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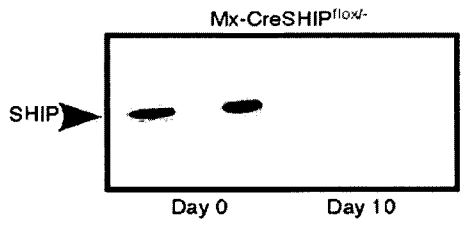
(54) Title: METHOD OF ABROGATING GRAFT VERSUS HOST DISEASE VIA INDUCED SHIP DEFICIENCY

FIG. 1a



(57) Abstract: Disclosed herein is method of treatment method wherein SHIP-deficiency is induced. The induction of SHIP deficiency leads to a rapid and significant expansion of myeloid suppressor cells (MySC) in peripheral lymphoid tissues. Consistent with expansion of MySC, splenocytes and LN cells from hosts with induced SHIP deficiency are significantly compromised in their ability to prime allogeneic T cell responses. These results demonstrate that SHIP regulates homeostatic signals for these immunoregulatory cells in adult physiology. Consistent with these findings, induction of SHIP deficiency prior to receiving a T cell replete BM graft abrogates acute GVHD. These findings indicate strategies that target SHIP could increase the efficacy and utility of allogeneic BMT and thereby provide a curative therapy for a wide spectrum of human diseases.

METHOD OF ABROGATING GRAFT VERSUS HOST DISEASE VIA INDUCED SHIP DEFICIENCY

CROSS REFERENCE TO RELATED APPLICATIONS

This application is a Non-Provisional Application of co-pending U.S. Provisional Application No. 60/890,565, filed February 19, 2007, and co-pending U.S. Provisional Application No. 60/890,568, filed February 19, 2007, which are incorporated herein by reference.

GOVERNMENT SUPPORT

This invention was made with Government support under Grant No. R01 HL72523 awarded by the National Institute for Health and the National Heart, Lung and Blood Institute. The Government may therefore have certain rights in the invention.

BACKGROUND OF THE INVENTION

Graft versus Host Disease (GvHD) is the leading cause of treatment related mortality in allogeneic bone marrow transplantation (BMT) protocols. Professional APC have been shown to play a critical role in priming allogeneic T cell responses that cause GvHD(1) and solid organ rejection.(2) A number of immunoregulatory cell types have been reported to reduce GvHD including natural suppressor cells,(3, 4) Treg cells,(5, 6) and $Gr1^+$ myeloid cells (MySC).(7, 8) Strategies to increase the numbers and/or activity of these regulatory cell types are viewed as a means to control GVHD in allogeneic BMT procedures, reduce autoimmune disease and prevent rejection of solid organ grafts.

The SH2-containing Inositol Phosphatase-1 (SHIP) is a 145 kDa protein that possesses 5' phosphatase activity and thus can hydrolyze the 5' phosphate on $PI(3,4,5,)P_3$ and 1,3,4,5-inositol-tetrakisphosphate (IP_4) that are products of PI-3-Kinase activity (9, 10). SHIP is primarily expressed in hematopoietic cells,(9, 10) but it can also be expressed in mouse embryonic fibroblasts.(11) The SHIP locus also encodes a stem cell specific isoform called s-SHIP that lacks the SH2 domain and is expressed by pluripotent stem cells and tissue-specific stem cells.(11, 12) SHIP's role in signal transduction allows it to regulate cell survival, proliferation, apoptosis and homeostasis of certain hematopoietic cell types,(7, 13-16) as well as primitive stem cell populations.(11, 12, 17, 18) Analysis of SHIP-deficient mice revealed that this protein also has a prominent role in the immune system.(7, 14, 15, 19) Significant pathologies have been observed in $SHIP^{-/-}$ mice including splenomegaly and an infiltration of myeloid cells into the lungs that contributes to their reduced life span. (13, 16, 20)

The inventors previously reported that the SH2-containing Inositol Phosphatase (SHIP) is critical for maintenance of NK receptor repertoire (NKRR) diversity.(15, 21) Disruptions of the NKRR caused by germline SHIP deficiency enhance engraftment of BM from donors with complete MHC mismatches.(15, 21) HSC in $SHIP^{-/-}$ mice exhibit spontaneous mobilization and reduced BM retention which may reduce the competitive barrier to engraftment by donor HSC in SHIP-deficient BMT recipients.(17) Furthermore, GVHD is reduced in SHIP-/- BMT recipients(15) due to a profound expansion of immunoregulatory myeloid suppressor cells (MySC) in secondary lymphoid tissues.(7)

SUMMARY OF INVENTION

In a first embodiment, the invention includes a method of enhancing transplant engraftment, abrogating acute GvHD, via induction of SHIP deficiency for a short period prior to allogeneic bone marrow transplant (BMT). As shown herein, the invention abrogates GvHD even in settings where donor and host have a complete MHC mismatch.

In another embodiment, the invention includes a treatment method wherein SHIP-deficiency is induced. The induction of SHIP deficiency leads to a rapid and significant expansion of myeloid suppressor cells (MySC) in peripheral lymphoid tissues. Consistent with expansion of MySC, splenocytes and LN cells from hosts with induced SHIP deficiency are significantly compromised in their ability to prime allogeneic T cell responses. These results demonstrate that SHIP regulates homeostatic signals for these immunoregulatory cells in adult physiology. Consistent with these findings, induction of SHIP deficiency prior to receiving a T cell replete BM graft abrogates acute GVHD. These findings indicate strategies that target SHIP could increase the efficacy and utility of allogeneic BMT and thereby provide a curative therapy for a wide spectrum of human diseases.

BRIEF DESCRIPTION OF THE DRAWINGS

For a fuller understanding of the invention, reference should be made to the following detailed description, taken in connection with the accompanying drawings, in which:

FIG. 1: Inducible ablation of SHIP expression in adult $MxCreSHIP^{flox}$ mice. (*a*) Western blot analysis of SHIP in whole cell lysates prepared from PBMCs of $MxCreSHIP^{flox/-}$ mice bled prior to polyI/C injection (Day 0), and Day 10 post polyI/C injection. (*b*) To confirm deletion of the SHIP WT allele, genomic DNA was prepared from PBMC of a polyI/C-treated $MxCreSHIP^{flox}$ mouse analyzed in (*a*) and was analyzed by a multiplex PCR assay that detects both WT or null SHIP alleles. Genomic DNA from $SHIP^{-/-}$, $SHIP^{+/-}$ and $SHIP^{+/+}$ mice was analyzed in parallel as positive and negative controls. (*a*) Western blot analysis of SHIP expression in

PBMC prepared from MxCreSHIP$^{flox/-}$ mice (#12-14) or a SHIP$^{flox/-}$ mouse that lacks the MxCre transgene (#5) following polyI/C-treatment (day 21). (*d*) Western blot analysis of SHIP expression in PBMC prepared from MxCreSHIP$^{flox/flox}$ mice (#2-4) or a SHIP$^{flox/flox}$ mice that lacks the MxCre transgene (#5-8) following polyI/C-treatment (day 21).

FIG. 2: A normal percentage of DC are found in secondary lymphoid tissues of SHIP-deficient MxCreSHIP$^{flox/-}$ mice. (*a, c)* Flow cytometry analysis of spleens and mesenteric LN from both poly-IC treated (MxCreSHIP$^{flox/-}$) and control (SHIP$^{flox/-}$) mice showed no significant difference in the percentage of DC. DC was comprised of cells stained B7.2^{+} and CD11c^{+} and lineage negative panel of antibodies (Lin^{-}) that consisted of NK1.1, CD3 and B220 to exclude NK cells, T cells and B cells. (*b, d)* The mean percentages of DC (B7.2^{+}CD11c^{+} Lin^{-}) were calculated for spleen (n=3) and LN (n=3) from MxCreSHIP$^{flox/-}$ and SHIP$^{flox/-}$.

FIG. 3: MySC expansion in SHIP deleted MxCreSHIP$^{flox/-}$ mice. (*a, c*) Cells from spleens and mesenteric LN of polyI/C treated MxCreSHIP$^{flox/-}$ mice and control SHIP$^{flox/-}$ mice were stained with Mac-1 (CD11b) and Gr-1 (Ly-6C/G). Cells were acquired by flow cytometry in order to evaluate the Mac-1^{+}, Gr-1^{+}, MySC population. (*b, d*) The absolute cell numbers of MySC (Mac-1^{+}Gr-1^{+}) were calculated for spleens (n=3) and LN (n=3) from poly(I/C)-treated MxCreSHIP$^{flox/-}$ and SHIP$^{flox/-}$ mice. (*e)* Splenocytes and LN cells from a MxCreSHIP$^{flox/-}$ mouse with partial SHIP ablation stained with Mac-1 and Gr-1 antibodies. *, $p < 0.05$; **, $p < 0.01$; (by two-tailed Student's t test).

FIG. 4: Induced SHIP deficiency compromises priming of allogeneic T cell responses by cells from secondary lymphoid tissues. (a) MySC purified from SHIP-deficient and WT spleens suppress a one-way MLR. 3H-Thymidine uptake by H2d BALB/C responders in the absence of irradiated stimulators (No Stimulators), in the presence of: WT (BL6) whole splenocytes (+/+ WS), SHIP-/- (BL6) whole splenocytes (-/- WS), +/+ WS (BL6) plus +/+ MySC (+/+ WS & +/+ MySC), +/+ WS (BL6) plus -/- MySC (+/+ WS & -/- MySC); [*$p < 0.05$; **$p< 0.01$ by a two-tailed Student's t-test]. This experiment is representative of two independent MLRs with purified SHIP-deficient MySC. (*b, c)* Splenocytes (*b*) and mesenteric LN (*c*) were prepared from mice that showed full deletion, partial deletion or no deletion of SHIP expression and were used as stimulators for allogeneic (Balb/c) responders in a one-way MLR assay. The bar graphs indicate the total ^{3}H-T uptake. ^{3}H-T was added to the MLR at 96 hr and the cells harvested for measurment of ^{3}H-T uptake 18 hr later. These experiments are representative of three independent MLRs with SHIP deficient mice and control mice. **, $p < 0.01$; ***, $p< 0.001$ (by two-tailed Student's t test).

FIG. 5: Induction of SHIP-deficiency in the host enhances survival in fully MHC mismatched BMT. (*a-c*) Western blot analysis of SHIP expression in PBMC of representative poly(I/C)-

treated SHIP$^{flox/flox}$ (*a*) and MxCreSHIP$^{flox/flox}$ (*b, c*) mice prior to BMT. The mouse in (*b*) is representative of the majority of the MxCreSHIP$^{flox/flox}$ cohort that had partial deficiency prior to transplant while (*c*) indicates one of the two animals in the cohort that had no detectable SHIP expression prior to BMT. All SHIP$^{flox/flox}$ and MxCreSHIP$^{flox/flox}$ mice were injected with 625□g polyI/C on days 1 and 4. On day 7 mice were bled and PBMC probed for SHIP and □-actin. On day 8 mice received BMT as described in the Methods. (*d*) Kaplan-Meier step-function of survival in the MxCreSHIP$^{flox/flox}$ (SHIP-deficient) (n=15) and SHIP$^{flox/flox}$ (SHIP-competent) (n=14) cohorts following allogeneic BMT. In parallel we performed syngeneic BMT on a cohort of C57BL6/J recipients as described in the Methods. (p=0.001, SHIP$^{flox/flox}$ allogeneic BMT vs. autologous BMT cohort; p=0.040, MxCreSHIP$^{flox/flox}$ allogeneic BMT vs. SHIP$^{flox/flox}$ allogeneic BMT cohort; p=0.232, MxCreSHIP$^{flox/flox}$ allogeneic BMT vs. autologous BMT cohort). (*e*) Analysis of weight in the SHIP-deficient and SHIP-competent cohorts post-transplant. (MxCreSHIP$^{flox/flox}$ vs. SHIP$^{flox/flox}$ cohort, p<0.05) (*f*) Average total GvHD score for the MxCreSHIP$^{flox/flox}$ and SHIP$^{flox/flox}$ BMT BMT cohorts per the rating system of Cooke *et al.*(25)

FIG. 6: Histopathological evidence of GVHD in mice that succumbed post-transplant. Formalin fixed tissue sections from all mice that succumbed in the BMT study described above were analyzed for evidence of GVHD in key target organs (skin, liver and small intestine) in a blinded fashion by a veterinary pathologist (RWE). All mice that succumbed showed histopathological evidence of GVHD. Examples of observed histopathology referrable to GVHD in the skin (*a*), liver (*c*) and small intestine (*e*) are shown, and compared to the skin (*b*), liver (*d*) and small intestine (*f*) of healthy C57BL6/J mice. (*a*) Skin of mice with GVHD showed pyknosis and vacuolation of epidermal cells in the basal layer and a mild lymphocytic infiltrate in the dermis, compared to unaffected skin of a C57BL6/J control (*b*). Liver of mice with GVHD (*c*) showed bile duct destruction and regeneration and a moderate, primarily lymphocytic infiltrate in portal areas with attendant destruction of hepatic parenchyma (between arrowheads & within inset), compared to the unaffected liver and portal triads (arrowhead, inset) of a C57BL6/J control (*d*). Small intestine of mice with GVHD (*e*) showed glandular destruction, moderate lymphocytic infiltrate, and focal loss of mucosa, compared to the unaffected intestine of a C57BL6/J control (*f*) (hematoxylin & eosin 200X, insets 400X).

FIG. 7: Donor hematopoietic engraftment and enhanced B cell repopulation in SHIP-deficient allogeneic BMT recipients. (*a*) Global donor hematopoietic repopulation in SHIP-deficient (MxCre^{+}) and SHIP-competent (MxCre^{-}) transplant recipients. (*b*) B lymphoid repopulation in the same mice as in (*a*). Donor repopulation was assessed at two months post-transplant by flow cytometric analysis of PBMC as previously described.(15) Donor and host repopulation

was assessed with H2d and H2b specific antibodies and appropriate lineage markers (B220, B cells; CD3, T cells, Mac1+Gr1, myeloid). The indicated p-values are for comparison of repopulation in the SHIP-deficient and SHIP-competent allogeneic BMT cohorts.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

To demonstrate the effectiveness of the invention, the inventors developed a genetic model in which SHIP deficiency can be induced in adult recipients using an inducible Cre transgene (23) on a $SHIP^{flox}$ background. This model was used to assess whether induction of SHIP deficiency can trigger an expansion of MySC in adult lymphoid tissues capable of repressing allogeneic T cell responses.

Here, the inventors disclose that deletion of SHIP leads to a significant increase in the MySC compartment in both spleen and lymph nodes of adult $MxCreSHIP^{flox}$ mice. In addition, splenocytes and LN cells from adult mice rendered SHIP-deficient lack the ability to prime allogeneic T cell responses in vitro.(24) Consistent with these in vitro findings, mice with induced SHIP-deficiency are protected from acute GVHD following a T cell replete allogeneic BMT. The results described herein demonstrate that SHIP is essential for MySC homeostasis during normal adult physiology and that targeting SHIP expression, or its activity in adult transplant recipients, can be used to modulate the activities of these myeloid regulatory cells for therapeutic purposes.

These findings further show that SHIP plays a critical role in the control of MySC homeostasis and function. Analysis of induced SHIP-deficiency in the $MxCreSHIP^{flox}$ model demonstrates SHIP controls MySC numbers during normal adult physiology. Consistent with this role, the following shows increased MySC function in secondary lymphoid tissues and reduced GVHD following induction of SHIP deficiency in adult mice. Suprisingly, induced SHIP-deficiency also enhances B lymphoid reconstitution in allogeneic BMT recipients.

The rapid expansion of the MySC compartment in secondary lymphoid tissues observed following induction of SHIP-deficiency indicates SHIP is regulating the numbers of these cells in response to normal homeostatic factors that promote their growth, survival and/or trafficking to secondary lymphoid tissues. Several growth factors are potentially involved in peripheral MySC homeostasis, including Flt3L, G-CSF and IL-4.(8, 27) Consistent with the findings of the inventors, SHIP is recruited to receptors for all of these ligands(28-31) and thus may oppose PI3K effector pathways triggered in response to these ligands that promote MySC survival and/or proliferation in secondary lymphoid tissues. Because SHIP can also limit chemotaxis in response to chemokines,(32) it is also possible that SHIP regulates

trafficking of MySC precursors into secondary lymphoid tissues. A distinct possibility is that SHIP regulates multiple signaling pathways in MySC.

In addition to regulating MySC numbers, SHIP-deficiency may also regulate their effector functions. This is suggested by previous findings that amplification of SHIP-deficient MySC did not compromise the ability of APC in spleen and LN to prime naïve, antigen-specific CD4 or CD8 T cell responses.(7) Others have shown that $Mac1^{+}Gr1^{+}$ MySC present in spleens of tumor bearing mice antagonize APC priming of antigen-specific CD8 T cell responses.(33) These disparate findings suggest the possibility that SHIP-deficient MySC are qualitatively different from their WT counterparts. Consistent with this possibility, the inventors show herein that purified SHIP-deficient MySC are more suppressive of allogeneic T cell responses than their WT counterparts on a per cell basis. The molecular mechanisms that underlie the functional differences between SHIP-deficient and SHIP-competent MySC could have important implications for tumor immunotherapy and allogeneic BMT protocols to treat malignancy where graft-vs.-tumor (GVT) responses by T cells play an important therapeutic role. Alternatively, the SHIP-deficient $Mac1^{+}Gr1^{+}$ cells decribed here and previously(7) could represent a MySC subset with distinct functional properties preferentially amplified by SHIP-deficiency in secondary lymphoid tissues.

An important, but unanticipated, finding of this study is the small but significant increase in donor B-lymphoid repopulation observed in SHIP-deficient allogeneic BMT recipients. As shown previously, humoral immune responses to T-dependent antigens are severely compromised following allogeneic BMT(34). Consistent with this seminal study in a murine allogeneic BMT model, recovery of B cell function and antibody responses to pathogens is frequently delayed or permanently compromised in clinical allogeneic BMT patients(35-38) and contributes to post-transplant morbidity.(39) The enhanced donor B cell repopulation observed in SHIP-deficient allogeneic BMT recipients may also contribute to the improved survival observed in these recipients. The mechanism responsible for enhanced donor B cell repopulation in SHIP-deficient hosts remains to be defined. However, residual host B lymphopoiesis was recently shown to limit donor B cell repopulation in an allogeneic murine BMT model(40) and that B lymphopoiesis is partially compromised by SHIP-deficiency.(22)

These findings demonstrate the usefulness of reversible SHIP inhibition strategies to abrogate GvHD in allogeneic BMT and potentially also host T cell responses that mediate graft rejection in solid organ transplantation. Although therapeutic agents currently exist to control such deleterious T cell activities, these have the drawback of being broadly inhibitory for T cell function and thus place the transplant recipient at risk for opportunistic infections, tumor relapse and secondary malignancies.(41-43) The inventors work, as well as that of

others, indicate SHIP-deficiency does not significantly impair antigen-specific T cell priming(7) or humoral responses to complex antigens.(14, 44) Thus, pursuit of reversible SHIP inhibition strategies could potentially provide a more selective form of immune suppression better suited for allogeneic transplant regimens. Although full and prolonged SHIP-deficiency clearly has deleterious consequences,(22) these findings show transplant success can be achieved with induced SHIP-deficiency even when a state of partial SHIP deficiency exists.

Most patients who could potentially benefit from an allogeneic BMT procedure are unable to find a donor with an appropriate HLA match. They must then forego a potentially efficacious therapy due to the heightened risk of GvHD from an unmatched donor. However, in murine allogeneic BMT recipients with induced SHIP-deficiency, they are protected from lethal acute GvHD despite a complete MHC mismatch with the donor. The findings of the inventors show the efficact of clinical BMT procedures involving a significant degree of HLA incompatibility. The application of induced SHIP-deficiency in this manner would profoundly increase the utility of this curative therapy in a wide spectrum of human maladies, including genetic, autoimmune and malignant disease.

Genetic model for the study of the impact of induced SHIP deficiency

To assess the impact that induction of SHIP-deficiency has on normal adult physiology, the inventors established the MxCreSHIP^{flox} model where either both SHIP alleles are floxed (MxCreSHIP$^{flox/flox}$ mice) or where one allele is floxed with the other having the germline SHIP mutation (MxCreSHIP$^{flox/-}$ mice). The inventors initially tested the feasibility of inducing SHIP deficiency in this model by treating two MxCreSHIP$^{flox/-}$ mice with poly(I/C) that activates transcription of the MxCre transgene. Western blot analysis of PBMC obtained immediately prior to poly(I/C) treatment or ten days later showed that ablation of SHIP expression was achieved in these MxCreSHIP$^{flox/-}$ mice with SHIP expression being undetectable by Western blot (Fig. 1*a*). These results indicate induction of SHIP expression is robust in the hematopoietic compartment of MxCreSHIP$^{flox/-}$ following poly(I/C) administration. The inventors confirmed deletion of the SHIP WT allele via PCR analysis of genomic DNA (Fig. 1*b*).

For the purpose of this invention, the inventors consider poly(I/C)-treated MxCreSHIP^{flox} mice to have "full" SHIP deficiency if no detectable SHIP allele or protein is present (Fig. 1). However, in some instances, poly(I/C)-treated MxCreSHIP^{flox} mice showed a decrease in SHIP protein expression by Western blot, but retain detectable expression of SHIP expression in PBMC (Fig. 1*c*). The inventors consider these latter mice to have "partial" SHIP deficiency. To date the analysis of induced SHIP deficiency in 25 MxCreSHIP^{flox} mice following three poly(I/C) injections showed that 17 mice exhibited full SHIP deficiency post-

treatment, while 7 exhibited partial SHIP deficiency with one mouse showing no significant SHIP deficiency as determined by Western blot analysis of PBMC. There appears to be no correlation with the presence of one floxed allele or two in achieving full SHIP deficiency in this model and therefore in the inventor's experience either MxCreSHIP$^{flox/flox}$ (Fig. 1*d*) or MxCreSHIP$^{flox/-}$ can be used interchangeably to induce SHIP deficiency in the adult. With the exception of one mice who died 10 days after initiation of poly(I/C) treatment, all poly(I/C) treated MxCreSHIP^{flox} mice survived to at least 21 days following the initiation of SHIP deficiency at which point they were euthanatized for analysis of their hematolymphoid compartment. The analysis of both full and partial SHIP deficiency is germane to the potential development of a clinically applicable SHIP inhibition strategy, and thus the inventors further analyzed MySC homeostasis and function in mice representative of both groups.

Dendritic cells are present in normal numbers, but MySC are increased in peripheral lymphoid tissues of adult mice with induced SHIP deficiency

The immune attack by donor T cells that is responsible for GvHD is triggered by host dendritic cells (DC) in peripheral lymphoid tissues that survive myeloablation.(1) In the inventor's previous study, the inventors showed that DC are present in normal numbers in the peripheral lymphoid tissues of SHIP$^{-/-}$ mice and that DC sorted from these tissues were capable of priming allogeneic T cells as efficiently as wild type APC.(7) In the MxCreSHIP^{flox} model the inventors show that DC are also present in normal numbers in the spleen (Fig. 2*a*) and LN (Fig. 2*c*) following induction of SHIP deficiency. Further statistical analysis of DC numbers in the spleen and LN of MxCreSHIP$^{flox/-}$ and control mice showed no significant difference in their representation in these tissues (Fig. 2*b* and Fig. 2*d*), respectively.

The inventors then assessed whether ablation of SHIP expression leads to a significant expansion of the MySC compartment in peripheral lymphoid tissues by analyzing the frequency of MySC in both the spleen and mesenteric LN by flow cytometry. The inventors found a significant expansion of the MySC compartment in both of these peripheral lymphoid tissues (Fig. 3*a* and Fig. 3*c*). The expansion of MySC in MxCreSHIP$^{flox/-}$ mice is approximately 5 to 10 fold in the spleen (Fig. 3*b*) and approximately 10 to 20 fold in LN (Fig. 3*d*) when compared to age-matched SHIP$^{flox/-}$ mice also treated with polyI/C. An MxCreSHIP$^{flox/-}$ mouse that was observed to have only a partial reduction in SHIP expression following poly(I/C) administration (#12 in Fig. 1*c*) also exhibited a significant expansion of the MySC compartment in both spleen and LN (Fig. 3*e*). Thus, induction of SHIP-deficiency for a short period can expand the MySC compartment and abrogate priming of allogeneic T cell responses and this can occur even when the hematopoietic compartment is partially SHIP-deficient.

Induction of SHIP deficiency in adult mice compromises priming of allogeneic T cells by peripheral lymphoid tissues

In the inventor's previous studies of germline SHIP deficiency, the inventors found that priming of allogeneic T cell responses, as measured by both proliferation and IL-2 production, was compromised in $SHIP^{-/-}$ spleens and LN.(7) The inventors proposed then that allogeneic T cell responses were compromised by the expanded numbers of MySC in these tissues.(7) To establish definitively that Mac1+Gr1+ MySC are the cell type that mediates suppression of allogeneic T cell responses in these tissues the inventors sorted Mac1+Gr1+ MySC from SHIP-deficient and WT spleens and tested their ability to suppress a one-way MLR (Fig. 4a). Addition of either SHIP-deficient or WT Mac1+Gr1+ MySC significantly suppressed the MLR (Fig. 4a). However, addition of an equal number of Mac1+Gr1- or Mac1-Gr1+ cells did not mediate significant suppression in the MLR ($p>0.05$). Interestingly, SHIP-/- MySC have greater suppressive ability on a per cell basis than WT MySC ($p<0.01$) (Fig. 4a), indicating SHIP-deficiency may increase both their representation in secondary lymphoid tissues and their suppressive capacity. Because MySC are also expanded in spleen and LN of adult mice following induction of SHIP deficiency, the inventors then examined whether allogeneic T cell priming by cells from these tissues was also compromised. Indeed, whole splenocytes and LN cells from mice with induced SHIP ablation were found to prime allogeneic T cell responses very poorly relative to cell preparations from similarly treated $SHIP^{flox/-}$ mice (Fig. 4*b* and Fig. 4*c*). The $MxCreSHIP^{flox/-}$ mice in Fig 3*a* and Fig. 3*c* showed expansion of MySC due to full deletion of SHIP expression while the $MxCreSHIP^{flox/-}$ mouse from Fig. 3*e* showed partial ablation of SHIP expression following Cre recombinase induction. Intriguingly, partially ablated mice still exhibit a significant expansion of their MySC compartment in the spleen and LN of these mice (Fig. *3e*) and significant repression of allogeneic T cell priming (Fig. 4*b* and *c*). Therefore, induction of SHIP-deficiency for relatively short periods can abrogate priming of allogeneic T cell responses in secondary lymphoid tissues even in instances when SHIP expression is not completely ablated.

Induction of SHIP deficiency in adult mice abrogates GvHD following transplant with a T cell replete, MHC mismatched BM graft

To test whether induced SHIP-deficiency could compromise allogeneic T cell responses in vivo the inventors established cohorts of $MxCreSHIP^{flox/flox}$ and $SHIP^{flox/flox}$ mice for GvHD analysis. Employing a poly(I/C) adminstration regimen utilized by Mikkola et al in $SCL^{flox/flox}$ mice(26) the inventors typically find that three consecutive injections of poly(I/C) are sufficient to render most adult $MxCreSHIP^{flox/flox}$ mice fully SHIP deficient (Fig. 1). However, two poly(I/C) injections typically triggers only partial SHIP deficiency in the overwhelming majority

of MxCreSHIP$^{flox/flox}$ mice. While this method was employed by the inventors for purposes of establishing the efficacy of the invention, the induction of SHIP deficiency could be achieved in BMT patients by other means, as will be appreciated by the skilled artisan, including systemic treatment with interfering RNAs (RNAi), small-hairpin RNAs or anti-sense oligonucleotides that target SHIP expression. Alternatively, SHIP enzymatic activity or signaling functions could be targeted or blocked by small molecules or low molecular weight compounds.

Since full SHIP deficiency might pose a significant threat to viability, the inventors chose to pursue this latter strategy to induce SHIP-deficiency. As expected most MxCreSHIP$^{flox/flox}$ mice had detectable, but reduced SHIP expression in their peripheral blood mononuclear cells (PBMC) 6 days after the initial poly(I/C) injection while two mice had no detectable SHIP expression (Fig. 5a-c). The former were considered to be partially SHIP-deficient, while the latter were considered fully SHIP-deficient. Seven days after the induction of SHIP deficiency, the inventors initiated a fully mismatched BMT procedure in both the poly(I/C)-treated MxCreSHIP$^{flox/flox}$ cohort and the identically treated SHIP$^{flox/flox}$ cohort. For BMT the mice were myeloablated by irradiation from a ^{137}Cs source (950cGy) and received 15x10^6 whole BM cells and 15x10^6 splenocytes from BALB/C (H2d) donors. The MxCreSHIP$^{flox/flox}$ and SHIP$^{flox/flox}$ transplant recipients are on a C57BL6/J (H2b) background and thus are completely mismatched to the donor at all major MHC loci. In parallel the inventors performed syngeneic BMT on a cohort of C57BL6/J (H2b) mice. Survival was monitored in all three BMT cohorts for 16 weeks post-transplant when acute GVHD is manifest. Survival in the syngeneic BMT cohort was 100%, 94% in the SHIP-deficient cohort (MxCreSHIP$^{flox/flox}$ + polyI/C) and 57% in the SHIP-competent cohort (SHIP$^{flox/flox}$ + polyI/C) (Fig. 5d). Comparison of survival in the SHIP-deficient and SHIP-competent allogeneic BMT cohorts by the Kaplan-Meier log-rank test indicated there was significant protection from acute and lethal GVHD when SHIP-deficiency was induced prior to transplant (p=0.040). As expected survival of the SHIP-competent cohort receiving allogeneic BMT was significantly reduced relative to the syngeneic BMT cohort (p=0.001). However, the Kaplan-Meier log-rank test indicated comparable survival in the SHIP-deficient allogeneic and syngeneic BMT cohorts (p=0.232) (Fig. 5d). These findings are consistent with the inventor's previous allogeneic BMT studies in germline SHIP$^{-/-}$ mice,(7, 15) but importantly they show that induction of SHIP deficiency in the adult just prior to a fully-mismatched, T cell replete transplant can provide protection from acute GvHD without significant toxicity.

In addition to survival, the inventors also monitored weight and clinical measures of GvHD post-transplant in the SHIP-deficient and SHIP-competent cohorts. Prolonged SHIP-deficiency causes wasting in germline SHIP-deficient mice due to a macrophage-mediated

consolidation of the lungs.(16, 22) However, induction of SHIP-deficiency for one week did not cause a significant drop in the weight of the SHIP-deficient cohort relative to the identically treated SHIP-competent cohort ($p=0.39$) (Fig. 5e). Despite starting at a comparable weight immediately prior to BMT, the weight of the SHIP-deficient cohort rebounded and increased significantly relative to that of the SHIP-competent cohort during the acute recovery phase of transplant ($p<0.05$) (Fig. 5e). Based on a scoring system that assesses different features of GVHD, including weight loss, skin integrity, fur texture, posture and activity(25), the SHIP-deficient cohort exhibited fewer or reduced manifestations of GVHD at 3 and 4 weeks post-transplant when acute GVHD is typically at its peak (Fig. 5f). Histopathological analysis of GVHD in key target organs (skin, liver and the gastrointestinal tract) confirmed the presence of GVHD in all mice that succumbed post-transplant (Fig. 6).

To confirm that mice were engrafted with donor BM, the inventors analyzed multi-lineage repopulation 8 weeks post transplant in the surviving mice. Flow cytometric analysis of peripheral blood mononuclear cells (PBMC) was used to determine donor BM contribution to the T, B and myeloid lineages (Fig. 7). This analysis showed that all mice in the SHIP-deficient and SHIP-competent allogeneic BMT cohorts had significant donor repopulation in all three lineages with no significant difference observed between the two cohorts in either T cell or myeloid repopulation (data not shown) or global hematopoietic repopulation ($CD45^+$ cells) (Fig. 7a). However, donor B lymphoid repopulation was significantly higher in the SHIP-deficient allogeneic BMT cohort relative to the SHIP-competent allogeneic BMT cohort (Fig. 7b).

Examples

Mice

Mice with germline transmission of a $SHIP^{flox}$ allele were previously created in our lab(15) and were maintained by intercrossing $SHIP^{flox/flox}$ mice (F10 to the C57BL6/J background). MxCre transgenic mice were purchased from Jackson Laboratories (Bar Harbor, ME). $SHIP^{flox/-}$ and MxCre/$SHIP^{flox/+}$ mice were mated to obtain progeny that are MxCre/$SHIP^{flox-}$ and $SHIP^{flox/-}$ on an C57BL6 background. MxCreSHIPflox/flox and SHIPflox/flox littermates were generated for the BMT study by intercrossing MxCreSHIPflox/+ and SHIPflox/flox mice. All studies were performed in accordance with the guidelines and approval of the Institutional Animal Certification and Use Committee (IACUC) at the University of South Florida.

Conditional Deletion of SHIP

MxCre/SHIP^{flox} mice were conditionally deleted for SHIP through the intraperitoneal injection of polyinosinic-polycytidylic acid (polyI/C) (Sigma). SHIP$^{flox/-}$ or SHIP$^{flox/flox}$ controls were treated in a similar fashion. Mice were injected 3 times with 625□g of polyI/C on days 1, 4, and 7. For GvHD studies mice were injected 2 times with 625□g of polyI/C on days 1 and 4 prior to BMT on day 8. The administration of polyI/C causes the in vivo production of interferon alpha and interferon beta, which activates the Mx1 promoter and Cre recombinase expression.(24) Cre recombinase specifically recognizes the loxP sites flanking the promoter and first coding exon of SHIP.(15)

PCR Confirmation of SHIP Deletion

For DNA analysis mice were bled post polyI/C injection and genomic DNA was isolated from PBMC using Qiagen's Dneasy Kit per the manufacturer's instructions. The multiplex PCR for SHIP was conducted as previously described.(15) The primers for the identification of deleted SHIP alleles are as follows: oIMR1084, 5'GCG GTC TGG CAG TAA AAA CTA TC3'; oIMR1085, 5'GTG AAA CAG CAT TGC TGT CAC TT3'; oIMR0042, 5'CTA GGC CAC AGA ATT GAA AGA TCT'3'; OIMR0043, 5'GTA GGT GGA AAT TCT AGC ATC ATC C3'. PCR reactions were performed using 3μl of DNA per reaction. The cycling conditions were step 1: 94°C for 3 minutes for denaturing. Step 2: 94°C for 30 seconds, 64°C for 30 seconds with –0.5°C per cycle, 72°C for 35 seconds. Step 2 was repeated 12 times. Step 3: 94°C for 30 seconds, 58°C for 30 seconds, 72°C for 35 seconds. Step 3 was repeated 25 times. Step 4: 72°C for 2 minutes. The holding temperature was 4°C. The samples were run on a 1% agarose gel in 0.5X TAE buffer at 100V for 25 minutes.

Western Blot Confirmation of SHIP Deletion

All mice were bled for Western blot analysis of SHIP expression. Red blood cells were first lysed to obtain PBMC (eBioscience, RBC lysis buffer) and then a lysate of PBMC was prepared with modified RIPA buffer (Upstate Cell Signaling). For immunoprecipitation of SHIP, the lysates were incubated with the mouse monoclonal SHIP antibody; P1C1 (Santa Cruz) followed by incubation with Protein A beads. The beads were centrifuged 10,000 RPM for 10 seconds and supernatants were loaded onto a 3-8% Tris-acetate gel. The gel was then transferred to Hybond-ECL nitrocellulose membrane (Amersham Biosciences, Piscataway, NJ). The membrane was blocked with 5% non-fat milk, 1xPBS, 0.1% Tween 20 and probed with 1mg/ml P1C1 (primary antibody) and anti-mouse IgG-HRP 1:80,000 (secondary antibody). SHIP protein was detected using Pierce's Super Signal West Femto chemiluminescent detection reagents. For analysis of SHIP expression on whole cell lysates 15□g of protein was loaded per lane.

Flow cytometry analysis

In all experiments, splenocytes and mesenteric LN cells from $SHIP^{flox/-}$ and $MxCreSHIP^{flox}$ mice were digested with Collagenase D (Roche Molecular Biochemicals, Indianapolis, IN) for 90 minutes at 37°C in order to increase yield. Collagenase D processed and RBC lysed splenocytes and collagenase D processed mesenteric LN cells were analyzed by flow cytometry with anti-NK1.1, anti-B220, anti-CD3, anti-CD11c and anti-B7.2 for identification of DC. In addition, MySC were identified using the anti-CD11b (Mac-1) and anti-Gr-1 (Ly6-G). All antibodies were purchased from BD Pharmingen (San Diego, CA).

Mixed leukocyte reaction (MLR)

Following red blood cell (RBC) lysis, $SHIP^{flox}$ and $MxCreSHIP^{flox}$ splenocytes or LN cells (stimulators) ($8x10^5$/well) were irradiated (2000 Rads) and co-cultured with Balb/c splenocytes or LN cells, (responders) ($4x10^5$/well) in a "one way" MLR assay. All cells were plated in triplicate in 96 well U-bottom plates (Costar, Cambridge, MA) containing RPMI complete medium for 4 days. Cells were pulsed with 1.0□Ci ^{3}H-Thymidine/well for 18 hours (post 96 hours of MLR assay setup). Cells were lysed and high MW weight DNA captured on glass fiber filtermates using an automated cell harvester (Packard, Meriden, CT). Incorporated ^{3}H-Thymidine was quantitated using a Packard TopCount NXT (Packard, Meriden, CT). Specific ^{3}H-Thymidine incorporation into genomic DNA was calculated as counts per minute (cpm) as the average of the mean (+ or – SEM) of triplicate wells. To analyze the suppressive potential of purified myeloid subsets using the "one-way" MLR assay described above, 3X104 sorted cells (Mac1+Gr1+, Mac1+Gr1- or Gr1+Mac1- cells) were added to each MLR well containing 4x105 stimulators (irradiated WT BL6 splenocytes) and 2x105 responders (Balb/c splenocytes).

BMT and GvHD analysis

All $SHIP^{flox/flox}$ and $MxCreSHIP^{flox/flox}$ mice were injected with 625□g polyI/C (i.p.) on days 1 and 4. On day 8 the mice received 950 Rads from a ^{137}Cs source as a single dose and the mice were then transplanted with $15x10^6$ BM cells and $15x10^6$ splenocytes from BALB/C (H2d) donors by retro-orbital injection. All recipients were on a C57BL6/J (H2b) background. Mice were maintained on autoclaved bedding, water and chow in microisolator cages for the duration of the study. The statistical significance of survival differences was assessed by the Kaplan-Meier log-rank test with $p<0.05$ considered significant. In parallel we also performed a syngeneic transplant on a cohort of C57BL6/J recipients using $15x10^6$ WBM cells and $15x10^6$ splenocytes from C57BL6/J donors. The clinical manifestations of GVHD were rated on a scale of 0-2 (0, no evidence of disease; 2, clear evidence of disease) and included

assessments of weight loss, posture, skin integrity, fur texture and activity as described by Cooke *et al.*(25) Three investigators (K.P., A.C. and WK) evaluated each mouse at each time point independently of each other and their scores were averaged.

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It will be seen that the advantages set forth above, and those made apparent from the foregoing description, are efficiently attained and since certain changes may be made in the above construction without departing from the scope of the invention, it is intended that all matters contained in the foregoing description or shown in the accompanying drawings shall be interpreted as illustrative and not in a limiting sense.

It is also to be understood that the following claims are intended to cover all of the generic and specific features of the invention herein described, and all statements of the scope of the invention which, as a matter of language, might be said to fall therebetween. Now that the invention has been described,

What is claimed is:

1. A method of abrogating graft versus host disease in a transplant recipient, comprising the step of suppressing expression of the SH2-containing Inositol Phosphotase-1in the recipient prior to the transplant.

2. The method of claim 1, comprising the step of fully suppressing expression of SH2-containing Inositol Phosphotase-1, whereby no SH2-containing Inositol Phosphotase-1allele or protein is detectable.

3. The method of claim 1, comprising the step of partially suppressing expression of SH2-containing Inositol Phosphotase-1, whereby a decrease in SH2-containing Inositol Phosphotase-1 protein expression is detectable.

4. The method of claim 1, wherein expression of the SH2-containing Inositol Phosphotase-1in the recipient is suppressed between about 1 and 7 days prior to the transplant.

5. The method of claim 1, wherein the transplant is selected from the group consisting of bone marrow transplants and solid organ transplants.

6. The method of claim 1, wherein the expression of the SH2-containing Inositol Phosphotase-1is reversibly suppressed.

7. A method of enhancing B cell recovery in a bone marrow transplant recipient, comprising suppressing expression of the SH2-containing Inositol Phosphotase-1in the recipient prior to the transplant.

8. The method of claim 7, comprising the step of fully suppressing expression of SH2-containing Inositol Phosphotase-1, whereby no SH2-containing Inositol Phosphotase-1allele or protein is detectable.

9. The method of claim 7, comprising the step of partially suppressing expression of SH2-containing Inositol Phosphotase-1, whereby a decrease in SH2-containing Inositol Phosphotase-1 protein expression is detectable.

10. The method of claim 7, wherein expression of the SH2-containing Inositol Phosphotase-1in the recipient is suppressed between about 1 and 7 days prior to the transplant.

11. The method of claim 7, wherein the expression of the SH2-containing Inositol Phosphotase-1is reversibly suppressed.

12. A method of alleviating immune suppression of a patient comprising the step of suppressing expression of the SH2-containing Inositol Phosphotase-1in the patient.

13. The method of claim 12, comprising the step of fully suppressing expression of SH2-containing Inositol Phosphotase-1, whereby no SH2-containing Inositol Phosphotase-1allele or protein is detectable.

14. The method of claim 12, comprising the step of partially suppressing expression of SH2-containing Inositol Phosphotase-1, whereby a decrease in SH2-containing Inositol Phosphotase-1 protein expression is detectable.

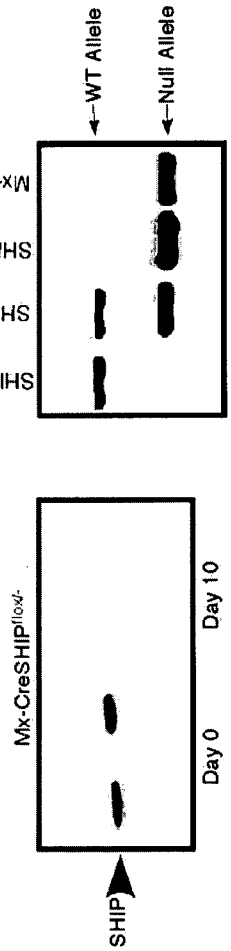


FIG. 1a

FIG. 1b

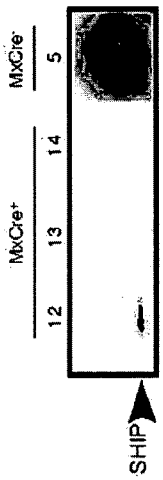


FIG. 1c

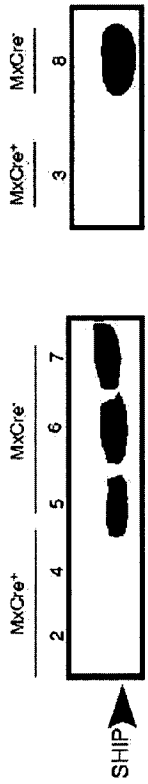


FIG. 1d

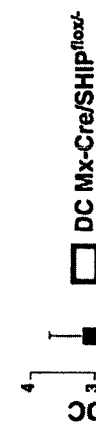


FIG. 2b

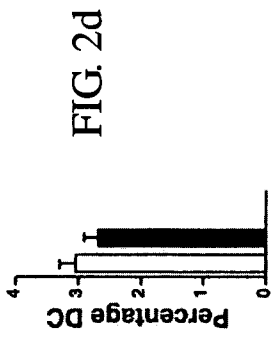


FIG. 2d

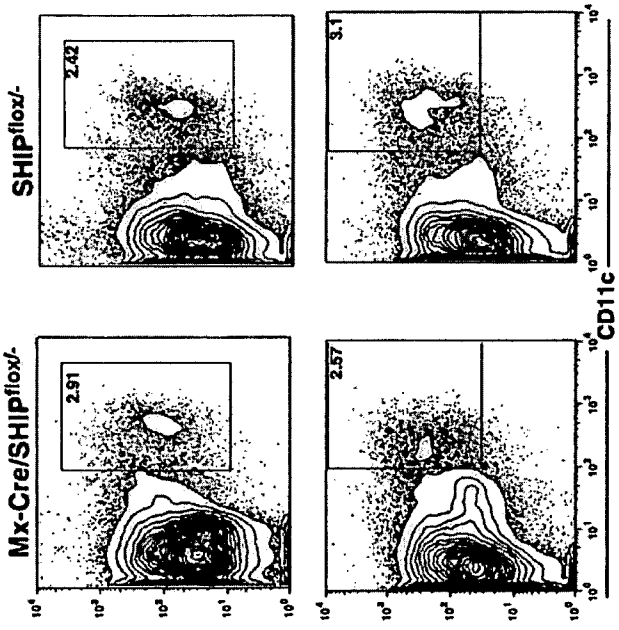


FIG. 2a

FIG. 2c

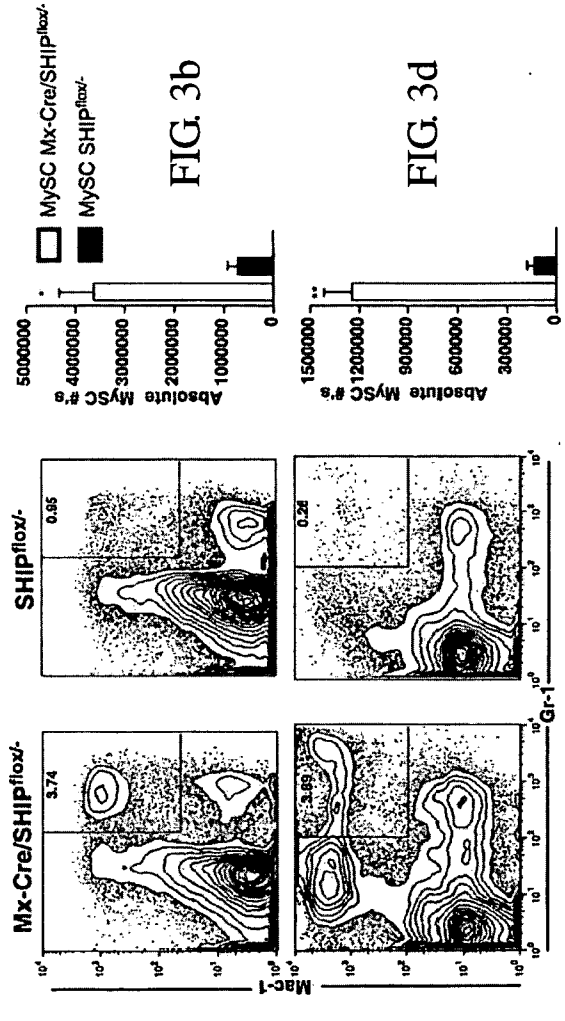


FIG. 3a

FIG. 3c

FIG. 3b

FIG. 3d

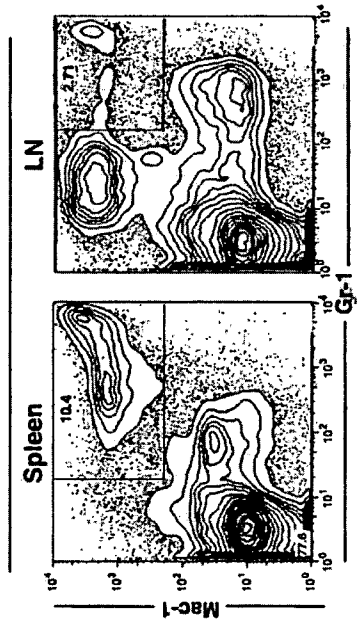


FIG. 3e

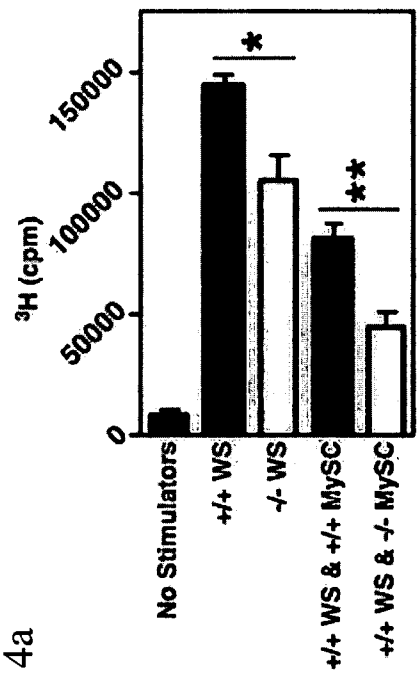


FIG. 4a

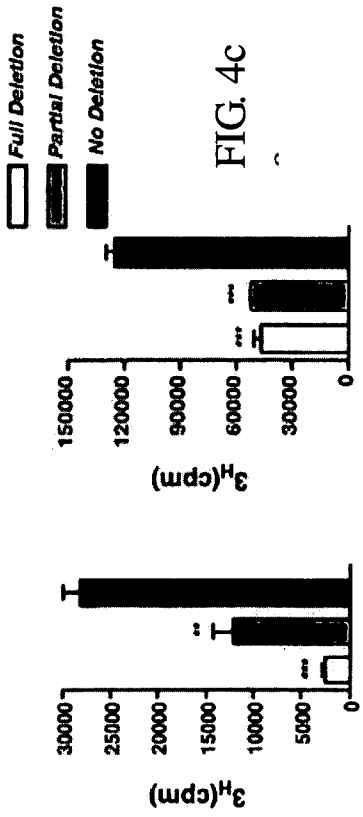


FIG. 4b

FIG. 4c

FIG. 5a FIG. 5b FIG. 5c

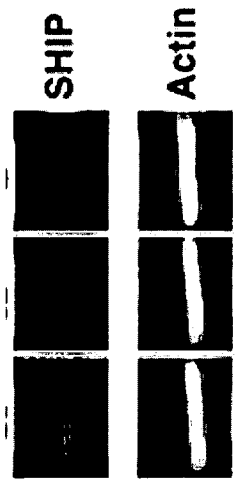
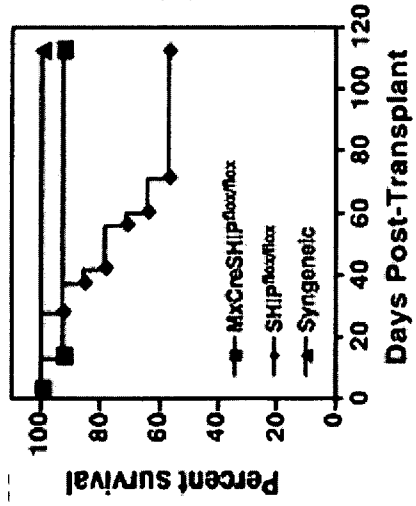


FIG. 5d



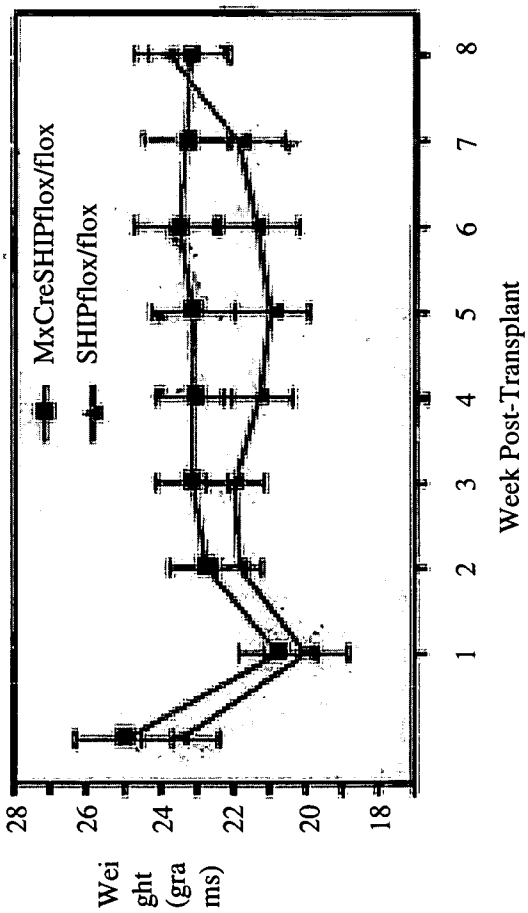

MxCreSHIPflox/flox
SHIPflox/flox
Weight (grams)
28
26
24
22
20
18
1
2
3
4
5
6
7
8
Week Post-Transplant

FIG. 5e

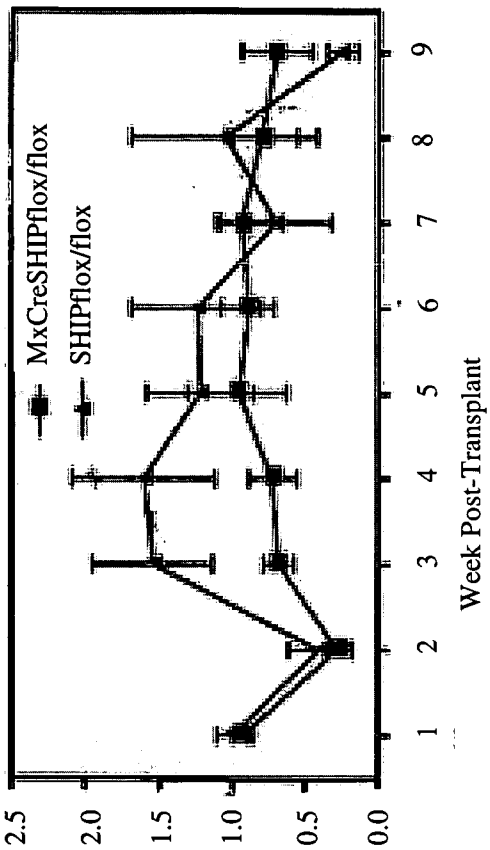

MxCreSHIPflox/flox
SHIPflox/flox
Avg. Gv HD Score
2.5
2.0
1.5
1.0
0.5
0.0
1
2
3
4
5
6
7
8
9
Week Post-Transplant

FIG. 5f

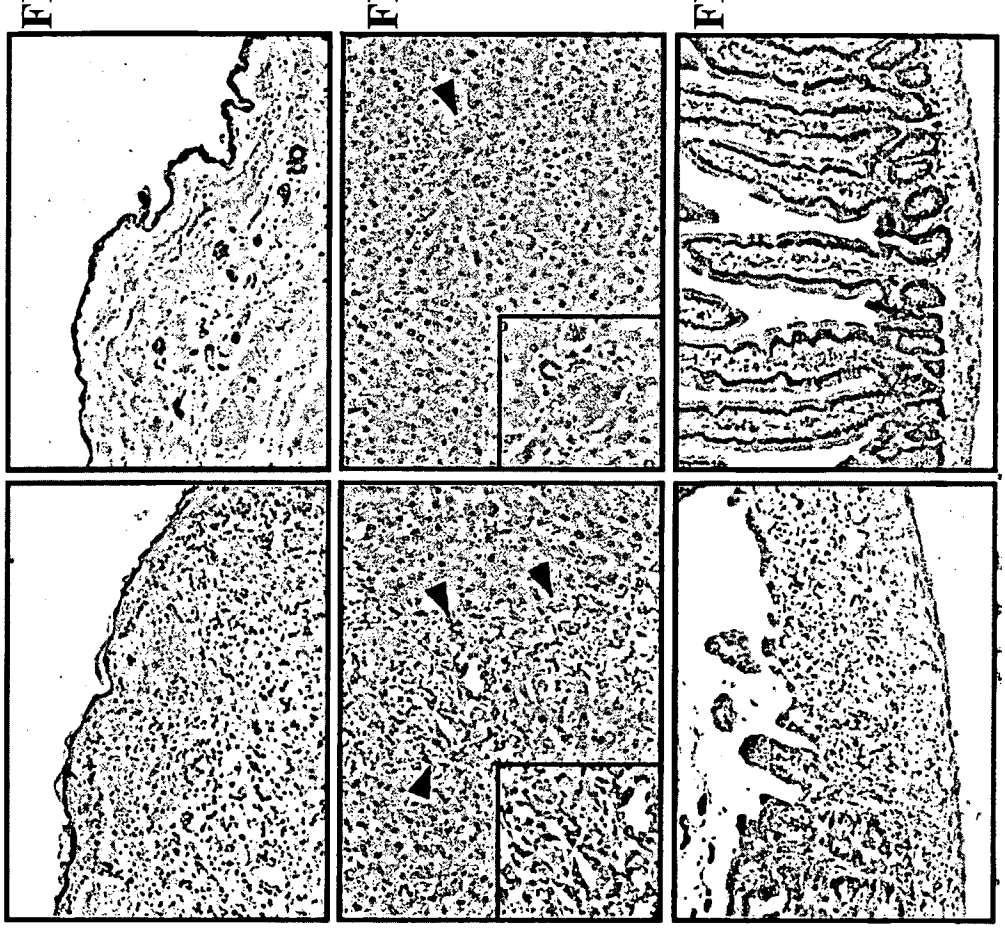


FIG. 6a FIG. 6b FIG. 6c FIG. 6d FIG. 6e FIG. 6f

FIG. 7b

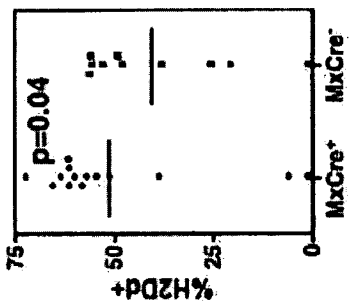
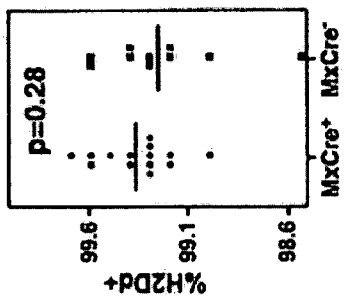

p=0.04
%H2Dd+
75
50
25
0
MxCre+
MxCre-

FIG. 7a


p=0.28
%H2Dd+
99.6
99.1
98.6
MxCre+
MxCre-

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 08/54263

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - A01N 43/04; A61K 31/70; C12N 5/06 (2008.04)
USPC - 514/44
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
USPC: 514/44

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC: 435/330; 424/93.2 (text search, see terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST (PGPB,USPT,EPAB,JPAB); Google/Scholar; PubMed (text search, see terms below)
Search Terms-SH2-containing inositol, SH2 domain-containing inositol, Src homology 2 domain-containing inositol, GVHD, graft versus host disease, phosphatase, bone marrow transplant, solid organ transplant, suppress, decrease, reduce.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2002/0137711 A1 (KERR) 26 September 2002 (26.09.2002) para [0020], [0034], [0036]-[0038], [0042], [0112]-[0113]; Fig. 1C, Fig. 1D, Fig. 8C	1-14

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 April 2008 (26.04.2008)	21 MAY 2008

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774