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Impaired T cell survival promotes mucosal inflammatory disease in SHIP1-deficient mice

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Abstract

T cells play a critical role in immune surveillance at mucosal surfaces. SHIP1$^{-/-}$ mice succumb to mucosal inflammatory disease that afflicts the lung and small intestine. The basis of this condition has not been defined. Here we show that SHIP1 is required for the normal persistence and survival of T cells in mucosal tissues. We find that CD4 and CD8 effector T cells are reduced, but Treg cells increased in the SI and lungs of SHIP1$^{-/-}$ and CD4CreSHIP$^{flox/flox}$ mice. Furthermore, a subset of T cells in the SI of SHIP1$^{-/-}$ mice are FasL+ and are more susceptible to extrinsic cell death. Mechanistic analyses showed that SHIP1 associates with the death receptor CD95/Fas and treatment with a Caspase8 inhibitor prevents SHIP1 inhibitor mediated T cell death. Notably, mucosal inflammation in SHIP1$^{-/-}$ mice is reduced by treatment with a Caspase8 inhibitor. We also find that the incidence of CD and pneumonia are significantly increased in mice with dual T and myeloid lineage SHIP1 deletion, but not in single lineage deleted mice. Thus, by promoting survival of protective T cells, thereby preventing an inflammatory myeloid response, SHIP1 maintains an appropriate balance of innate immune function at mucosal surfaces necessary for immune homeostasis.

Keywords

SHIP1; pneumonia; ileitis; Crohn's disease; T cells; adoptive T cell transfer (ACT); SHIP1 inhibitor; 3-α-aminocholestane (3AC); caspase 8; apoptosis

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INTRODUCTION

Previously we found that SHIP1-deficient hosts develop a profound ileitis that closely resembles human Crohn's Disease (CD) with many SHIP1$^{-/-}$ mice developing strictures and fissures in the terminal ileum[1]. The cause of this mucosal inflammatory disease was not defined in our initial study. However, we did observe a pronounced reduction of both CD4^{+} and CD8^{+} T cells accompanied by a predominantly neutrophilic infiltration of the small intestine in SHIP1$^{-/-}$ mice[1]. The terminal ileum is not the only inflamed mucosal site in the SHIP1$^{-/-}$ host as a myeloid consolidation of the lungs is routinely observed in both germline SHIP1$^{-/-}$ mice[2] and following ablation of SHIP1 expression in adult mice[3]. Thus, myeloid inflammatory disease at mucosal sites is thought to lead to the early demise of SHIP1$^{-/-}$ mice[1, 2]. These inflammatory processes are likely triggered by microbiological cues, as housing of SHIP1$^{-/-}$ mice in specific pathogen free (SPF) conditions, although failing to restore normal lifespan, does extend the survival of SHIP1$^{-/-}$ mice[4].

Although SHIP1$^{-/-}$ mice show a reduced frequency of T cells in the spleen[2], this is simply a reflection of their decreased representation due to an expansion of the myeloid compartment. In fact, the absolute number of splenic T cells was found to be normal in two different strains of SHIP1 mutant mice homozygous for distinct mutations, while their numbers were significantly increased in the mesenteric LN [5]. In addition, Treg cell numbers are significantly increased in both the spleen and LN of multiple SHIP1 mutant strains [5–7]. The above findings and the reduction in the number of T cells in the gut of SHIP1$^{-/-}$ mice indicates that the classical view of SHIP1 as solely an inhibitor of PI3K mediated survival can not be universally applied to all T cell types in all tissue locations. Adding to the complex role of SHIP1 in regulation of T cell survival is recent evidence that Jurkat T cells that have severely diminished SHIP1 expression are sensitive to FasL mediated apoptosis [8]. Thus, SHIP1 may have divergent roles in the control of T cell survival that vary with T cell type, tissue location or the mechanism of cell death (*e.g.*, intrinsic vs extrinsic pathway). A more nuanced understanding of this complexity, and particularly in normal physiology and different disease settings may then provide therapeutic insights for T cell mediated autoimmune diseases, T cell neoplasms and cancer treatment by adoptive transfer of T cells.

In certain forms of inflammatory bowel disease (IBD), including both Crohn's disease (CD) and ulcerative colitis (UC), autoreactive T cells that are resistant to apoptosis are thought to promote inflammation and tissue damage[9, 10]. Compelling evidence supporting this view has been elusive, however, with an alternate hypothesis being put forward that CD could be the result of an immune deficiency involving T cells and perhaps other inflammatory cell types[11]. Our finding of a selective T cell deficit in the small intestine of SHIP1$^{-/-}$ mice is consistent with this latter view[1]. Moreover, in chronically infected HIV patients where there is a severe depletion of intestinal CD4^{+} T cells, the incidence of mucosal inflammation in these patients is very high[12]. Importantly T cells from these patients are very sensitive to activation induced cell death (AICD) [13–15]. In addition, long-lived non-recirculating memory T cells that are Fas^{+} require highly efficient regulation to avoid incidental Caspase 8 activation to prevent depletion of these cells that are necessary for immune surveillance in the gut[16]. Maintaining a proper balance of survival vs. apoptosis in mucosal resident T cells is likely required then for immune surveillance at the mucosal barrier and thus for

maintenance of an appropriate balance of adaptive vs. innate immune function in the mucosa. Further elucidation of the mechanisms that determine survival vs. apoptosis in gut resident T cells is then critical for better understanding the basis of IBD in genetic conditions and during immune suppression like that which occurs in AIDS.

Due to the T cell deficit observed in the small intestine of SHIP1$^{-/-}$ mice, we proposed that SHIP1 signaling might be required for the persistence of effector T cells at mucosal sites. A selective defect in the ability of effector T cells to survive at mucosal sites would obviously also impair their capacity to participate in immune surveillance at these sites. We then hypothesized that decreased T cell effector function at mucosal sites in germline SHIP1-deficient mice would lead to an over-response by SHIP1-deficient neutrophils and other inflammatory myeloid cells to incitements like commensal microflora or crystal formation by bronchial epithelial cells culminating in severe, life-compromising tissue destruction by myeloid cells[1]. This hypothesis exemplifies the duality of SHIP1's role in immune cell signaling, manifesting as either an activator or inhibitor of immune function depending on the involved cell or receptor complex[17]. We show here that the impaired persistence of SHIP1$^{-/-}$ T cells is due to their enhanced susceptibility to Caspase 8 mediated cell death. Our findings thus represent a novel function of SHIP1 in promoting resistance of T cells to extrinsic cell death selectively at mucosal sites like the small intestine.

RESULTS

Selective reduction of T cells in the small intestine and lungs of SHIP1$^{-/-}$ mice

We investigated whether reduction in CD4 and CD8 T cells is restricted to the small intestine or whether the other inflamed mucosal site, lung, is also affected by SHIP1 deficiency. CD4 and CD8 T cells are significantly decreased in the small intestine and lungs of SHIP1$^{-/-}$ mice as compared to WT controls (Figure 1a & b). Because reduction in immu**no**suppressive regulatory T cells could potentially account for the inflammation observed in **the** small intestine and lungs of SHIP1$^{-/-}$ mice, we further examined their numbers in these inflamed tissues. The results showed that the CD4^{+}CD25^{+}FoxP$_3^{+}$ T regulatory (Treg) cells are significantly increased in the small intestine and lungs of SHIP1$^{-/-}$ mice (Figure 1c & d). Examination of CD4^{+}FoxP$_3^{-}$ (T effector cells) and CD4^{+}FoxP$_3^{+}$ (Treg cells) indicated that the ratio of CD4^{+}FoxP$_3^{-}$ to CD4^{+}FoxP$_3^{+}$ is reduced in both the SI and lungs of SHIP1$^{-/-}$ mice (Supplementary Figure S1 a,b), indicating that effector T cells are decreased but Treg cells are increased as a result of SHIP1 deletion. **This increase in Treg cells is also** consistent with the previous findings that peripheral (spleen and lymph node) Treg numbers and functions are increased in SHIP1$^{-/-}$ mice[5]. Thus, in spite of the increased frequency of Treg cells at mucosal sites like the lungs and SI of SHIP1$^{-/-}$ mice, these cells are incapable of abrogating inflammation mediated by myeloid elements that are refractory to control by Treg cells.

To test whether reduction of **mucosal** T cells is due to the inflammatory background of SHIP1$^{-/-}$ mice or whether it is due to an intrinsic function of SHIP1, a T cell specific deletion model of SHIP1, CD4CreSHIP$^{flox/flox}$, was examined. This analysis showed that frequency **and absolute numbers** of both CD4 and CD8 T cells is significantly reduced in CD4CreSHIP$^{flox/flox}$ mice as compared to SHIP$^{flox/flox}$ control**s in both the SI and lungs**

(Figure 2a,b). Reduction of both T cell lineages in CD4CreSHIP$^{flox/flox}$ mice was **selective for mucosal tissues** as no differences were observed in CD4 and CD8 T cell frequencies **or in the absolute numbers** in the spleens (Figure 2c) **or in lymph node (data not shown)** of CD4CreSHIP$^{flox/flox}$ mice These results demonstrate SHIP1 promotes the survival of mature T cells at mucosal sites like lung and SI in a T-lineage intrinsic fashion. Consistent with analysis of Treg cell numbers in germline SHIP1$^{-/-}$ mice, we also observed significant increase in the frequency of CD25^{+}**FoxP**$_3^{+}$ regulatory T cells in the SI, lung and spleen of CD4CreSHIP$^{flox/flox}$ **mice** (Figure 2d–f). **Additionally the absolute number of CD4^{+}CD25^{+}FoxP**$_3^{+}$ regulatory T cells was also increased in the SI and spleen of these mice (Figure 2d and 2f). Increased splenic Treg cell numbers is consistent with analysis of another T cell specific SHIP1 deletion mouse model LckCreSHIP$^{flox/flox}$ [18]. Taken together, our analysis demonstrates divergent roles for SHIP1 in the survival of effector T cells and Treg cells and particularly for T-lineage cells at mucosal sites like lung and small intestine.

Defective survival of SHIP1-deficient T cells

We further examined the requirement for SHIP1 in mucosal T cell persistence by developing a direct competition assay (DCA) for T cells that we and others have used to assess gene function in hematopoietic stem cells (HSC) [19]. In the T cell version of the DCA, CD45.1^{+}45.2^{+} hemizygous C57BL6 mice are sublethally irradiated and reconstituted with equal numbers of WT (CD45.2) and SHIP1$^{-/-}$(CD45.1) CD3^{+} T cells. One month later the mice are sacrificed and their lungs, small intestines **and spleen** analyzed by FACS to determine the contribution of WT, SHIP1$^{-/-}$ and host T cells to **these** compartments (Figure 3). The DCA assay revealed that SHIP1 expression is required for persistence of both CD4^{+} and CD8^{+} T cells in the lungs (Figure 3a) while only CD4^{+} T cells require SHIP1 for persistence in the lamina propria of the small intestine (SI) (Figure 3b). Interestingly, we also observed reduced number of SHIP1$^{-/-}$ CD4 and CD8 T cells in the spleens (Figure 3c) of these mice suggesting that in a competitive setting like the DCA assay, peripheral SHIP1$^{-/-}$ T cells are not able to homeostatically expand and survive as well as WT T cell s.

SHIP1 is not required for T cell trafficking to mucosal tissues

As SHIP1 deficiency resulted in reduced engraftment of T cells in mucosal tissues, we further examined whether the impaired numbers of SHIP1$^{-/-}$ T cells at these sites after competition is due to a defect in trafficking of SHIP1$^{-/-}$ T cells to mucosal sites rather than just a defect in their survival. To test this possibility equal numbers of WT (CD45.2) and SHIP1$^{-/-}$(CD45.1) CD3^{+} T cells were injected into sublethally irradiated CD45.1^{+}45.2^{+} hemizygous C57BL6 mice. After 16h, spleen, lungs, small intestine and blood were collected and flow cytometric analysis was performed to assess the contribution of WT and SHIP1$^{-/-}$ cells to the T cell compartment. The contribution of both CD4^{+} and CD8^{+} SHIP1$^{-/-}$ cells to the T cell compartment in all four tissues was significantly less than that of SHIP1-competent WT T cell competitors (Figure 4a–d). Further analysis of DAPI^{+} dead cells in the lungs and SI revealed that the frequency of SHIP1$^{-/-}$ T cells in the dead cell gate was significantly higher as compared to WT competitors (Supplemental Figure 2a–b). However in the non-mucosal tissue (spleen) the ratio of dead 45.1/45.2 cells was similar to live 45.1/45.2 cells (Supplemental Figure 2a–b), indicating SHIP1$^{-/-}$ T cells traffic with increased frequency to mucosal compartments, but then rapidly succumb at these sites.

Consistent with their enhanced trafficking to these sites, we observe a significantly higher frequency of $CXCR3^+CD4^+$T cells, in the spleen and mesenteric lymph nodes (mLN) of $SHIP1^{-/-}$ mice (Supplemental Figure 3a–d). CXCR3 promotes mucosal trafficking of peripheral lymphoid cells [20, 21]

SHIP1 deficiency promotes apoptosis of mucosal T cells

T cell numbers are reduced in the mucosal tissues but not in peripheral tissues of both $SHIP1^{-/-}$ **and CD4creSHIP**$^{fl/fl}$ mice. However, in the direct competition assay we find reduced persistence of T cells in both mucosal and peripheral tissues. To further understand this difference we performed apoptosis assay on mucosal (SI and lung) and peripheral (spleen) T cells. We **find** significantly increased apoptosis **of** $CD3^+$ T cells in both the small intestine and lung of $SHIP1^{-/-}$ **mice** as indicated by Annexin V and DAPI staining (Figure 5a, b). However, **no difference in the apopto tic frequency of** $SHIP1^{-/-}$ **splenic T cells was observed** (Figure 5c). Because the extrinsic cell death pathway is a major mechanism of T cell apoptosis, we further examined whether SHIP1 deficiency can alter CD95L (FasL) expression in lamina propria T cells of small intestine. Although no significant difference in Fas expression was observed (data not shown), a significant increase in the frequency of CD4 and CD8 T cells that express FasL in the SI of $SHIP1^{-/-}$ mice (Figure 5–d–e) was noted. FasL expressing T cells have been shown to engage Fas either on the same cell (*suicide*) or on other T cells (*fratricide*) and in so doing induce T cell death [22, 23]. We hypothesize that the subset of FasL expressing T cells in the lamina propria of $SHIP1^{-/-}$ mice selectively depletes effector T cells in the small intestine. Consistent with this, $SHIP1^{-/-}$ mice that exhibit a greater degree of T cell reduction in the small intestine also have higher frequency of $FasL^+$ CD4 and CD8 T cells (Supplemental Figure 4 a–b) further implicating FasL dysregulation in the reduction of SI T cells. We also find that the inappropriate expression of FasL by $SHIP1^{-/-}$ T cells is exclusive to the lamina propria of $SHIP1^{-/-}$ mice. Although we observe a slight increase in FasL surface density on splenic and lung $CD3^+$ T cells in $SHIP1^{-/-}$ mice, a distinct subset of $FasL^+$ T cells is not observed in either T cell compartment (Supplemental Figure 5a–b). We then examined FasL expression on regulatory T cells of the small intestine of $SHIP1^{-/-}$ **mice. Because in mice more than 95% of** $FoxP_3^+$ **T cells are also** $CD25^+$ [5]**we therefore examined FasL expression on** $CD3^+CD4^+CD25^+$ **(Treg) and** $CD3^+CD4^+CD25^-$ (Teff). Interestingly, a significant population of $SHIP1^{-/-}$ **regulatory T cells also expresses FasL** (Figure 5f) in small intestine. Enhanced expression of FasL on effector T cells has been reported to induce their death [24] whereas increased expression of FasL on Tregs has been shown to induce killing of effector T cells and other cell types like DC [25–28].

SHIP1 sets a threshold for Caspase 8 mediated cell death in T cells

Due to their selective depletion it is not feasible to obtain sufficient numbers of T cells from the small intestine of $SHIP1^{-/-}$ mice for *ex vivo* biochemical studies. Thus, we utilized HSB2, a human T cell line that expresses endogenous SHIP1 at normal levels as an alternative model to gain mechanistic insights into how SHIP1 regulates extrinsic T cell death. As anticipated, we find that the SHIP1 selective inhibitor 3AC [3] promotes Caspase 8 mediated cell death in HSB2 T cells. We find that 3AC treatment of HSB2 cells triggers a significant increase in Caspase 8 activation (Figure 6a) as well as FasL induction (Figure

6b). Importantly, we observe that the SHIP1 inhibitor-induced extrinsic cell death in HSB2 T cells is largely prevented by treatment with a Caspase 8 inhibitor prior to SHIP1 inhibition–demonstrating that SHIP1 inhibitor mediated cell death in T cells is preferentially through the Caspase 8 mediated extrinsic cell death pathway (Figure 6c). Interestingly, we also observed association of SHIP1 with Fas in HSB2 T cells, suggesting that interaction of SHIP1 with CD95/Fas may antagonize signaling by this death receptor and thereby set a threshold for Caspase 8 activation (Figure 6d). The absence of a SHIP1-mediated negative regulatory mechanism renders T cells more susceptible to Fas-FasL mediated cell death. These findings suggest two possible molecular roles for SHIP1 in preventing inappropriate activation of Caspase 8 in T cells (Figure 6e), and possibly in other immune cell types.

Caspase 8 inhibitor protects T cells in the mucosa and abrogates inflammation in SHIP1$^{-/-}$ mice

To assess whether the extrinsic cell death pathway was a major contributor to the demise of SHIP1$^{-/-}$ T cells *in vivo*, we treated SHIP1$^{-/-}$ mice beginning at 3 weeks of age with the Caspase 8 inhibitor, Z-IETD-FMK. The inhibitor and vehicle treated SHIP1$^{-/-}$ controls were then sacrificed at 6 weeks of age. There was grossly apparent diminution of anatomical pathology in both the SI and lungs of Caspase 8 inhibitor-treated mice (Figure 7a, 7b, left panels) compared to vehicle-administered controls. Importantly, we saw a profound recovery of viable CD3^{+} T cell numbers in the small intestine (Figure 7a) and lung (Figure 7b) of the Caspase 8 inhibitor treated SHIP1$^{-/-}$ hosts while the vehicle treated SHIP1$^{-/-}$ hosts exhibit the T cell paucity we routinely observe in the lamina propria of SHIP1$^{-/-}$ mice [1]. Thus, SHIP1-deficient T cells at mucosal sites exhibit selective sensitivity to extrinsic cell death mediated via Caspase 8. We then investigated whether mucosal inflammation results from selective deletion of SHIP1 in T cells or additional myeloid lineage specific deletion of SHIP1 is also required. As anticipated myeloid lineage-specific SHIP1 deficiency (LysMCreSHIP$^{flox/flox}$) alone did not result in **consolidating** pneumonia and CD-like ileitis in mice (Figure 7c). Additionally mice with **T cell** specific deletion of SHIP1 (CD4CreSHIP$^{flox/flox}$) also do not exhibit **mucosal inflammatory** disease, however a mouse model with a combined SHIP1 ablation in both myeloid cells and T cells (CD4LysMCreSHIP$^{flox/flox}$) exhibit significantly higher incidence of CD-like ileitis and consolidating pneumonia (Figure 7c–g). Taken together, the data suggests that failure of SHIP1$^{-/-}$ effector T cell responses accompanied by an increased infiltration of SHIP1$^{-/-}$ **myeloid cells results in mucosal inflammation in SHIP1$^{-/-}$ mice**.

DISCUSSION

An efficient balance of T cell proliferation and apoptosis is required to achieve a fully functional T cell compartment, which is essential in achieving a proper defense against pathogens and to prevent hyper immune response against self-antigens. In T cells the Fas-FasL mediated programmed cell death pathway is a fundamental mechanism that eliminates hyper reactive or autoreactive T cells and this regulation is important for immune tolerance [22, 29]. The susceptibility to Fas-FasL mediated cell death varies among different T cell subtypes, as naïve T cells are largely Fas negative and resistant to apoptosis, while effector and memory T cells are Fas positive and more sensitive to apoptosis [30]. The lamina

propria of the gut is mostly populated with Fas expressing effector and memory T cells and thus this compartment is acutely sensitive to Fas-mediated cell death [16]. The molecular basis of the survival of these Fas-FasL sensitive lamina propria T cells in spite of constant stimulation by commensal and pathogenic microorganisms present at the mucosal barrier is not fully understood. Our findings demonstrate an important role of the inositol phosphatase SHIP1 in promoting resistance by mucosal T cells to Fas-mediated cell death. Herein we demonstrate that SHIP1 may prevent Caspase 8 mediated cell death in T cells by two mechanisms that are not necessarily mutually exclusive: (1) SHIP1 associates with Fas to antagonize its activation of Caspase 8 and/or (2) SHIP1 inhibits inappropriate FasL induction (Figure 6e). We propose that one or both of these molecular roles is preferentially operative in SHIP1$^{-/-}$ lamina propria T cells, where T cells are chronically exposed to pathogens and thus more susceptible to Fas-mediated cell death. Hence, a concerted effect of both SHIP1 activities may prevent inappropriate depletion of T cells in mucosal sites like the SI. However, despite strong evidence for diminished SHIP1$^{-/-}$ T cell survival in the gut, defective trafficking of these cells to the gut may also contribute, in part, to decreased T effector cell numbers at mucosal sites in SHIP1$^{-/-}$ mice.

Interestingly we have observed depletion of effector T cells but not Treg cells in the lungs and SI of SHIP1$^{-/-}$ mice. In fact, FoxP$^{+}_{3}$CD25^{+} T cells are increased in the inflamed lamina propria and lungs of SHIP1$^{-/-}$ mice. Increased Treg cell numbers in mucosal tissues are consistent with the earlier reports of increased Treg cell numbers in the spleen and LN of SHIP1$^{-/-}$ mice [5–7]. In addition, SHIP1$^{-/-}$ Treg cells were found to be equally suppressive as WT *in vitro* and *in vivo*, including in a colitis model,[5, 7] indicating that mucosal inflammation does not result from a selective loss of Treg cells and their immunosuppressive properties at mucosal sites.

Our findings also reinforce the duality of SHIP1's role as both a terminator and a mediator of PI3K-mediated cell signaling [17]. In myeloid cells SHIP1 clearly has a pro- apoptotic role as SHIP1$^{-/-}$ mice exhibit myeloproliferative disorders with increased infiltration of myeloid cells in all the secondary lymphoid organs [2, 31]. In contrast, SHIP1 also functions to prevent Fas-FasL induced cell death in T cells by modulating Fas signaling as well as FasL induction. This observation is supported by the observed association of SHIP1 with Fas (CD95), increased frequency of FasL^{+} T cells in the SI of SHIP1$^{-/-}$ mice and increased expression of FasL caused by SHIP1 inhibition suggesting that SHIP1's function is both cell type and tissue dependent. Since the susceptibility to Fas-mediated apoptosis differs in naïve, effector, regulatory and memory T cells, it would be interesting to determine if SHIP1 is responsible for the differential sensitivity of these T cell subsets to Fas/FasL mediated programmed cell death.

Our finding that despite their expression of FasL, Treg cell numbers are increased in mucosal tissues of SHIP1$^{-/-}$ mice is consistent with other studies demonstrating that FasL^{+} Tregs are potent killers of effector T cells [25–28]. More recently such “killer Treg cells” were implicated in autoimmune diseases due to their enhanced ability to deplete effector T cells as compared to Treg cells that lack FasL [32–34]. Additionally the susceptibility of Treg cells to undergo FasL-mediated apoptosis has not been unequivocally demonstrated [25, 27, 28]. In the case of SHIP1-deficiency, we observed that SHIP1$^{-/-}$ Tregs do not undergo apoptosis at

an increased frequency and thus these the increased numbers of $FasL^+$ Treg cells are likely to contribute to the depletion of effector T cells that we observe in mucosal tissues of SHIP1-deficient mice.

In inflammatory bowel diseases (IBD) like ulcerative colitis and Crohn's disease it has been reported that exaggerated responses by T cells mediates tissue damage and inflammation. Moreover, T cells isolated from these patients have been shown to be resistant to Fas-mediated cell death [9, 10]. However in $SHIP1^{-/-}$ mice T cells do not cause inflammation, but rather the loss of effector T cell function due to their increased apoptosis combined with increased infiltration of myeloid cells results in inflammation. With the recent advances in IBD there is evidence that IBD is a consequence of an impaired acute inflammatory response[11, 35]. $SHIP1^{-/-}$ mouse model of IBD is consistent with the later view and therefore might used to define IBD states that result from T cell deficiency rather than T cell over-reaction.

We found that the human leukemic T cell line HSB2 is sensitive to extrinsic cell death triggered by a selective SHIP1 inhibitor, 3AC, indicating protection of T cells from extrinsic cell death may be a conserved function of human SHIP1/INPP5D. SHIP1 inhibitory compounds may therefore have therapeutic utility in IBD by selectively depleting auto-reactive T cells in mucosal tissues such as the small intestine and bowel[3, 36]. Recently T cell depletion strategies have been proposed to control inflammatory bowel diseases (IBD) mediated by autoreactive T cells [37]. Should these findings be extrapolated to the human setting, then treatment with SHIP1 inhibitory compounds might be used alone or in conjunction with such antibody depletion therapies to selectively deplete disease causing T cells at mucosal sites while sparing the bulk of the peripheral T cell pool.

METHODS

Mouse strains

The development and production of $SHIP1^{-/-}$(CD45.1) has been described previously[38]. $SHIP1^{\Delta IP/\Delta IP}$ mice were a kind gift of Dr. Jeffery Ravetch (Rockefeller University, NY) and for this study were maintained on the F2(129S/v × C57BL6) background described in Karlsson *et al*[39]. Mice with germline transmission of a $SHIP1^{flox}$ allele were generated previously in the laboratory[38] and propagated by intercrossing with $SHIP1^{flox/flox}$ mice (F_{10} to C57BL/6J background). LysMCre and CD4Cre transgenic mice were obtained from Jackson laboratory. $LysMCreSHIP^{flox/flox}$ and $CD4CreSHIP^{flox/flox}$ mice were created by intercrossing $SHIP^{flox/flox}$ with LysMCre or CD4Cre mice respectively. $CD4LysMCreSHIP^{flox/flox}$ mice were generated by intercrossing $CD4CreSHIP^{flox/flox}$ and $LysMCreSHIP^{flox/flox}$.

Gut and lung histopathological analysis

Gut and lungs were insufflated with 10% neutral-buffered formalin, the small intestines were rolled in segments to fit as Swiss rolls into cassettes for histological processing and microscopic evaluation. Tissues were fixed in 10% neutral-buffered formalin, dehydrated,

embedded in paraffin, sectioned at 3 μm and stained with H&E. Intestinal inflammation was scored as described previously[1].

Cell preparation and flow cytometry

Lungs were cut into small pieces and lymphocytes were isolated by incubating tissue in digestion buffer containing 5% FBS, 1.5mg/ml Collagenase and 40μg/ml in HBSS for 30 min at 37°C. For isolation of lamina propria T cells, Payer's patches were removed and the small intestines was opened longitudinally and cut into small pieces (1.5 cm). Epithelial cells were removed by incubating tissue (2X) in buffer containing 2mM EDTA and 5% FBS in HBSS for 20 min. Tissue was then digested with 1.5mg/ml Collagenase and 40μg/ml for 30 min at 37°C. Isolated cells were then layered on 40/80% percoll gradient (GE healthcare) and centrifuged for 20 min at 200 rpm. Cells at the interface of the gradient were collected. Single cell suspensions from spleen, lung and small intestine were incubated with anti-CD16/32 to block Fc receptor binding (BD Pharmingen (San Jose, CA) followed by staining with flourochrome-conjugated antibodies. All samples were acquired on an LSRII cytometer (Becton Dickinson) and analyzed using FlowJo software. Dead cells were excluded from the analyses following cytometer acquisition by exclusion of cells that stained positively for DAPI dye.

Mucosal T cell competition assay in CD45.1/2 hemizygous C57BL6 hosts

Spleens of CD45.1 SHIP1$^{-/-}$ and CD45.2 WT mice were harvested and CD3^{+} T cells were magnetically purified using a MACS CD3^{+} Cell selection Kit via tail vein injection. Prior to T cell transplant recipients CD45.1/2 hemizygous C57BL/6 mice received a single dose of 5.5Gy from an X-ray irradiator and then were adoptively transferred with 10×10^{6} WT (CD45.2) and 10×10^{6} SHIP1$^{-/-}$ (CD45.1) CD3^{+} T cells via tail vein injection. One month after ACT, the recipients were sacrificed for lung and small intestine harvest and the lymphocytes of these tissues assessed by flow cytometry.

SHIP1 inhibition (SHIPi) *in vitro*

HSB2 T cells were plated in 6 well plates in triplicate and treated with 7.5μM 3AC or vehicle (absolute ethanol). After 48h cells were incubated with CaspGLOW fluorescein active for 1h, harvested, washed and stained with Annexin V and propidium iodide (PI). Viable cells were gated on the basis of exclusion of PI and were analyzed for staining of Annexin V and active Caspase 8. For FasL induction, HSB2 cells after 48h treatment with 3AC (7.5μM) were washed and stained with anti-human CD178PE antibody (Ebioscience, San Diego, CA). Analysis of FasL expression on viable cells was performed on the basis of exclusion of DAPI^{+} cells. To assess Caspase 8 inhibitor mediated cell death rescue in 3AC treated cells, HSB2 cells were plated in triplicate and treated with either Caspase 8 inhibitor (Z-IETD-FMK) (50μM) or vehicle for 2h prior to 3AC (7.5μM) treatment. After 24h cells were washed and stained with Annexin V and PI. All samples were acquired on an LSRII cytometer (Becton Dickinson) and analyzed using FlowJo 9.4.1.

Fas-SHIP1 co-immunoprecipitation

HSB2 cells were washed twice with cold PBS and lysed in cell signaling IP lysis buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml Leupeptin and 1mM PMSF). Equal amount of protein was immunoprecipitated by using anti-Fas or an isotype control antibody (Santa Cruz, CA) along with protein A/G PLUS agarose beads (Santa Cruz, CA) overnight at 4°C. Immunoprecipitates were washed five times with cold lysis buffer and the pellet was resuspended in SDS sample buffer. Samples were heated at 100°C for 5 min and subjected to SDS PAGE. Association of SHIP1 with Fas was determined by probing with anti-SHIP1 (P1C1, Santa Cruz, CA) antibody.

Mucosal T cell competition in CD45.1/2 hemizygous C57BL6 hosts

Spleens of CD45.1 SHIP1$^{-/-}$ and CD45.2 WT mice were harvested and CD3$^+$ T cells were magnetically purified using a MACS CD3$^+$ Cell selection Kit via tail vein injection. Prior to T cell transplant recipients CD45.1/2 hemizygous C57BL/6 mice received a single dose of 5.5Gy from an X-ray irradiator and then were adoptively transferred with 10×10^6 WT (CD45.2) and 10×10^6 SHIP1$^{-/-}$ (CD45.1) CD3$^+$ T cells via tail vein injection. One month after ACT, the recipients were sacrificed for lung and small intestine harvest and the lymphocytes of these tissues assessed by flow cytometry as described above.

Caspase-8 inhibitor treatment of mice

SHIP1$^{-/-}$ mice were treated either with Z-IETD-FMK (5mg/kg) or vehicle (DMSO) three times each week for 3 weeks. Three weeks after initiation of the treatment, the SI and lungs were harvested, and lymphocytes isolated and then stained with anti-CD3 and DAPI dye. All samples were acquired on an LSR II cytometer and then analyzed using FlowJo 9.4.1.

Statistical analysis

The results are presented as the mean ± standard error of the mean. Statistical analysis was performed using two-tailed Students T-test. A p value < 0.05 was considered statistically significant.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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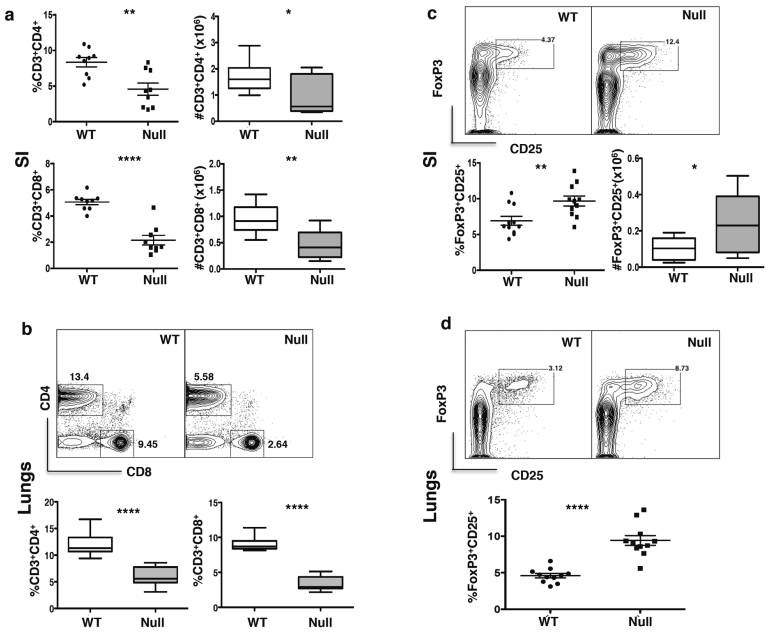

Figure 1.

SHIP1 deficiency **limits effector** T cells **and expand regulatory T cells** of small intestine and lungs. **(a)** Frequency and **absolute numbers** of live **CD3**$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells isolated from the lamina propria of small intestine of **germline SHIP1**$^{-/-}$ **mice (Null) and WT littermates**. Scatter plot (left panel) indicating frequency CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ T cells calculated on total live cells.(b) Flow cytometry plots representing frequencies of CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells from the lungs of germline SHIP1$^{-/-}$ mice (Null) and WT littermates. Box and whisker plot represents frequencies CD3$^+$CD4$^+$ and CD3$^+$CD8$^+$ T cells calculated on total live cells(c–d) Analysis of regulatory T cells of small intestine **(c)** and lungs **(d)**. Flow cytometry plots indicate frequency of CD25$^+$FoxP$_3$$^+$ cells gated on live CD3$^+$CD4$^+$ T cells and are shown in scatter plot (c & d; left panel) and box and whisker plot represents their absolute numbers (c; right panel). Data shown is mean ± SEM [****p<0.0001, **p<0.01, *p<0.05, Student's T-test].

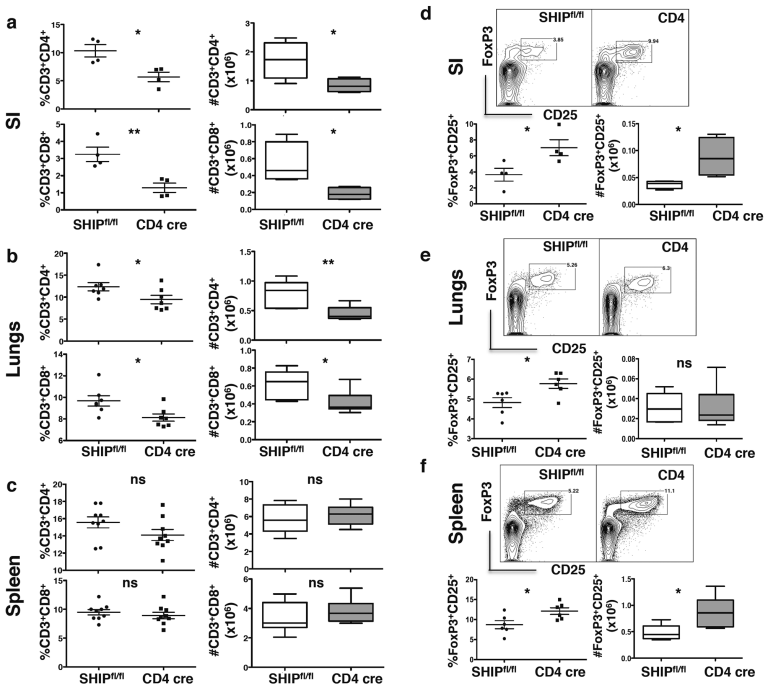

Figure 2.

T cell specific ablation of SHIP1 selectively reduce T cells in the mucosal tissues. (a–c) Frequency of live $CD3^+CD4^+$ and $CD3^+CD8^+$ T cells and their absolute numbers in the small intestine (SI) (a) lung (b) and spleen (c) of $CD4CreSHIP^{flox/flox}$ (CD4Cre) and $SHIP^{flox/flox}$ ($SHIP^{fl/fl}$) littermate. Scatter plot indicating frequency $CD3^+CD4^+$ or $CD3^+CD8^+$ T cells calculated on total live cells and box and whisker plot represents their absolute numbers (a–c; Right panel). (d–f) Regulatory T cell analysis in the SI (d), Lung (e) and spleen (f) of $CD4CreSHIP^{flox/flox}$ (CD4cre) and $SHIP^{flox/flox}$ ($SHIP^{fl/fl}$) controls. Flow cytometry plots indicate frequency of $CD25^+FoxP_3^+$ cells gated on live $CD3^+CD4^+$ T cells and are shown in scatter plot (left panel) and box and whisker plot represents their absolute numbers (right panel). Data shown is mean ± SEM [**p<0.01, *p<0.05, Student's T-test]. ns; non significant.

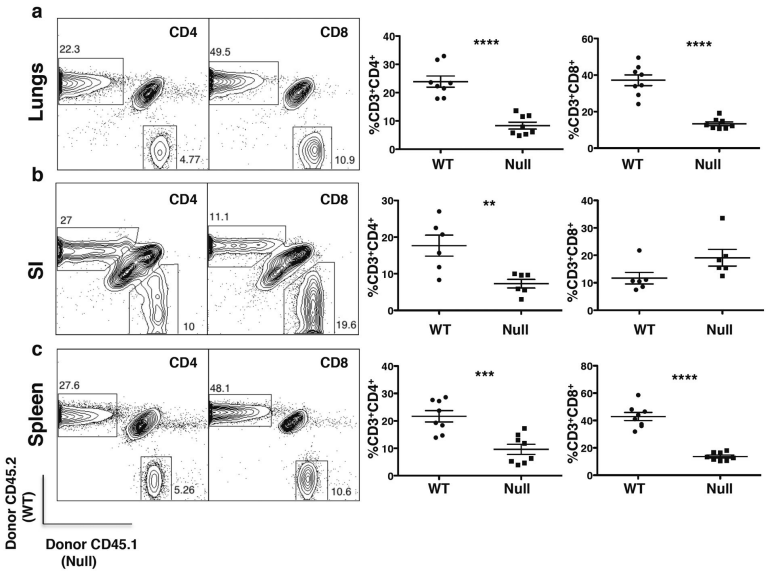

Figure 3.

Impaired persistence of SHIP1$^{-/-}$ T cells. CD45.1/2 hemizygous C57BL6 hosts were sub-lethally irradiated and co-transplanted with 10×10^6 WT (CD45.2) and 10×10^6 SHIP1$^{-/-}$ (CD45.1) CD3$^+$ T cells. One month later the contribution of host, WT and SHIP1$^{-/-}$ T cells to the lungs (**a**), SI (**b**) **and spleen (c)** was assessed by flow cytometry. Representative CD45.1 vs. CD45.2 contour plots are shown for all the tissues after gating on viable CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ cells as indicated. Scatter plots showing the frequency of SHIP1$^{-/-}$ (Null) and WT T cells populating the **total CD3$^+$**CD4$^+$ or **CD3$^+$**CD8$^+$ T cell pool in lung (**a**), SI (**b**) **and spleen (c)** are provided adjacent to the contour plots for their respective tissue. These results are pooled from two independent transplant experiments. [****p<0.0001, ***p<0.001,**p<0.01, Student's T-test]

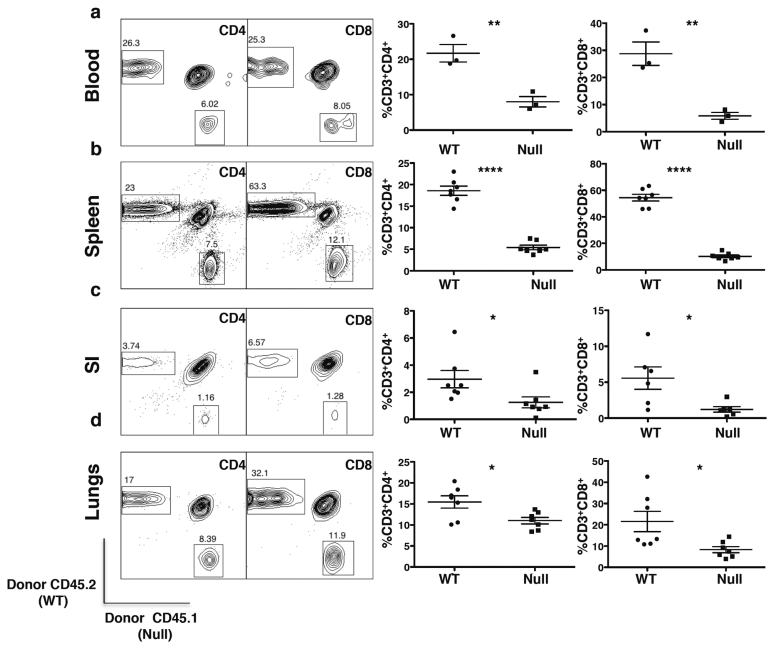

Figure 4.

SHIP1$^{-/-}$ T cells have defective survival but not defective trafficking. CD45.1/2 hemizygous C57BL6 hosts were sub-lethally irradiated and co-transplanted with 10×10^6 WT (CD45.2) and 10×10^6 SHIP1$^{-/-}$ (CD45.1) CD3$^+$ T cells. 16h post transplant spleen, lungs, small intestine and blood were collected and T cells from these tissues are analyzed by flow cytometry to assess the relative frequencies of WT and SHIP1$^{-/-}$ T cells. Representative CD45.1vs CD45.2 contour plots are shown for Blood (**a**), spleen (**b**), SI (**c**) and lungs (**d**) after gating on **viable** CD3$^+$CD4$^+$ or CD3$^+$CD8$^+$ cells as indicated (**Left panel**). Scatter plot to the right indicate the frequency of SHIP1$^{-/-}$ (Null) and WT T cells in the **CD3$^+$CD4$^+$** and **CD3$^+$CD8$^+$** T cell compartment in blood (**a**), spleen (**b**), SI (**c**) and lung (**d**). The results shown for all the tissues except blood are pooled data from two independent experiments. Data shown is mean ± SEM. [****p<0.0001, **p<0.01, *p<0.05, Student's T-test].

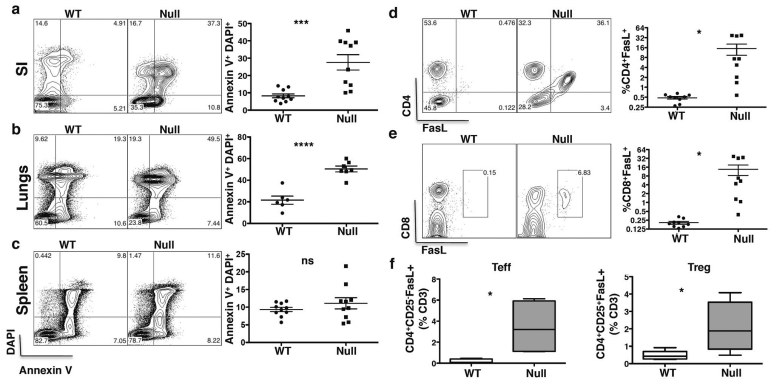

Figure 5.

SHIP1 deficiency promotes apoptosis **in mucosal tissues** and induces FasL expression on T cells. **(a–c)Apoptosis assay in SI, lung and splenic T cells**. T cells **from the SI, Lung and spleen of** WT and SHIP1$^{-/-}$ (Null) mice were stained with CD3, Annexin V and DAPI. Scatter plot represents the percentage of apoptotic dead cells (Annexin^{+}DAPI^{+}) **gated on CD3^{+} T** cells. (**d–e**) Analysis of FasL expression on small intestine T cells. Representative CD4 vs FasL (**d**) and CD8 vs FasL (**e**) contour plots of WT and SHIP1$^{-/-}$(Null) mice after gating on viable CD3 T cells. The scatter plot showing the frequencies of CD4^{+} FasL^{+} (**d, Right panel**) and CD8^{+} FasL^{+} (**e, Right panel**) cells gated on viable CD3 T cells. (f) Analysis of FasL expression on CD4^{+}CD25^{-} (effector T cells) and CD4^{+}CD25^{+} (regulatory T cells) in the small intestine (SI). The Box and whisker plot represent the frequency of CD4^{+}CD25^{-}FasL^{+} (right panel) or CD4^{+}CD25^{+}FasL^{+} cells (left panel) as a percentage of total viable CD3 T cells. Data shown is mean ±SEM. [******p<0.0001**,***p<0.001,*p<0.05 Student's T-test].

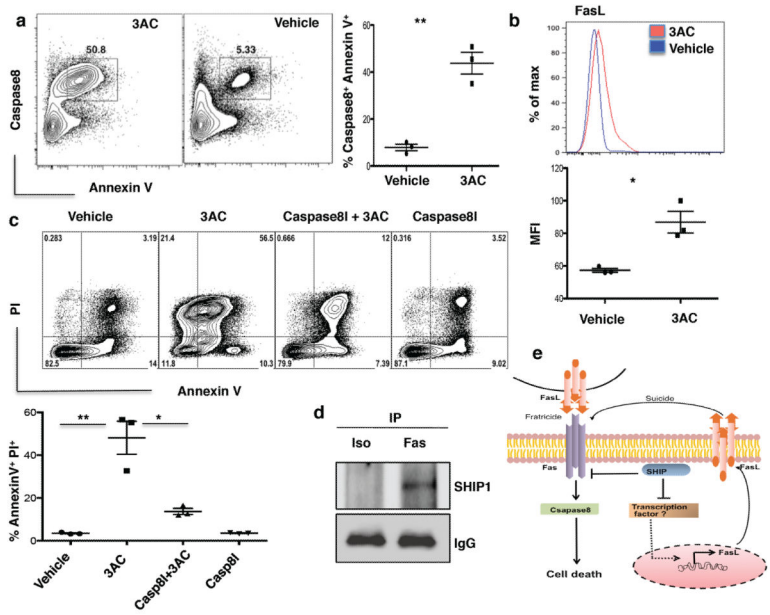

Figure 6.

SHIP1 negatively regulates extrinsic cell death by associating with the death receptor (Fas) and by inhibiting FasL induction. **(a)** SHIP1 inhibitor, 3AC promotes Caspase 8 mediated cell death in HSB2, a human T cell line. Cells were treated with 7.5 μM 3AC or vehicle (abs EtOH) for 48h followed by 1h incubation with CaspGLOW fluorescein active Caspase 8. Representative Caspase 8 vs. annexin V contour plot on viable cells (left) and scatter plot showing the frequency of Caspase8^{+} Annexin V^{+} (right). (**b)** FasL expression in HSB2 T cells after gating on viable cells following 48h treatment with 7.5 μM 3AC or vehicle by flow cytometry. (**c)** Analysis of cell death rescue by Caspase 8 inhibition. Cells were treated with 50μM of Caspase 8 inhibitor (Z-IETD-FMK) or vehicle for 2h followed by 24h treatment with 7.5 μM 3AC or vehicle. After 24h cells were stained and analyzed for Annexin V and PI staining by flow cytometry. Representative PI vs Annexin V contour plots for each treatment (left) and scatter plot for frequency of AnnexinV^{+}PI^{+} (right) are shown. Experiments were performed in triplicate. Results are representative of two independent experiments. Data shown is mean ± SEM [**p<0.001 *p<0.05, Student's T-test]. **(d)** Immunoblot analysis of association SHIP1 with Fas after immunoprecipitation with isotype control or Fas antibody in HSB2 cells. **(e)** Model summarizing regulation of Fas-mediated apoptosis by SHIP1 in T cells.

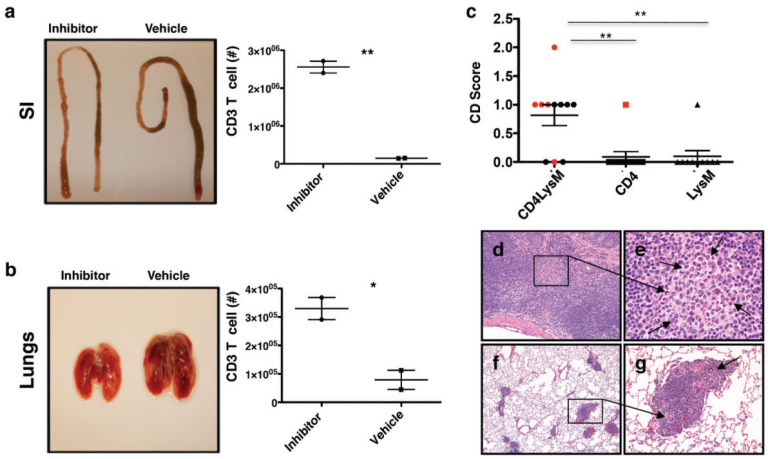

Figure 7.

Caspase 8 inhibitor protects SHIP1$^{-/-}$ mice from mucosal inflammation **(a,b)** SHIP1$^{-/-}$ mice were treated with the Caspase 8 inhibitor Z-IETD-FMK (5mg/kg) or vehicle three times a week over a 3 week period. Vehicle administered mice had **(a)** marked segmental thickening of the distal small intestine, characteristic of CD-like ileitis, compared to the SI of Caspase 8 inhibitor-treated mice **(a)**, and **(b)**, lungs of vehicle-administered mice were much firmer and enlarged, characteristic of consolidating pneumonia, compared to lungs of inhibitor-treated mice. The lymphocytes of the SI and the lung of either Z-IETD-FMK or vehicle treated SHIP1$^{-/-}$ mice were analyzed by flow cytometry for viable CD3^{+} T cells. (**c**) CD (Crohn's disease) score of CD4CreSHIP$^{flox/flox}$ (CD4), LysMCreSHIP$^{flox/flox}$ (LysM), and CD4LysMCreSHIP$^{flox/flox}$ (CD4LysM) mice. Red dots indicate mice that also develop pneumonia. (**d–g**) Histopathalogical analysis of CD4LysMCreSHIP$^{flox/flox}$ (CD4LysM) mice. CD4LysMCre mice developed (**d, e**) grade 1 Crohn's disease-like ileitis consisting of mild polymorphonuclear leukocyte infiltration of the lamina propria overlying and within Peyer's patch lymph nodules (**e, arrows**), and (**f, g**) mixed, multifocal pneumonia with infiltrating leukocytes and multinucleated giant cells (**g, arrows**) resulting in patchy pulmonary consolidation. Data shown is mean ±SEM [**p<0.01, *p<0.05, Student's T-test].