker of cellular degranulation, after cross-linking of their TCR and the costimulatory molecule CD28 (Fig. 1E). Thus, SHIPi treatment increases the responsiveness of both T and NK cells *in vivo* when stimulated via major activating receptors.

SHIPi acts as an immunotherapeutic

On the basis of the increased activating receptor signaling seen above, we sought to determine whether acute SHIPi treatment promotes *in vivo* clearance of tumor cells expressing a ligand for such receptors. We analyzed the ability of SHIPi treatment to increase host rejection of tumor cells *in vivo* using the C57BL/6-derived H2^b T cell lymphoma cell line RMA-Rae1. NK cell-mediated rejection of RMA-Rae1 cells requires perforin expression and is independent of IFN- γ production, and thus, this assay enables interrogation of a different NK cell effector function than that analyzed in Fig. 1 (30).

Mice were challenged with syngeneic tumor cells followed by, as described above, treatment with SHIPi or vehicle for two consecutive days. On the third day, peritoneal lavage was performed to evaluate tumor burden. In C57BL/6 hosts that have a fully functional adaptive immune system, we observed a marked reduction in lymphoma burden after SHIPi treatment (Fig. 2A). A similar reduction was also observed in *RAG1*^{-/-} hosts that lack B and T cells, and thus, acute rejection is not attributable in this assay to increased antitumor activity by B or T lymphocytes (Fig. 2B). To assess whether NK cells contributed to acute tumor rejection, we performed the same experiment using NK cell-depleted C57BL/6 hosts (Δ NK). SHIPi-treated hosts with an intact NK cell compartment had significantly greater tumor rejection com-

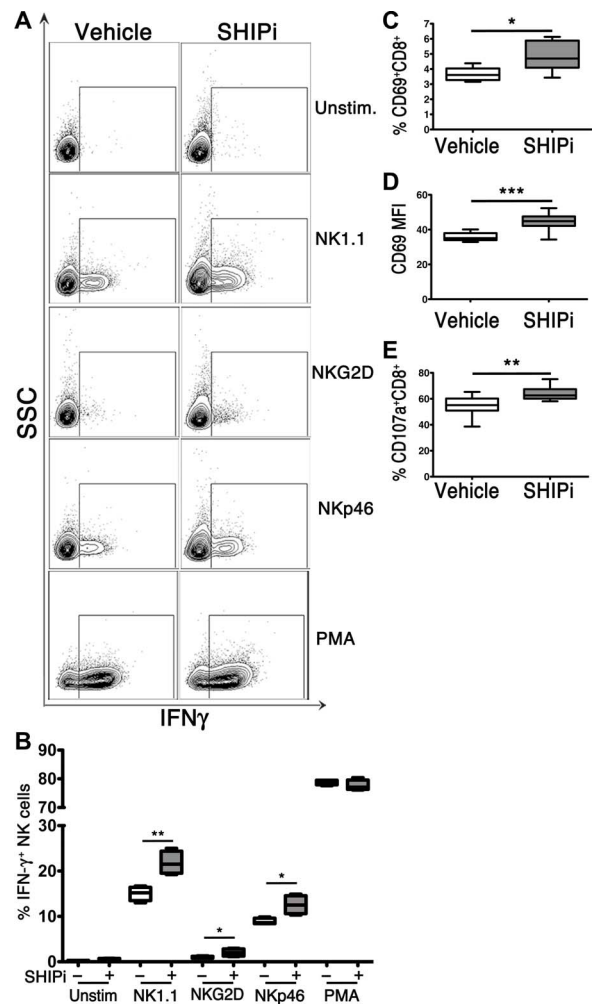


Fig. 1. NK cells and CD8 T cells exhibit enhanced activation after short-term *in vivo* SHIPi. (A and B) NK cells isolated from 2-day SHIPi- or vehicle-treated mice were stimulated by incubation on uncoated plates; on plates coated with antibodies against NK1.1, NKp46, or NKG2D; or on uncoated plates containing PMA and ionomycin (PMA). Representative flow plots (A) and box-and-whisker plots (B) show the frequency of NK cell production of IFN- γ determined by intracellular flow cytometry. (C and D) Frequency (C) and abundance (D) of CD69 among splenic, CD8⁺ T cells from 2-day SHIPi- or vehicle-treated mice. (E) T cells isolated from 2-day SHIPi- or vehicle-treated mice were stimulated by overnight incubation on plates coated with anti-CD3 and anti-CD28 antibodies. Anti-CD107a (LAMP-1) phycoerythrin (PE) antibody was added for the final 5 hours of incubation. Degranulation of CD8⁺ T cells was determined by analysis of CD107a expression by flow cytometry. NK cells are defined as NK1.1⁺CD3^ε⁻ and CD8⁺ T cells as NK1.1⁻CD3^ε⁺CD8⁺CD4⁻ or NK1.1⁻TCR β ⁺CD8⁺CD4⁻. Graphs are representative of at least two independent experiments with at least four SHIPi- and vehicle-treated mice per group (A to B), or pooled data from two independent experiments are shown (C to E). In (B), Unstim: $n = 4$, $P = 0.0962$ by unpaired t test with Welch's correction; NK1.1: $n = 4$, $P = 0.0038$; NKG2D: $n = 4$, $P = 0.0450$; NKp46: $n = 4$, $P = 0.0144$; and PMA: $P = 0.2945$, each by two-tailed Student's t tests. In (C), $n = 8$, $P = 0.0153$ by unpaired t test with Welch's correction. In (D) and (E), $n = 8$, $P = 0.0009$, and $n = 9$, $P = 0.0083$, each by two-tailed Student's t tests.

pared to SHIPi hosts depleted of NK cells before tumor challenge (Fig. 2C). This increase in tumor burden was not seen in vehicle-treated mice that were also depleted of NK cells (Fig. 2D). Regardless of whether mice were depleted of NK cells before tumor challenge,

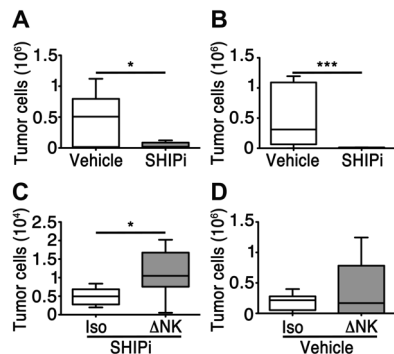


Fig. 2. SHIPi-treated mice have increased tumor rejection that requires NK cells. (A to D) Green fluorescent protein (GFP)–expressing RMA-Rae1 cells (5×10^5) were injected into C57BL/6 (A) or $RAG1^{-/-}$ (B) mice, or C57BL/6 mice that had either an antibody-mediated depleted (Δ NK) or intact (Iso) NK cell compartment (C and D). Mice were treated with either SHIPi or vehicle for 2 days, and tumor burden was determined by flow cytometric analysis of peritoneal cavity contents recovered by peritoneal lavage on the third day. Graphs represent pooled data from two independent experiments ($n \geq 4$ mice each condition in each experiment). (A) $P = 0.0433$ by Mann-Whitney U test; (B) $P = 0.0007$ by Mann-Whitney U test; (C) $P = 0.0153$ by unpaired t test with Welch's correction; (D) $P = 0.9188$ by Mann-Whitney U test.

SHIPi-treated mice had a profound reduction in tumor burden compared to vehicle-treated controls (Fig. 2, C and D). These results indicate that NK cells are required for SHIPi-induced acute tumor rejection, but that there may be additional mechanisms through which SHIPi mediates host protection from malignancy.

SHIPi increases NK cell numbers and maturation at the tumor site

Similar to mice with NK cell–specific deletion of SHIP1 that have NK cell lymphocytopenia (23), SHIPi-treated mice also have decreased numbers of splenic NK cells (Fig. 3A). However, we found that short-term inhibition of SHIP1 signaling with SHIPi significantly increased the number of NK cells present at the tumor site in the peritoneal cavity (Fig. 3B) and also significantly increased the terminal maturation of the peripheral NK cell compartment as assayed in the spleen (Fig. 3C). This terminally differentiated NK cell subset has increased cytolytic potential, and thus, their increased numbers in SHIPi-treated hosts are consistent with the increased tumor eradication observed after SHIPi treatment (31).

SHIPi enhances FasL-Fas–mediated killing of lymphoma cells

NK cells are able to directly kill target cells through several different mechanisms including both production of perforin and granzymes and induction of apoptosis via Fas-FasL signaling (32, 33). SHIPi-treated RMA cells express increased frequency of the death receptor CD95/Fas that initiates apoptosis via induction of caspase 8 after ligation by FasL/CD95L (fig. S1). SHIP1 was recently shown to be recruited to CD95/Fas to set a threshold for induction of caspase 8 and, thereby, a threshold for induction of apoptosis (21). We considered that this pathway might contribute to SHIPi-mediated killing of lymphoma cells in a host-extrinsic fashion and, possibly, even to NK cell–mediated clearance of tumor in vivo. To explore this possibility, we first examined whether SHIPi induces FasL, the death ligand on cytotoxic host cells present in the tumor site. We saw a marked increase in FasL abundance among both host NK cells and T cells at the tumor site in SHIPi-treated mice that was not

observed in vehicle-treated, tumor-challenged control mice (Fig. 3, D to F). Notably, a small but significantly increased frequency of splenic NK cells, but not T cells, also acquired FasL expression after SHIPi treatment (Fig. 3G).

Our data showed that NK cells are required for maximal reduction of tumor burden (Fig. 2); however, the large reduction in tumor burden among NK cell–deficient hosts treated with SHIPi indicated a possible direct chemotherapeutic effect of SHIPi on T cell lymphoma. This is consistent with what we have previously reported for multiple myeloma, myeloid leukemias, and T cell leukemia, and that which others have recently reported in precursor B cell acute lymphocytic leukemia (21, 25, 26, 34). We find that SHIPi is capable of directly killing RMA-Rae1 cells (Fig. 3H). We found a significant induction of apoptosis in SHIPi-treated RMA cells and increased activation of caspase 8, consistent with SHIPi lowering the threshold for Fas signaling (Fig. 3I), as we have previously shown in a human T cell leukemia (21). Therefore, the improved tumor clearance and survival after acute SHIPi treatment in vivo may result from both a chemotherapeutic effect and enhanced immune control.

SHIPi increases survival of tumor-bearing mice

The most clinically relevant endpoint in any cancer therapy study is not a measured decrease in tumor burden but, instead, a significant increase in survival promoted by the treatment. To determine whether the increased killing of tumor cells by hyperactivated NK and T cells, as well as increased apoptosis of tumor cells induced by SHIPi, results in increased survival of tumor-challenged hosts, we treated RMA-Rae1 tumor-bearing C57BL/6 hosts with either SHIPi or vehicle for two consecutive days each week followed by a 5-day cessation of treatment and then monitored survival (Fig. 4A). This pulsatile dosing strategy was chosen to avoid NK cell disarming and T cell exhaustion due to unattenuated activation as seen in mice with genetic or prolonged pharmacological ablation of SHIP1 signaling (23, 24, 29). We found that SHIPi-treated hosts had significantly increased survival compared to vehicle-treated controls (Fig. 4A). Notably, this tumor burden was uniformly lethal in vehicle-treated mice, whereas a subpopulation of SHIPi-treated mice had durable, long-term survival without any evidence of tumor burden (Fig. 4A), indicating that SHIPi may even be curative in a subset of tumor-challenged hosts.

SHIPi requires both NK and T cells to induce long-term survival after tumor challenge

To further define the capacity of SHIPi to control malignancy through immunomodulatory effects, and examine the roles of NK and T cells specifically, we examined the capacity of SHIPi to promote resistance to RMA-Rae1 lymphoma challenge in NSG, $TCR\alpha^{-/-}$, and NK cell–depleted mice. As before, mice received treatment with SHIPi or vehicle for two consecutive days each week followed by a 5-day cessation of treatment to prevent disarming and/or exhaustion. This treatment strategy was continued indefinitely, and survival was monitored. Tumor-challenged NSG mice (which are deficient in all lymphocyte lineages) had only a very modest increase in median survival in response to SHIPi therapy compared to vehicle-treated controls (Fig. 4A). Likewise, tumor-challenged SHIPi-treated $TCR\alpha^{-/-}$ mice (which lack a $TCR\alpha\beta^+$ T cell compartment) did not have improved survival rates compared to T cell–proficient C57BL/6 controls (Fig. 4A), indicating that maximal and sustained protection from tumor growth by SHIPi requires the presence of an intact immune system, specifically a lymphocyte compartment that includes $TCR\alpha$ -expressing T cells.

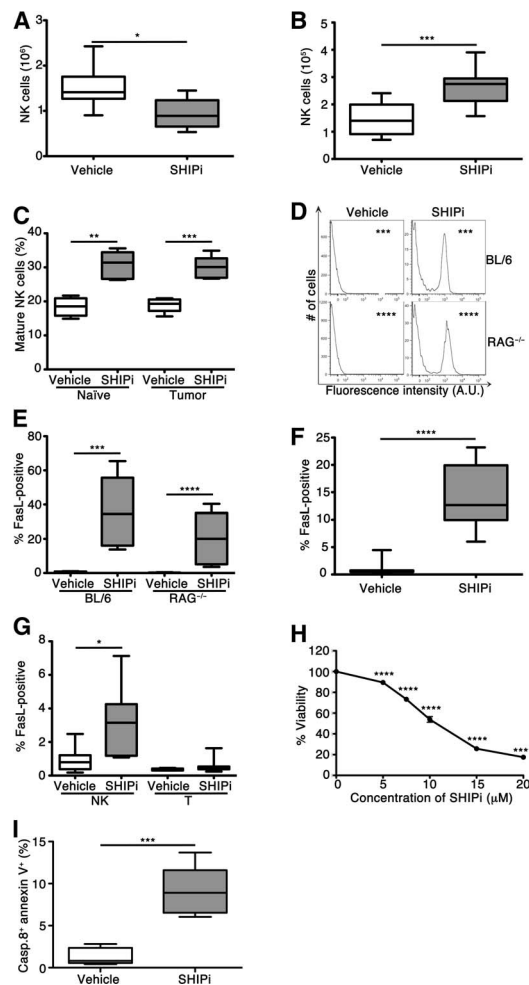


Fig. 3. SHIPi is a novel chemoimmunotherapeutic. (A) Flow cytometric assessment of absolute number of splenic NK cells isolated from mice the day after two consecutive days of treatment with SHIPi or vehicle. $*P < 0.05$ by two-tailed Student's *t* test. (B) Number of peritoneal NK cells recovered by peritoneal lavage from SHIPi- or vehicle-treated and tumor-challenged (5×10^5 RMA-Rae1 cells) C57BL/6 hosts. $***P < 0.001$ by two-tailed Student's *t* test. (C) Proportion of mature splenic NK cells (CD27⁺CD11b⁺) among total NK cells isolated from SHIPi- or vehicle-treated, naïve, or tumor-challenged C57BL/6 hosts. $**P < 0.01$, $***P < 0.001$ by two-tailed Student's *t* test. (D and E) Representative histograms (D) and box-and-whisker plots (E) of the number and percentage, respectively, of FasL-positive NK cells isolated from tumor-challenged C57BL/6 or *RAG1*^{-/-} mice, treated with either SHIPi or vehicle. $***P < 0.001$ by unpaired *t* test with Welch's correction, $****P < 0.0001$ by Mann-Whitney *U* test, compared with respective vehicle condition. A.U., arbitrary units. (F) Percent of FasL-positive T cells recovered by peritoneal lavage of SHIPi- or vehicle-treated, tumor-challenged mice. $****P < 0.0001$ by Mann-Whitney *U* test. (G) Percentage of FasL-positive splenic NK and T cells isolated from SHIPi- or vehicle-treated C57BL/6 mice. $*P < 0.05$ ($P = 0.0192$) by unpaired *t* test with Welch's correction (T cells, $P = 0.0659$ by Mann-Whitney *U* test). In (A) to (G), NK cells were detected by NK1.1⁺CD3e⁻ staining, and data are representative of at least two independent experiments in which $n \geq 4$ mice per treatment group. (H) RMA-Rae1 cell viability in vitro after exposure to SHIPi for 24 hours at the indicated doses. Viability is expressed as the frequency at a given concentration relative to solvent only-treated cells. Data are means \pm SEM, pooled from three independent experiments, each with six replicate wells per concentration. $****P < 0.0001$ by one-way analysis of variance (ANOVA) with Dunnett's multiple comparisons. (I) Box-and-whisker plots assessing apoptosis (annexin V) and caspase 8 activation in RMA cells after culture with SHIPi versus vehicle for 12 hours. Data are pooled from two independent experiments, each done in triplicate. $***P < 0.001$ by unpaired *t* test with Welch's correction.

To examine the requirement for specifically an intact NK cell compartment for SHIPi-mediated increased survival after tumor challenge, we challenged C57BL/6 hosts that were first depleted of NK cells. Here, before tumor challenge, hosts were injected either with NK cell-depleting anti-NK1.1 antibody (C57BL/6- Δ NK) or with antibody isotype control (C57BL/6-iso). NK cell depletion significantly compromised the survival of SHIPi-treated hosts relative to SHIPi-treated hosts pretreated with antibody isotype control (Fig. 4B), demonstrating that an intact NK cell compartment is also required for SHIPi to extend survival after tumor challenge. A previous report showed that mice receiving uninterrupted, extended-duration SHIPi treatment (for 6 days each week) have a disabled NK cell compartment as indicated by their inability to acutely reject a major histocompatibility complex I (MHC-I) mismatched bone marrow (BM) graft (24), a unique *in vivo* effector function of NK cells (35). To further explore whether NK cells contribute to SHIPi-mediated enhanced survival after tumor challenge, we examined the resistance to tumor challenge in hosts that received the same, extended-duration SHIPi treatment that we have previously shown disarms the NK cell compartment (24). We failed to observe increased survival of tumor-challenged C57BL/6 hosts receiving extended-duration SHIPi compared to vehicle-treated controls (Fig. 4C). Thus, consistent with our hypothesis that SHIP1 opposes NK cell activation to prevent disarming, sustained treatment with SHIPi disarms the NK cell compartment and effectively compromises host survival after tumor challenge as opposed to the transient and pulsatile SHIPi treatment strategy that induced NK cell activation and tumor rejection.

We then tested the effects of SHIPi in a genetic model in which SHIP1 is absent in a key target cell population of SHIPi—T cells (CD4CreSHIP^{fllox/fllox} mice). CD4CreSHIP^{fllox/fllox} mice treated with SHIPi after RMA-Rae1 challenge had a similar survival rate to that in SHIPi-proficient vehicle-treated mice (SHIP^{+/fllox} mice; Fig. 4D). Notably, SHIPi in the absence of a SHIP1-proficient T cell compartment does not compromise baseline host resistance to RMA-Rae1 lymphoma challenge. However, as expected, SHIPi treatment did enhance survival among lymphoma-challenged SHIP^{fllox/fllox} mice (which are SHIP1-sufficient) compared with either SHIPi-treated mice with T cell-specific SHIP1 deficiency (CD4CreSHIP^{fllox/fllox}) or vehicle-treated controls (SHIP^{+/fllox}; which are also SHIP1-sufficient) (Fig. 4D). Thus, SHIPi protection after tumor challenge is lost when the T cell compartment lacks the drug's molecular target (SHIP1) in T cells. Together, the findings thus far demonstrate that intermittent exposure to SHIPi enhances both NK and T cell function to promote host resistance to cancer and that the drug does so at least in part by targeting SHIP1 specifically in T cells.

Selective SHIP1 inhibition is required to promote improved tumor survival

There are two paralogs of SHIP: SHIP1, which is expressed in a limited range of tissues including hematopoietic cells, and SHIP2, which is expressed by a wide array of cell types. To determine whether SHIP2 may also play a role in host defense against malignancy as described above, we treated tumor-bearing mice with small-molecule inhibitors against SHIP2 as well. The small molecules shown to inhibit the SHIP1 and SHIP2 paralogs include those with a high degree of selectivity for SHIP1 [such as 3AC; (25)] and SHIP2 [such as AS1949490; (36)] or those that exhibit inhibitory activity for both isoforms ["pan-SHIP1/2 inhibitors" such as K118 and K149; (27, 37)]. Like 3AC, pan-SHIP1/2 inhibitors are also cytotoxic for RMA-Rae1 cells *in vitro* (fig. S2). We initially compared the pan-SHIP1/2 inhibitory compounds K118 and K149 to the SHIP1-selective compound SHIPi (3AC), and found that

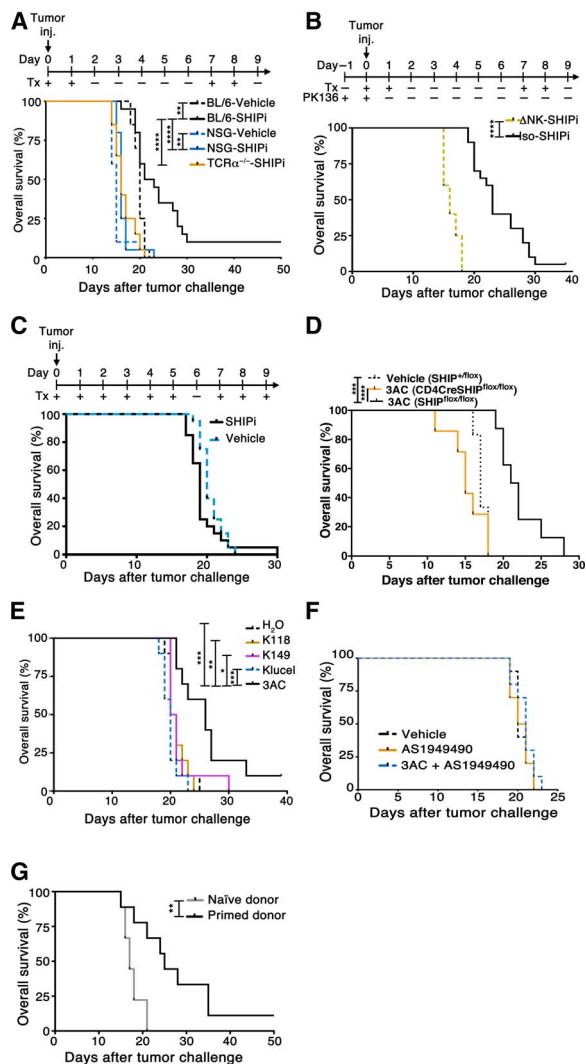


Fig. 4. SHIPi improves survival among tumor-bearing mice in a T cell- and NK cell-dependent manner. (A) Kaplan-Meier curves for overall survival in SHIPi- or vehicle-treated C57BL/6 and NSG mice, and in SHIPi-treated TCR $\alpha^{-/-}$ mice, each injected with 5×10^5 RMA-Rae1 cells. Assay and treatment schedule (Tx +) indicated in the schematic. (B) Kaplan-Meier curves for overall survival in SHIPi-treated NK cell-depleted C57BL/6 mice (Δ NK; yellow) and SHIPi-treated control C57BL/6 mice (Iso; black) injected with 5×10^5 RMA-Rae1 cells. PK136, NK1.1 antibody; Iso, isotype control antibody. (C) Kaplan-Meier curves for overall survival in chronic SHIPi- or vehicle-treated C57BL/6 mice injected with 10^5 RMA-Rae1 cells. (D) Kaplan-Meier curves for overall survival in CD4CreSHIP^{flox/flox}, SHIP^{+/-flox}, and SHIP^{flox/flox} mice that were injected with 5×10^5 RMA-Rae1 cells and treated with SHIPi (3AC) or vehicle as indicated. Assay and treatment schedule as shown in (A). (E) Kaplan-Meier curves for overall survival in C57BL/6 hosts injected with 1×10^5 RMA-Rae1 cells and treated with SHIPi (3AC; SHIP1 selective inhibitor), Klucel (3AC's vehicle), K118 or K149 (pan-SHIP1/2 inhibitors), or water (vehicle for K118 and K149). Assay and treatment schedule as shown in (A). (F) Kaplan-Meier curves for overall survival in C57BL/6 mice injected with 1×10^5 RMA-Rae1 cells and treated with AS1949490 (SHIP2 selective inhibitor), 3AC and AS1949490, or vehicle control. (G) Kaplan-Meier curves for overall survival in naïve C57BL/6 mice that received adoptive transfer of hematolymphoid cells (5×10^6 splenocytes and 5×10^6 BM cells) from either a SHIPi-treated long-term RMA-Rae1-challenged ("Primed") or naïve C57BL/6 donor before injection with 5×10^5 RMA-Rae1 cells. Data are each pooled from two independent experiments: total $n = 20$ (A to C), $n \geq 6$ (D), $n = 10$ (E and F), and $n = 9$ (G) mice in each condition. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$ by log-rank (Mantel-Cox) test.

despite the ability of K118 and K149 to target SHIP1, there was no protection afforded by either pan-SHIP1/2 inhibitory compound (Fig. 4E), whereas SHIPi again extended survival in the RMA-Rae1 lymphoma model as described above, including providing long-term survival in a subset of hosts. We considered then that simultaneous inhibition of both SHIP1 and SHIP2 may be deleterious to the immune response promoted by SHIPi. To test this, we performed a similar study in which RMA-Rae1 lymphoma-challenged mice were treated simultaneously with the highly selective SHIP2 inhibitor AS1949490 and SHIPi (selective for SHIP1). We found that treatment with AS1949490 offered no protection, and in agreement with our hypothesis, coinhibition of SHIP2 by AS1949490 abrogated the protective effect of SHIPi (Fig. 4F). These results further confirm that SHIPi has potent antitumor properties, but demonstrate that the beneficial effects of SHIP1 inhibition require paralogue-selective inhibition.

Immunological memory to tumor cells is present in surviving hosts

Given that SHIPi induced long-term survival among a subset of tumor-challenged mice, we hypothesized that SHIPi may have also induced formation of immunological memory capable of sustained and protective antitumor response that prevents relapse. To test for the continued presence of such immunity, we performed adoptive transfer of hemato-lymphoid cells from either a naïve donor C57BL/6 mouse or a tumor-challenged, SHIPi-treated, long-term surviving (herein called "primed") donor C57BL/6 mouse into naïve C57BL/6 hosts. These hosts (naïve to both treatment and tumor) were then challenged with tumor cells but did not receive SHIPi treatment. Recipients of primed hematolymphoid cells had significantly increased survival compared to recipients of naïve hematolymphoid cells after tumor challenge (Fig. 4G). These results indicate that immune cells in long-term survivors of tumor challenge that had been treated with SHIPi are capable of a protective and sustained response to the tumor cells.

SHIPi limits tumor growth in nonhematologic malignancies

We were then interested to know whether SHIPi is able to potentiate host rejection of solid tissue tumors in addition to hematological malignancy. Colorectal cancer (CRC) is the second leading cause of cancer mortality in the United States (38). Thus, we challenged mice with CT26, a spontaneous colon carcinoma that arose in BALB/c mice, and MC-38, a carcinogen-induced colon carcinoma identified in a C57BL/6 host. These are two prominent tumor models that are regularly featured in murine studies of immunotherapy; the growth of both tumors is subject to immune-mediated restriction, with T cell responses being a predominant mediator but with NK cells also contributing to reduced tumor growth (39–43). Unlike RMA-Rae1 lymphoma cells, CT26 and MC-38 CRC cells do not express SHIP1 (fig. S3) and thus are resistant to direct killing by the SHIP1-selective inhibitor 3AC (Fig. 5, A and B). Mice were challenged with subcutaneous injection of CT26 or MC-38 tumor cells into the right flank and then received either SHIPi or vehicle control for two consecutive days each week. We observed a significant reduction in tumor size and weight in both CT26 and MC-38 (Fig. 5, C to F) tumor models among the SHIPi-treated mice relative to vehicle-treated controls. Consistent with those results, histological analysis of tumors showed prominent zones of tumor cell death only in the tumors harvested from SHIPi-treated hosts (Fig. 5, G and H). Thus, because of its capacity to promote tumor immunity, SHIPi can also reduce the growth of epithelial tumor types that lack the expression of SHIP1.

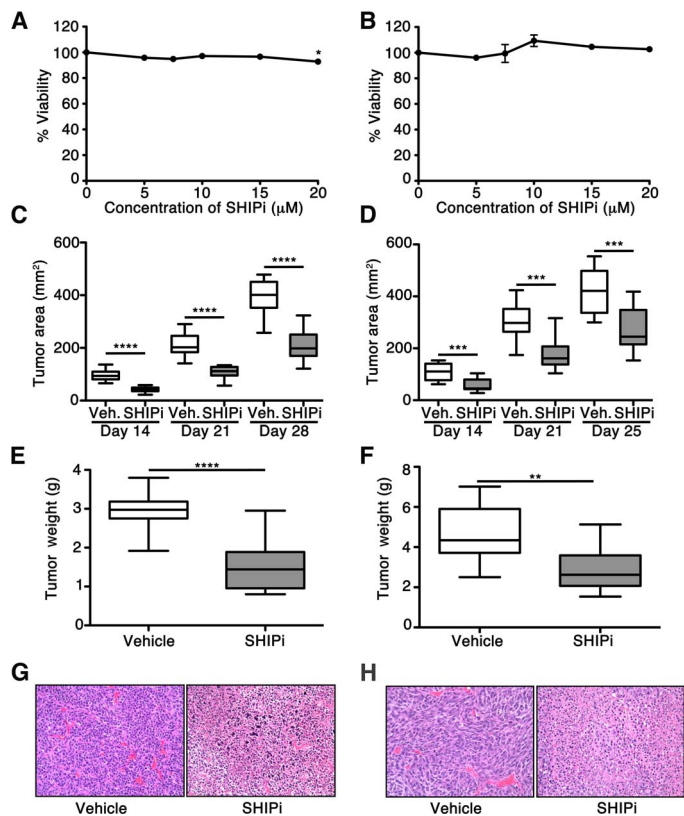


Fig. 5. SHIPi reduces nonhematopoietic tumor progression. (A and B) Viability in CT26 (A) and MC-38 (B) CRC cells after culture with SHIPi for 24 hours at the indicated doses relative to solvent-treated cells. Data are means \pm SEM, pooled from three independent experiments, each consisting of six replicate wells per concentration. (C to H) BALB/C and C57BL/6 mice were challenged with subcutaneous injection of CT26 (C, E, and G) or MC-38 (D, F, and H) colon carcinoma cells, respectively, in the right flank and then treated with either SHIPi or vehicle as described in Fig. 4A schematic. Tumor size was monitored over the duration of the experiment (C and D), and tumor weight (E and F) and hematoxylin and eosin (H&E) staining (G and H) were performed upon completion and dissection of the tumors. (A and B) $n = 18$ samples per group; $*P < 0.05$ and $**P < 0.01$ by one-way ANOVA with Dunnett's multiple comparisons. (C to F) Data are from one of two independent experiments; $n = 10$ mice per condition. $***P < 0.001$ and $****P < 0.0001$ by unpaired t test with Welch's correction (C, day 14) or two-tailed Student's t test (C, days 21 and 28, D, and E).

DISCUSSION

Chronic stimulation of T and NK cells results in hyporesponsiveness in an effort to maintain self-tolerance (44). This immunosuppression, in part, allows for progression and metastatic spread of malignancy because tolerant cytotoxic lymphocytes are unable to eradicate the disease (45, 46). Multiple strategies to activate the host immune system against malignancy are currently used in clinical practice or under investigation in clinical trials. Unfortunately, most other immunotherapeutics are either cellular- or protein-based therapies that are either difficult to administer (require injection or infusion), expensive, or both.

We have previously shown that sustained loss of SHIP1 signaling results in T cell dysfunction and NK cell hyporesponsiveness despite increased activation of signaling pathways that should promote effector function. Here, we show that transient and pulsatile inhibition of SHIP1 results not only in increased NK cell numbers and maturation but also in increased NK cell responsiveness, consistent with the disarming hy-

pothesis of NK cell education (6). In addition, we show that treatment with SHIPi in vivo promotes increased activation of and TCR responsiveness by CD8⁺ T cells. The increased responsiveness of cytotoxic lymphocytes promoted by SHIPi includes increased cytokine production, T cell responsiveness, and NK cell- and T cell-mediated clearance of tumor cells, resulting in significantly increased survival of tumor-bearing hosts and/or reduced tumor growth. These data demonstrate that SHIPi not only can directly induce apoptosis of malignant cells but also can activate an immune response that, in some cases, culminates in long-term immunological memory toward the tumor. Thus, this small-molecule inhibitor of SHIP1 (SHIPi) acts as a novel and potent chemoimmunotherapeutic capable of directly killing tumor cells while simultaneously promoting increased efficacy of NK and T cell anti-tumor responses. Additionally, its nature as a small molecule has numerous clinical advantages over current antibody- and cell-based immunotherapeutics, most critically cost and ease of administration, altogether affording SHIPi the potential to greatly improve the accessibility and effectiveness of cancer immunotherapy.

MATERIALS AND METHODS

Mice

C57BL/6, NSG, BALB/c, and TCR- $\alpha^{-/-}$ mice used in this study were purchased from either Jackson Laboratories or Taconic Biosciences. CD4CreSHIP^{fllox/fllox} (and SHIP^{+fllox} or SHIP^{fllox/fllox} littermates) and RAG1^{-/-} mice were bred and maintained in a pathogen-free environment at State University of New York (SUNY) Upstate Medical University. All animal experiments were approved by the SUNY Upstate Medical University Institutional Animal Care and Use Committee.

SHIPi

SHIPi was a solution of 3AC in 0.3% hydroxypropylcellulose (molecular weight, 60,000)/PBS (phosphate-buffered-saline) (w/v) (herein referred to as SHIPi). Mice received daily intraperitoneal injections of either 100 μ l of vehicle or 26.4 mg of 3AC per kilogram of body weight suspended in vehicle.

Ex vivo analysis of NK cell responsiveness

Mice received SHIPi or vehicle treatment at about the same time daily for 2 days. Plates were meanwhile coated overnight with anti-NK1.1 (PK136), anti-NKp46 (29A1.4), or anti-NKG2D (A10) (eBioscience). Splenocytes were then isolated and depleted of red blood cells (RBCs) (using 1 \times RBC lysis buffer; eBioscience) and then incubated in the presence of GolgiPlug (BD Biosciences) in each of the antibody-coated wells, in uncoated wells, or in uncoated wells containing PMA (1.67 μ g/ml) and ionomycin (1 μ g/ml) for 5 hours. Invitrogen's LIVE/DEAD Fixable Aqua Stain was used for dead cell exclusion. Surface receptors were stained using anti-CD49b (DX5), anti-NK1.1 (PK136), or anti-NKp46 (29A1.4) and anti-CD3e (145-2C11) antibodies (eBioscience). Cells were fixed and permeabilized using BD Cytotfix/Cytoperm kit (BD Biosciences). Intracellular staining was performed using an anti-IFN- γ (XMGI.2) antibody (eBioscience).

Ex vivo analysis of CD8⁺ T cell responsiveness

Mice received SHIPi or vehicle treatment at about the same time daily for 2 days. Splenocytes were stained with antibodies against NK1.1 (PK136), CD3e (145-2C11), TCR β (H57-597), CD4 (RM4-5), CD8 (53.6.7), and CD69 (H1.2F3). CD8⁺ T cells were defined as CD3e⁺CD8⁺CD4⁻NK1.1⁻ or TCR β ⁺CD8⁺CD4⁻NK1.1⁻. Surface expression of CD69 by CD8⁺

T cells was determined by flow cytometry. Degranulation was determined by analysis of CD107a (LAMP-1). Splenocytes harvested from 2-day SHIPi- or vehicle-treated mice were incubated overnight on plates coated with anti-CD3 ϵ (2 μ g/ml; 145-2C11, eBioscience) and anti-CD28 (5 μ g/ml; 37.51, eBioscience) antibodies. Anti-CD107a PE (1D4B) antibody (eBioscience) and GolgiStop (BD Biosciences) were added for the final 5 hours of incubation. CD107a expression was determined by flow cytometry.

In vivo NK and T cell antitumor targeting assay

Mice were injected with 5×10^5 RMA-Rae1 cells. RMA-Rae1 cells generated by retroviral transduction of RMA parental cells with an IRES-GFP vector were a gift from W. Yokoyama (Washington University in St. Louis). Mice then received 2 days of SHIPi or vehicle injections, with the first injection on the day of tumor challenge. On the third day, mice were sacrificed by CO₂ toxicity. The peritoneal cavity was washed with 10 ml and then 5 ml of cold PBS. Cells recovered from peritoneal lavage were stained with anti-NK1.1 (PK136), anti-NKp46 (29A1.4), anti-CD11b (M1/70), anti-GR-1 (RB6-8C5), anti-CD19 (1D3), and anti-CD3 ϵ (145-2C11) antibodies (eBioscience or BD Biosciences). Either LIVE/DEAD Fixable Aqua Stain or DAPI (4',6-diamidino-2-phenylindole) (Invitrogen) was used for dead cell exclusion.

Survival studies (SHIPi)

Mice were injected intraperitoneally with 5×10^5 RMA-Rae1 cells. Mice used in survival studies with NK cell depletion received two intraperitoneal injections of 200 μ g of anti-NK1.1 (PK136) 1 day before (day -1) and again the day of (day 0) tumor cell injection. Control mice received intraperitoneal injection of immunoglobulin G2a (IgG2a) antibody isotype control. The mice were then injected with SHIPi or vehicle on the same day and the day after tumor challenge (days 0 and 1) and repeated weekly (days 7 and 8, and so forth) until the mouse died. Assays testing chronic SHIPi induction of NK cell disarming were performed by intraperitoneal injection of 1×10^5 RMA-Rae1 tumor cells into C57BL/6 followed by treatment with SHIPi or vehicle for six consecutive days each week (starting with the day of tumor cell injection) until the mouse died.

Survival studies (K118, K149, and AS1949490)

C57BL/6 mice were injected with 100,000 RMA-Rae1 cells by intraperitoneal injection. Mice received two intraperitoneal injections of either of these compounds, one on the same day the tumor was injected and the other on the following day. Mice were injected twice a week for the duration of the experiment. K118 was dissolved in sterile water and injected at 10 mg/kg. K149 was dissolved in 5% dimethyl sulfoxide (DMSO) and injected at 10 mg/kg. AS1949490 (Tocris) was injected at 20 mg/kg in 5% DMSO. Where indicated in the figures, some mice received both 3AC and AS1949490 injections. Mice were monitored for survival daily.

Flow cytometry

Staining was performed as detailed in the relevant assay descriptions above. In all cases, Fc receptors were blocked with anti-CD16/CD32 (2.4G2) antibody (BD Biosciences) before staining. NK maturation was determined by staining splenocytes with anti-NK1.1 (PK136), anti-CD3 ϵ (145-2C11), anti-CD11b (M1/70), and anti-CD27 (LG.3A10) antibodies. Cells were analyzed using a BD LSRFortessa cell analyzer (BD Biosciences). Samples were analyzed using FlowJo 9.4.3 and typically involved the analysis of 0.5×10^6 to 1.0×10^6 events for each sample. NK cells were identified by expression of NK1.1, DX5, or NKp46 in

the absence of other lineage markers. Tumor cells were identified by expression of GFP. In all cases, cells were identified after electronic backgating on singlets and viable cells. For analysis of Fas surface density on RMA cells, cells were incubated with either SHIPi (21 μ M 3AC) or solvent alone for 12 hours before analysis. RMA cells were then stained with anti-Fas/CD95 (15A7) antibody (eBioscience).

Western blotting

Cells were pelleted by centrifugation at 350g for 5 min. Cell pellets were washed twice in PBS before resuspension in three pellet volumes of ice-cold lysis buffer [20 mM tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, and 1% Triton X-100] with 1 \times HALT Protease Inhibitor (Thermo Fisher Scientific) and 1 mM phenylmethylsulfonyl fluoride (Sigma-Aldrich). Lysates were incubated on ice for 10 min and centrifuged at 21,000g at 4°C for 10 min. Protein concentration in each cleared cell lysate was quantified using the BCA Protein Assay Kit (Thermo Fisher Scientific), according to the manufacturer's recommendation. Equal amounts of protein were separated by SDS-polyacrylamide gel electrophoresis on 4 to 15% gradient acrylamide gels (Bio-Rad Laboratories) using standard procedures. Proteins were transferred to nitrocellulose membranes using the Turbo Trans-Blot System (Bio-Rad Laboratories). The membranes were blocked in 5% nonfat dry milk in TBS with 0.1% Tween 20 (TBST) and probed with specific primary antibodies against SHIP1 and β -actin (clones PIC1 and C-4, respectively; Santa Cruz Biotechnology). After washes in TBST, blots were probed with horseradish peroxidase-conjugated goat anti-mouse IgG (Santa Cruz Biotechnology). Proteins were detected using SuperSignal West Pico Chemiluminescence Substrate (Thermo Fisher Scientific), and luminescence signal was acquired with ImageLab 5.1 software (Bio-Rad Laboratories) on the ChemiDoc XRS System (Bio-Rad Laboratories).

In vitro cell killing assay

RMA-Rae1 cells were plated at a density of 6×10^4 cells per well in 96-well plates in RPMI 1640 [American Type Culture Collection (ATCC)] supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich). CT26 and MC-38 cells were plated at a density of 1.8×10^4 cells per well in RPMI 1640 or Dulbecco's minimum essential medium supplemented with 1 mM sodium pyruvate (Thermo Fisher Scientific), 1 \times nonessential amino acids (Thermo Fisher Scientific), and 10% FBS, respectively. Cells were treated for 24 hours in the presence of 3AC or K118 at indicated doses in six replicate wells. CCK-8 cell viability assay (Dojindo Molecular Technologies Inc.) was performed as previously described (26). Cell viability is expressed as the percentage of solvent only-treated cells (1% ethanol in complete medium for 3AC and 0.5% DMSO in complete medium for K118). Mean \pm SEM was calculated from six replicate wells and pooled from three independent experiments.

Caspase 8 activation assay

Caspase 8 activation assay was performed using the CaspGLOW Fluorescein Active Caspase 8 Staining Kit as described previously (21). Briefly, RMA cells were treated with 21 μ M SHIPi or vehicle for 12 hours followed by incubation with FITC-IETD-FMK (eBioscience) for 1 hour. Cells were washed and stained for annexin V and propidium iodide (BD Biosciences) and analyzed by flow cytometry.

NK cell depletion assays

For NK cell depletion studies, mice received intraperitoneal injection of anti-NK1.1 (PK136) (200 μ g; Bio X Cell) for two consecutive days before tumor challenge experiments. The control group in all NK cell

depletion experiments received intraperitoneal injection of IgG2a antibody isotype control.

Assessment of antitumor immunological memory by SHIPi

Sublethally irradiated (5.5 Gy) C57BL/6 mice received RBC-lysed BM cells (5×10^6) and splenocytes (5×10^6) from naïve C57BL/6 donors or from SHIPi-treated, long-term surviving RMA-Rae1 tumor-challenged C57BL/6 donors. Three weeks after adoptive transfer of hematolymphoid cells, mice were challenged with 5×10^5 RMA-Rae1 tumor cells and monitored for survival without any additional treatment.

MC-38 and CT26 tumor studies

C57BL/6 and BALB/c mice were injected subcutaneously on the right flank with 5×10^5 MC-38 colon carcinoma cells (Kerafast) and 5×10^5 CT26.WT colon carcinoma cells (ATCC), respectively. The mice were then injected with SHIPi or vehicle on two consecutive days each week for the duration of the study, with the first injection given on the day tumors were injected. Tumor size was measured using a digital caliper and expressed as area (longest dimension multiplied by the perpendicular dimension). Tumor weight was determined by weighing dissected tumor specimen immediately after sacrifice. Tissues were H&E-stained and formalin-fixed (neutral buffered, 10% formalin solution; Sigma-Aldrich) and analyzed by a veterinary pathologist.

Statistical analysis

All statistical tests were performed using Prism (GraphPad) software as follows: Survival was analyzed using Mantel-Cox log-rank test. One-way ANOVA with Dunnett's multiple comparison test was used for analysis of in vitro tumor cell viability assays. Normality of the distribution of sample data was determined by the Shapiro-Wilk test wherever applicable. In case of loss of normality, the nonparametric Mann-Whitney *U* test was used. In case of normal distribution, variances of sample data were compared by *F* test to evaluate homoscedasticity. In case of loss of homoscedasticity, the Welch's *t* test was applied. All other samples were compared using two-tailed Student's *t* tests (GraphPad). For all graphs, **P* < 0.05, ***P* < 0.01, ****P* < 0.001, and *****P* < 0.0001.

SUPPLEMENTARY MATERIALS

www.sciencesignaling.org/cgi/content/full/10/500/eaam5353/DC1

Fig. S1. SHIPi increases the surface density of Fas (CD95) detected in RMA cell cultures.

Fig. S2. The pan-SHIP1/2 inhibitor K118 is cytotoxic against RMA-Rae1 cells.

Fig. S3. Abundance of SHIP1 in RMA-Rae1, MC-38, and CT26 cells.

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Dual enhancement of T and NK cell function by pulsatile inhibition of SHIP1 improves antitumor immunity and survival

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Finding the right balance in immunotherapy

To stimulate the immune system's activity against tumors, therapies often inhibit the regulatory pathways that suppress or counteract stimulatory signaling in immune cells. The phosphatase SHIP1 is one such target; however, chronic inhibition or genetic ablation of SHIP1 in mouse models of cancer fails to induce effective antitumor immunity, perhaps because chronic activation causes immune cell exhaustion. Gumbleton *et al.* found that, instead, a pulsatile regimen of SHIP1 inhibition not only enhanced the antitumor activity of critical populations of immune cells and extended the survival of mice with lymphoma and colon cancer but also induced immunological memory against the tumor cells. This treatment strategy may thus be effective at killing various types of tumors and preventing relapse in cancer patients.

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