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

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T cells have a critical role in immune surveillance at mucosal surfaces. SHIP1^{-/-} mice succumb to mucosal inflammatory disease that afflicts the lung and small intestine (SI). 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; however, Treg cells are increased in the SI and lungs of SHIP1^{-/-} and CD4CreSHIP1^{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 Caspase 8 inhibitor prevents SHIP1 inhibitor-mediated T-cell death. Notably, mucosal inflammation in SHIP1^{-/-} mice is reduced by treatment with a Caspase 8 inhibitor. We also find that the incidence of Crohn's disease (CD) and pneumonia is 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.

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 (SI) 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^{-/-} mice² 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 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 because of 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, whereas 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 indicate that the classical view of SHIP1 as solely an inhibitor of PI3K-mediated survival cannot 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.

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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 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 SI of SHIP1^{-/-} mice is consistent with this latter view.¹ Moreover, in chronically infected HIV patients in whom 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.^{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 the depletion of these cells that are necessary for immune surveillance in the gut.¹⁶ Maintaining a proper balance of survival vs. apoptosis in mucosa-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 functions 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 similar to that which occurs in AIDS.

Owing to the T-cell deficit observed in the SI 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 the 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 the mucosal sites in germline SHIP1-deficient mice would lead to an over-response by SHIP1-deficient neutrophils and other inflammatory myeloid cells to incitements such as 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 because of 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 such as the SI.

RESULTS

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

We investigated whether reduction in CD4 and CD8 T cells is restricted to the SI or whether the other inflamed mucosal site,

lung, is also affected by SHIP1 deficiency. CD4 and CD8 T cells are significantly decreased in the SI and lungs of SHIP1^{-/-} mice as compared with wild-type (WT) controls (**Figure 1a, b**). As reduction in immunosuppressive regulatory T cells could potentially account for the inflammation observed in the SI and lungs of SHIP1^{-/-} mice, we further examined their numbers in these inflamed tissues. The results showed that the CD4⁺CD25⁺FoxP3⁺ T regulatory (Treg) cells are significantly increased in the SI and lungs of SHIP1^{-/-} mice (**Figure 1c, d**). Examination of CD4⁺FoxP3⁻ (T-effector cells) and CD4⁺FoxP3⁺ (Treg cells) indicated that the ratio of CD4⁺FoxP3⁻ to CD4⁺FoxP3⁺ is reduced in both the SI and lungs of SHIP1^{-/-} mice (**Supplementary Figure S1a, b** online), 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 such as 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 in 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^{fllox/fllox}, was examined. This analysis showed that frequency and absolute numbers of both CD4 and CD8 T cells are significantly reduced in CD4CreSHIP^{fllox/fllox} mice as compared with SHIP^{fllox/fllox} controls in both the SI and lungs (**Figure 2a, b**). Reduction in both T-cell lineages in CD4CreSHIP^{fllox/fllox} 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 the lymph node (data not shown) of CD4CreSHIP^{fllox/fllox} mice. These results demonstrate that SHIP1 promotes the survival of mature T cells at mucosal sites such as the lung and SI in a T-lineage intrinsic manner. Consistent with the analysis of Treg cell numbers in germline SHIP1^{-/-} mice, we also observed significant increase in the frequency of CD25⁺FoxP3⁺ regulatory T cells in the SI, lung, and spleen of CD4CreSHIP^{fllox/fllox} mice (**Figure 2d–f**). In addition, the absolute number of CD4⁺CD25⁺FoxP3⁺ regulatory T cells was also increased in the SI and spleen of these mice (**Figure 2d, f**). Increased splenic Treg cell numbers are consistent with the analysis of another T-cell-specific SHIP1 deletion mouse model LckCreSHIP^{fllox/fllox}.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 such as the lung and the SI.

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.¹⁹ In the T-cell version of the DCA, CD45.1⁺45.2⁺ hemizygous C57BL6 mice are

Figure 1 SHIP1 deficiency limits effector T cells and expands 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 the small intestine of germline SHIP1^{-/-} mice (Null) and wild-type (WT) littermates. Scatter plot (left panel) indicating frequency CD3⁺CD4⁺ or CD3⁺CD8⁺ T cells calculated on total live cells. (b) Flow cytometry plots represent 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 of 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 the frequency of CD25⁺FoxP3⁺ 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 ± s.e.m. (*****P* < 0.0001, ***P* < 0.01, **P* < 0.05, Student's *t*-test).

sublethally irradiated and reconstituted with equal numbers of WT (CD45.2) and SHIP1^{-/-} (CD45.1) CD3⁺ T cells. One month later the mice are killed and their lungs, SIs, and spleen were analyzed with fluorescence-activated cell sorting 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**), whereas only CD4⁺ T cells require SHIP1 for persistence in the lamina propria of the 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 similar to the DCA assay, peripheral SHIP1^{-/-} T cells are not able to homeostatically expand and survive as well as WT T cells.

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

because of 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⁺CD45.2⁺ hemizygous C57BL/6 mice. After 16 h, the spleen, lungs, SI, 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 contributions of both CD4⁺ and CD8⁺ SHIP1^{-/-} cells to the T-cell compartment in all four tissues were significantly lesser than those of SHIP1-competent WT T-cell competitors (**Figure 4a–d**). Further analysis of 4',6-diamidino-2-phenylindole⁺ (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 with WT competitors (**Supplementary 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 (**Supplementary Figure 2a, b**), indicating that 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

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 CD4CreSHIP1^{fl/fl} (CD4Cre) and SHIP1^{fl/fl} (SHIP1^{fl/fl}) littermates. 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 CD4CreSHIP1^{fl/fl} (CD4Cre) and SHIP1^{fl/fl} (SHIP1^{fl/fl}) controls. Flow cytometry plots indicate frequency of CD25⁺FoxP3⁺ 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 ± s.e.m. (***P* < 0.01, **P* < 0.05, Student's *t*-test). ns, non significant.

observe a significantly higher frequency of CXCR3⁺CD4⁺ T cells in the spleen and mesenteric lymph nodes of SHIP1^{−/−} mice (Supplementary 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 the peripheral tissues of both SHIP1^{−/−} and CD4creSHIP1^{fl/fl} mice. However, in the DCA 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 SI and lung of SHIP1^{−/−} mice as indicated by Annexin V and DAPI staining (Figure 5a, b). However, no difference in the apoptotic frequency of SHIP1^{−/−} splenic T cells was observed (Figure 5c). As 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 the SI. 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

Figure 3 Impaired persistence of SHIP1^{-/-} T cells. CD45.1/2 hemizygous C57BL/6 hosts were sublethally irradiated and co-transplanted with 10×10^6 wild-type (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 using 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).

FasL in the SI of SHIP1^{-/-} mice (**Figure 5d, 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 SI. Consistent with this, SHIP1^{-/-} mice that exhibit a greater degree of T-cell reduction in the SI also have higher frequency of FasL⁺ CD4 and CD8 T cells (**Supplementary Figure 4a, 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 (**Supplementary Figure 5a, b**). We then examined FasL expression on regulatory T cells of the SI of SHIP1^{-/-} mice. As in mice > 95% of FoxP₃⁺ 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 the SI. 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 the killing of effector T cells and other cell types such as DC.^{25–28}

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

Owing to their selective depletion, it is not feasible to obtain sufficient numbers of T cells from the SI 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 3AC³ 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 before 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 the association of SHIP1

Figure 4 SHIP1^{-/-} T cells have defective survival but not defective trafficking. CD45.1/2 hemizygous C57BL/6 hosts were sublethally irradiated and co-transplanted with 10×10^6 wild-type (WT) (CD45.2) and 10×10^6 SHIP1^{-/-} (CD45.1) CD3⁺ T cells. Sixteen hours post transplant spleen, lungs, small intestine, and blood were collected and T cells from these tissues are analyzed using flow cytometry to assess the relative frequencies of WT and SHIP1^{-/-} T cells. Representative CD45.1 vs. 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 indicates 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 are mean $\pm$ s.e.m. (**** $P < 0.0001$, ** $P < 0.01$, * $P < 0.05$, Student's t-test).

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 killed 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, b, left panels) compared with vehicle-administered controls.

Importantly, we saw a profound recovery of viable CD3⁺ T-cell numbers in the SI (Figure 7a) and lung (Figure 7b) of the Caspase 8 inhibitor-treated SHIP1^{-/-} hosts, whereas 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^{fllox/fllox}) alone did not result in consolidating pneumonia and CD-like ileitis in mice (Figure 7c). In addition, mice with T-cell-specific deletion of SHIP1 (CD4CreSHIP^{fllox/fllox}) also do not exhibit mucosal inflammatory disease; however, a mouse model with a combined SHIP1 ablation in both myeloid cells and T cells (CD4LysMCreSHIP^{fllox/fllox}) exhibits significantly higher incidence of CD-like ileitis and consolidating pneumonia (Figure 7c–g). Taken together, the data suggest that failure of

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 wild-type (WT) and SHIP1^{−/−} (Null) mice were stained with CD3, Annexin V, and 4′,6-diamidino-2-phenylindole (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 plots 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 are mean ± s.e.m. (*****P* < 0.0001, ****P* < 0.001, **P* < 0.05 Student's *t*-test).

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 hyperimmune 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 naive T cells are largely Fas-negative and resistant to apoptosis, whereas 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 are 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 such as 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 the mucosal sites in SHIP1^{−/−} mice.

Interestingly, we have observed the depletion of effector T cells but not Treg cells in the lungs and SI of SHIP1^{−/−} mice. In fact, FoxP3⁺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

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 μ M 3AC or vehicle (abs EtOH) for 48 h followed by 1-h 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 Caspase 8⁺ Annexin V⁺ (right). **(b)** FasL expression in HSB2 T cells after gating on viable cells following 48-h treatment with 7.5 μ M 3AC or vehicle using flow cytometry. **(c)** Analysis of cell death rescue by Caspase 8 inhibition. Cells were treated with 50 μ M of Caspase 8 inhibitor (Z-IETD-FMK) or vehicle for 2 h followed by 24-h treatment with 7.5 μ M 3AC or vehicle. After 24 h, cells were stained and analyzed for Annexin V and PI staining using flow cytometry. Representative PI vs. Annexin V contour plots for each treatment (left) and scatter plot for frequency of Annexin V⁺ PI⁺ (right) are shown. Experiments were performed in triplicate. Results are representative of two independent experiments. Data shown are mean $\pm$ s.e.m. (** 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.

inflammation does not result from a selective loss of Treg cells and their immunosuppressive properties at the 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. As the susceptibility to Fas-mediated apoptosis differs in naive, effector, regulatory, and memory T cells, it would be interesting to determine whether 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 because of their enhanced ability to deplete effector T cells as

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 (5 mg kg⁻¹) 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 with the small intestine (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 with 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 using flow cytometry for viable CD3⁺ T cells. (c) CD (Crohn's disease) score of CD4CreSHIP1^{flox/flox} (CD4), LysMCreSHIP1^{flox/flox} (LysM), and CD4LysMCreSHIP1^{flox/flox} (CD4LysM) mice. Red dots indicate mice that also develop pneumonia. (d–g) Histopathological analysis of CD4LysMCreSHIP1^{flox/flox} (CD4LysM) mice. CD4LysMCre mice developed (d, e) grade 1 CD-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 are mean ± s.e.m. (***P* < 0.01, **P* < 0.05, Student's *t*-test).

compared with Treg cells that lack FasL.^{32–34} In addition, 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 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 IBDs such as UC and CD, it has been reported that exaggerated responses by T cells mediate 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 because of 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 be 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 that the 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 autoreactive T cells in mucosal tissues such as the SI and bowel.^{3,36} Recently, T-cell depletion strategies have been proposed to control IBDs 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) have been described previously.³⁸ SHIP1^{ΔIP/ΔIP} mice were a kind gift of Dr Jeffery Ravetch (Rockefeller University, New York, NY) and for this study were maintained on the F2(129Sv × C57BL6) background described in Karlsson *et al.*³⁹ Mice with germline transmission of a SHIP1^{flox} allele were generated previously in the laboratory³⁸ and propagated by intercrossing with SHIP1^{flox/flox} mice (F₁₀ to C57BL/6J background). LysMCre and CD4Cre transgenic mice were obtained from Jackson Laboratory (Bar Harbor, ME). LysMCreSHIP1^{flox/flox} and CD4CreSHIP1^{flox/flox} mice were created by intercrossing SHIP1^{flox/flox} with LysMCre or CD4Cre mice, respectively. CD4LysMCreSHIP1^{flox/flox} mice were generated by intercrossing CD4CreSHIP1^{flox/flox} and LysMCreSHIP1^{flox/flox}.

Gut and lung histopathological analysis. Gut and lungs were insufflated with 10% neutral-buffered formalin, and the SIs were rolled in segments to fit as Swiss rolls into cassettes for histological process-

ing and microscopic evaluation. Tissues were fixed in 10% neutral-buffered formalin, dehydrated, embedded in paraffin, sectioned at 3 μm , and stained with hematoxylin and eosine. 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% fetal bovine serum (FBS), 1.5 mg ml^{-1} Collagenase, and 40 $\mu\text{g ml}^{-1}$ in Hank's balanced salt solution (HBSS) for 30 min at 37 °C. For isolation of lamina propria T cells, Payer's patches were removed and the SIs were opened longitudinally and cut into small pieces (1.5 cm). Epithelial cells were removed by incubating tissue ($2 \times$) in buffer containing 2 mM EDTA and 5% FBS in HBSS for 20 min. Tissue was then digested with 1.5 mg ml^{-1} Collagenase and 40 $\mu\text{g ml}^{-1}$ for 30 min at 37 °C. Isolated cells were then layered on 40/80% percoll gradient (GE Healthcare, Piscataway, NJ), and centrifuged for 20 min at 200 r.p.m. Cells at the interface of the gradient were collected. Single-cell suspensions from the spleen, lung, and SI were incubated with anti-CD16/32 to block Fc receptor binding (BD Pharmingen, San Jose, CA) followed by staining with fluorochrome-conjugated antibodies. All samples were acquired on an LSRII cytometer (BD Pharmingen, San Jose, CA) and analyzed using the FlowJo software (Tree Star, Ashland, OR). 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 (Miltenyi Biotec Inc., San Diego, CA) via tail vein injection. Before T-cell transplant recipients CD45.1/2 hemizygous C57BL/6 mice received a single dose of 5.5 Gy 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 killed for lung and SI harvests and the lymphocytes of these tissues were assessed using flow cytometry.

SHIP1 inhibition *in vitro*. HSB2 T cells were plated in six-well plates in triplicate and treated with 7.5 μM 3AC or vehicle (absolute ethanol). After 48 h, cells were incubated with CaspGLOW fluorescein active for 1 h, 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 48-h treatment with 3AC (7.5 μM) 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; 50 μM) or vehicle for 2 h before 3AC (7.5 μM) treatment. After 24 h, 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 phosphate-buffered saline 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 $\mu\text{g ml}^{-1}$ Leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Equal amount of protein was immunoprecipitated by using anti-Fas or an isotype control antibody (Santa Cruz Biotechnology, Santa Cruz, CA) along with protein A/G PLUS agarose beads (Santa Cruz Biotechnology) overnight at 4 °C. Immunoprecipitates were washed five times with cold lysis buffer and the pellet was resuspended in sodium dodecyl sulfate sample buffer. Samples were heated at 100 °C for 5 min and subjected to

sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Association of SHIP1 with Fas was determined by probing with anti-SHIP1 (PIC1, Santa Cruz Biotechnology) antibody.

Caspase 8 inhibitor treatment of mice. SHIP1^{-/-} mice were treated either with Z-IETD-FMK (5 mg kg^{-1}) or vehicle (dimethylsulphoxide) three times each week for 3 weeks. Three weeks after initiation of the treatment, the SI and lungs were harvested, and lymphocytes were isolated and then stained with anti-CD3 and DAPI dye. All samples were acquired on an LSRII cytometer and then analyzed using FlowJo 9.4.1.

Statistical analysis. The results are presented as the mean $\pm$ s.e.m. Statistical analysis was performed using two-tailed Student's *t*-test. A *P*-value <0.05 was considered statistically significant.

SUPPLEMENTARY MATERIAL is linked to the online version of the paper at <http://www.nature.com/mi>

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DISCLOSURE

WGK and JDC are inventors on issued and pending patents concerning the modulation or detection of SHIP1 activity in human diseases. The remaining authors declared no conflict of interest.

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REFERENCES

- Kerr, W.G., Park, M.Y., Maubert, M. & Engelman, R.W. SHIP deficiency causes Crohn's disease-like ileitis. *Gut* **60**, 177–188 (2011).
- Helgason, C.D. *et al.* Targeted disruption of SHIP leads to hemopoietic perturbations, lung pathology, and a shortened life span. *Genes Dev.* **12**, 1610–1620 (1998).
- Brooks, R. *et al.* SHIP1 inhibition increases immunoregulatory capacity and triggers apoptosis of hematopoietic cancer cells. *J. Immunol.* **184**, 3582–3589 (2010).
- Smith, A.M. *et al.* Disordered macrophage cytokine secretion underlies impaired acute inflammation and bacterial clearance in Crohn's disease. *J. Exp. Med.* **206**, 1883–1897 (2009).
- Collazo, M.M. *et al.* SHIP limits immunoregulatory capacity in the T-cell compartment. *Blood* **113**, 2934–2944 (2009).
- Kashiwada, M. *et al.* Downstream of tyrosine kinases-1 and Src homology 2-containing inositol 5'-phosphatase are required for regulation of CD4+CD25+ T cell development. *J. Immunol.* **176**, 3958–3965 (2006).
- Locke, N.R. *et al.* SHIP regulates the reciprocal development of Tregulatory and Th17 cells. *J. Immunol.* **183**, 975–983 (2009).
- Charlier, E. *et al.* SHIP-1 inhibits CD95/APO-1/Fas-induced apoptosis in primary T lymphocytes and T leukemic cells by promoting CD95 glycosylation independently of its phosphatase activity. *Leukemia* **24**, 821–832 (2010).
- Atreya, R. *et al.* Blockade of interleukin 6 trans signaling suppresses T-cell resistance against apoptosis in chronic intestinal inflammation: evidence in crohn disease and experimental colitis *in vivo*. *Nat. Med.* **6**, 583–588 (2000).
- Mudter, J. & Neurath, M.F. Apoptosis of T cells and the control of inflammatory bowel disease: therapeutic implications. *Gut* **56**, 293–303 (2007).
- Marks, D.J., Rahman, F.Z., Sewell, G.W. & Segal, A.W. Crohn's disease: an immune deficiency state. *Clin. Rev. Allergy Immunol.* **38**, 20–31 (2010).

12. Olsson, J. *et al.* Human immunodeficiency virus type 1 infection is associated with significant mucosal inflammation characterized by increased expression of CCR5, CXCR4, and beta-chemokines. *J. Infect. Dis.* **182**, 1625–1635 (2000).
13. Kamat, A., Ancuta, P., Blumberg, R.S. & Gabuzda, D. Serological markers for inflammatory bowel disease in AIDS patients with evidence of microbial translocation. *PLoS ONE* **5**, e15533 (2010).
14. Sankaran, S. *et al.* Gut mucosal T cell responses and gene expression correlate with protection against disease in long-term HIV-1-infected nonprogressors. *Proc. Natl Acad. Sci. USA* **102**, 9860–9865 (2005).
15. Fevrier, M., Dorgham, K. & Rebollo, A. CD4 + T cell depletion in human immunodeficiency virus (HIV) infection: role of apoptosis. *Viruses* **3**, 586–612 (2011).
16. De Maria, R. *et al.* Functional expression of Fas and Fas ligand on human gut lamina propria T lymphocytes. A potential role for the acidic sphingomyelinase pathway in normal immunoregulation. *J. Clin. Invest.* **97**, 316–322 (1996).
17. Kerr, W.G. Inhibitor and activator: dual functions for SHIP in immunity and cancer. *Ann. N. Y. Acad. Sci.* **1217**, 1–17 (2011).
18. Collazo, M.M., Paraiso, K.H., Park, M.Y., Hazen, A.L. & Kerr, W.G. Lineage extrinsic and intrinsic control of immunoregulatory cell numbers by SHIP. *Eur. J. Immunol.* **42**, 1785–1795 (2012).
19. Despons, C., Hazen, A.L., Paraiso, K.H. & Kerr, W.G. SHIP deficiency enhances HSC proliferation and survival but compromises homing and repopulation. *Blood* **107**, 4338–4345 (2006).
20. Yuan, Y.H. *et al.* Chemokine receptor CXCR3 expression in inflammatory bowel disease. *Inflamm. Bowel Dis.* **7**, 281–286 (2001).
21. Papadakis, K.A. *et al.* Expression and regulation of the chemokine receptor CXCR3 on lymphocytes from normal and inflammatory bowel disease mucosa. *Inflamm. Bowel Dis.* **10**, 778–788 (2004).
22. Lynch, D.H., Ramsdell, F. & Alderson, M.R. Fas and FasL in the homeostatic regulation of immune responses. *Immunol. Today* **16**, 569–574 (1995).
23. Callard, R.E., Stark, J. & Yates, A.J. Fratricide: a mechanism for T memory-cell homeostasis. *Trends Immunol.* **24**, 370–375 (2003).
24. Sobek, V., Balkow, S., Korner, H. & Simon, M.M. Antigen-induced cell death of T effector cells *in vitro* proceeds via the Fas pathway, requires endogenous interferon-gamma and is independent of perforin and granzymes. *Eur. J. Immunol.* **32**, 2490–2499 (2002).
25. Baatar, D. *et al.* Human peripheral blood T regulatory cells (Tregs), functionally primed CCR4+ Tregs and unprimed CCR4-Tregs, regulate effector T cells using FasL. *J. Immunol.* **178**, 4891–4900 (2007).
26. Gorbachev, A.V. & Fairchild, R.L. CD4 + CD25 + regulatory T cells utilize FasL as a mechanism to restrict DC priming functions in cutaneous immune responses. *Eur. J. Immunol.* **40**, 2006–2015 (2010).
27. Strauss, L., Bergmann, C. & Whiteside, T.L. Human circulating CD4 + CD25highFoxp3 + regulatory T cells kill autologous CD8 + but not CD4 + responder cells by Fas-mediated apoptosis. *J. Immunol.* **182**, 1469–1480 (2009).
28. Singh, N. *et al.* CD4(+)CD25(+) regulatory T cells resist a novel form of CD28- and Fas-dependent p53-induced T cell apoptosis. *J. Immunol.* **184**, 94–104 (2010).
29. Nagata, S. & Golstein, P. The Fas death factor. *Science* **267**, 1449–1456 (1995).
30. Van Parijs, L. & Abbas, A.K. Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science* **280**, 243–248 (1998).
31. Liu, Q. *et al.* SHIP is a negative regulator of growth factor receptor-mediated PKB/Akt activation and myeloid cell survival. *Genes Dev.* **13**, 786–791 (1999).
32. Askenasy, N. Enhanced killing activity of regulatory T cells ameliorates inflammation and autoimmunity. *Autoimmun. Rev.* **12**, 972–975 (2013).
33. Kamnitz, A. *et al.* Killer Treg restore immune homeostasis and suppress autoimmune diabetes in prediabetic NOD mice. *J. Autoimmun.* **37**, 39–47 (2011).
34. Yolcu, E.S. *et al.* Immunomodulation with donor regulatory T cells armed with Fas-ligand alleviates graft-versus-host disease. *Exp. Hematol.* **41**, 903–911 (2013).
35. Glocker, E. & Grimbacher, B. Inflammatory bowel disease: is it a primary immunodeficiency? *Cell. Mol. Life Sci.* **69**, 41–48 (2012).
36. Fuhler, G.M. *et al.* Therapeutic potential of SH2 domain-containing inositol-5'-phosphatase 1 (SHIP1) and SHIP2 inhibition in cancer. *Mol. Med.* **18**, 65–75 (2012).
37. Yu, Q.T., Saruta, M. & Papadakis, K.A. Visilizumab induces apoptosis of mucosal T lymphocytes in ulcerative colitis through activation of caspase 3 and 8 dependent pathways. *Clin. Immunol.* **127**, 322–329 (2008).
38. Wang, J.W. *et al.* Influence of SHIP on the NK repertoire and allogeneic bone marrow transplantation. *Science* **295**, 2094–2097 (2002).
39. Karlsson, M.C. *et al.* Macrophages control the retention and trafficking of B lymphocytes in the splenic marginal zone. *J. Exp. Med.* **198**, 333–340 (2003).

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