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Expansion of Myeloid Suppressor Cells in SHIP-Deficient Mice Represses Allogeneic T Cell Responses[1]

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Previously we demonstrated that SHIP$^{-/-}$ mice accept allogeneic bone marrow transplants (BMT) without significant acute graft-vs-host disease (GvHD). In this study we show that SHIP$^{-/-}$ splenocytes and lymph node cells are poor stimulators of allogeneic T cell responses that cause GvHD. Intriguingly, SHIP$^{-/-}$ splenocytes prime naive T cell responses to peptide epitopes, but, conversely, are partially impaired for priming T cell responses to whole Ag. However, dendritic cells (DC) purified from SHIP$^{-/-}$ splenocytes prime T cell responses to allogeneic targets, peptide epitopes, and whole Ag as effectively as SHIP$^{+/+}$ DC. These findings point to an extrinsic effect on SHIP$^{-/-}$ DC that impairs priming of allogeneic T cell responses. Consistent with this extrinsic effect, we found that a dramatic expansion of myeloid suppressor cells in SHIP$^{-/-}$ mice impairs priming of allogeneic T cells. These findings suggest that SHIP expression or its activity could be targeted to selectively compromise T cell responses that mediate GvHD and graft rejection. *The Journal of Immunology,* **2004, 173: 7324–7330.**

Allogeneic bone marrow transplantation (BMT)[3] is an important treatment option in a variety of different cancers and genetic diseases. Graft-vs-host disease (GvHD) is a significant cause of morbidity and mortality in patients receiving allogeneic BMT. Clinical manifestations of GvHD consist of kyphosis, alopecia, skin lesions, diarrhea, as well as histopathologic changes in the skin, liver, and lymphohemopoietic compartment (1, 2). GvHD is promoted when donor T lymphocytes encounter host-derived APCs that express MHC Ags and costimulatory molecules (3, 4). APC consist of dendritic cells (DC), activated macrophages, and B cells (3). More importantly, T lymphocytes are the mediators of GvHD, but their activation after transplant is under the control of professional APC or DC (3, 4). Professional APC must supply an appropriate microenvironment for full activation of T lymphocytes that consists of presented Ags, cytokines, chemokines, and costimulatory molecules (4, 5).

SHIP is a 5′ inositol phosphatase that hydrolyzes the phospholipid product phosphoinositol 3,4,5-trisphosphatase of PI3K, which, in turn, regulates cell survival, proliferation, and differentiation (6). In this manner, SHIP influences the survival and/or function of numerous cell types, including myeloid cells (6, 7), osteoclasts (8), and NK cells (9). SHIP$^{-/-}$ macrophages and osteoclasts are deleterious to the host (6, 8). However, a potential benefit of SHIP deficiency is that SHIP$^{-/-}$ recipients accept allogeneic bone marrow (BM) grafts with a reduced incidence of GvHD (9). Although a disruption of the NK receptor repertoire in SHIP$^{-/-}$ mice accounts for their reduced resistance to engraftment of allogeneic BM, the mechanism that underlies their resistance to GvHD has not been elucidated (9).

Myeloid suppressor cells (MySC) have a myeloid morphology and express the markers Gr-1 (Ly6-G) and CD11b (Mac-1). MySC were previously referred to as natural suppressors that constituted a cell population of undefined phenotype (10). MySC are known to accumulate in lymphoid organs under conditions of intense immune activation, where they specifically inhibit T and B cell functions in vivo and in vitro (11, 12). In addition, MySC have been reported to inhibit peptide-specific, CD8-mediated immune responses in vitro and in vivo (13).

In this study we evaluated the ability of SHIP$^{-/-}$ splenocytes and lymph nodes (LN) to prime allogeneic T cell responses in vitro. We initially hypothesized that host APC from SHIP$^{-/-}$ mice may be defective in priming allogeneic T cell responses that culminate in GvHD. We found that SHIP$^{-/-}$ mice have a 10- to 20-fold increase in Gr-1^{+}Mac-1^{+} MySC in their LN and spleen relative to SHIP$^{+/+}$ mice and that this expansion suppresses the allogeneic T cell response in vitro. These findings may at least in part account for the reduced GvHD observed in SHIP$^{-/-}$ mice. Furthermore, these findings demonstrate that SHIP is essential for the control of MySC homeostasis.

Materials and Methods

Flow cytometric analysis

In all experiments SHIP$^{-/-}$ and SHIP$^{+/+}$ cells harvested from spleens or LN were analyzed by flow cytometry with anti-CD11c, anti-CD11b (Mac-1), anti-Gr-1 (Ly6-G), anti-B7.2, anti-IgM, anti-CD3, or anti-NK1.1 (14). All Abs were purchased from BD Pharmingen (San Diego, CA).

Longevity and post-transplant survival

Allogeneic BMT studies were conducted using 6- to 8-wk-old SHIP$^{-/-}$ and SHIP$^{+/+}$ mice on an F4(129/Sv × C57BL6) H2b background as recipients. As described previously, these hosts were irradiated with 9.5 Gy from a ^{137}Cs source, transplanted with 5×10^6 BALB/c (H2^d) whole BM cells, and monitored for signs of acute GvHD post-transplant as previously

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[3] Abbreviations used in this paper: BMT, bone marrow transplantation; BM, bone marrow; DC, dendritic cell; GvHD, graft-vs-host disease; LN, lymph node; MySC, myeloid suppressor cell; WS, whole splenocyte.

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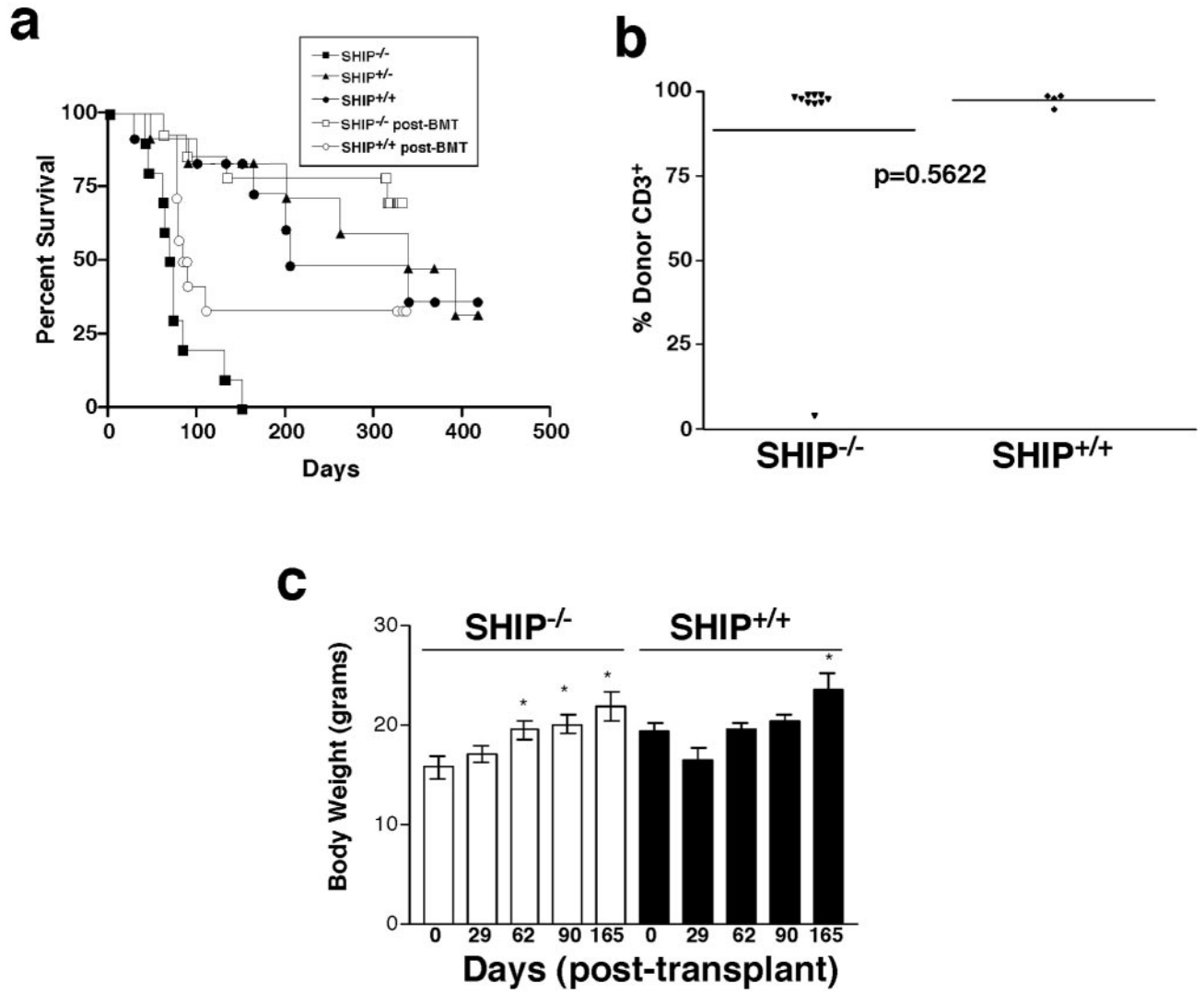

FIGURE 1. Long term survival, donor T cell repopulation, and weight gain in SHIP-deficient allogeneic BMT recipients. *a*, Allogeneic BMT endows $SHIP^{-/-}$ recipients with a normal life span. Shown is the age (days) of unmanipulated $SHIP^{-/-}$, $SHIP^{+/-}$, and $SHIP^{+/+}$ mice compared with that of $SHIP^{-/-}$ and $SHIP^{+/+}$ mice that received an allogeneic BMT from BALB/c mice ($p < 0.001$ for $SHIP^{-/-}$ vs $SHIP^{-/-}$ plus allogeneic BMT; $p < 0.001$ for $SHIP^{-/-}$ vs $SHIP^{+/-}$ and $SHIP^{+/+}$; $p = 0.0301$ for $SHIP^{-/-}$ plus allogeneic BMT vs $SHIP^{+/+}$ plus allogeneic BMT). $SHIP^{-/-}$ mice and their wild-type counterparts were transplanted between 42 and 56 days of life. *b*, Percent donor T cell chimerism in the $SHIP^{-/-}$ and $SHIP^{+/+}$ BMT cohorts in *a* as measured by flow cytometric analysis of PBMC at 30 wk post-transplant. *c*, Average body weight of $SHIP^{-/-}$ and $SHIP^{+/+}$ BMT recipients from *a* measured on the indicated days posttransplant or pretransplant (day 0). The posttransplant body weights that are significantly higher than the pretransplant weight for that cohort are indicated (*, $p < 0.05$).

described (9). BM donors were not exsanguinated before BM harvest, because this eliminates peripheral T cells from whole bone marrow grafts that are necessary for acute GVHD in this transplant setting (15–17). Before transplant, mice were housed in a nonbarrier, but specific pathogen-free, facility at the Moffitt Research Center vivarium. Water, bedding, and chow are not autoclaved in this facility for conventionally housed mice. Filter tops are present on the cages, but cage changes take place in an open room. After irradiation, mice were transferred to high efficiency particulate air filter isolator units, where bedding, water, and chow are autoclaved. Autoclaved drinking water was also acidified. Mice remained in high efficiency particulate air isolators for 1 mo post-transplant and then were returned to conventional housing for the duration of the study. The statistical significance of survival differences was assessed by the Kaplan-Meier log-rank test. Donor chimerism in transplant recipients was assessed by staining PBMC with lineage-specific Abs (B220, CD3, and Mac1/Gr1) and an anti-H2d Ab (BD Pharmingen) and by flow cytometric analysis.

MLR

After RBC lysis, $SHIP^{-/-}$ and $SHIP^{+/+}$ splenocytes or LN cells (stimulators; 8×10^5/well) were irradiated (2000 rad) and cocultured with BALB/c splenocytes or LN cells, (responders; 4×10^5/well) in a one-way MLR assay (18). All cells were plated in triplicate in 96-well, U-bottom plates (Costar, Cambridge, MA) containing RPMI 1640 complete medium for 4 days. Cells were pulsed with 1.0 μCi/well [^{3}H]thymidine for 18 h. Cells were harvested on glass-fiber filtermates using an automated cell harvester (Packard Instrument, Meriden, CT). Incorporated [^{3}H]thymidine was counted using a TopCount NXT (Packard Instrument). Specific [^{3}H]thymidine incorporation was calculated in cpm as the average of the mean ± SEM of triplicate wells.

MHC class I and II Ag presentation experiments

Splenocytes (5×10^5) from OT-I mice (19) (responders) were incubated with OVA peptide (micrograms per milliliter), pulsed irradiated $SHIP^{-/-}$ and $SHIP^{+/+}$ splenocytes, or LN cells (1×10^5). Splenocytes (5×10^4) from OT-II mice (20) were incubated with 3 μg of OVA peptide-pulsed, irradiated $SHIP^{-/-}$ and $SHIP^{+/+}$ splenocytes or LN cells (1×10^5). Three micrograms of OVA peptide or 100 μg of whole OVA were incubated with $SHIP^{-/-}$ and $SHIP^{+/+}$ splenocytes or sorted $SHIP^{-/-}$ and $SHIP^{+/+}$ DC (3×10^3) and OT-II splenocytes in triplicate wells in 96-well, U-bottom plates (Costar) containing RPMI 1640 complete medium for 4 days. Cells were pulsed with 1.0 μCi/well [^{3}H]thymidine for 18 h, then harvested on

glass-fiber filtermates using an automated cell harvester (Packard Instrument). Incorporated [^{3}H]thymidine was counted using a TopCount NXT (Packard Instrument). Specific [^{3}H]thymidine incorporation was calculated in cpm as the average of the mean ± SEM of triplicate wells.

Cytometric bead analysis Th1/Th2 cytokine production

Two days after initiation of the MLR or Ag presentation assay, 100 μl of supernatant was collected, and IL-2 or IFN-γ levels were measured by the Th1/Th2 cytometric bead array assay (BD Pharmingen) according to the manufacturer's instructions (21).

DC and macrophage sorting

CD11c$^+$B7.2$^+$Lin$^-$ DC were sorted from SHIP$^{-/-}$ and SHIP$^{+/+}$ splenocytes using the following Lin panel: NK1.1, B220, CD3, Thy1.2, Ter119, and Gr-1 (14). SHIP$^{-/-}$ and SHIP$^{+/+}$ macrophages for add-back experiments were sorted based on the following phenotype: Mac-1$^+$NK1.1$^-$CD3$^-$B220$^-$.

MLR with purified DC and macrophages

Sorted CD11c$^+$B7.2$^+$Lin$^-$ DC from SHIP$^{-/-}$ and SHIP$^{+/+}$ spleens (3×10^3) were irradiated and cultured with BALB/c responder (2×10^5) cells. For macrophage add-back experiments, sorted and irradiated SHIP$^{-/-}$ and SHIP$^{+/+}$ DC (3×10^3) were incubated with sorted Mac1$^+$NK1.1$^-$CD3$^-$B220$^-$ macrophages (3×10^4) along with BALB/c responders (2×10^5) in the MLR. [^{3}H]thymidine uptake measurements were conducted as in the one-way MLR described above.

MLR with Gr-1-depleted splenocytes

SHIP$^{-/-}$ and SHIP$^{+/+}$ splenocytes were depleted of Gr-1$^+$ MySC and granulocytes by incubation with purified Gr-1(L6-G) mAb (0.5 μg/10^6 cells) and were lysed by addition of rabbit complement (Cedarlane Laboratories, Westbury, NY) at 37°C for 30 min. The cell suspension was then pelleted on a Ficoll (Amersham Biosciences, Mountain View, CA) gradient to remove dead cells. Gr-1-depleted SHIP$^{-/-}$ and SHIP$^{+/+}$ splenocytes were irradiated and used as stimulators in a one-way MLR as previously described.

Statistical analysis

All in vitro experiments described in this study are representative of at least three independent analyses. All MLR, Ag presentation, cytometric bead array, and flow cytometry results were analyzed with two-tailed Student's *t* test using PRISM4 software (GraphPad, San Diego, CA). Differences were considered significant at $p < 0.05$.

Results

SHIP$^{-/-}$ splenocytes and LN cells prime allogeneic T cell responses very poorly

Previously we found that SHIP$^{-/-}$ hosts permit engraftment of allogeneic BM with reduced incidence of acute GvHD relative to SHIP$^{+/+}$ mice (9). Long term follow-up of survival revealed that allogeneic BMT endows SHIP$^{-/-}$ mice with a life span indistinguishable from that of unmanipulated SHIP$^{+/+}$ or SHIP$^{+/-}$ littermates (Fig. 1*a*), indicating that the transplant also protects them from myeloproliferative disease, to which these mice typically succumb by 8–12 wk of life. Moreover, repopulation of T cells (Fig. 1*b*), B cells, and myelogranulocytic lineages (data not shown) was, on the average, ≥88% for all lineages at 30 wk post-transplant. SHIP mice also showed no post-transplant weight loss and gained weight more rapidly after transplant than their SHIP$^{+/+}$ counterparts (Fig. 1*c*), indicating that they thrive post-transplant. Taken together, these findings indicate fully MHC-mismatched marrow grafts can repopulate all lineages, including T cells, in the majority of SHIP-deficient recipients without significant acute or chronic GvHD. These transplant findings also demonstrate that SHIP deficiency in nonhemopoietic organs does not significantly impair longevity.

Shlomchik et al. (14) showed that host-derived APC are responsible for the initiation of GvHD. Thus, we hypothesized that SHIP-deficient mice might be relatively resistant to GvHD, because they fail to prime allogeneic T cells present in the incoming graft. Thus, we assessed the ability of SHIP$^{-/-}$ splenocytes and LN cells to prime allogeneic T cell responses (proliferation and cytokine production) using the MLR assay. When irradiated whole splenocytes (WS) from SHIP$^{-/-}$ mice (C57BL/6) were used as stimulators for BALB/c splenocytes in a one-way MLR, we found that SHIP$^{-/-}$ cells stimulated allogeneic T cell proliferation very poorly compared with SHIP$^{+/+}$ WS (Fig. 2*a*). The same disparity was found when SHIP$^{-/-}$ LN cells were used as stimulators for BALB/c LN responders (Fig. 2*b*). Consistent with poor priming of T cell proliferation in the MLR, SHIP$^{-/-}$ WS primed IL-2 production at significantly reduced levels relative to SHIP$^{+/+}$ WS (Fig. 2*c*). In addition, we found that SHIP$^{-/-}$ H2k splenocytes and LN cells failed to efficiently prime allogeneic responses by H2d (BALB/c), H2b (C57BL/6), and H2r (RIIIS/J) responders (Fig. 2*d* and data not shown). This latter finding indicates that inefficient priming of allogeneic T cells by SHIP$^{-/-}$ splenocytes is probably extrapolated to a broad spectrum of donor/host pairings in allogeneic transplantation.

SHIP$^{-/-}$ splenocytes and LN cells can prime CD4$^+$ and CD8$^+$ Ag-specific T cells

The inability of SHIP$^{-/-}$ splenocytes and LN cells to present and prime a robust allogeneic T cell response could potentially be explained by a general defect in APC function or a reduction in their numbers. However, a severely disabled or reduced APC compartment seems unlikely, because no overt defects in adaptive immune responses have been reported in these mice (22, 23). In fact, despite a reduction in the number of mature B cells in the periphery of SHIP$^{-/-}$ mice, humoral responses to complex protein Ags appear to be unimpaired by SHIP deficiency (22, 23). Nonetheless, we assessed the ability of SHIP$^{-/-}$ APC to prime naive T cell responses directly by comparing the ability of SHIP$^{-/-}$ and SHIP$^{+/+}$ splenocytes and LN cells to prime Ag-specific proliferation by naive CD4$^+$ and CD8$^+$

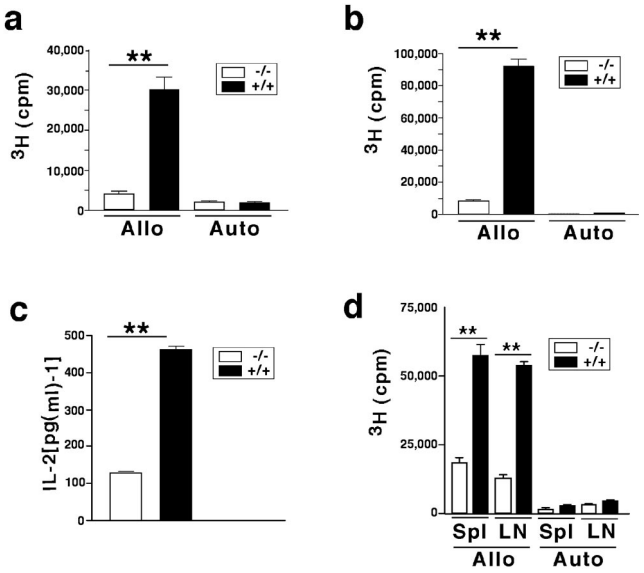

FIGURE 2. Priming of allogeneic T cell responses is selectively compromised in SHIP$^{-/-}$ hosts. *a*, MLR using irradiated SHIP$^{-/-}$ and SHIP$^{+/+}$ splenocytes (8×10^5) to stimulate splenocytes from BALB/c mice (4×10^5; Allo). *b*, MLR using irradiated SHIP$^{-/-}$ LN cells (8×10^5) to stimulate BALB/c responder cells (4×10^5; Allo). *c*, IL-2 levels in the SHIP$^{-/-}$ and SHIP$^{+/+}$ MLR from *a*. *d*, MLR using irradiated SHIP$^{-/-}$ and SHIP$^{+/+}$ B10.H2k splenocytes or LN cells (8×10^5) to stimulate BALB/c H2Dd splenocytes or LN cells (4×10^5), respectively (Allo). SHIP$^{+/+}$ or SHIP$^{-/-}$ responders were mixed with irradiated, autologous stimulators to assess background proliferation (Auto). **, $p < 0.01$ (by two-tailed Student's *t* test).

T cells in vitro. Consistent with the analysis of Ab responses to specific Ags in SHIP$^{-/-}$ mice (22, 23), we found that SHIP$^{-/-}$ WS or LN cells prime naive Ag-specific CD8$^+$ and CD4$^+$ T cell responses as efficiently as SHIP$^{+/+}$ WS (Fig. 3). Presentation of MHC class I- or class II-restricted epitopes to syngeneic T cells by SHIP$^{-/-}$ WS stimulated CD8$^+$ and CD4$^+$ T cells to proliferate at levels comparable to those produced by SHIP$^{+/+}$ WS (Fig. 3, *a* and *c*). In addition, SHIP$^{-/-}$ WS prime Ag-specific production of the effector cytokine, IFN-γ, by both CD8$^+$ and CD4$^+$ T cells at levels equal to or greater than those produced by Ag-pulsed SHIP$^{+/+}$ WS (Fig. 3, *b* and *d*). Thus, APC priming of naive T cell responses to specific class I- or II-restricted epitopes is not impaired by SHIP deficiency.

SHIP$^{-/-}$ DC can successfully prime allogeneic T cell responses

DC are professional APC capable of priming allogeneic T cell responses in vitro (14, 18) and in vivo (24–26). Therefore, we examined whether DC numbers were reduced in the spleen and LN of SHIP$^{-/-}$ mice or whether there was some qualitative defect in their ability to prime allogeneic T cell responses. FACS quantitation of CD11c$^-$Mac-1$^+$ myeloid cells showed an increase in the spleen and LN of SHIP$^{-/-}$ mice (Fig. 4, *a* and *b*). In fact, on the contrary, the numbers of CD11c$^+$Mac-1$^+$ DC were significantly increased in peripheral LN of SHIP$^{-/-}$ mice, but not in spleen (Fig. 4*b*). We then compared the ability of purified CD11c$^+$B7.2$^+$ Lin$^-$ DC from both SHIP$^{+/+}$ and SHIP$^{-/-}$ mice to prime allogeneic T cell responses. The comparison showed that SHIP$^{-/-}$ DC are equally effective at priming an MLR (Fig. 4*c*), demonstrating that SHIP$^{-/-}$ DC are not functionally impaired with respect to priming of allogeneic T cell responses. However, an aliquot of SHIP$^{-/-}$ WS from which the DC were purified exhibited defective priming of allogeneic T cells (Fig. 4*c*), consistent with previous MLR assays.

SHIP$^{-/-}$ DC can process and present MHC class II-restricted T cell epitopes

To confirm that SHIP$^{-/-}$ DC are functional in their ability to process and present Ag, whole splenocytes and purified DC were pulsed with 3 μg of OVA peptide or 100 μg of whole OVA (Fig. 4, *d* and *e*), then incubated with OT-II transgenic splenocytes (19). This analysis revealed that purified SHIP$^{-/-}$ DC can present $OVA_{323–339}$ peptide and whole OVA to OVA-specific T cells as efficiently as SHIP$^{+/+}$ DC. However, SHIP$^{-/-}$ WS do not process whole OVA and present it to OVA-specific T cells as well as SHIP$^{+/+}$ WS. Because DC numbers are normal or increased in lymphoid tissues of SHIP$^{-/-}$ mice, and their ability to prime allogeneic T cell responses and present Ag is unimpaired, we postulate that SHIP deficiency suppresses priming of allogeneic responses due to an extrinsic effect by another cell type present in SHIP$^{-/-}$ lymphoid tissues. In addition, our studies with whole OVA (Fig. 4, *d* and *e*) indicate there is also an extrinsic effect that partially impairs DC processing and presentation of class II-restricted T cell epitopes.

MySC are overrepresented in SHIP$^{-/-}$ lymphoid organs

MySC can suppress allogeneic T cell responses in vitro and in vivo (27). Others have reported a substantial increase in myeloid cells in the BM and peripheral organs of SHIP$^{-/-}$ mice (6). Thus, we examined the composition of the myeloid compartment in peripheral lymphoid tissues of SHIP$^{-/-}$ mice. We found that the number of Mac-1$^+$CD11c$^-$ monocyte/macrophages was substantially increased in both the spleen and LN of SHIP$^{-/-}$ mice (Fig. 4, *a* and *b*). Further analysis (Fig. 5*a*) revealed that Mac-1$^+$Gr-1$^+$ MySC numbers were disproportionately increased in both the spleen and LN of SHIP$^{-/-}$ mice (Fig. 5, *a* and *b*). Significant increases in other myelogranulocytic cell types (Mac-1$^+$Gr-1$^-$ and Gr-1$^+$Mac-1$^-$ subsets) were also evident in spleen and LN of SHIP$^{-/-}$ mice. However, the ~20-fold increase in the representation of MySC was the most profound increase in any myelogranulocytic subset present in peripheral lymphoid tissues of SHIP$^{-/-}$ mice (Fig. 5*b*).

SHIP$^{-/-}$ MySC suppress allogeneic T cell responses in vitro

We speculated that an increase in a myeloid cell type, such as MySC, might impair priming of allogeneic T cell responses in SHIP$^{-/-}$ lymphoid tissues. To initially assess this, we added purified SHIP$^{-/-}$ Mac-1$^+$ cells to purified SHIP$^{-/-}$ or SHIP$^{+/+}$

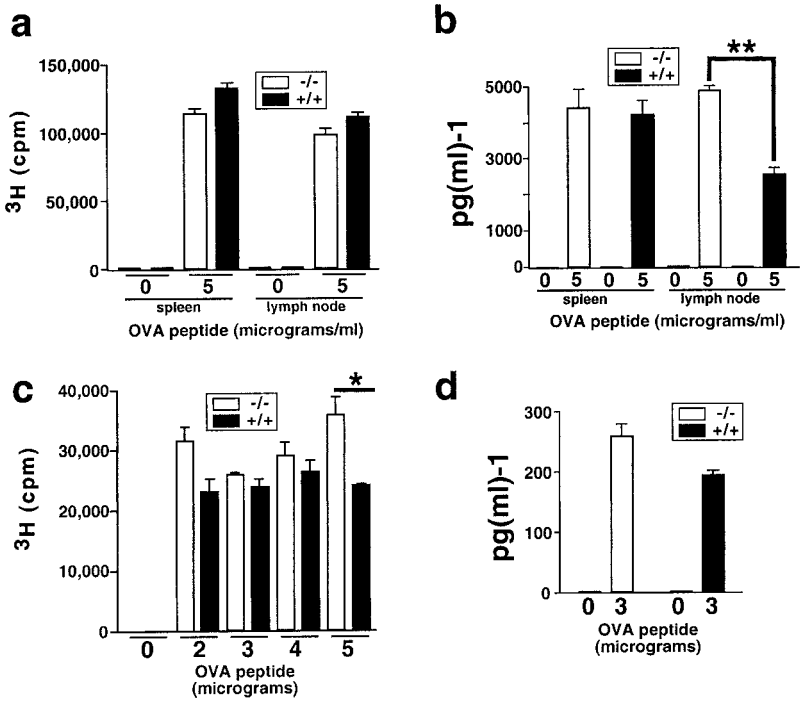

FIGURE 3. Priming of naive CD4$^+$ and CD8$^+$ T cells is unaffected by SHIP deficiency. *a*, Proliferation of anti-OVA transgenic CD8 T cells in response to OVA-peptide pulsed SHIP$^{-/-}$ and SHIP$^{+/+}$ splenocytes and LN. *b*, Production of IFN-γ by anti-OVA transgenic CD8 T cells in response to OVA peptide-pulsed SHIP$^{-/-}$ and SHIP$^{+/+}$ splenocytes and LN. *c*, Proliferation of anti-OVA transgenic CD4$^+$ T cells in response to OVA peptide-pulsed SHIP$^{-/-}$ and SHIP$^{+/+}$ splenocytes. *d*, Production of IFN-γ by anti-OVA transgenic CD4$^+$ T cells to OVA peptide-pulsed SHIP$^{-/-}$ and SHIP$^{+/+}$ splenocytes. *, $p < 0.05$; **, $p < 0.01$ (by two-tailed Student's *t* test).

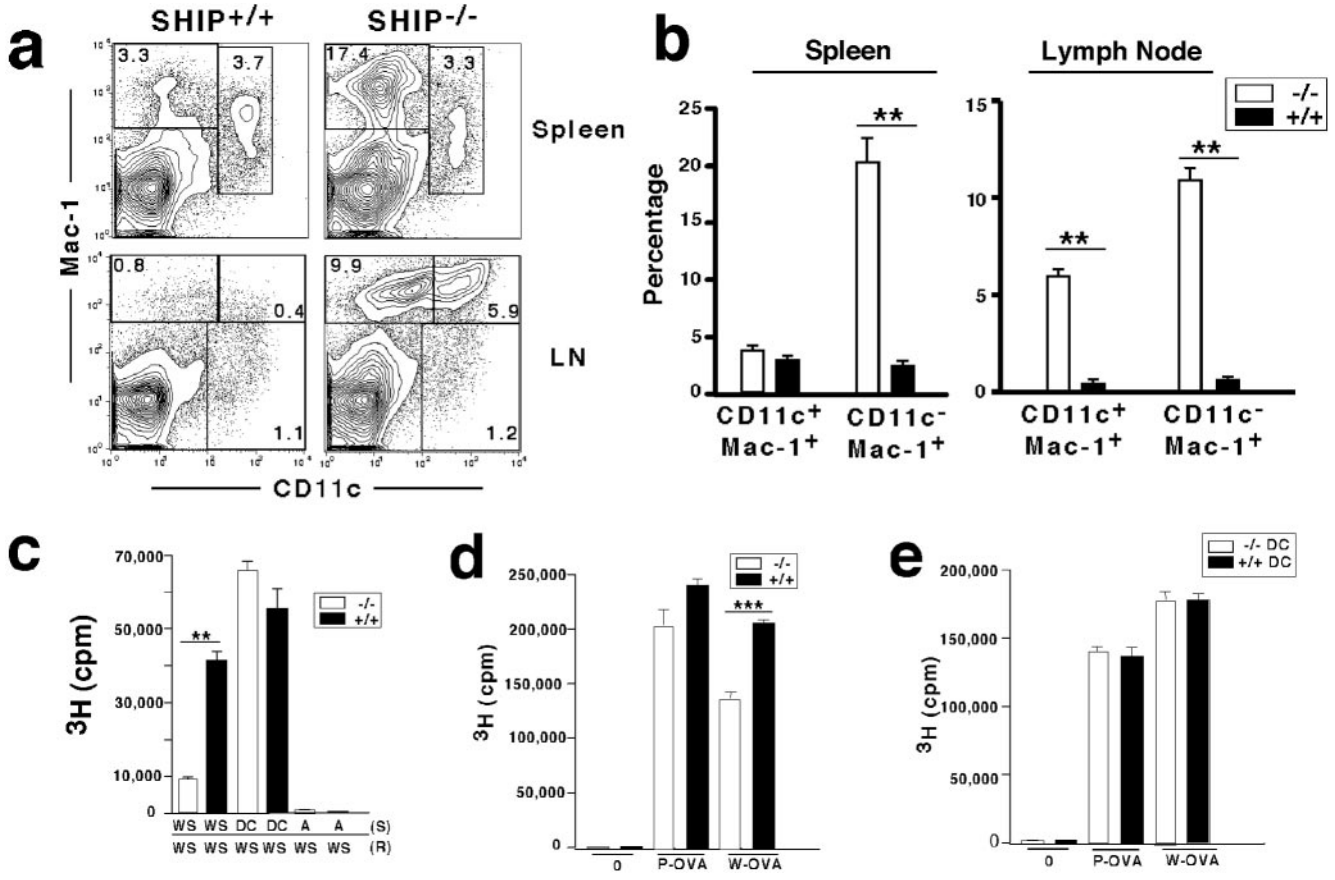

FIGURE 4. Deficient priming of allogeneic T cells by SHIP$^{-/-}$ secondary lymphoid tissues is extrinsic to DC. *a*, CD11c^{-}Mac-1^{+} myeloid cells are increased in the spleens and LN of SHIP$^{-/-}$ mice. CD11c^{+}Mac-1^{+} DC are increased only in the LN, not the spleen, of SHIP$^{-/-}$ mice, as indicated by FACS analysis. *b*, The mean percentages of myeloid cells (Mac-1^{+}CD11c^{-}) and DC (CD11c^{+}Mac-1$^{+)}$ were calculated for spleen and LN from four or five SHIP$^{-/-}$ and SHIP$^{+/+}$ mice. *c*, MLR using irradiated SHIP$^{-/-}$ and SHIP$^{+/+}$ WS (8×10^5) or purified DC (CD11c^{+}B7.2^{+}Lin^{-}; 3×10^3) used as stimulators (S) incubated with WS responders (R) from BALB/c mice (4×10^5 or 2×10^5). WS SHIP$^{-/-}$ and SHIP$^{+/+}$ responders (R) were cultured with irradiated SHIP$^{-/-}$ and SHIP$^{+/+}$ autologous stimulators (A) to assess background proliferation. *d* and *e*, SHIP$^{-/-}$ and SHIP$^{+/+}$ WS (*d*) and sorted DC (*e*) were incubated with 3 μg of OVA$_{323-339}$ peptide (P-OVA) or 100 μg whole OVA (W-OVA) along with OVA-specific OT-II transgenic T cells in Ag presentation assays, respectively. **, $p < 0.01$; ***, $p < 0.001$ (by two-tailed Student's *t* test).

DC and found that the presence of SHIP$^{-/-}$ Mac-1^{+} cells significantly repressed priming of allogeneic T cells by DC of either genotype (Fig. 5*c*). Conversely, the addition of Mac-1^{+} cells from SHIP$^{+/+}$ mice had no impact on the priming of allogeneic T cells by DC of either genotype, demonstrating that suppression caused by SHIP$^{-/-}$ Mac-1^{+} cells is not simply due to the presence of Mac-1^{+} cells (Fig. 5*c*), but, rather, to an inappropriate activity or cell type over-represented in the Mac-1^{+} population of SHIP$^{-/-}$ mice. To further delineate the cell type responsible for the suppression, we then depleted Gr-1^{+} cells from WS before using them as stimulators in an MLR. The removal of Gr-1^{+} cells from SHIP$^{-/-}$ WS restored their ability to prime an MLR to levels equivalent to or higher than those primed by SHIP$^{+/+}$ WS (Fig. 5*d*). In addition, the removal of Gr-1^{+} cells from SHIP$^{+/+}$ WS resulted in better priming of allogeneic T cells than in non-Gr-1-depleted SHIP$^{+/+}$ WS in the MLR (Fig. 5*d*). These results suggest that SHIP$^{+/+}$ Mac-1^{+}Gr-1^{+} cells are also suppressive, but that their low abundance in peripheral lymphoid tissues is not sufficient to mediate profound suppression as they do in SHIP$^{-/-}$ lymphoid tissues, where Mac-1^{+}Gr1^{+} cells are expanded ~20-fold. Taken together, the Mac-1^{+} cell add-back and Gr-1 depletion studies indicate that the increased representation of Mac-1^{+}Gr-1^{+} MySC in SHIP$^{-/-}$ peripheral lymphoid tissues can mediate significant suppression of allogeneic T cell responses. This is one potential mechanism for the relative resistance of these mice to GvHD.

Discussion

In this study we demonstrate that SHIP regulates the homeostasis of MySC. We observed a 10- to 20-fold increase in MySC in both the spleen and LN of SHIP$^{-/-}$ mice compared with SHIP$^{+/+}$ mice. Consistent with the loss of MySC homeostasis in lymphoid tissues, there was a negative impact on priming of allogeneic T cell responses under these conditions. Other investigators have shown that depletion of MSC in vitro or in vivo can reverse the depression of CD8^{+} T cell function, thus confirming the important role of MySC in the induction and maintenance of immunosuppression during vigorous immune responses (28–30).

In addition, we found that purified DC from SHIP$^{-/-}$ mice can prime allogeneic T cells and present specific Ag to T cells in vitro using either OVA peptide or whole OVA, suggesting that there is no overt intrinsic effect on DC function in SHIP$^{-/-}$ mice. However, SHIP$^{-/-}$ WS were partially impaired in their ability to process whole OVA and present it to Ag-specific T cells in vitro. These results suggest that SHIP$^{-/-}$ MySC may also dampen allogeneic T cell responses indirectly by altering the ability of DC to effectively process and present Ags. This is a novel function for

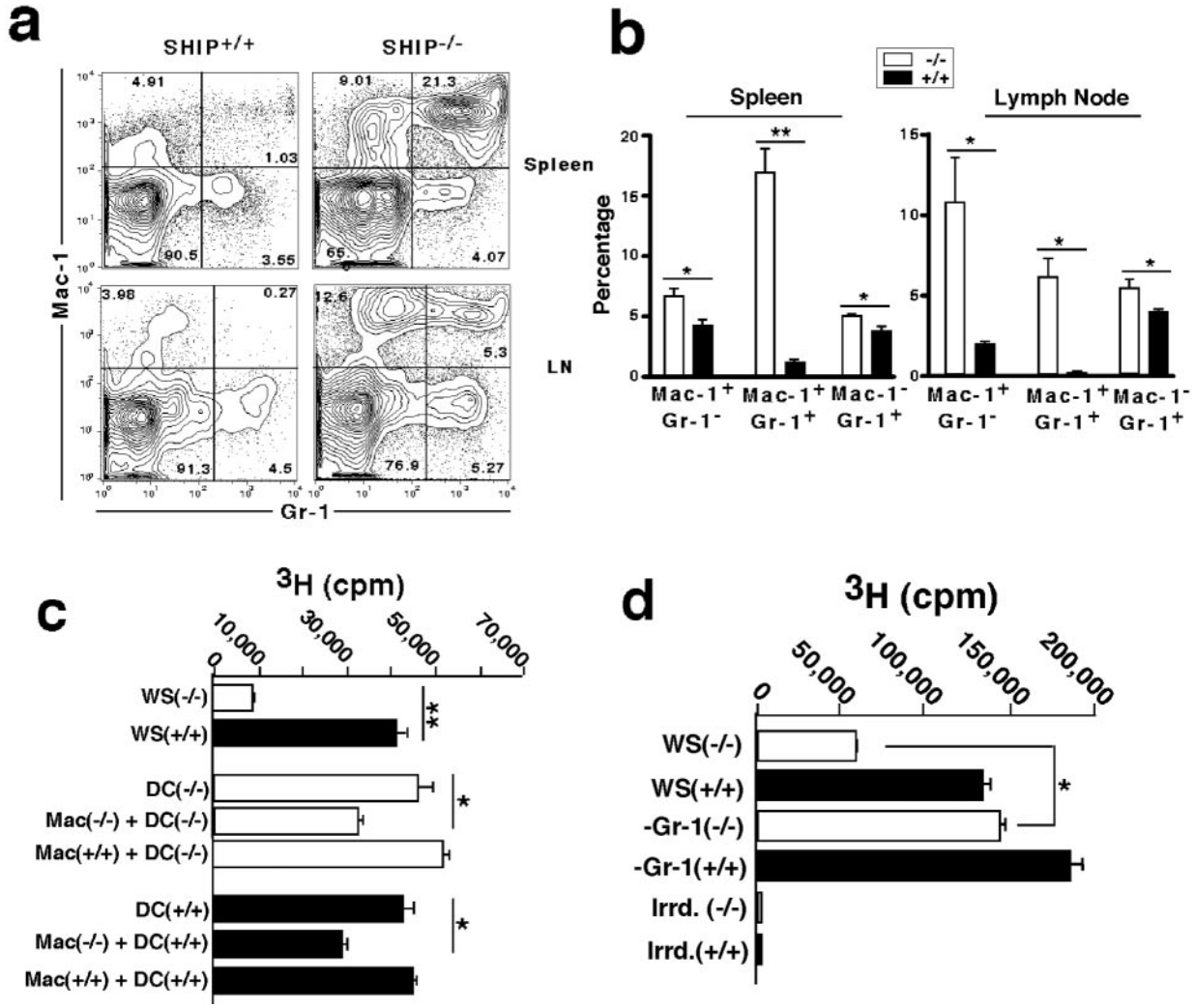

FIGURE 5. SHIP$^{-/-}$ MSC suppress the ability of DC to prime allogeneic T cell responses. *a*, FACS analysis of myelogranulocytic cell types shows that Mac-1^{+}Gr-1^{+} MySC are increased in the spleens and peripheral LN of SHIP$^{-/-}$ mice. *b*, The mean percentages of MySC (Mac-1^{+}Gr-1^{+}), macrophages (Mac-1^{+} Gr-1^{-}), and granulocytes (Mac-1^{-}Gr-1^{+}) were calculated in spleens and LN from four or five SHIP$^{-/-}$ and SHIP$^{+/+}$ mice. *c*, SHIP$^{-/-}$ Mac-1^{+} cells repress DC priming of allogeneic T cell responses. BALB/c WS responders (2×10^5) were incubated with irradiated SHIP$^{-/-}$- or SHIP$^{+/+}$-purified DC (3×10^3) to which purified macrophages (Mac-1^{+}; 3×10^4) were added. BALB/c WS responders (4×10^5) were incubated with SHIP$^{-/-}$ and SHIP$^{+/+}$ WS (8×10^5). *d*, Irradiated, Gr-1-depleted (−Gr-1), and nondepleted SHIP$^{-/-}$ and SHIP$^{+/+}$ splenocytes (8×10^5) were coincubated with BALB/c splenocyte responders (4×10^5) in an MLR assay. Irradiated (Irrd.) SHIP$^{-/-}$ and SHIP$^{+/+}$ WS were cultured alone as controls for autoproliferation. *, $p < 0.05$; **, $p < 0.01$ (by two-tailed Student's *t* test).

MySC that merits additional investigation to determine whether MySC alter Ag processing and presentation pathways in DC or whether they compete with DC for uptake of Ag. In this regard, it is of interest that phagocytosis may be enhanced in SHIP$^{-/-}$ macrophages (31).

MySC are known to release free radicals capable of blocking T lymphocyte activation or survival (27). In addition, MySC are known to produce several soluble mediators, including inhibitory cytokines, PGs, reactive oxygen intermediates, hydrogen peroxide, and NO, all of which can dampen immune responses. NO is a well-known soluble factor that inhibits T cell function (32). In myeloid cells, NO is produced by inducible NO synthase during the conversion of L-arginine to L-citruline. Inducible NO synthase mRNA and protein are induced by a number of cytokines, including IFN-γ and TNF-α in macrophages (27). We are currently investigating NO production in MySC, granulocytes, and macrophages in SHIP$^{-/-}$ lymphoid organs along with other potential immune-suppressive factors.

Biochemical pathways that are essential for MySC homeostasis have not been fully investigated. Therefore, we are currently delineating the signal transduction pathways that may be involved in the loss of MySC homeostasis in SHIP$^{-/-}$ mice. We speculate that PI3K/Akt signal transduction pathways that promote cell survival as well as pathways leading to NO production may be dysregulated in SHIP$^{-/-}$ myeloid and granulocytic cells. Another explanation that may account for the immune suppression and inhibition of Ag presentation we observed is that MySC may decrease T cell expression of CD3ζ (33). A decrease in CD3ζ can mediate significant suppression of Ag-specific T cell responses (33). One other possible explanation for the immune suppression of allogeneic T cell responses we observed in SHIP$^{-/-}$ WS MLR is that MySC may release cytokines that can specifically down-regulate cell surface expression of MHC class II. Therefore, the down-regulation of MHC class II on the surface of MySC or other cells will interfere with their ability to properly present Ag (34).

These findings indicate that blockade of SHIP activity or its

expression could preferentially antagonize deleterious allogeneic T cell responses, while still permitting epitope-specific responses to pathogens and residual tumor cells. Immunosuppressive strategies currently exist to control organ rejection and GvHD. However, in many cases these regimens put transplant patients at risk for life-threatening infections from opportunistic pathogens and tumor relapse (28, 35). Our results suggest that SHIP inhibition might selectively hamper deleterious allogeneic T cell responses while leaving Ag-specific T cell responses critical for post-transplant recovery and graft-vs-tumor effects intact. Consistent with this hypothesis, we have recently observed that MySC are profoundly increased after temporal ablation of SHIP expression in adult MXCre/SHIP^{flox} mice, and this increase is correlated with profound suppression of allogeneic T cell priming in vitro (T. Ghansah, K. Paraiso, and W. Kerr, unpublished observations). In addition, a SHIP blockade strategy might be applied to allogeneic organ transplantation, where the host-vs-graft response of T cells must be suppressed without severely impairing adaptive immunity.

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