Targeting Syk Activated B-cells in murine and human chronic graft-versus-host disease

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Short title: Chronic GVHD suppression by Syk inhibition

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Key Points

1) Syk is required for increased B cell activation and cGVHD generation and maintenance.

2) The Syk inhibitor Fostamatinib can treat murine cGVHD and increase human cGVHD B cell death.
Abstract

Novel therapies for chronic graft-versus-host disease (cGVHD) are needed. Aberrant B cell activation has been demonstrated in mice and humans with cGVHD. Having previously found that human cGVHD B cells are activated and primed for survival, we sought to further evaluate the role of the spleen tyrosine kinase (Syk) in cGVHD in multiple murine models and human peripheral blood cells. In a murine model of multi-organ system, non-sclerodermatous disease with bronchiolitis obliterans where cGVHD is dependent on antibody and germinal center (GC) B cells, we found that activation of Syk was necessary in donor B cells but not T cells for disease progression. BM-specific Syk deletion in vivo was effective in treating established cGVHD as was a small molecule inhibitor of Syk, fostamatinib which normalized GC formation and decreased activated CD80/86+ dendritic cells. In multiple distinct models of sclerodermatous cGVHD, clinical and pathological disease manifestations were not eliminated when mice were therapeutically treated with fostamatinib, though both clinical and immunological effects could be observed in one of these scleroderma models. We further demonstrated that Syk inhibition was effective at inducing apoptosis of human cGVHD B cells. Together these data demonstrate a therapeutic potential of targeting B-cell Syk signaling in cGVHD.
**Introduction**

The development of cGVHD is a major complication following allogeneic hematopoietic stem cell transplantation. Discovery of new therapies has been limited by the absence of murine models that closely represent the clinical human disease and pathogenesis\(^1\),\(^2\). Herein we use multiple mouse models to assess the effectiveness of therapies during cGVHD. We utilize a mouse model of antibody-dependent, multi-organ system cGVHD, previously demonstrated to mimic several aspects of human cGVHD pathology, with the exception of sclerodermatous cGVHD\(^3\),\(^4\) and three models of sclerodermatous cGVHD with skin manifestations\(^5\),\(^6\).

While the exact mechanisms of cGVHD remain unknown, recent studies have elucidated a role for antibody production by B-cells. The increase of B-cells in the germinal center (GC) has been shown to be necessary for the development of an antibody-facilitated multi-organ system cGVHD model that includes bronchiolitis obliterans (BO)\(^3\). Spleen tyrosine kinase (Syk) is activated by B-cell receptor (BCR) engagement. After antigen-BCR engagement, Syk is phosphorylated at Y348, allowing for B-cell survival and proliferation. Increased proximal BCR signaling was recently described in cGVHD patient B cells.\(^7\)

Syk also affects myeloid cell [macrophages; neutrophils and dendritic cells (DCs)] including phagocytosis, signal transduction via activating FcRs, and antigen-uptake, internalization and upregulation of costimulatory molecules in DCs.\(^8\) Syk also has been demonstrated to have an impact on cell-migration of monocytes.\(^9\) Given the function of Syk in myeloid cells and the critical role of
macrophages and DCs in cGVHD\textsuperscript{5,10}, Syk inhibition may have additional advantages of treating cGVHD via macrophage and DC effects.

We sought to determine whether B-cell Syk activation was a critical component in cGVHD pathophysiology. First, we demonstrated that Syk was hyper-responsive in B-cells during cGVHD and that Syk was necessary in B- but not T-cells for murine cGVHD progression. Next, we demonstrated that the deletion of Syk in donor BM cells \textit{in vivo} in mice with established cGVHD with BO was able to reverse disease, a finding that was phenocopied by the \textit{in vivo} molecular inhibition of Syk with fostamatinib. Inhibition of Syk decreased the frequency of GCs and expression of the activation costimulatory molecules CD80 and CD86 on CD11c\textsuperscript{+} cells \textit{in vivo}. In multiple distinct models of sclerodermatous cGVHD, clinical and pathological disease manifestations were not eliminated when mice were therapeutically treated with fostamatinib, though both clinical and immunological effects could be observed in one of the sclerodermatous cGVHD models. Human cGVHD B cells had increased death when treated with fostamatinib, demonstrating BCR-activated B cells can be preferentially targeted. These data together suggest that Syk could be a novel therapeutic for cGVHD patients.
Materials and Methods

Mice

C57Bl/6 (B6; H2b) mice were purchased from the National Cancer Institute. B10.BR (H2k) mice were purchased from Jackson Laboratories. Female C57Bl/6 (B6) (H-2b, CD45.2), and B6D2F1 (H-2b/d, CD45.2) mice were purchased from the Animal Resources Center. BALB/c, B10.D2-Thy1.2 and B10-.D2-Thy1.1 mice were propagated in the animal facility at the Johns Hopkins University Cancer Research Building I. The Syk fl/fl x ERT2-cre mice were provided by RAC from Columbia University. Deletion of Syk (Syk KO) occurred with administration of tamoxifen (Sigma) PO for 5 days and confirmed by Western Blot. Mice were housed in a specific pathogen-free facility and used with the approval of each institution’s animal care committee.

BMT

For model 1 (systemic cGVHD with BO, MHC disparate), B10.BR recipients were conditioned with cyclophosphamide and total body irradiation (8.3 Gy). Recipients received 10x10^6 T cell depleted (TCD) bone marrow (BM) cells from B6 wildtype (WT) or BM cells from donors that were induced to delete Syk by tamoxifen administration. Cohorts received no supplemental T cells or purified splenic T-cells (0.1x10^6) from WT vs KO donors\(^3\). In one experiment, as indicated, mice were given donor BM from Syk fl/fl x ERT2-cre or WT mice with or without purified T cells; on D28 post-BMT, mice were given tamoxifen to delete Syk from donor BM cells. For model 2 (sclerodermatous cGVHD, semi-allogeneic
recipients), on day -1, B6D2F1 recipient mice received total body irradiation (11 Gy; ¹³⁷Cs source), split into 2 doses separated by 3 hours to minimize gastrointestinal toxicity⁵. On day 0, recipients underwent transplantation with 5 x 10⁶ BM with or without 1 x 10⁶ purified T cells from B6 mice. Non-GVHD control groups were injected with TCD BM grafts. For model 3 (sclerodermatous cGVHD with G-CSF mobilized splenocytes, MHC disparate), on day –1, mice received total-body irradiation (1Gy; ¹³⁷Cs source), delivered as in model 2. On day 0, B6 mice each received 25 x 10⁶ donor splenocytes from G-GSF–mobilized BALB/c donors. Spleen were depleted of T cells as previously described for non-GVHD control groups¹¹. Transplanted mice were monitored daily, and those with GVHD clinical scores of ≥6¹⁰,¹¹ were sacrificed, and the date of death registered as the next day in accordance with institutional animal ethics committee guidelines. For model 4 (sclerodermatous cGVHD, minor histocompatibility antigen disparate), a single lethal irradiation dose of 7.75 Gy was administered using a ¹³⁷Cs irradiator. Animals were reconstituted with 10⁷ B10.D2-Thy1.2 TCD BM cells (10⁷) alone or supplemented 1.8 x 10⁶ CD4⁺ and 0.9 x 10⁶ CD8⁺ Thy1.1⁺ T cells, reflecting T cells found in 1.2 x 10⁷ B10.D2 donor splenocytes, a dose that reproducibly induces GVHD⁶. Purified populations of donor T cells were obtained using T cell isolation kits (Dynabeads, Invitrogen, Carlsbad, CA).

Where indicated, cGVH recipients were given R788 (30 mg/Kg/animal/twice PO daily) or 0.1% carboxymethylcellulose vehicle from d28-56 (BO model 1), D14-28 (B6 into B6D2F1, scleroderma, model 2), days 7-21 (G-CSF mobilized Balb/c splenocytes into B6 recipients, scleroderma model 3),
or B10.D2 into Balb/c (experiment 1, D21-35; experiment 2, D14-28). Both pro-
drug, R788, and its active metabolite, R406 (both known as fostamatinib) were
kindly provided by Rigel Pharmaceuticals. Lung function was assessed as
previously described.\textsuperscript{12}

**Patient Samples**

Samples were obtained from patients following written informed consent in
accordance with the Declaration of Helsinki. Viably frozen peripheral blood
samples from patient with either active clinical manifestations of cGVHD or no
active cGVHD were randomly selected from cell banks for study (patient
characteristic details are shown in (Supplemental Table 1). The Institutional
Review Boards at the University of North Carolina Chapel Hill, Duke University
Medical Center, and the Dana-Farber Cancer Institute approved all studies.

**Analysis of Syk Activation and *In Vitro* Effects of Syk Inhibition**

For analysis of Syk phosphorylation in murine cGVHD, B-cells were purified from
murine cGVHD spleens or BM only controls on D60 in the cGVHD/BO model and
activated by 5 \( \mu \)g/mL anti-IgM Fab (Jackson Research), fixed with BD Cytofix
Fixation Buffer and permeabilized with BD Perm Buffer III. Cells were
subsequently stained with anti-Syk Y348 PE (eBioscience) as previously
described\textsuperscript{13}.

For analysis of the effects of *in vitro* Syk inhibition, cryopreserved PBMCs
from cGVHD or non-cGVHD control patients were thawed and allowed to rest
overnight. The Syk inhibitor, R406, (0.01 or 0.1 \( \mu \) M) or DMSO control (0.1%) for
48 hours at 37°C. Apoptosis was measured by gating on CD19^+ or CD19^- cells and co-staining with Annexin V and 7-AAD.

**Flow Cytometry**

GC B cells and CD11c^+ cells were labeled with anti-CD19 (Clone: eBio1D3), anti-CD11c (Clone: N418), anti-CD80 (Clone:16-10A1), anti-CD86 (Clone: GL1), anti-MHCII (Clone: M5/114.15.2), anti-GL7 (Clone: GL-7) (eBioscience), anti-CD4 (clone: RM4-5) anti-CD95 (Clone: Jo2) (BD Biosciences), anti-F4/80 (Clone: BM8), anti-CXCR4 (Clone: L276F12), anti-Ly6G (Clone: 1A8), anti-CD3 (Clone:145-2C11), anti-Ly6C (Clone: HK1.4), anti-CD8 (Clone: 53-6.7), and anti-CD11b (Clone: M1/70), (biolegend) were analyzed on BD LSRFortessa. Ficolled human PBMC were stimulated and stained for Annexin V as previously described.\(^{14,15}\)

**Frozen tissue preparation, pathology and immunofluorescence** Organs harvested were embedded in Optimal Cutting Temperature compound, snap frozen, and stored at -80°C. GC detection and pathologic scoring were accomplished as previously described\(^4\). At various times post transplantation, GVHD target tissues were harvested, fixed in 10% formalin for 24hrs, embedded in paraffin, and processed to generate 5µm-thick sections. H&E sections of skin were examined in a blinded fashion using a semi quantitative scoring system for GVHD as previously published.\(^4,16\) Samples were scored from 0 to 4 for
epidermal and dermal inflammation, dermal fibrosis and subcutaneous fibrosis, epidermal apoptosis, and loss of subcutaneous fat.

Statistics
Group comparisons of pathology, pulmonary function tests, cell counts and flow cytometry data were analyzed by Student t-test or one-way ANOVA.
Results

Syk phosphorylation is increased during murine cGVHD and is necessary in donor BM derived cells for the development and maintenance of cGVHD.

To determine if Syk activation is important in murine cGVHD, purified B cells (Figure 1) from day 60 cGVHD spleens were assayed for Syk phosphorylation. Purified B cells from cGVHD mice were activated with anti-IgM and the amount of phosphorylated Syk at Y348 was measured. There was an increase in the percentage pSyk in both BCR stimulated B-cells and unstimulated B-cells in cGVHD (Figure 1). These data extend a recent report that human B-cells have increased pSyk during cGVHD7.

To test whether Syk is necessary for cGVHD progression, mice were transplanted with WT or induced Syk KO BM with WT T-cells and analyzed for disease on day 60. Mice receiving Syk KO BM and WT T-cells did not develop cGVHD pulmonary dysfunction compared to those receiving WT BM and T-cells (Figure 2 A). Since Syk is necessary for the proliferation of B-cells following antigen stimulation17, we analyzed the spleens of transplanted mice to determine if there was a defect in the maintenance of Syk KO BM-derived B-cells. We found that total B-cell frequency in mice receiving Syk KO BM was decreased 8-fold (Figure 2B). These data are consistent with the dependency of activated B-cells on Syk for proliferation and survival, and a requirement for activated donor-derived BM B-cells in cGVHD pathogenesis3, 4.

In contrast to Syk dependence in BM derived cells, the addition of Syk KO donor T-cells did not attenuate the functional manifestations of BO (with the
exception of resistance) compared to Syk WT T cell transplanted mice (Figure 2C). In addition cGVHD pathology in the lungs and liver were similar in recipients of Syk WT vs KO T cells (Figure 2D). While T cell effects after Syk inhibition were previously described in acute\textsuperscript{18} and sclerodermatous cGVHD\textsuperscript{19}, in this systemic cGVHD model with BO and without scleroderma, donor T-cell Syk was not fully required for cGVHD development.

To determine whether Syk function in BM-derived B cells was essential for sustaining cGVHD in mice with established disease, recipients were given donor BM from Syk fl/fl x ERT2-cre or WT mice with or without purified T cells. On D28 post-BMT, mice were given tamoxifen. Induced deletion of Syk in donor BM cells significantly improved all pulmonary function parameters to levels of non-cGVHD controls (Figure 3).

**Therapeutic pharmacological inhibition of Syk decreases pathology in a murine BO model of cGVHD but had little clinical therapeutic effect in scleroderma models of cGVHD.**

Fostamatinib is a potent small molecule inhibitor of Syk. Studies in rheumatoid arthritis have demonstrated efficacy with fostamatinib in randomized phase II clinical trials\textsuperscript{20, 21}. Fostamatinib was notably safe in patients treated for non-Hodgkin’s lymphoma, including some who had received prior autologous HCT\textsuperscript{22}. Additionally, Leonhardt et. al.\textsuperscript{18} demonstrated that Syk inhibition decreased costimulatory molecules on antigen-presenting cells and increased survival of mice during acute GVHD while preserving anti-tumor and anti-viral
immunity. In addition, other studies have demonstrated the importance of BCR signaling for the development of murine cGVHD.23

Having demonstrated the requirement for Syk in donor BM cells in mice with established cGVHD and BO, we next sought to determine whether drug treatment with fostamatinib in mice with established cGVHD would be similarly efficacious as Syk deletion in donor BM cells.3,4 Mice receiving fostamatinib beginning on D28 had restoration of pulmonary function, similar to the healthy transplanted BM only controls (Figure 4A). Improvement in pulmonary function correlated with a reduction in cGVHD pathology in the lung (Figure 4B). The number of GC reactions in the spleen was decreased in fostamatinib versus vehicle treated cGVHD mice (Figure 4C). This was highlighted by a decrease in frequency of splenic GC B-cells in fostamatinib treated cGVHD mice, matching BM only controls (Figure 4D). These data demonstrate the importance of Syk in B cell signaling during active disease.

Previous reports demonstrated a decrease in activation of CD11c+ cells18 in mice with acute GVHD treated with fostamatinib. In the BO cGVHD model we found increased expression of costimulatory molecules CD80 and CD86 on CD11c+ spleen cells compared to BM only transplanted mice. When these cGVHD mice are treated with fostamatinib beginning D28, we found a significant decrease in the frequency of CD11c+ cells expressing CD80 and CD86 though without affecting MHC class II expression (Figure 4 E-G). These data highlight a potentially beneficial alternative effect of Syk inhibition CD11c+ myeloid cells during cGVHD.
In order to determine whether the effects of Syk inhibition attenuate murine sclerodermatous cGVHD in our hands, we utilized three different cGVHD scleroderma models involving lethally irradiated recipients: B6 into B6D2F1 model (model 2); G-CSF mobilized BALB/c splenocytes into B6 (model 3), and a minor-mismatch model of B10.D2 into Balb/c (model 4). In the B6 into F1 model (fostatinib, D14-28) and in the G-CSF-mobilized Balb/c into B6 models (fostatinib, D7-21), the frequency of splenic CD19+ B cells in vehicle and fostatinib treated cGVHD were >10- and 5-fold fold lower than non-cGVHD, BM only controls in both the B6 into B6D2F1 and G-CSF Balb/c into B6 models, respectively (Figure 5 A and Supplemental Figure 1A). Neither a significant beneficial clinical therapeutic nor pathological effect as reflected by total skin inflammation score was seen in either model (Figure 5 B and Supplemental Figure 1B). However, we found a significant decrease in peripheral blood CD11b+F4/80+ and Ly6C^hi monocytes numbers in the B6 into B6D2F1 model after treatment with fostamatinib in the B6 into B6D2F1 model (Figure 5 C-E), which was also seen in the G-CSF mobilized Balb/c into B6 model but did not reach statistical significance (Supplemental Figure 1 C-E).

In the B10.D2 into Balb/c model of scleroderma, fostamatinib treatment from D.21-35 did not impact skin disease or clinical score (not shown). However, beginning fostatinib treatment 1 week earlier on D14, as used by Huu et. al.19, did significantly improve these parameters on D19 and there was a reduction in the both mean skin score from 3 to 1 and the GVHD clinical score from 6 to 4 in the fostatinib vs vehicle treated controls on D35, the end of the study (Figure 6 A and
B). In the latter experiment, there also was a decrease in total numbers of viable cervical and mesenteric lymph node (but not splenic) B cells and frequency of CD11b+ cervical lymph node cells. CXCR4+ T cells in the spleen and cervical lymph node were also decreased in fostamatinib vs vehicle treated cGVHD mice (Figure 6 C-G), suggesting an impairment to the overall immune response and migration of T cells and myeloid cells to tissues. Taken together, these data suggest that early post-transplant treatment of sclerodermatous cGVHD may attenuate disease progression.

Fostamatinib increases apoptosis in B cells purified from cGVHD patients.

To determine if human cGVHD B-cells are more susceptible to Syk blockade, human peripheral blood (Supplementary Table 1) were treated in vitro with R406, the active form of R788. B cells from patients with active cGVHD had increased apoptotic and total cell death compared to patients with inactive or no cGVHD after R406 (Figure 7). These data, consistent with work by Allen et. al, reveal that R406 preferentially kills cGVHD B cells via apoptosis. Global targeting of B cells with rituximab has been met with mixed success possibly due to altered B cell homeostasis perpetuated in some patients. We now demonstrate that constitutively activated B cells can be selectively targeted in cGVHD by Syk inhibition (Figure 7B). Consistent with the lack of a requirement for Syk in donor T cells in the cGVHD BO model, fostamatinib did not induce apoptosis in the non-B cell population (Figure 7C). In 3 of samples from each patient group, T-cell specific anti-CD3 antibody was included to stimulate T-cell proliferation in the
presence of R406; no increase in T-cell death was observed under these conditions. Together, these data point to the therapeutic potential of fostatinib and pSyk targeting in cGVHD patients.

**Discussion**

Here, we demonstrated that pSyk was upregulated in murine cGVHD B cells in a multi-organ system model of cGVHD with BO that is dependent upon donor T cell support of antibody isotype switching and immunoglobulin deposition. Such cGVHD B cells were hyper-responsive to B cell activation signals *in vitro*. Syk function in donor BM-derived B cells but not T cells was required for cGVHD generation and Syk deletion in donor BM cells in mice with established cGVHD on D28 post-BMT reversed disease. A small molecule inhibitor of pSyk, fostamatinib that has been tested in the clinic for autoimmune diseases, was able to treat established cGVHD when therapy was initiated on D28. In cGVHD patients, previous studies have shown that cGVHD B cells have high levels of pSyk and in the current study, we now show that B cells from patients with active cGVHD but not B cells from patients without active cGVHD was induced into apoptosis *in vitro* by the active metabolite of fostamatinib. Whereas fostamatinib was highly effective in reversing cGVHD in the BO model, clinical and immunological effects in sclerodermatous cGVHD appeared to be more modest under the conditions tested.

It is unclear if B cells are intrinsically or extrinsically affected during cGVHD. B cells might increase their activation state based on pathogen activating molecular patterns released during conditioning treatment and
activating toll-like receptors on B cells. In contrast there is potential that increased activating factors such as B cell activating factor (BAFF) are causing B cells to increase response from factors such as Syk since BAFF receptor may be reliant on Syk for its signaling.\textsuperscript{26} The requirement of Syk in BM derived B cells for cGVHD development in the BO model also allowed us to elucidate a potential mechanism of disease. BCR stimulation with antigen leads to phosphorylation of proximally located Syk to initiate down-stream signaling for B cell survival and proliferation. Syk signaling is necessary for survival of B cells during the GC reaction. The decrease in B cells after Syk-deficient BM transplant in the BO model suggests a mechanistic link between BCR-antigen engagement that requires Syk for B cell promotion and perpetuation. The absence or relatively modest effects of fostamatinib in sclerodermatous cGVHD is consistent with the findings that there were few splenic B cells present in our studies in fostamatinib or vehicle treated sclerodermatous cGVHD mice when analyzed 4-5 weeks post-transplant. These B cell data contrast to the significantly increased splenic GC B cell frequency seen in cGVHD mice with BO.\textsuperscript{3, 4}

Studies of cGVHD patient peripheral blood have demonstrated an important role of B cells in development of disease. The production of auto- and allo-antibodies has previously been associated with the development of cGVHD in patients.\textsuperscript{27-30} The dysregulation of B cells could be due to the significant increase in BAFF present during cGVHD.\textsuperscript{15, 31} B cells in patients with cGVHD were more sensitive to Syk inhibition compared to B cells from patients without active cGVHD, consistent with our previous finding that these cells have
increased activation of Syk and the proximal BCR signalosome\textsuperscript{7}. We demonstrated that these cells had an increase in cell death rates compared to controls. Like BCR-activated B cell lymphomas\textsuperscript{32}, only those cGVHD B cells activated through the BCR pathway are presumably susceptible to killing at low drug concentration. Together these data demonstrate the relative selective ability of fostamatinib to target the activated B cells in cGVHD patients.

The role of Syk in donor T cells was demonstrated to be of little importance in the development of murine cGVHD in the BO model. However in the minor antigen-disparate sclerodermatous cGVHD model, we did find a decrease in the expression of CXCR4 on T cells when mice with established clinical cGVHD were treated with R788, consistent with Huu et al.\textsuperscript{19} who also demonstrated increased pSyk in T cells on day 14 post-transplant also showed a reduction in proliferation of T cells from cGVHD mice when exposed \textit{in vitro} to the active fostamatinib metabolite, R406 and mice (4-6 per group) treated with fostamatinib \textit{in vivo} exhibited ~1 week shift in cGVHD onset and progression when treatment was initiated on day 14 post-transplant, resulting in significantly lower average skin scores and body weight between days 21-42 post-transplant.\textsuperscript{19} The decrease in migration of T cells to effector tissues could have a beneficial effect on the development of disease. Thus, while treatment did not fully restore health in our study in the B10.D2 into Balb/c mode, there was a decrease in the progression of disease associated with a lowered frequency of CXCR4\textsuperscript{+} T cells that may contribute to disease reduction.
Despite the apparent lack of Syk dependency of donor T cells in cGVHD with BO, we demonstrate that the Syk inhibitor fostamatinib is capable of blocking the cGVHD GC response that is dependent upon T follicular helper cells and GC B cells. These data point to a T cell independent effect of fostamatinib in abrogating the cGVHD GC response, which is essential for cGVHD generation and progression in the BO model.

In addition to targeting B cells during cGVHD, there is evidence that antigen-presenting cells are susceptible to fostamatinib treatment. For example, in an acute GVHD model, Leonhardt et. al. showed that fostamatinib reduced costimulatory molecule expression and disrupted cytoskeletal organization, along with blocking T cell proliferation and migration, resulting in acute GVHD amelioration with preservation of graf-versus-leukemia and anti-viral immunity. Consistent with other reports that inhibition of Syk can decrease costimulatory molecules expression, we demonstrated in vivo decrease in the frequency of activated (CD80+, CD86+) DCs in mice with cGVHD and BO.

Although we could not discern effects of fostamatinib on the total skin inflammatory scores in either MHC-disparate models of sclerodermatous cGVHD, in the B6 into B6D2F1 model, fostamatinib significantly decreased the absolute number of CD11b+F4/80+ monocytes and the inflammatory Ly6C hi but not the Ly6C lo non-inflammatory subset in peripheral blood. These data are interesting in light of the role of Syk in macrophages and monocytes and our recent study implicating monocytes and macrophages in cGVHD generation and maintenance in the two scleroderma cGVHD models as well as in the BO model.
Notably, there was a significant decrease in CD11b⁺ cells in the cervical lymph node of fostamatinib treated mice in the minor antigen disparate B10.D2 into Balb/c sclerodermous cGVHD model. Monocyte and macrophages may contribute to cGVHD in these models by antigen-presentation capacity or the elaboration of pro-fibrogenic proteins such as TGFβ. Thus, the impact of fostamatinib in cGVHD models may depend in part as to the stage of disease progression as well as the dominant underlying mechanisms for disease pathogenesis.

In summary, our data clearly indicate by biochemical and genetic and pharmacological approaches a role of Syk in donor BM-derived B cells in a multi-organ system model of cGVHD with BO. Further we have translated these findings into a therapeutic using a pharmacological small molecule Syk inhibitor, fostamatinib, that has progressed through phase II clinical trials. Fostamatinib ameliorated disease in mice with cGVHD and BO and has biological effects in some models of scleroderma. These preclinical murine and human data point to consideration of clinical trials of fostamatinib or other Syk inhibitor alone or in combination with other similar agents for the treatment of cGVHD.
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Author contributions

RF designed experiments, performed experiments, and wrote the paper. JLA, LL, KPM, KP, JP, KAA, JD, and JCP designed and performed experiments. AP-M performed histological analyses, discussed experimental design, and edited the paper. PAT performed experiments and edited the paper. JSS, WJM, GRH, KPM, LL, IM, JK, CSC, RJS, and JR designed experiments and edited the paper; NJC and RAC provided reagents, discussed experiments and edited the paper, SS and BRB designed experiments and edited the paper.

Conflict of interest

No conflicts of interest are known.
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Figure Legends

**Figure 1:** Syk activation during cGVHD. B cells were purified from the spleens of healthy control (BM Only) or cGVHD (BM + T) mice and stimulated with anti-IgM at 5 μg/mL. Splenocytes from B10.BR mice transplanted with B6 BM and low numbers of T cells were analyzed for phosphorylated Syk at Y348 after *ex vivo* stimulation by 5 μg/mL of anti-IgM* p < 0.5; Error bars represent SEM, n=8 representative data from 2 experiments.

**Figure 2:** Presence of Syk in BM derived B cells but not donor T cells is necessary for development of cGVHD pathology in BO model. A) Day 60 pulmonary function tests of mice transplanted with taxomifen induced Syk KO BM and WT T cells. B) Frequency of B cells in transplanted mice on D60 after transplant. C) Pulmonary function tests from mice transplanted with WT BM and either WT T cells or Syk KO T cells. D) Pathology scores from lung and liver of mice transplanted with Syk KO T cells. * p < 0.5; ** p < 0.01. Error Bars represent SEM, Representative data from 3 independent experiments with n=8 per group.

**Figure 3:** Genetic deletion of Syk during active disease prevents pathogenic pulmonary function. Mice were transplanted with either WT or Syk fl/fl x ERT2-Cre BM. On D8 mice were treated with Tamoxifen for 5 days to delete Syk in donor BM derived cells. A) Pulmonary function tests of mice on day 60. * p < 0.5; ** p < 0.01. Error Bars represent SEM, n=10 per group.
Figure 4: Inhibition of Syk by R788 decreases cGVHD in murine BO model.
A) Day 56 pulmonary function tests on mice treated with 30 mg/Kg of R788 twice daily from D28-56. B) GVHD pathology scores from the lungs of mice on D60. C) Number of GCs present in situ in spleens of mice. D) Frequency of GC B cells (gated on CD19⁺GL7⁺CD95hi) present in the spleens of transplanted mice on day 60. E-G) Frequency of CD11c cells expressing CD80, CD86 and MHC Class II in the spleens of transplanted mice on day 60. * p < 0.5; ** p < 0.01; *** p< 0.001. Error Bars represent SEM, Representative data from 3 independent experiments with n=8 per group.

Figure 5: Fostamatinib does not alter skin inflammation in B6 into B6D2F1 model of sclerodermatous GVHD but decreases peripheral blood Ly6C⁹ monocytes. A) Frequency of CD19⁺ B cells in spleens of mice on day 21 in vehicle treated (grey bars), R788 treated (black bars) and non-GVHD controls (white bars). B) Total inflammation score. C) Absolute number of CD11b⁺ F4/80⁺ peripheral blood monocytes. Peripheral monocytes were examines for D) Ly6C high or E) Ly6C low expression. * p < 0.5; ** p < 0.01; Error bars represent SEM, n=6 per group.

Figure 6: Fostamatinib has little clinical therapeutic benefit in the B10.D2 into Balb/c sclerodermatous cGVHD model but decreases expression of CXCR4 on CD11b⁺ and CD4⁺ cells. A) Clinical GVHD scores or b) Skin GVHD
Scores in vehicle treated (grey squares) or R788 treated (black circles) in B10.D2 into Balb/c model. C) Frequency of viable CD11b+ cells and D) expression of CXCR4 on CD11b+ cells. E) Absolute number CD19+ B cells and F) expression of CXCR4 on B cells G) expression of CXCR4 on CD4+ cells. * p < 0.5; Error bars represent SEM. n=5 per group.

Figure 7: Increased apoptosis in human B cells when treated with R788. (A) Representative flow plots of human PBMC with or without cGVHD. Consistent with previous work36 unmanipulated, untreated cGVHD B cells had superior survival compared to B cells from patients without disease. Peripheral blood mononuclear cells from patients without cGVHD (n = 6, open circles) and with cGVHD (n = 6, filled squares) treated with R406 (0, 0.01, and 0.1 μM) as indicated for 48 h. Apoptotic B cells were defined as CD19+ Annexin V+ 7AAD− cells (B) or as CD19 negative non-B cells (C). Fold increase in apoptosis by R406 divided by PBS is depicted. Data are median +/- range pooled from 2 independent experiments. * p < 0.5; ** p < 0.01; *** p < 0.001.
Figure 1

Unstimulated vs. anti-IgM stimulation in BM- and BM + T-primed splenic B cells. Flow cytometry analysis showing activation of pSYK in B cells. The percentage of pSYK+ cells is significantly increased in BM + T-primed splenic B cells compared to BM-primed cells. * indicates statistical significance.
Figure 6

A. Clinical GVHD Score

B. Skin GVHD Score

C. Viable CD11b+ Cells

D. CXCR4 in CD11b+ Cells (Total)

E. Viable B cells

F. CXCR4 in B cells

G. CXCR4 in CD4+Thy1.1+ T Cells (%)

[Graphs and data showing the changes in GVHD scores, CD11b+ cell viability, CXCR4 expression in different cell types over time after transplant.]
Targeting Syk Activated B-cells in murine and human chronic graft-versus-host disease