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Targeting Transcription Factors XBP-1 and Fli-1 as Novel Translational Strategies to Control Graft-Versus-Host Disease

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Targeting Transcription Factors XBP-1 and Fli-1 as Novel Translational Strategies to Control Graft-Versus-Host Disease

by

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Dedication

The work presented here in this dissertation is dedicated to my late Grandfather, Eugene Schutt, who led a full and wonderful life, but still was taken from the world too early due to leukemia in 2014. During my most challenging times over these last several years, I have often drawn inspiration from the possibility that my work during graduate school may one day help someone like him in the future.

It is also dedicated to my supportive and loving parents Jeff and Kim Schutt who were always there for me during the worst and the best times and guide me through life's challenges.

My wife Maribel Vazquez also deserves an important dedication, as she has always encouraged and supported me throughout our time together, and her kindness and smile are always able to brighten even the darkest days. I enthusiastically look forward to starting our new life together and pursuing both of our goals and dreams.

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Abstract

Hematopoietic stem-cell transplantation (HCT) is a curative procedure for hematological malignancies, but chronic graft-versus-host disease (GVHD) remains a major complication after allogeneic HCT. Because donor B cells are essential for chronic GVHD (cGVHD) development and B cells are sensitive to endoplasmic reticulum stress, we hypothesized that the IRE-1α/XBP-1 pathway is required for B-cell activation and function in cGVHD. Here, we used mice deficient of XBP-1 specifically in B cells, and recipients transplanted with grafts containing XBP-1–deficient B cells displayed reduced cGVHD compared with controls that was associated with reduced B-cell activation and production of alloreactive antibodies. Prophylactic administration of B-I09 (an IRE-1α/XBP-1 inhibitor) also reduced cGVHD without compromising GVL effect against chronic myelogenous leukemia.

Although B cells play an important role in chronic GVHD development, effector T cells are still primary drivers of the disease. We investigated the T-cell specific role of a second transcription factor, Fli-1, in GVHD pathogenesis. Ablation of two critical exons of the *fli-1* gene in donor-derived T cells was associated with significant reduction of disease development in allo-HCT models of cGVHD and aGVHD. This reduction was associated with increased regulatory T cells (Tregs) and decreased IFN-γ^+, IL-17A$^+$, and T_{FH} T cells in lymphoid organs. We also demonstrated that low-dose camptothecin exhibits action as a potent Fli-1 inhibitor, both *in vitro* and *in vivo* against mouse and human Fli-1. Utilization of drugs that targeted Fli-1 was able to prevent cGVHD development and reverse already established cGVHD. Camptothecin also prevented aGVHD while preserving the GVL effect against P815 mastocytoma. Further, camptothecin reduced human T-cell proliferation both *in vitro* and *in vivo,* while also reducing GVHD in a human xenograft model.

These findings in combination suggest that XBP-1 is a critical factor regulating the B-cell component of chronic GVHD development while Fli-1 is a critical factor involved in the T-cell

component. Many transcription factors are also notoriously difficult to inhibit, however, we identified that both XBP-1 and Fli-1 could be targeted using pharmaceuticals, making these factors promising translational strategies for reducing GVHD in the clinical setting, while also preserving the GVL effect of the donor graft.

Chapter 1: Background Information

Note to Reader

Some portions of this chapter refer to information that has been previously published (Schutt et al., 2018, Blood Advances) and have been utilized with permissions from the publisher (See Appendix A).

1.1 Allogeneic hematopoietic stem cell transplantation (Allo-HCT)

Allogeneic hematopoietic stem cell transplantation (allo-HCT) is often performed to cure malignant hematological malignancies such as leukemia, myeloma, and lymphoma, where the efficacy of allo-HCT lies within the ability of donor lymphocytes contained within the graft to destroy remaining tumor cells following myeloablative and or chemotherapeutic conditioning regimens which aim to eliminate the majority of the malignant cells (1) . Further, additional uses for allo-HCT include curing sickle-cell anemia (2)and for patients who have bone marrow failure, commonly caused by aplastic anemia (3). As of 2014, more than 8,000 allogeneic bone marrow or peripheral blood transplants are performed in the United States annually, and exceed 30,000 annually when combined with other Western countries. The rate of allogeneic transplants also continue to rise to match the increasing frequencies of malignancies such as leukemia in the general population (4-6). Many unique and novel uses for allo-HCT continue to develop over time as technology and knowledge of the biology of transplantation improves. Intriguingly, a case report recently showed that allo-HCT was able to cure a patient of his Schizophrenia (7) , suggesting along with other studies that this disease can be mediated by immune system dysfunction. Thus, it is likely that the number of allo-HCT procedures will continue to rise with time as new and exciting applications for this curative procedure are implemented.

1.2 Beneficial effects of Allo-HCT: GVL

In proliferative disorders, such as leukemia, the purpose of the conditioning regimen followed by allo-HCT is to destroy already existing tumor cells, and further prevent development of additional tumor cell growth by eliminating the patient's existing stem cells that primarily reside in the bone marrow, and replace the patient's bone marrow cells with a fresh source of non-malignant stem cells . The transplant then causes a phenomenon whereby intact immune cells present within the fresh donor graft are able to recognize and eliminate the recipient malignant cells, which is referred to as the graft-versus-malignancy (GVM), or in terms of cancer and leukemia, the graft-versus-tumor (GVT) and graft-versus-leukemia (GVL) effect , respectively (8). Human leukocyte antigens (HLA) play a key role in GVT/GVL responses, as T cells that recognize foreign (non-self) HLA molecules expressed on encountered cells will attempt to kill these cells (9). $CD4^+$ and $CD8^+$ T cells react to HLA-mismatched cells differently, where $CD8^+$ T cells release cytotoxic factors to lyse the cell or induce its apoptosis via cytokines such as granzymes and perforins. On the other hand, $CD4^+$T cells can release diverse types of multi-functional cytokines such as IL-4 and IL-17 that can activate APC's, the innate immune system, as well as provide help to $CD8^+$ T cells (10). Thus, strategies employed to maintain or preserve the GVL ability of transplanted cells is required to ensure a successful long-term allo-HCT outcome.

1.3 Detrimental effects of Allo-HCT: GVHD

Although allo-HCT is typically an effective and curative procedure, its success is often undermined by the development Graft-versus-Host Disease (GVHD), which remains the prominent cause of morbidity and mortality in allo-HCT patients. After allo-HCT, GVHD is known to form in two distinct phases: 1) In the acute form of GVHD (aGVHD), the conditioning regimen causes activation of recipient antigen presenting cells (APC) that stimulate T-cell activation and cytokine production leading to host tissue damage, organ failure, and mortality. 2) In the chronic form of GVHD (cGVHD), the same process is implicated, but with an important role for donor B cells, which act as potent stimulators for donor T cells and produce alloreactive-antibodies that bind to host tissues and lead to tissue inflammation, damage, and organ failure. cGVHD in particular occurs in between 30%-70% of allo-HCT recipients, and its development is the primary cause of late-stage transplant-related morbidity and mortality, despite available prophylactic strategies and treatments (11, 12). Thus, determining biological mechanisms of how allogeneic T cells and B cells interact after allo-HCT can provide novel targets for therapeutic intervention to reduce the incidence and severity of GVHD in the clinic.

1.4 Role of Adaptive Lymphocytes in GVHD

Although innate lymphocytes such as natural killer (NK) cells and neutrophils have been documented to play important roles in GVHD pathogenesis, most previous studies on GVHD have focused on elucidating the role of the adaptive immune system, consisting of T cells and B cells.

1.4.1 CD4+ T cells

During the priming stage of T-cell activation, depending on different signaling and cytokine elements present in their microenvironment, alloreactive CD4+ T cells typically undergo differentiation into several different subsets, including Th1, Th2, Th17, and Treg. Classical Th1 cells express T-bet master transcription factor and predominantly produce IL-2, IFN-γ, and TNF-α; Th2 cells express GATA3 master transcription factor and produce IL-4, IL-5, and IL-13; Th17 cells express RORγT master transcription factor and produce IL-17A or IL-17F, IL-21, as well as IL-22; Treg express FoxP3 master transcription factor and produce IL-10, TGFβ, Granzymes and perforins (13).

Th1 cells have classically been shown to be pathogenic in acute GVHD development (14), although complete absence of IFN-γ using knock-out studies showed opposing findings that some IFN-γ signaling was required to suppress GVHD development (15, 16). Mechanisms of action for the pathogenicity of Th1 cells include indirect methods such as secretion of IL-2 which can promote CD8+ T-cell survival and proliferation, or more direct cytotoxic functions which include production of TNFα and expression of Fas-ligand that can cause target cell apoptosis of host tissues (17)

Th2 cells can either be regulatory or pathogenic in the acute form of GVHD. Reports have identified enhanced Th2 infiltration into skin of aGVHD patients and that cytokines produced by Th2 cells cause organ fibrosis and damage in certain host tissues such as skin and lung (18, 19). However, Th2 cells have also been shown to play regulatory roles and become suppressive role against alloreactive T cells resulting in reduced aGVHD development (14, 20).

Th17 cells have been documented to be pathogenic in both acute and chronic forms of GVHD, where the primary mechanisms of action include aggravation of the Th1 response as well as recruitment of innate immune cells to GVHD target tissues to exacerbate inflammation,

fibrosis, and organ damage (21-23). Further, Th17 cells produce IL-21 which can promote B cells to differentiate into pathogenic antibody-producing plasma cells as well as promote naïve CD4+ T cells to differentiate into T follicular helper (T_{FH}) cells which appear to play an important role in cGVHD development and or maintenance (17, 24).

T_{FH} cells, while still relatively new and controversial in the field of GVHD, have been shown to be elevated in peripheral blood of patients with ongoing chronic GVHD (24). Further, murine studies by our group and others have shown that T_{FH} cells are elevated in mice with ongoing chronic GVHD (25-29). However, another group recently showed evidence that T_{FH} cells may not required for cGVHD disease development or maintenance (30), however the role of this cell subset in GVHD still remains to be fully elucidated. Because T_{FH} cells have plasticity to adopt Th1-, Th2-, or Th17-like features, they possess a potent ability to induce B-cell humoral responses by producing a variety of cytokines such as IFN-γ, IL-4, IL-17, and IL-21 which can activate naïve B cells, allow them to differentiate into germinal center B cells and plasma cells, as well as induce class switching to different immunoglobulin groups (13).

Treg cells have a clearly defined role in GVHD, where either elevation of their number or augmentation of their suppressive capacity is beneficial in both murine models and human GVHD (21, 31). Tregs can exert their suppressive functions against their target effector T cells by a variety of mechanisms, including: secretion of inhibitory cytokines IL-10 and TGFβ; secretion of cytolytic molecules such as granzymes and perforins; contact-dependent methods including CTLA4-mediated suppression of effector T cells; IL-2 absorption from the immune microenvironment to outcompete effector T cells for this critical growth and survival cytokine (32, 33).

1.4.2 CD8+ T cells

CD8+ T cells play a pivotal role in allo-HCT, as they are highly effective cytotoxic cells that can recognize and eliminate malignant cells that form the primary disease of the patient such

as leukemia or lymphoma. However, CD8+ T cells alone are sufficient to cause GVHD development even in the absence of CD4+ T cells in some cases (34, 35). CD8+ effector T cells—depending on the co-stimulatory signals and cytokine signaling they receive—can produce a variety of cytokines including IFN-γ, IL-4, TNF-α, Granzyme B, perforins, GM-CSF, and IL-17 (36). CD8+ T cells also upregulate a cell-surface receptor programmed cell death protein 1 (PD-1) upon T-cell receptor (TCR) engagement via cognate antigen (37). If the target antigen is able to be successful cleared, then CD8+PD-1+ T effector T cells can reduce PD-1 expression and can convert to memory CD8+ T cells, however, when allogeneic nonhematopoietic antigen from the allo-HCT recipient tissues is continuously present, CD8+ T cells continue to express high levels of PD-1 and become functionally exhausted during acute GVHD (38). While this process is well defined in acute infection and aGVHD, the role of PD-1 expression in cGVHD is less clear. In certain chronic inflammatory diseases, CD8+ T cells that express high levels of PD-1 were found to not exhibit functional exhaustion and were capable of producing high levels of IFN-γ, suggesting that a chronic inflammatory environment may allow for continuous activation and effector function of CD8+ T cells without induction of exhaustion (39).

Similar to CD4+ T cells, CD8+ T cells can also differentiate into a FoxP3+ subset of CD8+ regulatory T cells. CD4+ Tregs are efficient at suppressing effector T cells and reducing GVHD, however this can result in leukemia relapse at the expense of reduced GVHD. However, if both CD4+ Tregs and CD8+ Tregs are used in a combinational therapy, they are effective at reducing GVHD without compromising the GVL effect (40).

1.4.3 B cells

Similar to dendritic cells and macrophage, which are classically viewed as professional antigen presentation cells (APC's), B cells also possess a high capacity to process and present

antigens to cognate T cells during GVHD development. Not only can B cells present antigen to T cells, but upon activation they also can highly express co-stimulatory molecules such as CD86, CD40, and OX40L which deliver powerful activating signals to their cognate T cells (41). Further, similar to different T-cell subsets, B cells can also differentiate into multiple effector subsets. B cells differentiated to produce IFN-γ and IL-12 are defined as B-effector 1 (Be1) and those that produce IL-4, IL-5, and IL-13 are defined as B-effector 2 (Be2). Upon encountering cognate T cells, Be1 or Be2 cells can deliver a combination of co-stimulatory surface receptor signals (e.g., CD86) together with either IFN-γ/IL-12 or IL-4 cytokines to promote differentiation of naïve CD4+ T cells into Th1 or Th2, respectively (42, 43). However, the specific roles of Be1 and Be2 cells in cGVHD still remains to be elucidated.

In addition to their cytokine capacities, B cells can also differentiate into germinal center (GC) B cells which facilitate clonal expansion of effector T cells (29). While there is not a clear consensus for the role of germinal center B cells in cGVHD (13), our group and others have found that they are increased in mice with ongoing moderate to severe cGVHD compared to mice which received BM only as a negative control (14-16). Germinal center B cells themselves may not necessarily be pathogenic or be required for GVHD induction, but they could be an important biomarker for the step between naïve B cell activation and differentiation into plasma cells that are indeed pathogenic in cGVHD. However, germinal center B cells form in the T-cell dependent response which requires co-stimulation from cognate T cells, but it is also possible for plasma cells to differentiate directly from naïve B cells via a T-cell independent response (44). Thus, depending on if the majority of B cells adopt a T-cell dependent or T-cell independent pathway of activation to differentiate into plasma cells may determine whether germinal center B cells play any important role in this process during GVHD.

The role of plasma cells in cGVHD is a relatively novel area of study, however it is now clear that alloreactive antibodies produced by donor-derived plasma cells are pathogenic in

cGVHD. The mechanism of action for the allo-antibodies involves recognition via Fc receptors expressed on recipient macrophage, which induce macrophage polarization into inflammatory subsets that can secrete PDGF and TGF-β1 that led to collagen deposition and fibrosis in cGVHD target organs such as lung and skin (20). Similar to mouse models of cGVHD where it was found that antibodies derived from alloreactive donor B cells can directly exacerbate cGVHD pathogenicity (45), patients with active cGVHD have been shown to exhibit elevated serum concentrations of autoreactive antibodies that could promote pathogenic CD4+ T cell differentiation and survival (46).

1.5 Current Clinical Strategies and Therapies for GVHD: Targeting B cells

Pathogenic B cells in cGVHD have gained a high level of attention in recent years, and therapies which can target B cell activation or function have shown great promise toward the amelioration of cGVHD development in humans. Inhibition of Toll-ligand receptor (TLR) signaling, including TLR7, TLR8, and TLR9 signaling on B cells utilizing chloroquine treatment strategies has shown good results against GVHD development in both mice and humans (47, 48). Intuitive therapy such as targeting the main B-cell surface receptor, CD20, was able to prevent cGVHD development, but was unsuccessful at reversing cGVHD that had already been established (49). A key signaling component of B-cell receptor signaling, spleen tyrosine kinase (Syk), has also been found to be highly activated in B cells of mice and patients with cGVHD, and efforts to target Syk were successful at reducing murine cGVHD (50). Another critical signaling component of the B-cell receptor cascade is Bruton's tyrosine kinase (BTK). Previous research has identified that BTK is required for cGVHD development, as mice with BTK deficient mice failed to develop cGVHD after allo-HCT, and a pharmacological agent, ibrutinib, which could inhibit BTK on B cells, and IL-2 T-cell inducible kinase (ITK) on T cells showed the

ability to reverse already established cGVHD in mouse models (51). Our group also found similar efficacy of ibrutinib in mouse models of cGVHD, but with a stronger effect on cGVHD prophylaxis as opposed to reversal of already established cGVHD (25). Importantly, studies using ibrutinib in pre-clinical mouse models were able to be translated effectively to human clinical trials, where ibrutinib showed a high response rate for reversing already established cGVHD in patients (52, 53).

1.6 IRE-1α/XBP-1 pathway in immune cells

One potential unexplored strategy for combatting cGVHD involves targeting endoplasmic reticulum stress (ER stress) and the unfolded protein response (UPR). This approach has been shown as promising in the treatment of several hematological malignancies including chronic lymphocytic leukemia (CLL), lymphoma, and myeloma (54, 55). The UPR is a mechanism employed by all cells of the immune system in order to cope with cell stress and to avoid stress-induced apoptosis. The three primary regulators of the UPR are IRE-1, PERK, and ATF6. When activated, IRE-1α converts unspliced XBP-1 (XBP-1u) mRNA into spliced XBP-1 (XBP1s) mRNA via its ribonuclease activity. Spliced XBP-1 (XBP-1s) mRNA is a precursor to a potent nuclear transcription factor that is up regulated following the activation of IRE-1α. XBP-1s mRNA is subsequently translated into XBP-1s protein, which acts as a nuclear transcription factor regulating genes for protein folding, protein degradation, and UPR function (56, 57). XBP-1s was thus demonstrated to involved in the development and function of several immune cell subsets including plasma cells (58), dendritic cells (59, 60), and eosinophils (61). However, up until a study conducted by our group (26), there was little to no research elucidating the role of the IRE-1α/XBP-1 pathway in cGVHD. Shortly after the work published by our group on IRE-1α/XBP-1 in cGVHD, a separate group also published similar findings except their study

specifically determined the role of IRE-1α/XBP-1 in aGVHD, and found that targeting the IRE-1α/XBP-1 pathway suppressed human dendritic cell co-stimulation of alloreactive T cells without impairing the GVL effect (62).

1.6.1 XBP-1 in regulation of inflammatory cytokines

Using mice deficient for XBP-1 on hematopoietic cells, a previous study found that XBP-1 was required for optimal production of IL-6 by macrophage in response to TLR2 or TLR4 stimulation. Both IL-6 cytokine and mRNA levels were reduced as a consequence of XBP-1 deficiency, suggesting that XBP-1 regulates IL-6 at the transcriptional level. TNF and IL-1β cytokines also showed similar reductions at the mRNA level due to XBP-1 deficiency. The role of XBP-1 in transcriptional regulation of macrophage cytokines was further confirmed using chromatin-immunoprecipitation (ChIP) which found that XBP-1 was able to effectively bind to gene promoters for *Il6* and *Tnf* following stimulation with TLR agonists (63). A second group also found that in a macrophage leukemia cell line, that the IRE-1α/XBP-1 pathway regulated IL-1β and TNFα cytokine production (64). This phenomenon was not only limited to macrophage, as another group found that leukemic B cells deficient for XBP-1 also showed reduced IL-6 secretion upon stimulation with cognate antigen (65).

1.6.2 Role of XBP-1 in plasma cell development and differentiation

Early reports found that XBP-1s was essential for plasma cell differentiation and function *in vitro*, and that $Rag2^{-/-}$ mice bearing XBP-1 deficiency also had greatly diminished presence of plasma cells (66-69). At first it was believed that accumulation of misfolded proteins, particularly

unfolded IgM, was responsible for induction of IRE-1α activation and XBP-1s up regulation (68). However, later research determined that XBP-1s can be induced independently of accumulation of unfolded proteins and IgM, and is instead important in regulation of BCR signaling and migration of plasmablasts to the bone marrow niche due to failure of CXCL12/CXCR4 signaling events (65).

1.6.3 IRE-1/XBP-1 Regulation of production and secretion of plasma cell antibodies

Upon encounter of T-cell dependent or independent antigens, B cells become activated in order to produce cytokines and antibodies designed to execute antigen clearance. This activation process leads to a large amount of protein synthesis, which if left unchecked, leads to accumulation of unfolded proteins within the endoplasmic reticulum (ER) causing B-cell ER stress that can result in cell apoptosis if the stress is not eventually alleviated (70). It was then found that XBP-1 is one the key factors involved in the UPR to facilitate proper folding and secretion of plasma cell antibodies. The initial paradigm for the role of XBP-1 in the UPR was thus that upon accumulation of unfolded proteins, especially IgM, IRE-1α became activated to splice XBP-1 into its active transcription factor XBP-1s to facilitate the UPR and alleviate the unfolded protein load (58, 66, 67, 69). However, additional findings showed that XBP-1 splicing events preceded accumulation of unfolded proteins and that XBP-1 splicing could occur in the absence of unfolded IgM (65). It was then later determined that lack of XBP-1 was not solely responsible for reduction in secretion of antibodies, but that instead in the absence of XBP-1, IRE-1α becomes hyperactive and exhibits IRE1-dependent decay of mRNA (RIDD) activity that cleaves μ secretory Ig chains. Thus, while XBP-1 is still important for plasma cell differentiation and survival, RIDD activity exerted by activated IRE-1 in the absence of XBP-1 has now been implicated as the mechanism for reduction in secretory immunoglobulin (71-73).

1.6.4 IRE-1α/XBP-1 in T cells

While it was initially found that XBP-1 played no appreciable role in T-cell differentiation or function (66), future studies using conditional XBP-1 KO mice discovered crucial roles for XBP-1 activity in T cells. One of the first groups to undertake research specifically on T-cell XBP-1 showed evidence that CD8+ T cells exhibit significant levels of XBP-1 splicing during bacterial and viral infection (74). It was then eventually discovered using genomic studies that IRE-1α/XBP-1 signaling plays a crucial role in positive regulation of T-helper cell proliferation and response to ER stress by upregulation of cellular components that could relieve stress induced by accumulation of a large amount of secretory proteins (75). Further study into individual T-cell subsets revealed that the IRE-1α/XBP-1 pathway particularly plays an important role in Th2 cell cytokine production and pathogenicity in an allergic airway inflammation model (76). Similar to Th2 cells, another group discovered that XBP-1 was essential for optimal production IL-17 by Th17 cells under conditions of ER stress, and that XBP-1 specific deficiency on T cells and B cells (Rag1 Cre) led to significantly curtailed EAE development which was associated with reduced IL-17 levels found in draining lymph nodes of mice (77). Although research on the role of the IRE-1α/XBP-1 in Treg is sparse, a recent report identified that the ER stress response mediated via IRE-1α is a negative regulator of Treg suppressive function and stability. This report also found that Treg-specific deletion of an E3 ligase, Hrd1, that serves to functionally suppresses the ER stress response, led to spontaneous colitis and multi-organ effector T-cell infiltration and inflammation. The effect on Hrd1 deletion was able to be reversed by blockade of IRE-1α kinase activity using KIRA6, but not blockade of its ribonuclease activity using STF-083010 (78). Although the role of IRE-1α in Tregs was determined by this study, the role of XBP-1 in Treg differentiation and function still remains to be elucidated.

1.7 IRE-1α/XBP-1 in autoimmunity

Evidence for the role of the IRE-1α/XBP-1 pathway in autoimmunity was first described when *in situ* hybridization was utilized to show that the XBP-1 gene was highly expressed within the inflamed synovium of rheumatoid arthritis patients, and that the XBP-1 expression co-localized to infiltrated plasma cells (66). Using an IRE-1α inhibitor, 4μ8C, was then accordingly shown to reduce arthritis in an animal model (79). When conditional KO mice became a more commonly used technique sometime after this initial discovery, the same group used conditional KO mice that were XBP-1 deficient specifically in B cells mediated via XBP-1$^{flox/flox}$ CD19Cre. Using these B-cell specific XBP-1 KO mice, this group induced SLE in either control mice or XBP-1$^{flox/flox}$CD19Cre+ mice sing a peptide immunization model that can cause autoimmune symptoms consisting of autoantibody production and immune complex deposits within kidney glomeruli. It was found using this SLE model that mice with B-cell XBP-1 deficiency developed significantly less immune complexes in both the brain and kidney and had decreased serum anti-dsDNA antibodies compared with XBP-1$^{flox/flox}$CD19Cre- controls (58).

The IRE-1α/XBP-1 pathway has also been shown to be implicated in vitiligo development, where melanocytes treated with phenols that can chemically induce vitiligo were shown to have increased XBP-1s expression and was associated with increased IL-6 and IL-8 cytokine production (80). Further, an increased UPR response and increased IRE-1α/XBP-1 activation as a consequence of reactive oxygen species (ROS) accumulation in keratinocytes of vitiligo patients was associated with an increased production of CXCL16 that enhanced recruitment and skin infiltration of autoreactive CD8+ T cells (81). Similarly, inhibition of IRE-1α/XBP-1 pathway using 4μ8C—an inhibitor of IRE-1α—led to reduced fibrosis of the liver and skin in mouse models of scleroderma, mediated in part through reduction in TGF-β. It was then shown that inhibition of IRE-1α using 4μ8C was able to reduce fibrosis in this scleroderma model (82).

1.8 IRE-1α/XBP-1 in cancer

Targeting the IRE-1α/XBP-1 pathway has been shown to be an exciting strategy for treating multiple myeloma (54) and chronic lymphocytic leukemia (CLL) (55). It was also demonstrated that triple-negative breast cancer (TNBC) relies upon IRE-1α/XBP-1 signaling for a cell-survival adaptation to increased ER stress caused by the cancer microenvironment, and that the increased XBP-1 response correlated with reduced TNBC patient survival (83).

Not only is IRE-1α/XBP-1 signaling important directly in tumor cells, but also in the immune cells that infiltrate the tumor microenvironment to attempt to eliminate the tumor. In this regard, it was recently shown that T cells infiltrating ovarian cancer tumors undergo ER stress and XBP-1 splicing which impaired their ability to uptake glucose in order to produce inflammatory cytokines and eliminate tumor cells. In this study, mice with T cells deficient for XBP-1 had lower tumor volume and significantly increased survival compared to controls (84). Similarly, it was found that tumor resident dendritic cells also undergo significant XBP-1 splicing, and as a result of sustained ER stress accumulate lipid droplets that suppress co-stimulation of tumor infiltrating T cells. siRNA mediated silencing of either IRE-1 or XBP-1 rescued the ability of tumor resident dendritic cells to co-stimulate tumor infiltrating T cells, and significantly extended survival of mice with ovarian cancer (60). Thus, for some types of cancer including multiple myeloma, leukemia, breast cancer, and ovarian cancer, targeting the IRE-1/XBP-1 pathway could be a promising translational strategy to reduce disease burden (85).

1.9 Current Clinical Strategies and Therapies for GVHD: Targeting T cells

Since T cells are a primary driving force in the initiation of GVHD, several therapies which attempt to target T-cell signaling or eliminate alloreactive T cells have been proposed and tested in both murine models and in the clinical setting (86). A promising therapy that has been

implemented in the clinic is administration of antithymocyte globulin (ATG) shortly before or after transplant in order to deplete alloreactive T cells (87, 88). Ablation of alloreactive T cells has also been accomplished using a broad immune suppressant and chemotherapeutic agent, cyclophosphamide (Cy). Post-transplant administration of Cy has thus been shown to reduce GVHD incidence and severity in the clinic due to its suppression of T cells (89-91). However, due to the broad immunosuppressive effects of Cy, especially on activated T cells, concerns of increased infection rates and an intact GVL response have been raised and remain a challenging obstacle to overcome (92).

To avoid the problematic broad immunosuppressive effects imposed by treatments such as Cy, many studies investigating specific T-cell signaling components have been conducted to attempt to reduce T-cell activation without causing excessive suppression and deletion of donor T cells. Therapies currently employed in the clinic to reduce aGVHD severity include Janus kinase 1 (JAK1) and JAK2 inhibitors such as ruxolitinib. One of the main mechanisms of inhibitors targeting JAK signaling is reduction of inflammatory cytokines produced by T cells and dendritic cells (93, 94). However, the pitfall of this particular agent can include development of severe anemia and cytopenia in patients (95).

Tregs are critical negative regulators of GVHD development, thus several strategies have been devised to selectively augment Treg expansion and or function in patients. One such therapy was administration of low-dose IL-2, which allowed preferential expansion of Tregs and showed reduced cGVHD incidence and severity (96, 97).

While implemented primarily as a B-cell targeting therapy through its action on BTK, ibrutinib also inhibits ITK expressed on T cells (51). The dual inhibition of both pathogenic B cells and T cells via ibrutinib could explain the promising results seen in cGVHD patients (53). Indeed, our group found that in experimental settings devoid of B cells using donor cells from B-cell deficient mice, ibrutinib was able to significantly reduce aGVHD and prolong survival,

suggesting that a portion of the mechanism of action also relies on ITK inhibition on T cells in cGVHD (25).

1.10 Fli-1 in cancer and autoimmunity

Given the encouraging results of both pre-clinical and clinical studies involving targeting of specific T-cell signaling pathways in GVHD, insights into novel pathways that regulate alloreactive T cells are highly valuable in the study of GVHD in order to provide improved therapeutics.

One such signaling pathway that may play a key role in alloreactive T cells is the E26 transformation-specific (ETS) transcription factor family member Friend Leukemia Virus Integration 1 (Fli-1). In regard to immune cells, Fli-1 is highly expressed on T- and B-lymphocytes, myeloid cells, and dendritic cells. Research involving Fli-1 has been focused primarily on elucidating its role in Ewing's sarcoma(98, 99), leukemia(100-102), other cancers(103-106), and in systemic lupus erythematous (SLE)(107-110).

Retroviral overexpression of *fli-1* in T cell progenitor cells led to initiation of uncontrolled T-cell proliferation and pre-T-cell lymphoblastic lymphoma mediated by NOTCH-1 mutations in T cells (111). A Fli-1 protein inhibitor, camptothecin, was able to impair tumor growth in multiple erythroleukemia cell lines *in vitro* and in Friend Murine Leukemia virus (F-MuLV) induced erythroleukemia *in vivo*(112). Similarly, global transgenic overexpression of *fli-1* also caused B cell proliferation, autoimmunity, and exacerbated lupus symptoms in mice, which was able to be ameliorated via germ line heterozygous genetic ablation of the *fli-1* gene (107-109).

1.11 Fli-1 in T-cell differentiation and function

While research involving the role of Fli-1 in T cells is limited, some critical reports have identified that Fli-1 is an important regulator of T-cell functions or that it can regulate several target genes within non-T cells that are involved in important T-cell functions.

Fli-1 was identified early to be one of two important candidate genes (the other Ets1) that regulated T-cell fractions in the nonobese diabetic (NOD) mouse model(113). Notably, an early study on Fli-1 also discovered that germ-line heterozygous mutation of Fli-1 resulted in normal thymus development, but homozygous mutation resulted in significantly reduced thymocyte numbers that was attributed to defects in pre-thymic T-cell progenitors (114). Further findings confirmed these studies that Fli-1 may be important in murine T cells and found that retroviral overexpression of *fli-1* in T cell progenitor cells led to perturbed CD4+ and CD8+ populations in the thymus and initiation of uncontrolled T-cell proliferation and pre-T-cell lymphoblastic lymphoma mediated by Notch-1 mutations in T cells. Mechanistically, Fli-1 was shown in this study to be able to directly bind promoter regions in the Notch-1 gene and facilitate its expression (111).

Fli-1 has not been necessarily studied in normal primary mouse T cells to date, but T cells recovered from mice bearing a *Fas* mutation (SLE prone mice) in combination with Fli-1 heterozygous deficiency have been characterized to reveal that Fli-1 plays an important role in several pro-inflammatory T-cell effector molecules CXCR3, IL-6, and C16-Ceramide in T cells that were correlated with lupus (SLE)(115-117). Although not studied specifically in T cells, Fli-1 was found to positively regulate expression of the miR17-92 cluster in small cell lung cancer (118). Although still not known to date in T cells, Fli-1 was shown as a direct negative regulator of SH-2 containing inositol 5' polyphosphatase 1 (SHIP-1) in erythroid cells(119). SHIP-1 itself was shown to be an important inhibitor of PI3K signaling which is implicated in T follicular helper cell (T_{FH}) differentiation(120) and T-cell immunity (121). These previously listed factors

have been shown by our group and others to play important roles in GVHD pathogenesis (122-127).

While nearly all of the studies involving Fli-1 in T cells and immune cells have been performed in mouse models, it was only recently identified that Fli-1 regulates expression of NANOGP8 mRNA and NANOGP8 protein in human Jurkat T-cell leukemia cells, and that shRNA-mediated knockdown of Fli-1 led to reduced NANOGP8 expression as well as reduced proliferation of Jurkat cells (128). Thus, this finding is one of the first to demonstrate that Fli-1 may play an important role in human T-cell proliferation., although earlier studies identified that Fli-1 protein was rapidly down regulated in human T cells upon PMA/Ionomycin stimulation, but returned to baseline levels after 48 hours of stimulation (129).

Despite these previous findings, to date there has been no research that connects Fli-1 as a link between T-cell immune tolerance in the context of allo-HCT and GVHD.

1.12 Role of Fli-1 in non-T cells

1.12.1 Fli-1 in B cells

Research on the role of Fli-1 in B-lymphocytes is also limited, although it has been clearly established that Fli-1 regulates SLE development (130), in which B cells play a crucial role due to autoantibody production. An early study also identified that Fli-1 contributed, together with Erg3, to proper function of the immunoglobulin heavy-chain enhancer region in B cells (131). Using heterozygous Fli-1 deficient mice, a previous report found also that Fli-1 regulated B-cell proliferation in response to B-cell mitogens (132). It was then also discovered that Fli-1

positively regulated several target genes important for B-cell differentiation and function including Igα, Pax-5, E2A, and Egr-1, while negatively regulating Id1 and Id2 (133).

1.12.2 Fli-1 in myeloid cells

A more recent study identified that in mice lacking a key region of the Fli-1 gene, the C-terminal transcriptional activation domain (CTA), both splenic and peripheral blood frequencies of conventional dendritic cells (cDC), plasmacytoid DC (pDC), and macrophage were increased suggesting that the CTA region of the Fli-1 gene negatively regulates monocyte development in mice (134). A previous report also indicated that Fli-1 positively regulated neutrophilic granulocyte and monocyte populations, while negatively regulating the natural killer (NK) population in peripheral blood of mice (135).

1.12.3 Fli-1 in erythrocytes

Fli-1 was shown to be highly expressed in hematopoietic progenitor cells, and was found to be important in the differentiation of erythroblasts the committed erythroid lineages (136-138). Thus, Fli-1 is critically involved in hematopoiesis of the erythroid lineage.

1.13 Fli-1 translocation and oncofusion

1.13.1 EWS/Fli-1

Ewing's sarcoma, a cancer targeting bone development that occurs primarily in pediatric patients, was found to be highly correlated with a genomic translocation of the EWS gene with the Fli-1 gene, leading to the fusion of the two genes that could encode a protein termed EWS/Fli-1 that is implicated in causing the disease (98). Not only did the EWS/Fli-1 oncofusion protein play a pathogenic role in Ewin's sarcoma, but also in the development of myeloid/erythroid leukemia using a Cre mediated transgenic overexpression of EWS/Fli-1 (139).

However, whether this oncofusion protein occurs in settings of allo-HCT and GVHD remains to be explored, and whether functions and targets of the hybrid protein can be compared to the single EWS or Fli-1 proteins individually in lymphocytes is unclear, deserving additional study.

1.14 Fli-1 targeting drugs

1.14.1 Camptothecin

Camptothecin (CPT) was originally discovered and isolated in the 1960's from the bark of the tree Camptoehca acuminate, which is native to China (140). A study from the early 1990's found that CPT was remarkably effective at reducing Friend virus-induced erythroleukemia, such that a single dose of 1mg/kg CPT together with the virus capable of inducing erythroleukemia was able to provide robust protection against disease development

(141). Up until the study performed by Y-J Li et al., 2012, the only mechanism of action known for CPT was inhibition of topoisomerase I , (Top 1) one of six enzymes dedicated to DNA replication (112, 142). CPT was tested in the 1950's through the 1970's in clinical trials for melanoma, stomach, and bladder cancer, in addition to specific types of leukemia (143-146). Clinical trials of the original lactone form of CPT were largely ceased after the 1970's due primarily to the nature of its low water-solubility along with side effects such as myeloid cell suppression, GI tract toxicity, and cystitis (147). However, new derivates of the original form of CPT were designed —irinotecan and topotecan—which showed lower toxicities in patients compared to the original lactone form of CPT and have since been FDA approved for treatment of metastatic colorectal cancer (148, 149).

A relatively recent article by Li et al., 2017 discuss that while successful efforts to develop novel CPT-derivatives for cancer treatment are measured based their ability to effectively inhibit topoisomerase I (Top 1) as the primary mechanism of action, this may not be the most effective strategy (150). This group studied a new CPT-derivative (FL118) that was effective both *in vitro* and *in vivo* against multiple colorectal cancer lines in xenograft models, and that the inhibition of tumor growth was not dependent on Top 1 inhibition, but instead through down regulation of multiple oncogenic genes associated with tumor survival (151). Another group also showed that the CPT-derivative topotecan affected gene expression in neurons due to both Top I dependent and independent mechanisms. Further, Wolff et al., 2018 found that both CPT and the CPT-derivative irinotecan exhibited Top 1-independent mechanism of action against alveolar rhabdomyosarcoma mediated via inhibition of a histone methyltransferase (KMT1A)(152). These studies together suggest that CPT and its derivatives can utilize secondary mechanisms of action besides Top I inhibition in order to reduce tumor growth (153).

Accordingly, a 2012 study by Li et al., found that CPT was able to inhibit the Fli-1 protein dose-dependently beginning at a 50 nM concentration in CB3 mouse erythroleukemia

cells. In the same study, in order to assess the level of specificity of CPT against Fli-1 an erythroleukemia cell line was infected with a Fli-1 overexpressing vector that increased the amount of functional Fli-1 protein present. Using this Fli-1 overexpressing cell line, this study found that it took significantly higher concentrations of CPT to reduce leukemic cell growth in the Fli-1 overexpressing cells compared to empty vector controls, suggesting that CPT at least partially mediated its anti-leukemia effects through Fli-1 inhibition(112). Further, this group identified that the mechanism of Fli-1 inhibition by CPT was not based on transcriptional downregulation of Fli-1, as Fli-1 mRNA was unchanged between vehicle and CPT treated cells, suggesting that CPT regulated Fli-1 protein expression through a post-transcriptional mechanism that remains unknown. Given that some CPT-derivatives are FDA-approved and are in clinical use today, insight into their mechanisms of action beyond Top 1 inhibition would be highly valuable for design and development of future anti-cancer therapies (150).

1.14.2 Etoposide

The study by Li et al., 2012 not only identified CPT as a potent Fli-1 inhibitor, but also found that etoposide was an effective Fli-1 inhibitor in erythroleukemia cell lines, albeit Fli-1 inhibition was demonstrated at a higher IC_{50} compared to CPT (112). Unlike CPT, etoposide is known to be a selective topoisomerase II (Top 2) inhibitor(154). Etoposide is currently FDA-approved for treatment of small cell lung cancer (155).

Thus, while both CPT-derived drugs and etoposide are each used in the clinical setting based on their capacity to inhibit topoisomerase I or II, previous reports (112, 151, 153)—along with our unpublished data studying Fli-1—suggest that these drugs may be utilizing Fli-1 as an important mechanism of action beyond topoisomerase inhibition in order to exert their biological effects.

Chapter 2: Inhibition of the IRE-1α/XBP-1 Pathway Prevents Chronic GVHD and Preserves the GVL Effect in Mice

Note to Reader

Material from this chapter has previously been published elsewhere (Schutt et al., Blood Advances 2018, 2(4): 414-427). For permissions from the publisher, see Appendix B.

2.1 Introduction

Chronic graft-versus-host disease (cGVHD) remains a prominent cause of allogeneic hematopoietic stem cell transplantation (allo-HCT)-related morbidity and mortality even with available treatments. Despite this, the most effective treatment for hematological malignancies including leukemia, lymphoma, and myeloma is allo-HCT. Although there has been progress in understanding acute GVHD development, mechanisms responsible for development of cGVHD are less understood, and remain a major obstacle in providing optimal allo-HCT therapies.

One potential unexplored strategy for combatting cGVHD involves targeting the endoplasmic reticulum (ER) stress response. This approach is promising in the treatment of hematological malignancies (54, 55, 156, 157). The ER stress response is employed by many types of immune cells to cope with cell stress to avoid apoptosis(58, 59, 61, 68, 158-160). The three primary regulators of the ER stress response are IRE-1α, PERK, and ATF6(70). IRE-1α is

particularly critical for the function of plasma cells (56, 57, 161). When activated, IRE-1α converts unspliced XBP-1 (XBP-1u) mRNA into spliced XBP-1 (XBP1s) mRNA via its ribonuclease activity. XBP-1s is subsequently translated into XBP-1s protein, which acts as a transcription factor regulating genes for protein folding, protein degradation, and UPR function (56, 57). Non-canonical functions such as binding to promoters of genes encoding inflammatory cytokines IL-6 and TNF in macrophages demonstrate the multifunctional nature of this protein (63).

Here, we utilize both genetic and pharmacological approaches to uncover the role of the IRE-1α/XBP-1 pathway in pre-clinical mouse models of cGVHD, and present a potential therapeutic strategy to prevent cGVHD that is applicable in patients after allo-HCT.

2.2 Materials and Methods

2.2.1 Mice

Female B10.D2 (H-2^d, CD229.1^-) were purchased from Jackson Laboratory (Bar Harbor, ME); BALB/c (H-2^d, CD229.1^+), B6.Ly5.1 (H-2^b, CD45.1), and B6D2F1 (H-$2^{b/d}$) were purchased from Charles River Laboratories (Wilmington, MA); B10.BR (H-2^k) were purchased from Jackson Laboratory and bred in a specific pathogen free (SPF) facility at the Medical University of South Carolina (MUSC, Charleston, SC). B-cell conditional XBP-1 knock-out (KO) strain (XBP-$1^{fl/fl}$CD19-Cre^+) and littermate wild-type (WT) control strain (XBP-$1^{fl/fl}$CD19-Cre^-) were generated by crossing XBP-$1^{fl/fl}$ mice with CD19-Cre mice on a B6 background described previously (65, 162). Experimental animals were housed in the American Association for Laboratory Animal Care–accredited Animal Resource Center at MUSC (Charleston, SC). All animal experiments were approved by the MUSC Institutional Animal Care and Use Committee.

2.2.2 Allogeneic bone marrow transplantation (BMT)

T-cell depletion (TCD-BM) or T- and B-cell depletion (TBCD-BM) of bone marrow was performed for donor strains as described previously (25). In B6 to BALB/c and B10.D2 to BALB/c models, recipients were monitored with cGVHD clinical scoring system described previously (25). On day 30 or 60 post-transplant, recipient spleens and trunk-skin were collected for flow cytometry analysis, and skin paraformaldehyde fixed and sectioned for H&E staining. An independent pathologist scored skin sections for cGVHD as described previously (25). In B6 to B10.BR model, recipients were given 120 mg/kg intraperitoneal (i.p.) cyclophosphamide on days -3, -2 and sub-lethal irradiation (700cGy, X-ray source) on day -1 prior to bone marrow transplantation (BMT) on day 0 as described previously (29). Unfractionated splenocytes from either XBP-1 WT or XBP-1 KO donors were pooled with respective TCD-BM from each strain and injected IV at a dose of 5 x 10^6 TCD-BM plus 0.15 x10^6 splenocytes per mouse. B6 to B6D2F1 acute GVHD model was described previously (163).

2.2.3 Treatment with B-I09

B-I09, a small-molecule inhibitor for the IRE-1α RNase, was developed and tested for inhibiting the expression of XBP-1s in vitro and in vivo, described previously (55). B-I09 was dissolved in Dimethyl sulfoxide (DMSO) at a concentration of 50-100 μM, syringe filter sterilized, aliquoted and stored at -80^{o}C. Aliquots were brought to 3.33 mg/ml with 1X phosphate buffered saline (PBS) and animals were injected i.p. with 150 μl per mouse (25 mg/kg) 2-4 hours prior to allo-BMT or on day-21 after BMT for treatment, and followed with once daily injections until day-21 or day-42, respectively.

2.2.4 GVL response

The blast-crisis chronic myelogenous leukemia (BC-CML) model was generated previously (164, 165) and was a gift from Dr. Shlomchik's lab. B10.D2 donor-grafts were given to lethally irradiated BALB/c recipients with or without BC-CML cells at a dose of 1 x 10^6 BC-CML cells per mouse together with TCD-BM (5 x 10^6 per mouse) with or without whole splenocytes (5 x 10^6 per mouse). Mice given TCD-BM plus BC-CML cells treated with either vehicle or B-I09 alone without splenocytes were used as controls. Peripheral blood from recipients was collected periodically starting 14 days after transplant, and splenocytes collected on day-60 and analyzed via flow cytometry for expression of $CD11b^+$ and GFP^+ BC-CML cells.

2.2.5 FlexiVent pulmonary function tests

B10.BR recipients were anesthetized with isoflurane, weighed, and subjected to tracheostomy in order to evaluate lung function using a flexiVent device (SciReq, Montreal, QC, Canada) as described previously (29). Mice were ventilated at a tidal volume of 10 ml/kg body weight, a positive end-expiratory pressure of 3 cmH2O, and 150 breaths/minute (default). Spontaneous respiration was suppressed by intraperitoneal injection of pancuronium bromide (0.08 mg/kg) and maintenance of the mice on 5% isoflurane. Compliance (Crs; ease of lung extension), airway resistance of the respiratory system as a whole (Rrs; level of lung constriction) as well as quasi-static compliance (Cst; pressure of the lungs at a given volume), alveolar constriction (G; energy required to expand lung tissue), and elastance (H; rigidity or stiffness) of the lungs from each animal were determined using the Mouse Mechanics Scan script and flexiWare software version 7.6.

2.2.6 Flow cytometry and serum IgG detection

Splenocytes were analyzed for surface proteins and intracellular cytokines using standard flow cytometric protocols as previously described (25, 166, 167). Protocol for isolation of lymphocytes from lungs and trunk skin was adapted from a previous publication (168). The following Abs were used for staining: Fixable Live/Dead yellow (BD Biosciences), anti-TCRβ-FITC or -PE-Cy7 (Clone: H57-597), anti-CD4–FITC, or –V450 (RM4-5), anti-CD8α–FITC, or –allophycocyanin-cy7 (53-6.7), anti-PD-1-PE or -PerCpCy5.5 (J43), anti-CXCR5-PE-Cy7 (SPRCL5), anti-B220–V450 (RA3-6B2), Annexin V-PE and 7AAD Apoptosis staining kit (BD Biosciences), anti-CD80–PE-Cy7 (16-10A1), anti-CD86–PE-Cy5 (GL1), anti-Fas (Jo2), anti-GL7-Alexa 647 (GL7), anti-IgM-PE-Cy7 (RMM-1), anti-CD229.1–Biotin or -PE (30C7), anti-CD11c-PE-Cy7 (N418), anti-CD11b-PerCpCy5.5 (M1/70) purchased from BD Biosciences, anti–IFN-γ–PE or -Per-cp5.5 (XMG1.2; BD Biosciences), anti–IL-12p35–eflour660 (4D10p35) and IL-12p40 Biotin (eBioscience), and the appropriate unstimulated controls to guarantee intracellular staining specificity. Cell isolates were analyzed using Diva software, LSR II (BD Biosciences, San Jose, CA), and FlowJo (TreeStar, Ashland, OR). Enzyme-linked immunosorbent assay (ELISA) using an IgG capture antibody (Biolegend) or dsDNA coated plates to measure serum total IgG and anti-dsDNA IgG antibodies was carried out as described previously (25).

2.2.7 Statistics

Data presentation and statistical analyses were carried out using Prism 6 software (GraphPad) and graphed as mean±SEM. For cGVHD clinical scores, all animals were examined for cGVHD clinical signs on day 0 as a reference point, and non-reference point clinical score data were analyzed using a Mann-Whitney test to determine any statistical significance between

groups ($p<0.05$), or an unpaired two-tailed Student's *t* test was used to analyze a single time point at the experiment endpoint. For all other data, differences among experimental groups were compared using an unpaired two-tailed Student's *t* test to determine any statistical significance ($p<0.05$) between two groups, or when comparing more than two groups a One-way ANOVA using Bonferroni multiple comparison's test was performed to determine any statistical significance ($P<0.05$) unless otherwise stated.

2.3 Results

2.3.1 B-cell specific XBP-1 impairs pulmonary function in a bronchiolitis obliterans (BO) model of cGVHD

XBP-1 regulates processes in B cells such as BTK phosphorylation, BCR activation, and plasma cell development (55, 58, 65, 68, 169) which are implicated in pathogenesis of cGVHD.(25, 45, 51, 170, 171) We hypothesized conditional deletion of XBP-1 on donor allogeneic B-lymphocytes would impair B-cell activation and development of cGVHD. To test this hypothesis, we utilized a BO model of pulmonary cGVHD (B6 to B10.BR) mediated by donor T cells and antigen presenting cells (APC's).(29) Recipients transplanted with allogeneic grafts from B-cell specific XBP-1 conditional knock-out (XBP-1 KO) donors displayed less body weight loss compared to those that received wild-type (XBP-1 WT) grafts at several time points **(Fig. 1A)**. The recipients of XBP-1 KO donor-grafts also developed reduced pulmonary cGVHD compared to those of XBP-1 WT donor-grafts as indicated by measurements of pulmonary mechanics. The recipients of XBP-1 KO grafts demonstrated significantly decreased respiratory resistance and increased lung compliance **(Fig. 1B,C)**. These recipients also displayed increased static compliance, reduced alveolar constriction, and reduced lung elastance indicating less injured lungs compared to those of XBP-1 WT grafts **(Fig. 1C)**. In agreement with previous

reports, at baseline we characterized the B-cell specific XBP-1 KO and XBP-1 WT donor cells and found no gross differences or abnormalities in the B-cell compartment (data not shown).(55, 58, 65) Collectively, these data indicate that B-cell intrinsic XBP-1s plays a pathogenic role in the development of pulmonary cGVHD.

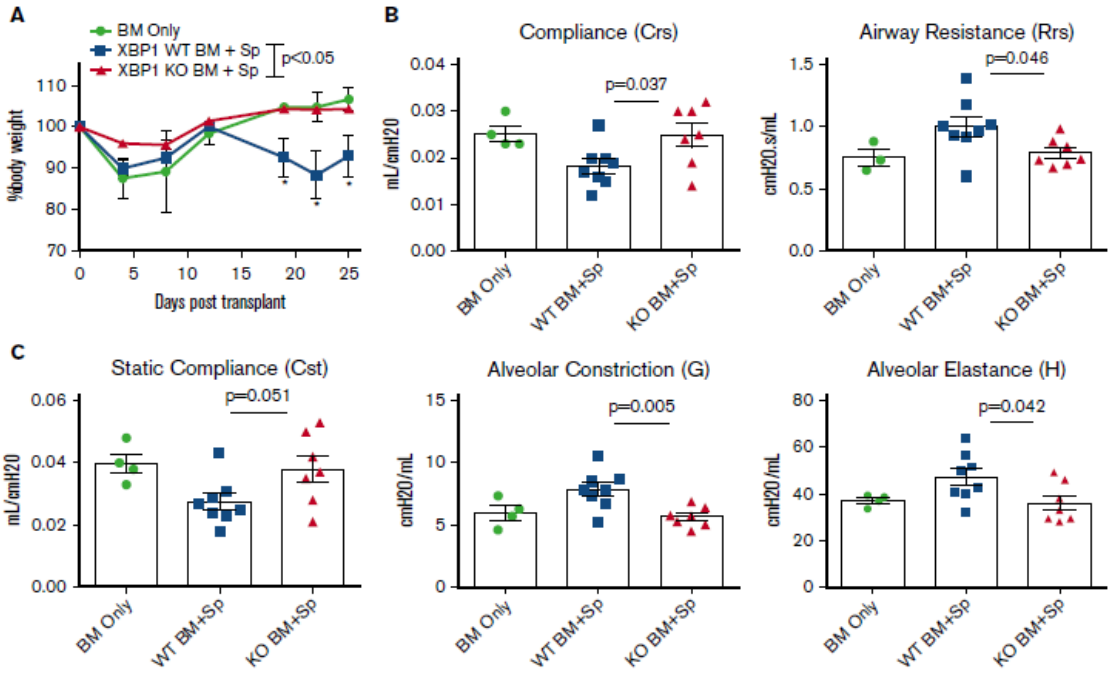

Figure 2.1 B-cell specific XBP-1 impairs pulmonary function in a BO model of cGVHD.

B10.BR recipients were treated twice with cyclophosphamide (120mg/kg) 1x per day on days -3,-2 and sub-lethally irradiated on day -1 before transplant. On day 0, mice were transplanted with TCD-BM (5 x 10^6 per mouse) from XBP-$1^{flox/flox}$CD19Cre- (n=10) or XBP-$1^{flox/flox}$CD19Cre+ donors (n=9) on a B6 background with (n =19) or without (n = 4) whole splenocytes at 0.15x10^6 per mouse. Mice were monitored for body weight loss until experiment endpoint **(A)**, where recipients were anesthetized, given tracheostomy, and subjected to lung function analysis using a SciReq flexiVent device 28 days after transplant **(B-C)**. Data from B-C are pooled from two separate experiments. Panel A shows representative body weight loss from two replicate experiments. Statistics were performed using two-tailed Student's *t*-tests of each indicated time-point where a p value of <0.05 indicates statistical significance and an asterisk indicates statistical significance between XBP-$1^{flox/flox}$CD19Cre- and XBP-$1^{flox/flox}$CD19Cre+ groups: *p<0.05.

2.3.2 XBP-1 decreases B-cell recovery and promotes the TFH cell subset after allo-BMT

We further investigated potential mechanisms of the impact of XBP-1 on cGVHD using the B6 to B10.BR model. On day 28 after allo-BMT, recipients transplanted with XBP-1 KO donor-grafts had higher frequencies of donor-derived B cells in the spleens **(Fig. 2A-B)**, which correlated with reduced frequencies of the splenic follicular helper T-cell (T_{FH}) cell subset **(Fig. 2A,C)**. Isolated lung lymphocytes showed that recipients of XBP-1 KO grafts had significantly increased frequencies of donor-derived B cells in the lungs compared to XBP-1 WT recipients **(Fig. 2D-E)**. The XBP-1 KO donor B cells within the recipient lungs were also characterized by significantly lower frequencies of apoptotic markers Annexin V and 7AAD compared to donor XBP-1 WT B cells **(Fig. 2D,F)**. Together, these results indicate that XBP-1 contributes to impaired reconstitution of B cells in lymphoid and non-lymphoid organs after allo-BMT, at least in part due to apoptosis of B cells in non-lymphoid organs such as the lungs. Furthermore, deficiency of XBP-1 in B cells also reduced differentiation of T_{FH} cells, a subset attributable to cGVHD pathogenesis.(24, 27, 28)

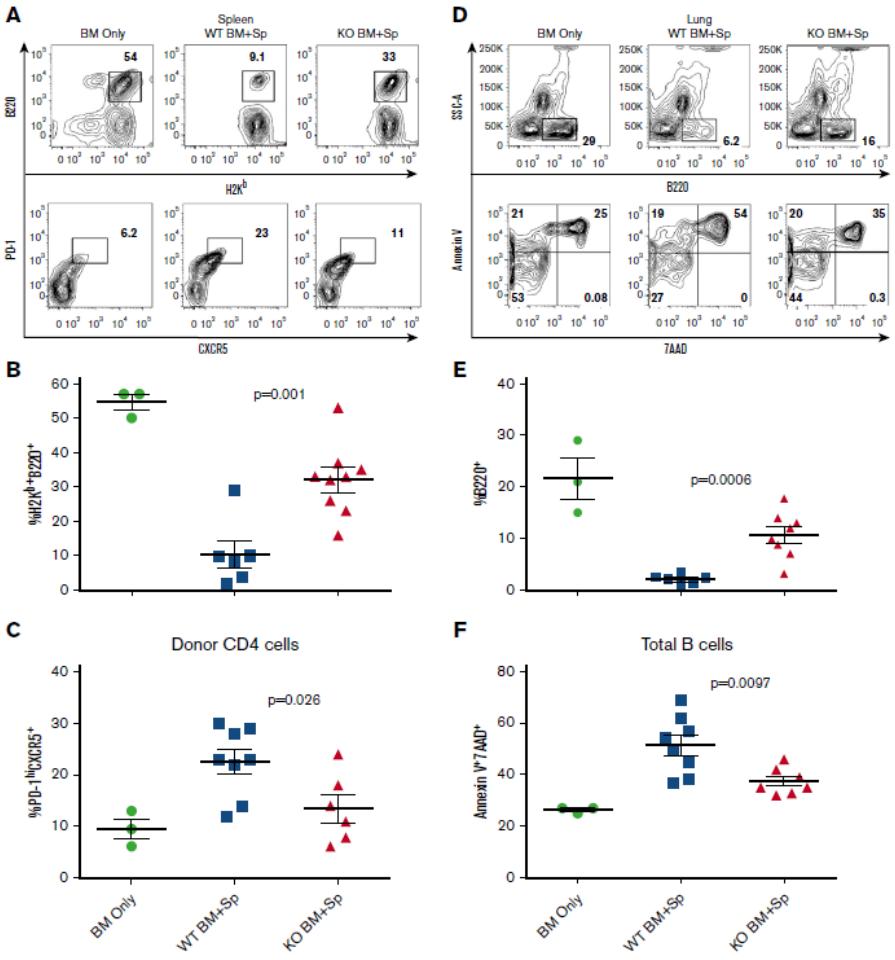

Figure 2.2 XBP-1 decreases B-cell recovery and promotes the T_{FH} cell subset after allo-BMT. B10.BR recipients were treated twice with cyclophosphamide (120mg/kg) 1x per day on days -3,-2 and sub-lethally irradiated on day -1 before transplant. On day 0, mice were transplanted with TCD-BM (5 x 10^6 per mouse) from XBP-$1^{flox/flox}$CD19Cre- (n=10) or XBP-$1^{flox/flox}$CD19Cre+ donors (n=9) on a B6 background with (n =19) or without (n = 4) whole splenocytes at 0.15x10^6 per mouse. On day 28 after allo-HCT, recipient mice were euthanized and spleens excised and processed into single-cell suspensions for flow cytometry analysis of $B220^+$ B cells **(A-B)** and donor $CD4^+$ T_{FH} cells ($PD\text{-}1^{hi}CXCR5^+$) **(A,C)**. Lungs were excised and lymphocytes were isolated and stained for $B220^+$ B cells **(D-E)** and apoptotic B cells (Annexin V^+7AAD^+) **(D-F)**. Data shown in A,D are individual flow cytometry plots representative of each group. Data shown in B,C,E,F are pooled from two replicate experiments. A p value of <0.05 indicates statistical significance.

2.3.3 XBP-1 mediates B-cell activation and cGVHD pathogenicity

We utilized a previously described second model of cGVHD (B6 to BALB/c) to further investigate the role of XBP-1 in cGVHD.(25, 172) Recipients given XBP-1 KO

donor-grafts developed significantly reduced cGVHD compared to those that received XBP-1 WT donor-grafts indicated by cGVHD clinical scores **(Fig. 3A)**. Within the clinical scores, recipients of XBP-1 KO donor-grafts showed reduced diarrhea, eye inflammation and conjunctivitis, and improved posture and fur integrity. Similar to B6 to B10.BR model, XBP-1 KO recipients displayed a significant increase in the frequency of splenic B cells compared to XBP-1 WT donor-graft recipients 30 days after BMT **(Fig. 3B)**. In XBP-1 KO donor-graft recipients, B cells displayed significantly lower expression of CD86 **(Fig. 3C)** and lower percentage of $Fas^{+}GL7^{+}$ cells **(Fig. 3D-E)**, suggesting a reduction in costimulatory activity and germinal center (GC) B cells compared to those of XBP-1 WT B cells, respectively. B cells in recipients of XBP-1 KO grafts expressed significantly higher levels of surface IgM compared to those in XBP-1 WT recipients **(Fig. 3D,F)**. We then measured serum autoantibodies against double-stranded DNA (dsDNA) via ELISA, and recipients of XBP-1 KO grafts had significantly decreased levels of serum anti-dsDNA IgG compared to those of WT grafts **(Fig. 3G)**. B cells in the recipients of XBP-1 KO grafts produced significantly lower levels of IL-12p35 **(Fig. 3H-I)** and IFN-γ **(Fig. 3H,J)** compared to B cells from those of XBP-1 WT grafts. Taken together, these data indicate that cGVHD was reduced in the absence of XBP-1 in donor B cells, and that B-cell intrinsic XBP-1 contributes to a diverse repertoire of B-cell functions, such as activation, differentiation, and cytokine production in the context of allo-BMT.

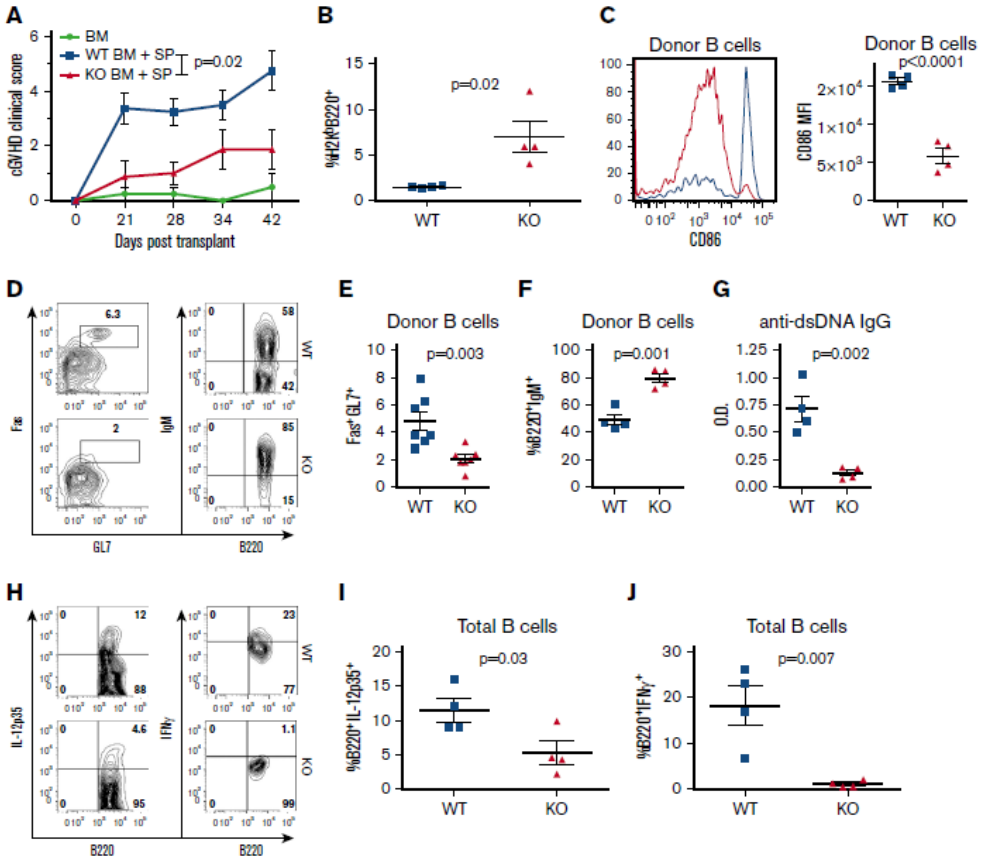

Figure 2.3 XBP-1 mediates B-cell activation and cGVHD pathogenicity. Lethally irradiated BALB/c mice were transplanted with TCD-BM (5 x 10^6 per mouse) from XBP-$1^{flox/flox}$CD19Cre- (n=21), XBP-$1^{flox/flox}$CD19Cre+ (n=15), or TBCD-BM B6Ly5.1^+ (n=10) donors on a B6 background with (n=40) or without (n=6) between 0.5-1x10^6 whole splenocytes. Mice were monitored for cGVHD clinical scores until day 60 after allo-HCT and statistics were performed using a Mann-Whitney test of the entire time-course **(A)**. Subsets of recipient mice were euthanized on day 30 and spleens excised and processed into single-cell suspensions for flow cytometric analysis of $B220^+$ B cells **(B)**, CD86 expressing B cells **(C)**, germinal center B cells (Fas^+GL7^+) **(D-E)**, and B-cell surface IgM **(D,F)**. Serum from peripheral blood was collected on day 30 and assayed for anti-dsDNA autoantibodies using ELISA **(G)**. On day 30, 2x10^6 recipient splenocytes from each mouse were stimulated with LPS, PMA, and Ionomycin and B cells were intracellularly stained and analyzed for IL-12p35 and IFNγ cytokine production **(H-J)**. Data shown in A is representative clinical scoring of 3 replicate experiments. Data in B-D and F-J was collected from one subset of mice (n=10) out of n=40 recipients. Data in E is pooled from two independent experiments. A p value of <0.05 indicates statistical significance.

2.3.4 B-cell XBP-1 modulates T-cell activation during cGVHD

We then asked whether or how XBP-1 deficiency in B cells impacts T cells during cGVHD development using the B6 to BALB/c cGVHD model. Corresponding with our long-term data, mice that received XBP-1 KO donor-grafts had significantly lower cGVHD clinical

scores compared to those that received B-cell XBP-1 WT grafts **(Fig. 4A)**. This result was associated with reduced surface expression of PD-1 on donor CD8 T cells compared to those from the recipients of XBP-1 WT grafts **(Fig. 4B-C)**. Recipients of XBP-1 KO grafts also had significantly lower frequencies of donor-derived IFN-γ-producing CD4 **(Fig. 4B,D)** and CD8 **(Fig. 4B,E)** T cells in their spleens compared to those of XBP-1 WT grafts. In agreement with our data from the B6 to B10.BR model, frequencies of the T_{FH} cell subset were also reduced in the recipients of XBP-1 KO grafts compared to those of XBP-1 WT grafts **(Fig. 4F)**.

In acute GVHD setting, transplant of XBP-1 KO B cells, relative to XBP-1 WT B cells, was associated with significant reduction of IFNγ-producing $CD4^+$ and $CD8^+$ cells and IL-17A-producing $CD4^+$ T cells in the spleens of mice given acute GVHD in B6D2F1 recipients. However,T cells in mesenteric lymph nodes or gut were unaffected and more importantly acute GVHD pathology was not impacted **(Fig. S1A)**, consistent with literature showing that B cells do not significantly contribute to acute GVHD.(46) By day-14 post-transplant, no death was observed in any groups tested, and body weight loss was comparable between the recipients with WT versus KO grafts, **(Fig. S1B)**. Collectively, these data suggest the ER stress response mediated by XBP-1 specifically in B cells can significantly alter the activation status of both $CD4^+$ and $CD8^+$ T cells as well as differentiation of T_{FH} cells during chronic GVHD, but not necessarily in acute GVHD.

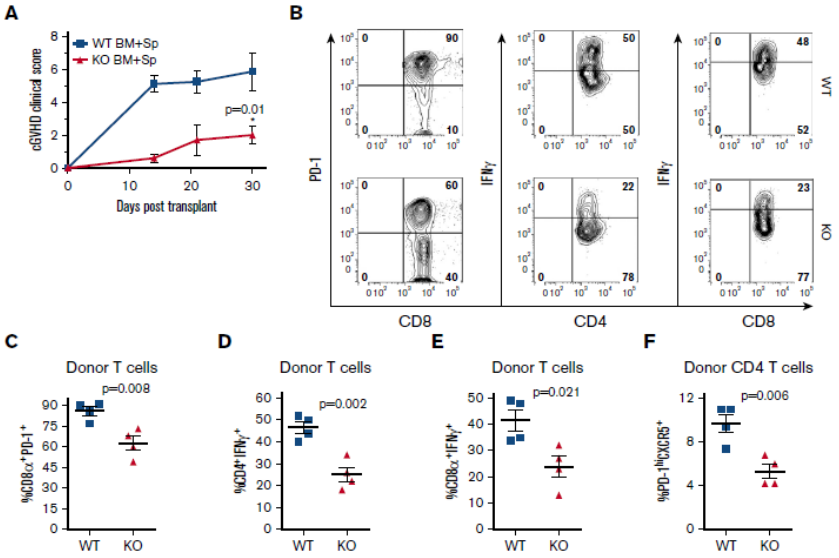

Figure 2.4 B-cell XBP-1 modulates T-cell activation during cGVHD. Lethally irradiated BALB/c mice were transplanted with T and B-cell depleted bone marrow (TBCD-BM) (5 x 10^6 per mouse) from B6Ly5.1$^+$ mice (n=10) with 1x10^6 whole splenocytes from XBP-1$^{flox/flox}$CD19Cre- (n=5) or XBP-1$^{flox/flox}$CD19Cre+ (n=5), donors on a B6 background. Mice were monitored for cGVHD clinical scores after allo-HCT **(A)** (indicated statistical significance is a two-tailed Student's *t* test performed for the day 30 time-point). The recipient mice were euthanized on day 30 and their spleens excised and processed into single-cell suspensions for direct flow cytometric analysis of donor H2K^b PD-1 expressing CD8$^+$ T cells **(B-C)**, or were stimulated with LPS, PMA, and Ionomycin to detect IFNγ producing CD4$^+$ **(B,D)** and CD8$^+$ T cells **(B,E)**. Data in A-F was collected from one cohort of mice (n=10). A p value of <0.05 indicates statistical significance.

2.3.5 Pharmacological inhibition of IRE-1α/XBP-1 prevents cGVHD

We then tested the ability of a potent IRE-1α inhibitor, B-I09, to prevent cGVHD. B-I09 blocks RNAse activity of IRE-1α and prevents its ability to splice XBP-1 mRNA into the active XBP-1 transcription factor.(55) We tested this inhibitor as a prophylactic in MHC-mismatched BMT-model (B6 to BALB/c), and as both a prophylactic and a treatment in MHC-matched BMT- model of cGVHD (B10.D2 to BALB/c), where skin damage is the most prominent feature. We found that recipients prophylactically injected with B-I09 exhibited significantly reduced cGVHD clinical scores compared to those injected with vehicle in both MHC-mismatched **(Fig. 5A)** and MHC-matched BMT models **(Fig. 6A)**. Within the clinical scores, the recipients treated with B-I09 showed

reduced skin damage, diarrhea, eye inflammation and conjunctivitis and improved fur integrity and mobility. Although Th1 and Th17 cells in the spleens and peripheral lymph nodes were not impacted (data not shown), antibody-secreting plasma cells were significantly reduced in recipient spleens by B-I09 prophylaxis compared with vehicle controls in B6 to BALB/c model as measured by surface syndecan-1 (CD138) and intracellular IgG1 on day-50 after BMT **(Fig. 5B-C)**. Images showing disease status in mice prophylactically treated with vehicle or B-I09 using the B10.D2 to BALB/c model were taken on day-45 post-transplant **(Fig. 6B)**. On day-60 after allo-BMT, skin biopsies were excised for pathologic analysis **(Fig. 6C)**, indicating that recipients treated with B-I09 displayed significantly reduced skin pathology scores consisting of reduced epidermal thickening, fat loss, inflammation, follicular loss, and dermal fibrosis parameters compared to vehicle recipients **(Fig. 6C,D)**. Although the use of B-I09 was highly effective at preventing cGVHD when used as a prophylactic, a delayed treatment strategy was not able to ameliorate already established cGVHD when treatment was initiated at day 21 post-transplant **(Fig. 6A)**. We then found that the recipients given B-I09 prophylaxis had significantly lower frequencies of donor-derived $CD4^+$ cells (**Fig. 6E-F**), $CD8^+$ cells (**Fig. 6E,G**), and $CD11c^+$ cells in their skin compared to those treated with vehicle (**Fig. 6E,H**). In agreement with our data from using B-cell conditional KO mice (**Fig. 4**), splenic donor $CD8^+$ T cells from recipients treated with B-I09 displayed reduced PD-1 expression on day 60 compared to those treated with vehicle control, although not statistically different **(Fig. 6I)**. Collectively, these results indicate that targeting the IRE-1α/XBP-1 pathway pharmacologically is able to prevent development

of sclerodermatous cGVHD, primarily through the reduction of infiltration of donor T cells and DCs into the skin.

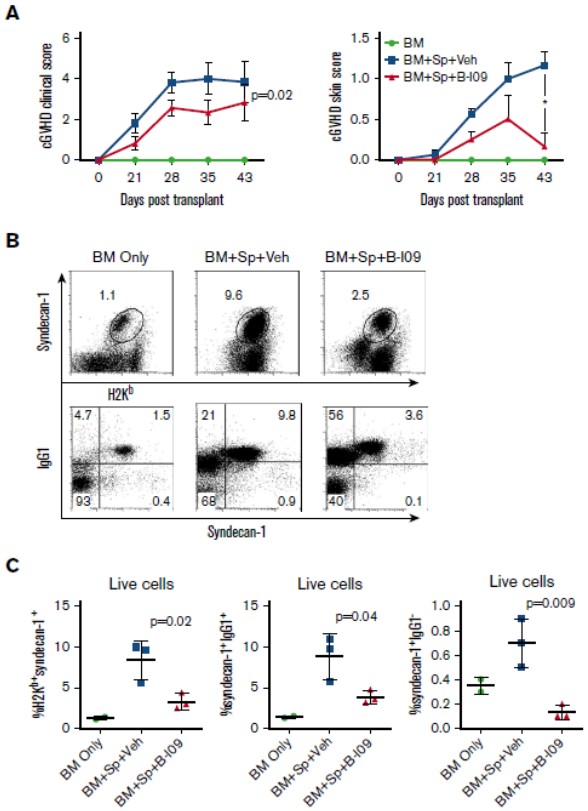

Figure 2.5 Pharmacological inhibition of IRE-1α/XBP-1 prevents cGVHD in MHC-mismatched BMT model. B6 to BALB/c BMT was carried out as in figure 3. The recipients were left without treatment (n=4) or i.p. injected daily with DMSO vehicle or with B-I09 at a dose of 25 mg/kg starting on day 0 and continued for 3 weeks (n=8 per group). BMT recipients (n=19) were monitored for cGVHD clinical scores and skin scores **(A)**, and euthanized on day 30 (n=4 per group) or 50 (n=4 per group) after transplant and spleens were subjected to flow cytometric analysis for surface expression of syndecan-1 and intracellular IgG1. Representative flow plots from day 50 for each group are depicted in **(B)**. Data from each group is quantified in **(C)**. Overall cGVHD clinical scores for the entire experimental time-course were analyzed using a paired t test. For skin cGVHD clinical scores, a two-tailed Student's *t*-test was performed for the final time-point. A p value of <0.05 indicates statistical significance. Data were collected from n=19 mice from one representative experiment.

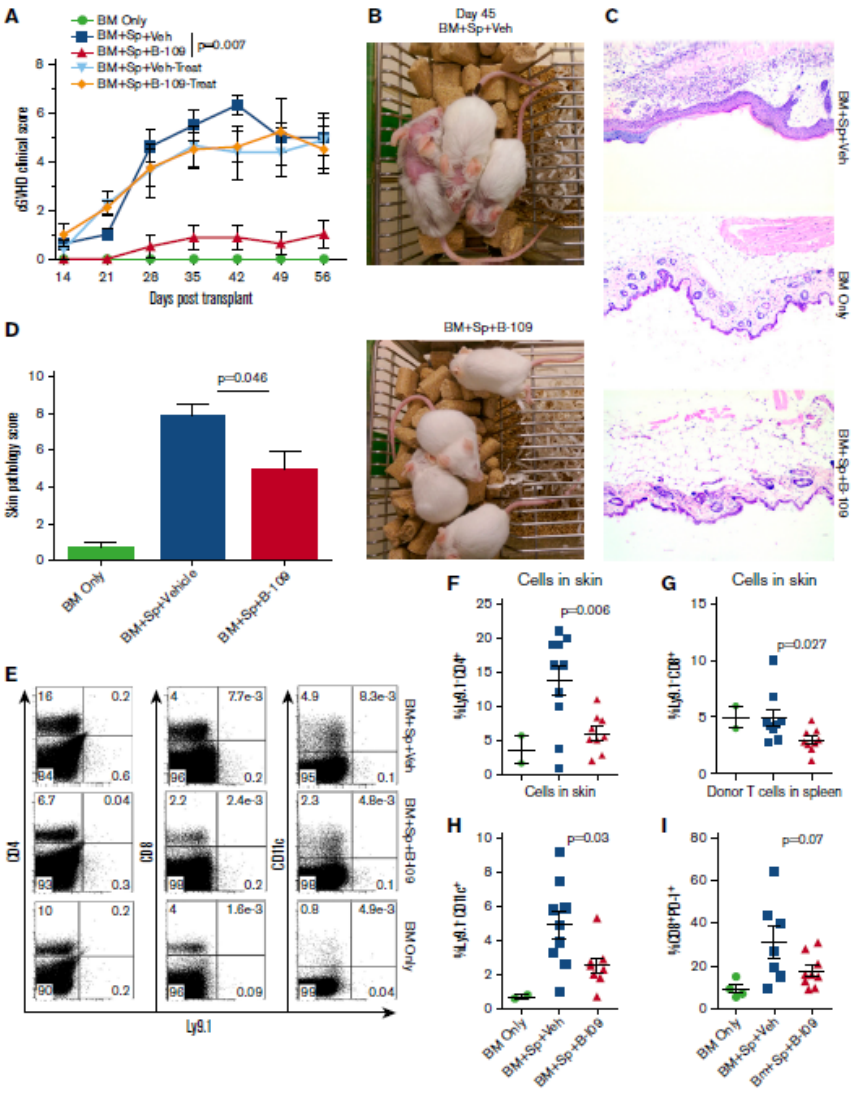

Figure 2.6 Pharmacological inhibition of IRE-1α/XBP-1 prevents cGVHD in MHC-matched BMT model. Lethally irradiated BALB/c recipients were transplanted with TCD-BM ($5x10^6$/mouse) from B10.D2 donors with (n=30) or without (n=4) whole splenocytes at $5x10^6$/mouse. Groups were either given no treatment (n=4), daily i.p. injection of DMSO vehicle alone starting on day 0 and continued for 3 weeks (Days 0-21 n=10) or beginning on day 21 (Days 21-42 n=5), or were i.p. injected with B-I09 at a dose of 25 mg/kg beginning at day 0 and continued for 3 weeks (Days 0-21 n=10) , or beginning on day 21 (Days 21-42 n=5). Recipient mice were monitored for cGVHD clinical scores **(A)** until experiment endpoint on day 60. On day 45, images were taken of prophylactically treated vehicle and B-I09 groups **(B)**. Skin biopsies were sectioned and stained with H&E **(C)** and analyzed by an independent pathologist for signs of cGVHD skin damage **(D)**. On day 60, mice were euthanized and spleens and trunk skin were excised for processing into single cell suspension for flow cytometric analysis of donor $CD229.1^-$ ($Ly9.1^-$) CD4, CD8, and CD11c lymphocyte skin infiltrates **(E)**. Quantification of CD4 **(F)** CD8 **(G)** and CD11c **(H)** cells in skin are shown. Splenic donor $CD8^+$ T cells were analyzed via flow cytometry for expression of PD-1 **(I)**. Data shown in A-C are representative of two separate experiments. Data shown in D are pooled from two replicate experiments. A p value of <0.05 indicates statistical significance. Data in E are representative flow plots from two separate experiments which are quantified as pooled data in F-I. Statistical analysis of cGVHD clinical scores was performed using a Mann-Whitney test of the entire experimental time-course. A p value of <0.05 indicates statistical significance.

2.3.6 XBP-1 is required for B-cell activation and differentiation

To further ask how XBP-1 affects B-cell activation and differentiation in general, B cells were activated with LPS + IL-4 *in vitro*. B cells deficient for XBP-1 differentiated into $Fas^{+}GL7^{+}$ GC B cells at a significantly lower frequency (**Fig. S2A-B**) and expressed lower CD86, ICOS-L, and MHCII (**Fig. S2A-C**) as compared to WT B cells. Inhibition of XBP-1 with B-I09 also reduced GC B-cell differentiation and expression of costimulatory molecules although less profound than XBP-1 deficiency (**Fig. S2A-B**). Consistent with our observation *in vivo* (**Fig. 3**), XBP-1 KO B cells secreted significantly lower total IgG antibodies into cell culture media, which was phenocopied by XBP-1 inhibition with B-I09 (**Fig. S2D**). Furthermore, both XBP-1 deficiency and treatment with B-I09 impaired the ability of B cells to produce IL-4/5 and IL-12p40 cytokines after stimulation when compared to XBP-1 WT B cells or vehicle controls (**Fig. S2E-F**). Collectively, these *in vitro* studies suggest that XBP-1 plays a critical role in B-cell activation and differentiation into cytokine-secreting or GC B cells, and that B-I09 can phenocopy multiple aspects of XBP-1 deficiency in B cells.

2.3.7 Prophylactic B-I09 administration does not impair the GVL effect

Here, we utilized a transplantable blast-crisis chronic myelogenous leukemia (BC-CML) model to test the impact of B-I09 administration and XBP-1 inhibition on the GVL effect. In this model, allogeneic T cells contained in donor B10.D2 splenocytes mediate an anti-tumor response against tumor cells expressing disparate minor histocompatibility antigens as the host (BALB/c).(164) As expected, recipients that received allogeneic BM

alone without splenocytes developed GFP-expressing CML cells in both vehicle and B-I09 treated conditions **(Fig. 7 A-B)**. The recipients that received allogeneic BM plus splenocytes and treated with vehicle control did not display any signs of tumor growth. Similarly, the recipients that received allogeneic BM plus splenocytes and treated with B-I09 displayed potent anti-tumor responses with no evident impairment of GVL activity in peripheral blood measured until day-35 **(Fig. 7 A-B)** or in spleen measured on day-60 **(Fig. 7C)**. Although B-I09 treatment alone without splenocytes did not suppress CML growth in peripheral blood or the spleens, these recipients did not succumb to tumor mortality compared to recipients of vehicle alone without splenocytes which displayed 33% tumor mortality (data not shown). To test how XBP-1 affects B-cell mediated antigen presentation, we compared ability of XBP-1 WT versus KO B cells to stimulate allogeneic T cells *in vitro*. Under this condition, XBP-1 in B cells had no effect on T-cell proliferation, activation, and cytolitic potential (Granzyme B) **(Fig. S3)**. These data support that inhibition of the IRE-1α/XBP-1 pathway may not impair the GVL effect.

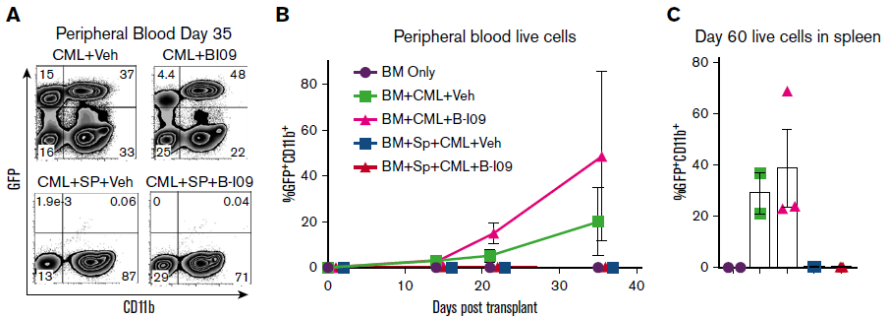

Figure 2.7 Prophylactic B-I09 administration does not impair the GVL effect. Lethally irradiated BALB/c recipients were transplanted with TCD-BM from B10.D2 donors with (n=20) or without (n=13) whole splenocytes. Mice were also co-transplanted with either no blast crisis chronic myeloid leukemia cells (BC-CML) (n=4), or $1x10^6$ BC-CML splenocytes (n=29). Groups subsequently either received no treatment (n=4), daily i.p. injection of DMSO vehicle (n=16), or 25 mg/kg B-I09 (n=13) on day

0 and continued for 3 weeks. Peripheral blood was collected and analyzed for GFP^+CD11b^+ expressing BC-CML cells via flow cytometry **(A)** periodically after transplant until day 35, and is shown as a time-course of GFP^+CD11b^+ cells in each group **(B)**. On day 60, mice were euthanized and splenocytes were analyzed for presence of BC-CML cells indicated by GFP^+CD11b^+ expression **(C)**. Flow cytometry plot from A is representative of 2 replicate experiments. Data from B and C are pooled from two replicate experiments.

2.4 Discussion

Here, targeting XBP-1 specifically in B cells using a conditional knock-out strategy was sufficient to prevent cGVHD in two pre-clinical mouse models. Prophylactic administration of an IRE-1α/XBP-1 pathway inhibitor (B-I09) during allo-BMT prevented cGVHD development in both MHC-matched and MHC-mismatched cGVHD models with minimal toxicity. *In vitro* assays revealed that XBP-1 mediates optimal TLR4 signaling in B cells and is required for generation of IL-12p40-producing B effector 1 cells and IL-4/5-producing B effector 2 cells in response to LPS and IL-4 stimulation.

Reduction of cGVHD in recipients of XBP-1 KO donor-grafts was characterized by improved pulmonary function tests suggesting XBP-1 contributes to pulmonary fibrosis. Lung trichrome staining showed no significant differences in Type I collagen deposition between groups (data not shown), suggesting lung function might be mediated by other types of collagen rather than Type I as previously described.(173, 174) XBP-1-deficient B cells also exhibited reduced apoptosis in lungs but not in the spleens, in agreement with the literature demonstrating that GVHD patients have impaired B-cell

reconstitution attributable to active GVHD or treatments for GVHD, in addition to Fas- and IFNγ-mediated apoptosis of B cells by donor T cells.(12, 68, 175-179)

Costimulation of T cells via CD86 expressed by APC's delivers a strong costimulatory signal to cognate T cells, and has been shown to be critical for development of cGVHD.(41, 180, 181) In light of our *in vivo* and *in vitro* data on costimulatory molecules and given previous reports that IL-4 rapidly induces XBP-1 gene expression through STAT6 signaling(68), we propose that XBP-1 promotes the expression of costimulatory molecules CD86, MHCII, and ICOS-L via IL-4/STAT6-dependent signaling through IL-4.(182) Although it is known that IL-4/STAT6 signaling regulates transcription of CD86 and MHCII genes in response to IL-4,(183-185) the role of XBP-1 in this B-cell process has not been clearly established. Activated B cells that produce cytokines have previously been shown to enhance Th1 differentiation and promote autoimmunity.(41-43) IL-12p40 mediates cGVHD as shown previously(186, 187) and IL-4/5 drives fibrosis.(17) In our study, XBP-1-deficient B cells produced significantly less IL-12p40 and IL-4/5 cytokines after LPS and IL-4 stimulation, suggesting that XBP-1 regulates both type 1 and 2 effector B-cell differentiation. Although some of our B-cell data can be connected to an intrinsic defect of BCR signaling and S1P1 from XBP-1 deficiency(65), we have demonstrated that XBP-1 also plays a critical role in several 'innate' B-cell functions that do not rely on classical activation of the BCR, such as IL-12 and IL-4/5 cytokine production after TLR4 and IL-4 stimulation.(188)

Differentiation of B cells into GC B cells has been shown to be highly correlated with cGVHD development.(27, 28) XBP-1-deficient B cells displayed reduced

differentiation into GC B cells both *in vivo* and *in vitro* and B-I09 treatment of B cells *in vitro* also demonstrated this effect. Antibodies from donor B cells have recently been directly correlated with cGVHD development.(45) Here, XBP-1-deficient B cells secreted less antibodies in response to both *in vitro* and *in vivo* stimuli, and both *in vitro* and *vivo* treatment with B-I09 phenocopied this effect. XBP-1 KO B cells also engaged in less class-switch recombination (CSR) after allo-BMT compared to XBP-1 WT B cells, where higher retention of surface IgM was consistent with reduced levels of serum anti-dsDNA IgG, consistent with a previous report that XBP-1 KO B cells express less activation-induced cytidine deaminase (AID).(65) Because reduction in antibody secretion and plasma cell numbers can potentially cause vulnerability to pathogens, targeting XBP-1 should likely be considered as a short-term strategy to prevent cGVHD. Nevertheless, a comparable strategy using ibrutinib to target B cells did not cause infections for the majority of patients.(52)

Follicular helper T cells (T_{FH}) are a T-cell subset that has recently been shown to be correlated with cGVHD pathogenesis.(27, 28)Although the role of T_{FH} and GC B cells in murine models of cGVHD is still under debate(30, 45), Forcade et al. recently indicated a key role of T_{FH}, which strongly correlated with cGVHD in human patients.(24) Accordingly, in our study murine recipients of XBP-1 KO B cells displayed an indirect impact on T_{FH} cell differentiation, where donor T_{FH} cells were reduced in the spleens. $CD8^+$ T cells are also critically involved in cGVHD, where secretion of inflammatory cytokines and cytolytic molecules can led to tissue fibrosis and organ failure.(20, 170) In our study, recipients of XBP-1 KO donor-grafts or recipients treated with an IRE-1α/XBP-1 pathway inhibitor B-I09 displayed reduced chronic $CD8^+$ T-cell

activation, indicated via lower PD-1 surface expression, a co-inhibitory receptor that can serve as a marker of T-cell receptor ligation and chronic T-cell activation as well as T-cell exhaustion.(37, 189) This finding also occurred in conjunction with reduced intracellular production of IFNγ in both $CD4^+$ and $CD8^+$ T cells in the recipients of XBP-1 KO donor-grafts. These findings suggest that reduction in PD-1 on $CD8^+$ T cells does not necessarily exacerbate GVHD as reported previously by others.(190)

One of the most notable features of human cGVHD is skin damage and scleroderma which occurs in up to 75% of cGVHD patients (191). Thus, we sought to test if targeting XBP-1 would also be efficacious in a minor-antigen mismatched cutaneous model of cGVHD. Using this model, we found that blocking the IRE-1α/XBP-1 pathway with an inhibitor, B-I09, dramatically reduced sclerodermatous cGVHD. In contrast, delayed treatment with B-I09 did not suppress cGVHD, and XBP-1 KO B cells did not impact acute GVHD development, suggesting that the IRE1-α/XBP-1 pathway mediates cGVHD pathogenicity during the transition from the acute to chronic phase. Supporting our findings, a recent report found experimental-autoimmune-encephalitis (EAE) development was significantly delayed in mice prophylactically treated with an ER stress inhibitor (TUDCA); however EAE was not ameliorated with delayed administration due to no effect on early Th17 differentiation.(77) In our study, prophylactic B-I09 reduced infiltration of donor $CD4^+$, $CD8^+$, and $CD11c^+$ lymphocytes into recipient skin. This result could be attributed to XBP-1-dependent defects in dendritic cell-derived type I interferon signaling which regulates T-cell survival(160, 192) , from chemotactic signals such as CXCL16 produced by non-hematopoietic cells, also regulated by XBP-1 (81), or from antibody-mediated cutaneous infiltration of

pathogenic Th17 cells.(45, 77) In spleens, frequency of total granulocytes was significantly reduced after B-I09 administration in the B10.D2 to BALB/c model (data not shown), in agreement with previous reports indicating that XBP-1 is critically involved in the differentiation and recruitment of granulocyte subsets into tissues leading to inflammatory fibrosis,(61, 193, 194) and that these subsets are involved in cGVHD.(195, 196) Current leading therapies for cGVHD directly target B cells or T cells(20, 171) whereas the current study and previous literature suggest that inhibition of the IRE-1α/XBP-1 pathway via B-I09 could directly impact other cell subsets involved in cGVHD development in addition to B cells and T cells(77), such as dendritic cells(160), granulocytes(61), and even non-hematopoietic cells.(197)

The ultimate goal of the hematopoietic stem cell transplantation field is to cure cancers such as leukemia without producing deleterious GVHD. Along these lines, prophylactically targeting the IRE-1α/XBP-1 pathway using B-I09 or using XBP-1 KO B cells as APC's *in vitro* did not impair the GVL effect or anti-tumor functions of T cells in agreement with previous reports that XBP-1 deficiency had little to no direct impact on T-cell responses against infection and did not impair proliferation, activation, or differentiation of Th1 and Th2 cells.(63, 66, 77, 198) Further, administration of B-I09 in a previous study was able to directly inhibit tumor growth of B cell leukemia, lymphoma, and multiple myeloma.(55) Thus, inhibition of IRE-1α/XBP-1 *in vivo* likely does not have a direct impact on anti-tumor functions in T cells.

Taken together, our results demonstrate that in the context of allo-BMT, XBP-1 regulates a diverse repertoire of B-cell functions via antibody-dependent and antibody-independent mechanisms that correlate with the alleviation of cGVHD. Furthermore, systemic

administration of an IRE-1α/XBP-1 pathway inhibitor, B-I09, is able to prevent disease progression and severity in two pre-clinical models of cGVHD. Inhibition of the IRE-1α/XBP-1 pathway with B-I09 was also able to preserve the GVL activity against BC-CML and produced a durable and long-lasting anti-tumor response in recipients. These results provide strong rationale for additional investigation of the potential for targeting the ER stress response mediated by the IRE-1α/XBP-1 pathway to prevent GVHD while also preserving the GVL effect after allo-HCT in the clinic.

2.5 Abstract

Hematopoietic stem cell transplantation (HCT) is a curative procedure for hematological malignancies, but chronic graft-versus-host disease (cGVHD) remains a major complication after allogeneic HCT. Because donor B cells are essential for cGVHD development and B cells are sensitive to endoplasmic reticulum (ER) stress, we hypothesized that the IRE-1α/XBP-1 pathway is required for B-cell activation and function and for the development of cGVHD. To test this hypothesis, we used conditional knock-out mice deficient of XBP-1 specifically in B cells. Recipients transplanted with donor grafts containing XBP-1–deficient B cells displayed reduced cGVHD compared with controls. Reduction of cGVHD correlated with impaired B-cell functions, including reduced production of anti–double-stranded DNA immunoglobulin G antibodies, CD86, Fas, and GL7 surface expression, and impaired T-cell responses, including reduced interferon-γ production and follicular helper T cells. In a bronchiolitis obliterans cGVHD model, recipients of transplants containing XBP-1–deficient B cells demonstrated improved pulmonary function correlated with reduced donor splenic follicular helper T cells and increased B cells compared with those of wild-type control donor grafts. We then tested if XBP-1 blockade

via an IRE-1α inhibitor, B-I09, would attenuate cGVHD and preserve the graft-versus-leukemia (GVL) effect. In a cutaneous cGVHD model, we found that prophylactic administration of B-I09 reduced clinical features of cGVHD, which correlated with reductions in donor T-cell and dendritic cell skin infiltrates. Inhibition of the IRE-1α/XBP-1 pathway also preserved the GVL effect against chronic myelogenous leukemia mediated by allogeneic splenocytes. Collectively, the ER stress response mediated by the IRE-1α/XBP-1 axis is required for cGVHD development but dispensable for GVL activity.

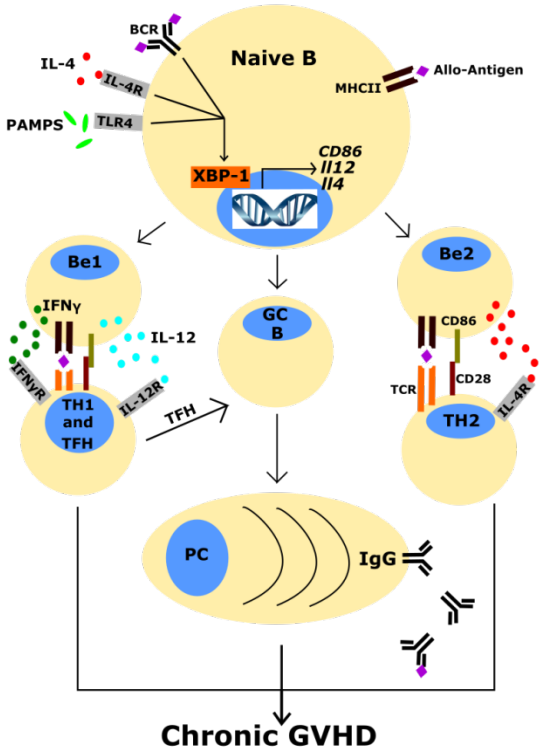

Figure 2.8 Graphical Abstract : Inhibition of the IRE-1α/XBP-1 Pathway Prevents Chronic GVHD and Preserves the GVL Effect in Mice

Chapter 3: Fli-1: A Druggable Transcription Factor that Regulates T-cell Immunity and Experimental GVHD

3.1 Introduction

Currently, the most effective treatment in the clinic for hematological malignancies including leukemia, lymphoma, and myeloma is allogeneic hematopoietic stem cell transplantation (allo-HCT), where the source of cells in the graft are from unrelated donor bone marrow (BM) or peripheral blood (PB) that contain lymphocytes that directly recognize and kill malignant cells within the host, termed as the graft-versus-tumor effect (GVT) or graft-versus-leukemia effect (GVL). A detrimental side effect of allo-HCT that occurs in between 30%-70% of transplant recipients is chronic GVHD (cGVHD). cGVHD is the primary cause of late-stage transplant-related morbidity and mortality despite available prophylactic strategies and treatments (11, 12, 199). Strategies that can prevent and or treat GVHD without necessarily compromising GVL would further enhance the efficacy and availability of allo-HCT.

One potential candidate target which is relevant to both leukemia and the immune response that has not been investigated in GVHD pathogenesis is the transcription factor Fli-1. Research involving Fli-1 has been focused primarily on elucidating its role in Ewing's sarcoma(98, 99), leukemia(100-102), other cancers(103-106), and in systemic lupus erythematous (SLE) where it was shown that Fli-1 is aberrantly expressed in humans and mice with SLE(107-110). While Fli-1 has been relatively understudied specifically in primary lymphocytes, especially T cells, it was indeed previously demonstrated that retroviral overexpression of fli-1 in T-cell progenitor cells led to initiation of uncontrolled T-cell proliferation and pre-T-cell lymphoblastic lymphoma mediated by NOTCH-1 mutations(111), and that T cells from germ-line

fli-1 heterozygous deficient mice bearing the Faslpr mutation showed that Fli-1 positively associated with inflammatory factors CXCR3, IL-6, and C16-ceramide(115-117); these listed factors also can play important roles in GVHD pathogenesis(117, 122-124, 126, 200). Fli-1 was also identified to be one of two candidate genes (the other Ets1) that regulated T-cell fractions in nonobese diabetic (NOD) mouse model(113). Although not known to date in T cells, Fli-1 was shown as a direct negative regulator of SH-2 containing inositol 5' polyphosphatase 1 (SHIP-1) in erythroid cells(119). SHIP-1 itself was shown to be an important inhibitor of PI3K signaling which is implicated in T follicular helper cell (TFH) differentiation(120) and T-cell immunity (121).

Additionally, global transgenic overexpression of fli-1 was associated with increased B cell proliferation, autoimmunity, and exacerbated lupus symptoms in mice, which was able to be ameliorated via germ line heterozygous genetic ablation of the fli-1 gene (107-109). Further, a demonstrated cancer chemotherapeutic, camptothecin (CPT), was shown to be a potent Fli-1 protein inhibitor (by both the current study and others (112)), and was able to impair tumor growth in multiple erythroleukemia cell lines in vitro and in Friend Murine Leukemia virus (F-MuLV) induced erythroleukemia in vivo (112, 141). Additionally, multiple cancer types that are targeted with allo-HCT such as AML, lymphomas, and other hematopoietic malignancies have been shown to express high Fli-1 mRNA, suggesting that targeting Fli-1 in these cancer types could be beneficial to reduce their growth (201).

Despite these previous findings, to date there has been no research to our knowledge that directly implicates Fli-1 as a link between immune tolerance and anti-leukemia immunity in the context of allo-HCT. Further, whether Fli-1 plays an important role in the antigen-specific T-cell response has not been addressed until the current study. Here, we utilized a genetic strategy to target Fli-1 activity specifically on T cells and determined the role of Fli-1 in experimental T-cell mediated GVHD models. We then employed known pharmacological agents with demonstrated

Fli-1 inhibitory activity in pre-clinical allo-HCT mouse models of aGVHD and cGVHD, as well as in a humanized xenograft model of GVHD, and show that reducing Fli-1 expression or activity may be an important translational concept for reducing pathogenesis of GVHD without impairing the GVL response.

3.2 Materials and Methods

3.2.1 Mice

Female and male BALB/c (H-2^{d}), C57BL/6 (B6).Ly5.1 (H-2^{b}, CD45.1), B6.Ly5.2 (H-2^{b}, CD45.2) and (BALB/c x DBA2)F1 (B6D2F1, H-$2^{b/d}$) mice were purchased from Charles River Laboratories (Wilmington, MA). $Rag1^{-/-}$ (H-2^{b}) mice were purchased from Jackson Laboratories (Bar Harbor, ME). Fli-$1^{flox/flox}$ mice on B6 background were a gift from Dr. Zhang's group (202). T-cell conditional deletion of *fli-1* exons 3 and 4 was mediated by a Cre/Lox system utilizing the *CD4* promoter. Homozygous *fli-1* exon 3 and 4 deletion (referred to as Fli-$1^{flox/flox}$) was mediated via Fli-$1^{flox/flox}$CD4Cre^{+}, heterozygous *fli-1* exon 3 and 4 deletion (referred to as Fli-$1^{flox/wt}$) was mediated via are Fli-$1^{flox/wt}$CD4Cre^{+}, and wild-type controls (referred to as Fli-$1^{wt/wt}$) were Fli-$1^{flox/flox}$CD4Cre^{-}, Fli-$1^{flox/wt}$ CD4Cre^{-}, or Fli-$1^{wt/wt}$CD4Cre^{+}. Fli-$1^{flox/flox}$*CD4*Cre+ mice were also crossed with Marilyn transgenic mice (described previously (31)) to generate HY-antigen specific T cells with reduced Fli-1 activity. All strains were maintained in a specific pathogen-free facility at the Medical University of South Carolina (MUSC, Charleston, SC). Experimental animals were housed in the American Association for Laboratory Animal Care–accredited Animal Resource Center at MUSC. All animal experiments were approved by the MUSC Institutional Animal Care and Use Committee.

3.2.2 Allogeneic bone marrow transplantation (Allo-BMT)

In B6 to BALB/c cGVHD model, recipients were lethally irradiated (700cGy, X-ray source from Precision X320) on day -1 or 0, and infused with allogeneic B6 unfractionated splenocytes (0.5×10^6) from either Fli-$1^{wt/wt}$, Fli-$1^{flox/wt}$, or Fli-$1^{flox/flox}$ donors and supplemented with T-cell depleted bone marrow (TCD-BM) (5×10^6) from each respective strain (or from B6.Ly5.1^+ donors in certain experiments) and injected I.V. within 24h after irradiation. TCD-BM protocol was described in detail previously (25, 26). BALB/c recipients were monitored weekly with the cGVHD clinical scoring system described previously(25). On days 40-60 post-transplant, recipients were sacrificed and spleens, peripheral lymph nodes, and thymus were collected for flow cytometry analysis, and GVHD target organs were paraformaldehyde fixed and sectioned for hematoxylin and eosin (H&E) staining. In B6 to BALB/c aGVHD model, conditions were similar, except instead of total splenocytes, a dose of 0.5×10^6 total T cells was infused and all recipients were given B6.Ly5.1+ TCD-BM. An independent pathologist scored histology sections for cGVHD, aGVHD, and colitis as described previously (25). B6 to B6D2F1 acute GVHD and GVL model using P815 mastocytoma cells was also used for pharmacological studies and was described previously (40).

3.2.3 Graft-versus-leukemia (GVL) response

Wild-type B6 donor grafts were given to lethally irradiated B6D2F1 recipients with or without 5,000 P815 cells per mouse together with TCD-BM (5×10^6 per mouse) with or without 3×10^6 purified total T cells. Mice given TCD-BM plus P815 cells were treated with either vehicle (DMSO) or camptothecin (0.25mg/kg q.o.d) alone without donor T cells were used as

controls. Recipients were subjected to bioluminescent imaging (IVIS200 System, Perkin Elmer) at periodic time points as shown following allo-BMT to assess P815 growth and relapse.

3.2.4 Colitis Model

Rag1$^{-/-}$ mice on a B6 background were given syngeneic transplant of 1 x 10^6 naïve CD4$^+$ T cells (CD4$^+$CD8$^-$CD25$^-$CD44$^-$) isolated from spleen and lymph nodes from either Fli-1$^{wt/wt}$, Fli-1$^{flox/wt}$, or Fli-1$^{flox/flox}$ mice. Mice were followed weekly for colitis clinical score (described previously (124)) and on day 60 post T-cell transfer, mice were sacrificed and colonic sections were stained with H&E and scored for colitis via an independent pathologist using previously established criteria (203).

3.2.5 Treatment with Fli-1 inhibiting drugs camptothecin and etoposide

Camptothecin (CPT) and etoposide (ETO) are known chemotherapeutics via their inhibition of topoisomerase enzymatic activity but have demonstrated potent off-target effects against Fli-1 protein level. CPT in particular can deplete Fli-1 protein on multiple different human and mouse cell lines at very low concentrations (shown in the current study and by other groups (112)). Each drug in its powder form was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 3mg/mL (CPT), 8mg/mL (ETO), aliquoted and then stored at −80°C. For CPT, aliquots were brought to 0.1 mg/mL with DMSO, and animals were injected i.p. with 50 μL per mouse (0.25mg/kg) 2 to 4 hours prior to allo-BMT as prevention or on day 28-30 after BMT for treatment, and followed with every other day injections until days 14 or 28 (prevention) or from

days 28-56 (treatment). For ETO, aliquots were brought to 0.8 mg/mL with a vehicle solution containing 30% PEG300 and sterile nanopure H_2O, and was injected i.p with 50µL (2mg/kg) or 100µL (5mg/kg) on day 0 and every other day until day 14.

3.2.6 Topoisomerase I enzymatic activity assay

Primary wild-type mouse T cells were polyclonally activated via anti-CD3 (plate-bound 2µg/mL) and anti-CD28 (soluble 2µg/mL) and cultured with DMSO (vehicle) or low-doses (15nM, 30nM) or high doses (500nM, 1µM) of CPT for 48h. On day 2, whole-cell lysates were extracted from T-cell cultures, and fresh lysates containing endogenous topoisomerases were incubated with Topoisomerase I reaction buffer and supercoiled plasmid DNA for 30 min at 37ºC. Reaction products were then run on 0.8% agarose gel to determine ability of endogenous topoisomerase I to relax supercoiled plasmid DNA as described previously (204).

3.2.7 Flow cytometry, ELISA, and Western Blotting

Splenocytes, thymocytes, and lymph node cells were analyzed for surface proteins and intracellular cytokines using standard flow cytometric protocols as previously described (25, 26). The following antibodies were used for staining: Fixable Live/Dead yellow (BD Biosciences, San Jose, CA), anti-TCRβ–FITC or -PE–Cy7 (Clone: H57-597), anti-CD4–FITC, or –V450 (RM4-5), anti-CD8α–FITC, or -allophycocyanin-cy7 (53-6.7), anti-PD-1–PE or –PerCpCy5.5 (J43), anti-CXCR5–PE-Cy7 (SPRCL5), anti-B220–V450 (RA3-6B2), Annexin V-PE and 7AAD Apoptosis staining kit (BD Biosciences), anti-CD86–PE-Cy5 (GL1), purchased from BD Biosciences/

Intracellular straining antibodies: anti–interferon-γ (IFN-γ) Per-cp5.5 (XMG1.2);, IL-17A PE-Cy7 or APC, IL-4 PE, IL-5 PE, FoxP3 APC or PE (BD Biosciences). Cell isolates were analyzed using Diva software, LSR II (BD Biosciences), and FlowJo (TreeStar, Ashland, OR). Enzyme-linked immunosorbent assay (ELISA) using an IL-2 capture antibody (BD) and IL-2 biotin detection antibody (BD) was used to measure IL-2 from cell-culture supernatants. Primary rabbit anti-human/mouse Fli-1antibodies used for western blots were either produced in house from Dr. Zhang or purchased from Abcam (cat# ab133485) and mouse anti-β-actin was purchased from Sigma Aldrich (cat# A2228). Secondary antibodies were either anti-rabbit or anti-mouse directly conjugated to HRP or to fluorescent markers IRDye680RD (cat# 925-68070) or IRDye 800CW (cat# 925-32211).

3.2.8 qRT-PCR and RNAseq

Total RNA was extracted from cells using TRIzol reagent according to its protocol and converted to cDNA using iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad). qRT-PCR was carried out using ssoAdvanced Universal SYBR Green Supermix (Bio-Rad) and reactions were amplified and quantified on the Bio-Rad CFX96 instrument. qRT-PCR primers used are provided in Supplemental methods Table 1. RNA was further purified from TRIzol extracted samples using RNEasy column purification (Qiagen) according to the company protocol and used for RNAseq carried out by Novogene (Sacramento, CA) according to their company protocol: RNAseq downstream analysis was performed using a combination of programs including STAR, HTseq, Cufflink and Novogene wrapped scripts. Alignments were parsed using Tophat program and differential expressions were determined through DESeq2/edgeR. Reference genome and gene model annotation files were downloaded from

genome website browser (NCBI/UCSC/Ensembl) directly. Indexes of the reference genome was built using STAR and paired-end clean reads were aligned to the reference genome using STAR (v2.5). STAR used the method of Maximal Mappable Prefix(MMP) which can generate a precise mapping result for junction reads. (For DESeq2 with biological replicates) Differential expression analysis between two conditions/groups (two biological replicates per condition) was performed using the DESeq2 R package (2_1.6.3). DESeq2 provide statistical routines for determining differential expression in digital gene expression data using a model based on the negative binomial distribution. The resulting P-values were adjusted using the Benjamini and Hochberg's approach for controlling the False Discovery Rate(FDR). Genes with an adjusted P-value <0.05 found by DESeq2 were assigned as differentially expressed.

3.2.9 Statistics

Data presentation and statistical analyses were carried out using Prism 8 software (GraphPad) and graphed as mean ± standard error of the mean. For cGVHD clinical scores, all animals were examined for GVHD clinical signs on day 0 as a reference point, and non-reference point clinical score data were analyzed using mixed model tests to determine any statistical significance between groups ($P < .05$). Murine survival data of GVHD was analyzed with a log-rank (Mantel-Cox) test to determine any statistical significance between groups ($P < .05$). Fisher's exact test was performed on day 60 of the xenograft model to determine difference in survival between groups. In BALB/c model of acute GVHD, clinical scoring in this model of aGVHD data was treated as 'last observation carried forward' (LOCF) for any mouse in any group that succumbed to mortality prior to experimental endpoint (day 80 post-BMT). For all other data, differences between only two experimental groups were compared using an unpaired

2-tailed Student *t* test to determine any statistical significance ($P < .05$), or when comparing >2 groups, a 1-way analysis of variance (ANOVA) using Tukey or Bonferroni correction for multiple comparisons was performed to determine any statistical significance ($P < .05$).

3.3 Results

3.3.1 Characteristics of fli-1 conditional knock-out (KO) mice

The Fli-1flox/flox mouse strain was shown previously to effectively reduce Fli-1 mRNA and protein levels via Cre-mediated recombination on the Tie2(Tek) promoter (202). We verified the ability of the CD4 promoter based Cre/Lox system to mediate effective fli-1 recombination in T cells. By using a PCR probe specific for the consensus LoxP sites present near exon 3 and exon 4, where, as expected, we found complete deletion of the exon 4 LoxP site in Fli-$1^{flox/flox}$*CD4*Cre+ T cells compared to Fli-$1^{flox/wt}$*CD4*Cre- T cells which contained both LoxP sites, but no *CD4*Cre recombinase expression, indicating effective cell-specific *fli-1* recombination occurred in the presence of *CD4*Cre (Supplemental Figure 1 A). In this Cre/Lox system, *fli-1* exon 4 LoxP site is cleaved while exon 3 LoxP site is maintained after recombination (202). Further, we found significantly reduced Fli-1 mRNA level in T cells from Fli-$1^{flox/wt}$ and Fli-$1^{flox/flox}$ mice compared to Fli-1^{WT} controls (Supplemental Fig. 1 B). Notch-1 is a signaling component known to be essential for IL-2 production as well as GVHD development (205-208) and because Fli-1 has already been shown to positively associate with Notch-1+ mutations in pre-T cell lymphoblastic lymphoma (pre-TLL) (111), we investigated whether this phenomenon would also apply to murine activated primary T cells using qRT-PCR. Within 48h after polyclonal T-cell activation,

reduced Fli-1 activity was associated with reduction in Notch1 mRNA levels in both Fli-1$^{flox/wt}$ and Fli-1$^{flox/flox}$ T cells (Supplementary Figure 1 B). Fli-1 is also a known regulator of Ship-1 levels in transformed erythroid cells (119), prompting us to examine Ship-1 gene expression, where we found that Fli-1$^{flox/flox}$ but not Fli-1$^{flox/wt}$ T cells had significantly lower *Inpp5d* (Ship-1) mRNA compared to Fli-1^{WT} T cells. These qPCR data indicated that genetic ablation of *fli-1* exons 3 and 4 causes a loss of function and transcriptional activity of Fli-1 as previously described (202).Fli-1 protein was found to be moderately reduced in resting and polyclonally activated T cells with *fli-1* exon 3 and exon 4 genetic deletion via western blot (Supplemental Fig. 1 C).

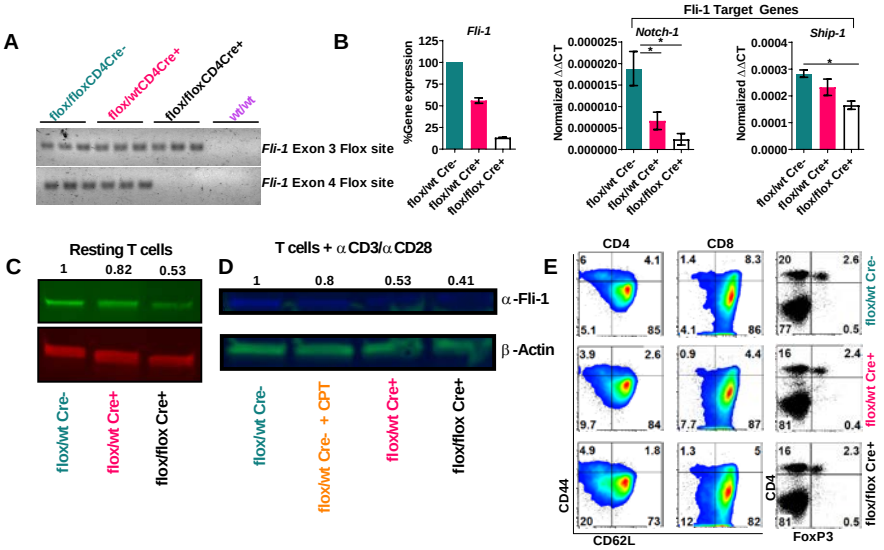

Figure 3.1 Characteristics of fli-1 conditional knock-out (KO) mice. PCR probes specific to the consensus LoxP sequence flanked by primers specific to either Fli-1 exon 3 flox site or exon 4 flox site were used to determine the efficiency of Cre-recombinase mediated recombination of Fli-1 exons. Genomic DNA was isolated from purified naïve CD4^{+} T cells of Fli-1$^{wt/wt}$, Fli-1$^{flox/wt}$, or Fli-1$^{flox/flox}$ mice and amplified using LoxP probes specific for exon 3 and exon 4 **(A)**. qRT-PCR primers designed to detect Fli-1, Notch-1, or Ship-1 gene expression were used to amplify cDNA converted from total mRNA of *in vitro* polyclonally stimulated T cells from the indicated genotypes **(B)**. Western blot analysis of Fli-1 and β-actin loading control performed on lysates from resting purified T cells **(C)** and polyclonally activated purified T cells after 48h stimulation **(D)**. Some activated T-cell cultures were incubated with 15nM CPT for 48h to assess impact of CPT on Fli-1 protein level of activated T cells (lane 2). Splenocytes from the indicated donor genotypes were stained with the indicated surface or intracellular markers to determine potential differences in the baseline T-cell phenotype. Across >6 donor mice, no significant differences were observed in naïve/memory/central memory, or nTreg populations **(E)**. Data in A, C, D represent an individual experiment, data in B represent two independent experiments. *p<0.05.

3.3.2 T-cell specific Fli-1 mediates cGVHD development

To determine the role of Fli-1 in allo-HCT, we utilized a well-established pre-clinical cGVHD model. Under cGVHD conditions, using donor marrow and splenocyte grafts from Fli-$1^{flox/wt}$ mice resulted in improved survival and a striking reduction in cGVHD clinical score of murine recipients, but not in the recipients given Fli-$1^{flox/flox}$ or Fli-$1^{wt/wt}$ grafts (Fig. 1 A-B). Between days 40-60 after BMT, thymic reconstitution—a key marker of GVHD progression and severity—was examined in these BMT recipients. Here, we found that recipients given Fli-$1^{flox/wt}$ grafts had superior $CD4^+CD8^+$ thymic reconstitution compared to the recipients given either Fli-$1^{flox/flox}$ or Fli-$1^{wt/wt}$ grafts when analyzing both frequency and absolute number via flow cytometry (Fig. 1 C-E). Because CD4 and CD8 double-positive cells are generated from T-cell progenitors that migrate from bone marrow (209), we tested if Fli-1 deficiency played a role in the conversion of T-cell progenitors to double-positive thymocytes by performing splenocyte and marrow chimeric BMT. Here, recipients subjected to cGVHD conditions were transplanted with either Fli-$1^{wt/wt}$, Fli-$1^{flox/wt}$, or Fli-$1^{flox/flox}$ splenocytes (CD45.2) but each group received the same wild-type marrow bearing a congenic marker (CD45.1). Under these conditions, recipients given Fli-$1^{flox/flox}$ splenocytes and wild-type marrow had significantly increased frequencies of $CD4^+CD8^+$ thymocytes and had lower cGVHD clinical score compared to recipients that received Fli-1^{WT} splenocytes and wild-type marrow, yet recipients that received Fli-$1^{flox/wt}$ splenocytes and wild-type marrow grafts still had the lowest cGVHD clinical scores (Supplemental Fig. 2 A). Comparison of the matched splenocyte and marrow graft results to the chimeric graft results suggested that T-cell progenitors from Fli-$1^{flox/flox}$ marrow had a reduced ability to undergo normal $CD4^+CD8^+$ thymic reconstitution after BMT, reflecting worse cGVHD clinical score, whereas the recipients of Fli-$1^{flox/wt}$ grafts had improved thymic reconstitution, reflecting low cGVHD clinical scores (Supplemental Fig. 2 B-C). These data indicate that while heterozygous Fli-1 mutation did

not impact thymus development, homozygous Fli-1 mutation lead to a significant reduction in normal thymic T-cell frequencies, in agreement with previous observations by Melet et al. (114). Thus, while reduction of Fli-1 activity on mature T cells may be beneficial toward reducing cGVHD, at least some Fli-1 activity may contribute to conversion of T-cell progenitors into $CD4^+CD8^+$ thymocytes after allo-BMT.

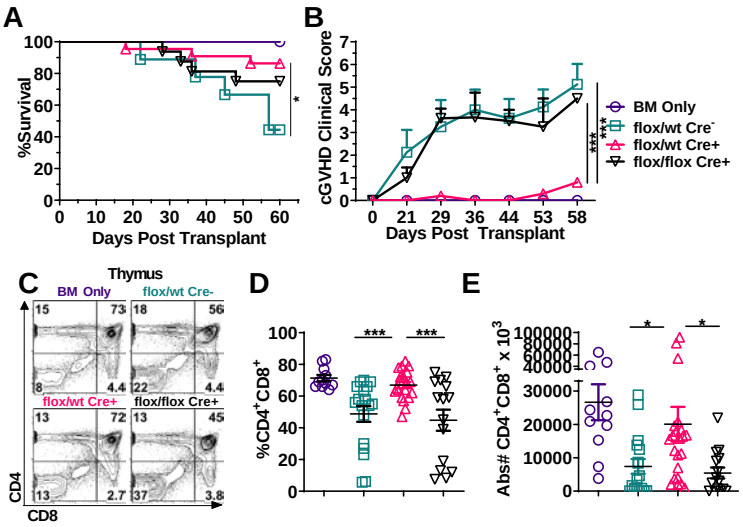

Figure 3.2 T-cell specific Fli-1 mediates cGVHD development. Lethally irradiated BALB/c mice were transplanted with 5 x 10^6 TCD-BM and 0.5 x 10^6 total splenocytes from Fli-1^{WT}, Fli-$1^{flox/wt}$, or Fli-$1^{flox/flox}$ donors. % cumulative survival **(A)** and representative chronic GVHD clinical scores **(B)**. Representative flow cytometry plots of $CD4^+CD8^+$ thymocytes from experimental endpoint (days 45-60) **(C)** together with the cumulative frequency **(D)** and absolute number **(E)**. Data represent six independent experiments. *p<0.05, **p<0.01, ***p<0.001.

3.3.3 Fli-1 dynamically inhibits Treg and promotes T-cell IFN-γ, IL-17A, and TFH-like responses *in vivo*

To further understand how Fli-1 was able to regulate T cells to control cGVHD disease development, we examined different types of T-cell subsets and T-cell phenotypes within a secondary lymphoid organ (spleen) and in peripheral lymph nodes (pLN) of mice at late time points after BMT. Recipients given matched Fli-$1^{flox/wt}$ as well as Fli-$1^{flox/flox}$ splenocyte and marrow grafts had enhanced donor-derived $CD4^+$ but not $CD8^+$ reconstitution compared to recipients given Fli-1^{WT} grafts in their spleens. Among these experimental groups, only recipients

given Fli-1$^{flox/wt}$ grafts had significantly higher B-cell (B220^{+}) reconstitution (Fig. 2 A). We consistently found that lower frequencies of donor-derived splenic PD-1^{+}CXCR5^{+} expressing CD4^{+} T cells, commonly referred to as T-follicular helper (T_{FH}) cells, in recipients of either Fli-1$^{flow/wt}$ or Fli-1$^{flox/flox}$ grafts than those in the recipients of Fli-1$^{wt/wt}$, although only the difference between Fli-1$^{flow/wt}$ and Fli-1$^{wt/wt}$ was statistically significant. A similar phenomenon of reduced PD-1 expression was also observed on CD8^{+} T cells (Fig. 2 B-C). These data suggest that Fli-1 may contribute toward differentiation of T_{FH} cells and CD8^{+} T-cell activation. Within pLN's, we found significant reductions in frequencies of donor-derived CD4^{+} T cells that produced IFN-γ in the recipients of Fli-1$^{flox/wt}$ or Fli-1$^{flox/flox}$ grafts, but only Fli-1$^{flox/wt}$ T cells demonstrated significantly reduced frequencies of CD4^{+}IL-17A^{+} T cells. Frequencies of donor-derived CD4^{+}FoxP3^{+} Tregs were also increased in the recipients of Fli-1$^{flox/wt}$ or Fli-1$^{flox/flox}$ grafts, but only significantly in the Fli-1$^{flox/flox}$ group compared to the Fli-1^{WT} group (Fig. 2 D). Using the chimeric model, we found the marrow-derived cells in the recipients given Fli-1$^{flox/wt}$ grafts also showed significant reduction in the PD-1^{+}CXCR5^{+} T_{FH}-like population, while also showing a significant trend of increased PD-1^{+}CXCR5^{+}→CD4^{+}FoxP3^{+} T-follicular regulatory (T_{FR})-like cells compared to Fli-1^{WT} group (Supplemental Fig. 2D). In both spleen and lymph nodes, the recipients given Fli-1$^{flox/wt}$ T cells and WT marrow had significantly reduced frequencies of CD4^{+}IL-17A^{+} T cells compared to those of Fli-1 $^{flox/wt}$ grafts. Frequencies of CD4^{+}IFN-γ^{+} T cells were also reduced in spleen and lymph nodes of the recipients given Fli-1$^{flox/wt}$ grafts, although reductions were restricted to marrow-derived T cells. The recipients given Fli-1$^{flox/wt}$ grafts showed a significant trend of increased CD4^{+}FoxP3^{+} Tregs within the marrow-derived compartment compared to Fli-1$^{wt/wt}$ group in their peripheral lymph nodes (Supplemental Fig. 2 E-G). Taken together, these findings demonstrate that Fli-1 plays an important and dynamic role in regulating the presence of pathogenic CD4^{+}IFN-γ $^{+}$ T_H1, CD4^{+}IL-17A^{+} T_H17, PD-1^{+}CXCR5^{+} T_{FH}, and protective CD4^{+}FoxP3^{+} T_{reg} subsets in lymphoid organs of the recipients with cGVHD.

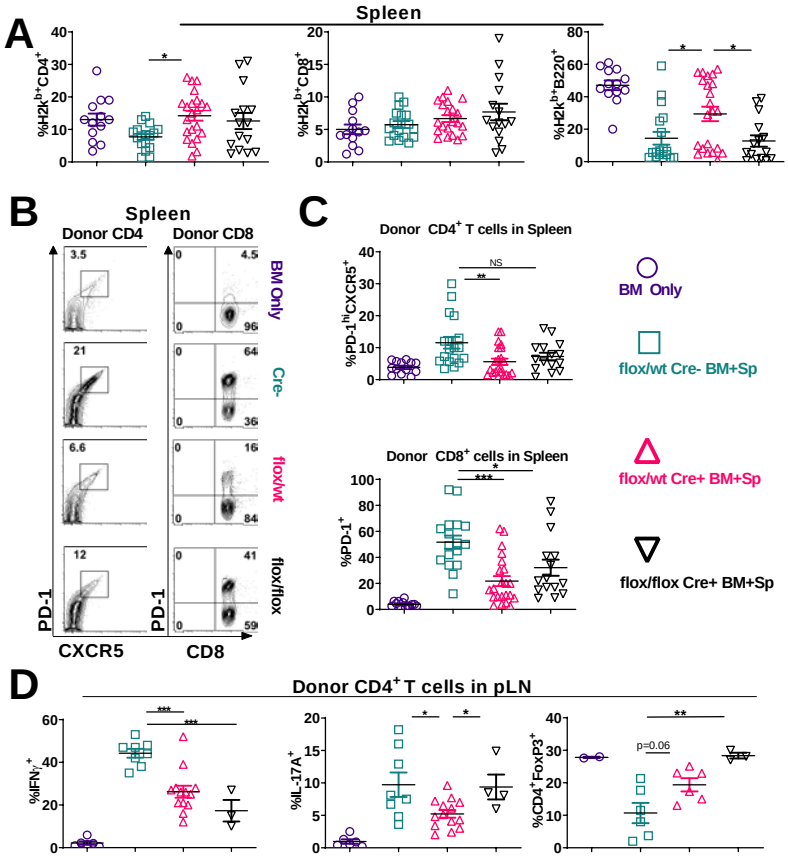

Figure 3.3 Fli-1 dynamically inhibits Treg and promotes Th1, Th17, and TFH responses *in vivo.* Spleens and peripheral lymph nodes were collected from BMT recipients and analyzed at experimental endpoints via flow cytometry. Frequency of donor ($H2K^b$) $CD4^+$, $CD8^+$, and $B220^+$ cells from spleen **(A)**. Representative flow cytometry plots **(B)** and cumulative frequencies of donor $CD4^+$→$PD\text{-}1^+CXCR5^+$ or $CD8^+PD\text{-}1^+$ populations in recipient spleens **(C)**. Cell isolates from peripheral lymph nodes of BMT recipients and frequencies of $IFN\gamma^+$, $IL\text{-}17A^+$, and $FoxP3^+$ cells from donor $CD4^+$ compartment **(D)**. Data in A-C represent six independent experiments, D represents three independent experiments. *p<0.05, **p<0.01, ***p<0.001

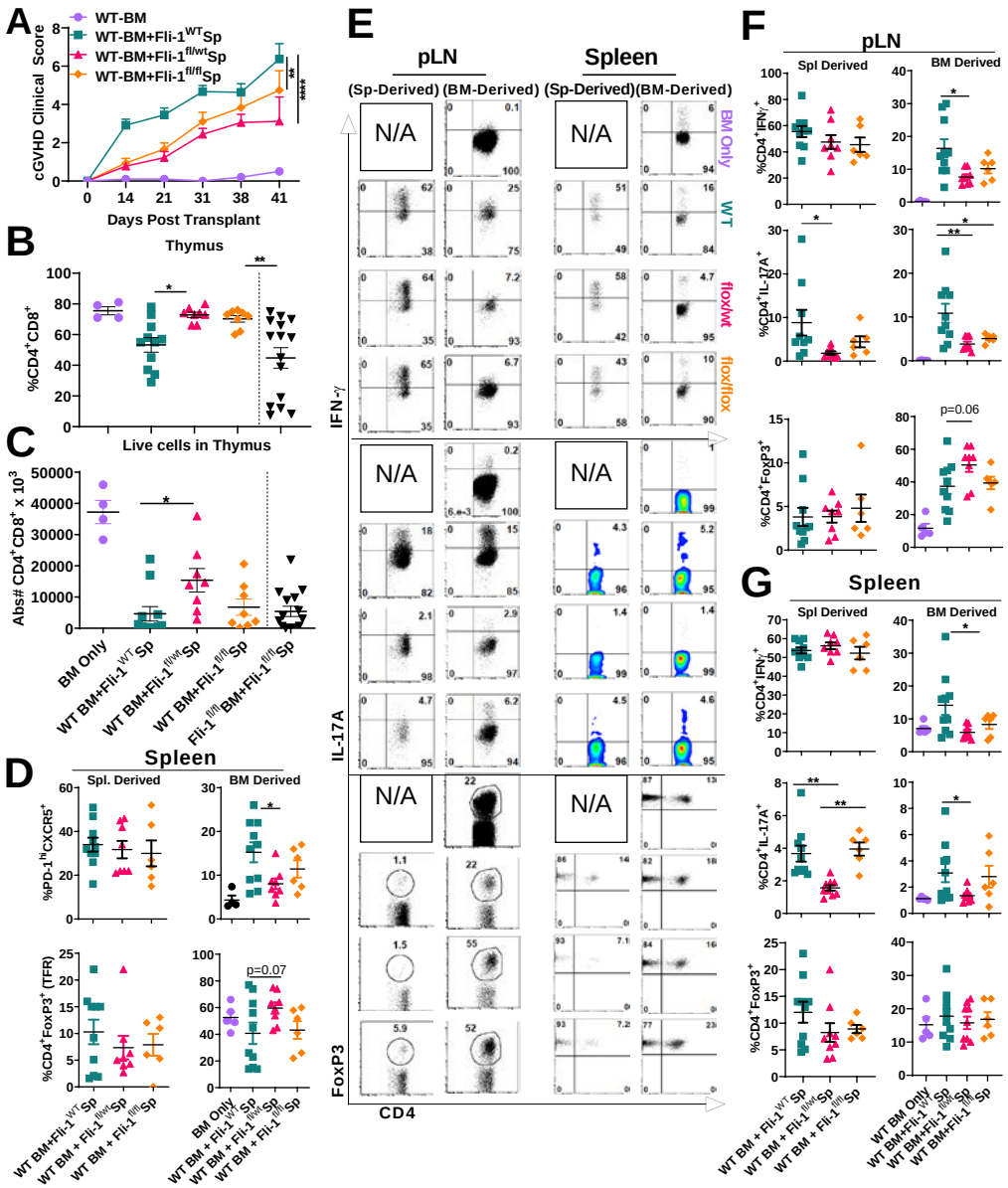

Figure 3.4 Homozygous and heterozygous reduced activity of Fli-1 on BM-derived T cells dynamically impacts cGVHD. Similar experiments were performed as in Fig. 2, except that donor BM source for all BALB/c recipients was changed to WT CD45.1^{+} B6 donors, supplemented with or without 0.5 x 10^{6} donor splenocytes from Fli-1$^{wt/wt}$, Fli-1$^{flox/wt}$, or Fli-1$^{flox/flox}$ donors. cGVHD clinical score was monitored following BMT **(A)**. Cumulative thymic CD4^{+}CD8^{+} populations were analyzed ~day 40 post transplant via flow cytometry **(B-C)**. The data in Fli-1$^{flox/flox}$BM+Fli-1$^{flox/flox}$ Sp in B-C was derived from experiments in figure 2 to compare to the mice that received Fli-1^{WT} BM and Fli-1$^{flox/flox}$Sp (WT BM+Fli-1$^{flox/flox}$Sp). Splenocytes of recipients were analyzed for the indicated T_{FH}-like and T_{FR} surface and intracellular markers and cumulative frequencies are shown **(D)**. Representative flow cytometry plots show splenocyte derived (CD45.1-) or BM-derived (CD45.1+) donor T-cell populations within peripheral lymph

nodes (pLN) or spleen **(E)**. The first four plots (IFNγ+) are labeled with the corresponding genotype, and the order is the same in subsequent sets of 4 plots. pLN **(F)** and spleens **(G)** of recipients were analyzed for the indicated intracellular cytokines and cumulative frequencies are shown. Data represent two independent experiments. *p<0.05, **p<0.01, ***p<0.001.

3.3.4 Fli-1 inhibits antigen-specific iTreg function while promoting IL-2 secretion and Th17 differentiation *in vitro*

Little is known about the potential roles of Fli-1 in normal primary T-cell biology, thus we decided to create a TCR transgenic (TCRtg) mouse strain paired with our Fli-$1^{flox/flox}$CD4*Cre* strain in order to study the role of Fli-1 in antigen-specific T-cell responses. $CD4^+$ T cells from these TCRtg mice are only able to respond to HY-peptide (31). Thus, to study the effects of Fli-1 on the antigen-specific T-cell response, we polarized $CD4^+$ T cells with HY-peptide and different cytokine cocktails to induce Th1, Th17, or iTreg differentiation. Strikingly, we found that both Fli-$1^{flox/wt}$ and Fli-$1^{flox/flox}$ TCRtg cells had enhanced iTreg ($CD25^+FoxP3^+$) differentiation and expression of iTreg functional molecules (CD25, CD39, CD73, and NRP-1) compared to TCRtg Fli-$1^{wt/wt}$ iTregs on culture day 3 and 4 (Supplemental Fig. 3 A-B). Further, both Fli-$1^{flox/wt}$ and Fli-$1^{flox/flox}$ TCRtg cells had significant reduction in intracellular production of IL-17A compared to Fli-$1^{wt/wt}$ TCRtg cells under Th17 polarizing conditions, suggesting Th17 differentiation was impaired due to reduction in Fli-1 activity (Supplemental Fig. 3 C-D). In agreement with this finding, we also found that mRNA levels of the *Il17a* gene in polyclonally stimulated non-TCRtg Fli-$1^{flox/wt}$ and Fli-$1^{flox/flox}$ T cells were significantly reduced compared to Fli-$1^{flox/wt}$ T cells (data not shown). The frequency of IFN-γ producing T cells in both Fli-$1^{flox/wt}$ and Fli-$1^{flox/flox}$ TCRtg groups was similar or even elevated compared to Fli-$1^{wt/wt}$ group under Th1 polarizing conditions; however, both Fli-$1^{flox/wt}$ and Fli-$1^{flox/flox}$ cultures had significantly lower overall T-cell differentiation

(%TCRvβ6^{+}CD4^{+}) compared to Fli-1$^{wt/wt}$ cultures, suggesting IFN-γ production was preserved but T-cell growth or survival was reduced (data not shown). To evaluate the impact of Fli-1 on T-cell growth and survival, we tested the abundance of IL-2 cytokine secreted into culture media from Th17 and Th1 polarizing cultures. We found that culture supernatants from both Fli-1$^{flox/wt}$ and Fli-1$^{flox/flox}$ cultures had significantly reduced levels of IL-2 in Th17 and Th1 conditions, suggesting that Fli-1 regulates antigen-specific T-cell IL-2 production (Supplemental Fig. 3 E-F). Together, these results suggest that Fli-1 contributes toward the enhancement of Th17 polarization while suppressing iTreg differentiation. These differences in antigen-specific T-cell activation resulting from reduced Fli-1 activity prompted us to further investigate the role of Fli-1 on T cells *in vivo*.

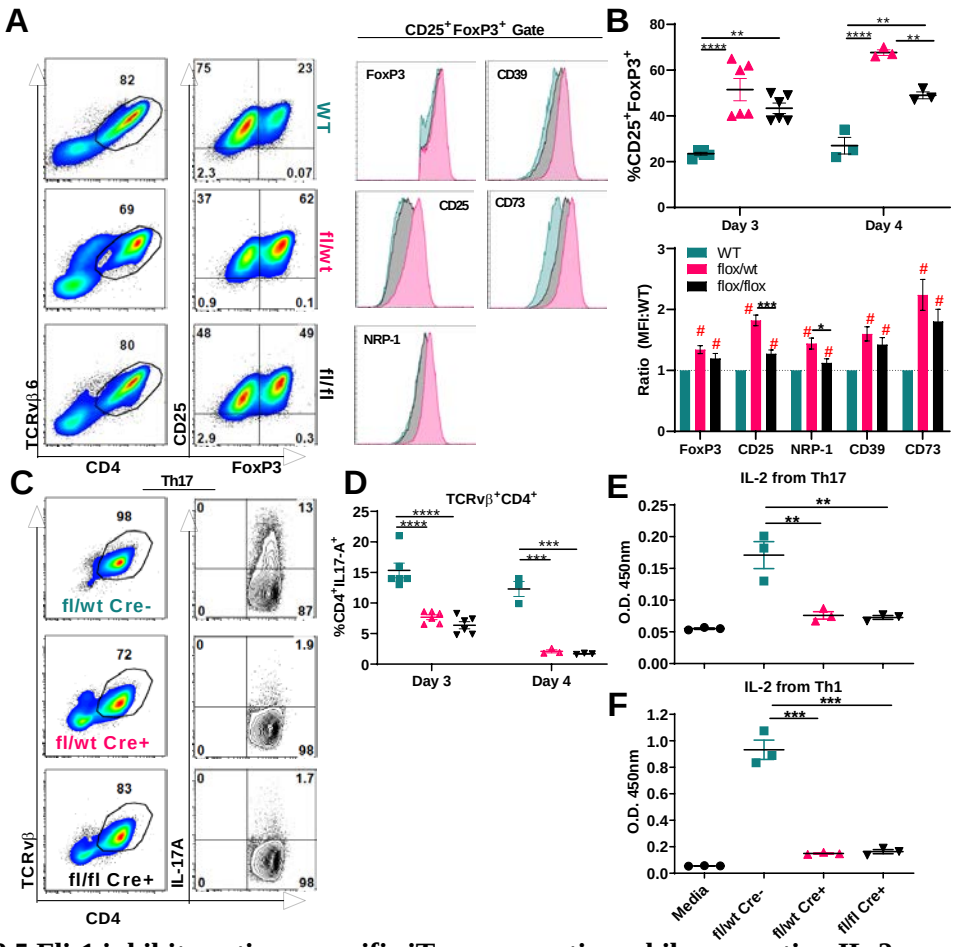

Figure 3.5 Fli-1 inhibits antigen-specific iTreg generation while promoting IL-2 secretion and Th17 differentiation *in vitro*. CD8 and CD25-depleted splenocytes from Fli-1$^{wt/wt}$, Fli-1$^{flox/wt}$, and Fli-1$^{flox/flox}$ TCR-tg mice were polarized into iTreg using 0.5μg/ml HY-peptide, 5ng/ml TGF-β, and 2ng/ml IL-2. Cultures were analyzed on days 3 and 4 for frequency of iTreg (TCRvβ6+CD4+CD25+FoxP3+) **(A)** and surface Treg functional molecules CD39, CD73, and NRP-1 **(B)**. Cumulative Fli-1$^{wt/wt}$ MFI values for CD25, FoxP3, NRP-1, CD39, and CD73 were set to "1" as a baseline comparison, and the ratio of Fli-1$^{flox/wt}$ and Fli-1$^{flox/flox}$ MFI values over Fli-1$^{wt/wt}$ values was calculated and graphed. Hashtag symbol indicates statistical significance ($p<0.05$) between Fli-1WT and indicated groups was reached within each individual experiment. The same TCRtg splenocytes were polarized into Th17 via addition of 0.5ug/ml HY-peptide, 10μg/ml α-IFN-γ, 10ng/ml IL-6, and 5ng/ml TGF-β or into Th1 via 0.5μg/ml HY-peptide, 2ng/ml IL-12, and 10ng/ml IFN-γ and analyzed on days 3 and 4 for frequency of Th17 cells (TCRvβ+CD4IL-17A+) **(C)** and cumulative results are graphed in **(D)**. Prior to re-stimulation, culture supernatants from each condition were collected and assayed for IL-2 cytokine abundance using ELISA **(E-F)**. Data shown in A-B represent three independent experiments, C-F represent two independent experiments. *p<0.05, **p<0.01, ***p<0.001, ****, p<0.0001.

3.3.5 Fli-1 regulates T-cell pathogenicity in aGVHD

CD4$^+$ IFN-γ$^+$ and or IL-17A$^+$ T cells play critical roles in aGVHD pathogenesis, which prompted us to determine if Fli-1 also can contribute to acute GVHD development. First, we examined early T-cell activation and proliferation using *in vivo* mixed lymphocyte reaction

(MLR) model of aGVHD and found that donor Fli-1$^{flox/wt}$ CD4^{+} cells produced significantly lower IFN-γ compared to both Fli-1$^{wt/wt}$ and Fli-1$^{flox/flox}$ CD4^{+} cells (Fig. 3 A). Using a well-established aGVHD murine model, we utilized the donor splenocyte and marrow chimera strategy as described above to determine the role of Fli-1 in aGVHD. Consistently, we found that recipients of either Fli-1$^{flox/wt}$ or Fli-1$^{flox/flox}$ grafts were able to significantly increase survival rates and reduce aGVHD clinical scores compared to those that received Fli-1$^{wt/wt}$ grafts. Among all three experimental groups, the recipients of Fli-1$^{flox/wt}$ grafts showed the lowest aGVHD clinical scores and pathological damage in liver, small intestine, and colon (Fig. 3 B-D and F). In agreement with the aGVHD scores and pathology, we found that on day 14 after BMT, T cells derived from Fli-1$^{flox/wt}$ donor grafts produced lower intracellular levels of IFN-γ in both CD4^{+} and CD8^{+} compartments compared to Fli-1$^{wt/wt}$ and Fli-1$^{flox/flox}$ donor T cells in recipient mesenteric lymph nodes (mLN) (Fig. 3 E and G), a lymphoid organ that can closely reflect gut T-cell migration and activation (210, 211).

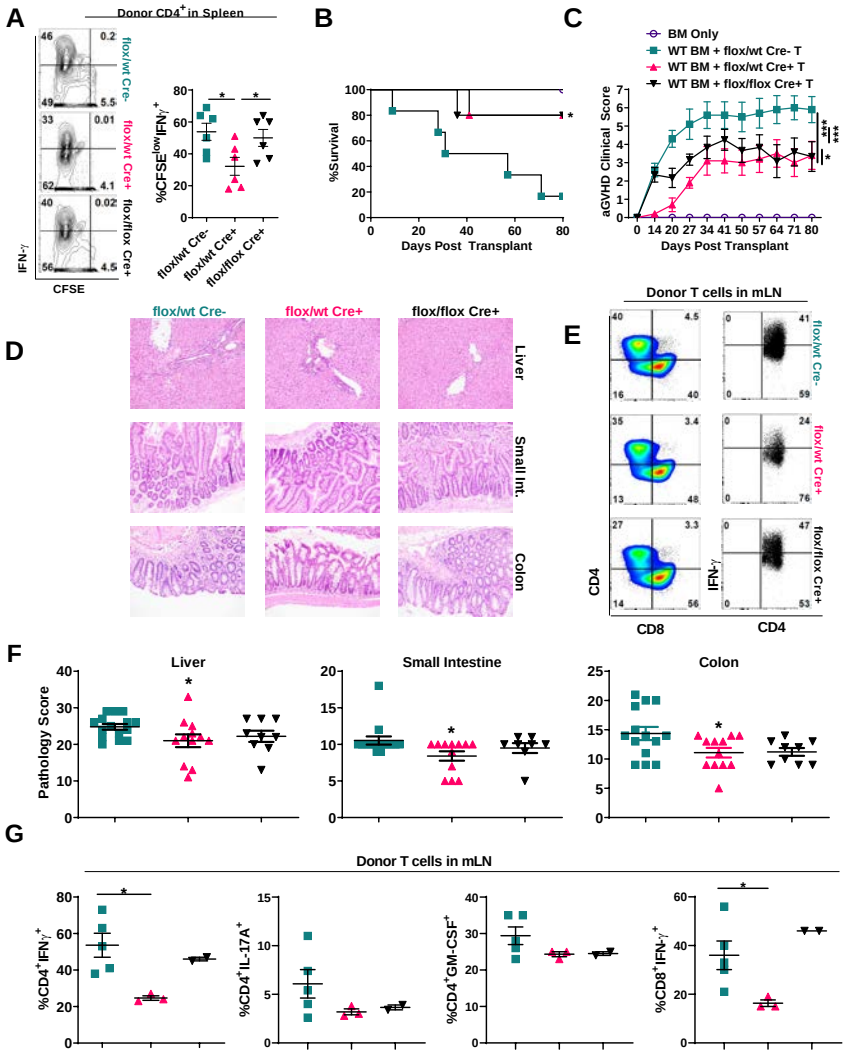

Figure 3.6 Fli-1 regulates T-cell pathogenicity in aGVHD. Purified T cells from spleen and lymph nodes of Fli-1$^{wt/wt}$, Fli-1$^{flox/wt}$, or Fli-1$^{flox/flox}$ were CFSE labeled and infused into lethally irradiated BALB/c mice at 2 x 10^6 per mouse. Day 4 representative flow cytometry plots and cumulative frequencies of proliferated (CFSE^{low}) donor$^+$CD4$^+$ cells producing IFNγ **(A)**. Lethally irradiated BALB/c mice were transplanted with 5 x 10^6 TCD-BM from CD45.1 B6 donors supplemented with or without 0.5 x 10^6 purified total T cells from spleen and lymph nodes of Fli-1$^{wt/wt}$, Fli-1$^{flox/wt}$, or Fli-1$^{flox/flox}$ donors. aGVHD representative survival percentage **(B)** as well as representative acute GVHD clinical scores **(C)**. On day 14 after BMT, indicated tissues sections were H&E stained for pathologic scoring **(D)**. Mesenteric lymph nodes (mLN) were analyzed for donor T-cell populations producing IFNγ, IL-17A, or GM-CSF. Representative flow cytometry plots display IFNγ producing T cells in mLN **(E)**, and cumulative pathological scores are shown **(F).** Frequencies of each indicated donor T-cell population in mLN **(G)**. Data in A-F represent 2-3 independent experiments. Data in G was collected from one set of mice belonging to three independent experiments *p<0.05, **p<0.01, ***p<0.001.

Indeed, alloreactive T cells are highly implicated in causing or exacerbating gut damage during GVHD (212). To extend our study beyond allogeneic response, we utilized the classical syngeneic T-cell transfer model of colitis to determine if Fli-1 contributes to T-cell mediated gut damage. In support of our aGVHD findings, we saw that both Fli-1$^{flox/wt}$ and Fli-1$^{flox/flox}$ naïve T cells had a reduced ability to induce colitis, where Fli-1$^{flox/wt}$ T cells showed the least pathogenicity in colitis development (Supplemental Fig. 4 A). In agreement with reduced colitis scores, mice given Fli-1$^{flox/wt}$ had a significant trend of reduced pathological damage in the colon compared to mice given Fli-1$^{wt/wt}$ T cells (Supplemental Fig. 4 B). Cumulatively, these data suggest that Fli-1 dynamically contributes to IFN-γ^+ producing T cells in both spleen and mLN during aGVHD development, and that Fli-1 may be a critical regulator of the ability of T cells to induce gut damage.

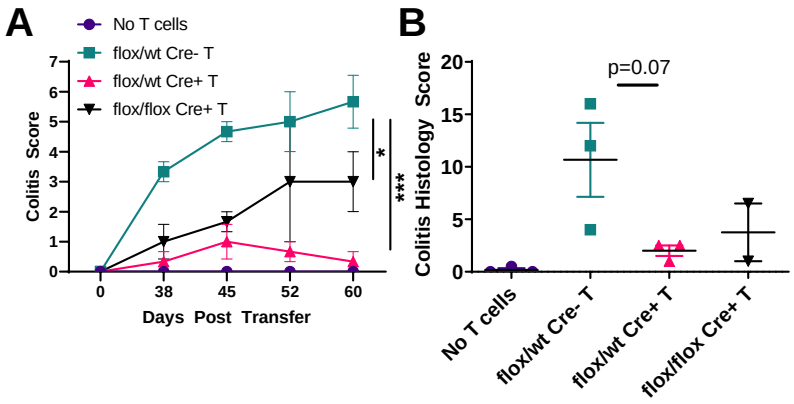

Figure. 3.7 Fli-1 regulates T-cell pathogenicity in murine colitis. Rag1-/- deficient hosts on a B6 background were transplanted with 1 x 10^6 naïve CD4$^+$ T cells (CD8-CD25-CD44-CD62L+) isolated from Fli-1wt/wt, Fli-1flox/wt, or Fli-1flox/flox donors. Colitis clinical score **(A)** and pathologic scores of H&E stained colon sections were analyzed from mice 60 days after T-cell transfer **(B)**. Data represent an individual experiment.

3.3.6 Fli-1 contributes to regulation of genes involved in Treg and effector T-cell development and function

To expand beyond the few target genes already known to be either positively or negatively regulated by Fli-1, we utilized next generation RNAseq of purified T cells during their peak expansion phase (day 14 post-BMT) isolated from spleens of aGVHD mice transplanted with either Fli-1^{WT}, Fli-1$^{flox/wt}$, or Fli-1$^{flox/flox}$ grafts. In agreement with long-term experiments, we saw at the 2 week time-point that reduced Fli-1 activity was associated with a significant reduction in aGVHD clinical scores (Supplemental Fig. 5 A). Sequencing revealed both large downregulated and upregulated gene clusters in Fli-1$^{flox/wt}$ T cells compared to both Fli-1^{WT} and Fli-1$^{flox/flox}$ T cells (Supplemental Fig. 5 B). Fli-1$^{flox/wt}$ T cells showed multiple upregulated genes that are involved in Treg differentiation and function, including *Foxp3, Cd36 (213), Tgfβ1*, *Il10ra (214) , and Inpp5d* (SHIP-1) that we confirmed with qRT-PCR (Supplemental Fig. 5 C). Further, 1$^{flox/wt}$ T cells also showed several downregulated genes related to effector T-cell function, including ten genes that encode for various components of the TCR (*Trbv4* was tested as one representative TCR gene for qRT-PCR), *Ifnγ*, *Il21*, *Fas*, *Gpr18 (215, 216)* , and *Sema7a (217)* that we also confirmed via qRT-PCR (Supplemental Fig. 5 D). Taken together, these results suggest that upon T-cell activation with alloantigen, Fli-1 can dynamically regulate multiple genes correlated with both cellular activation and inflammatory pathways, as well as the anti-inflammatory regulatory T-cell differentiation pathway which could contribute toward and suppress GVHD development, respectively.

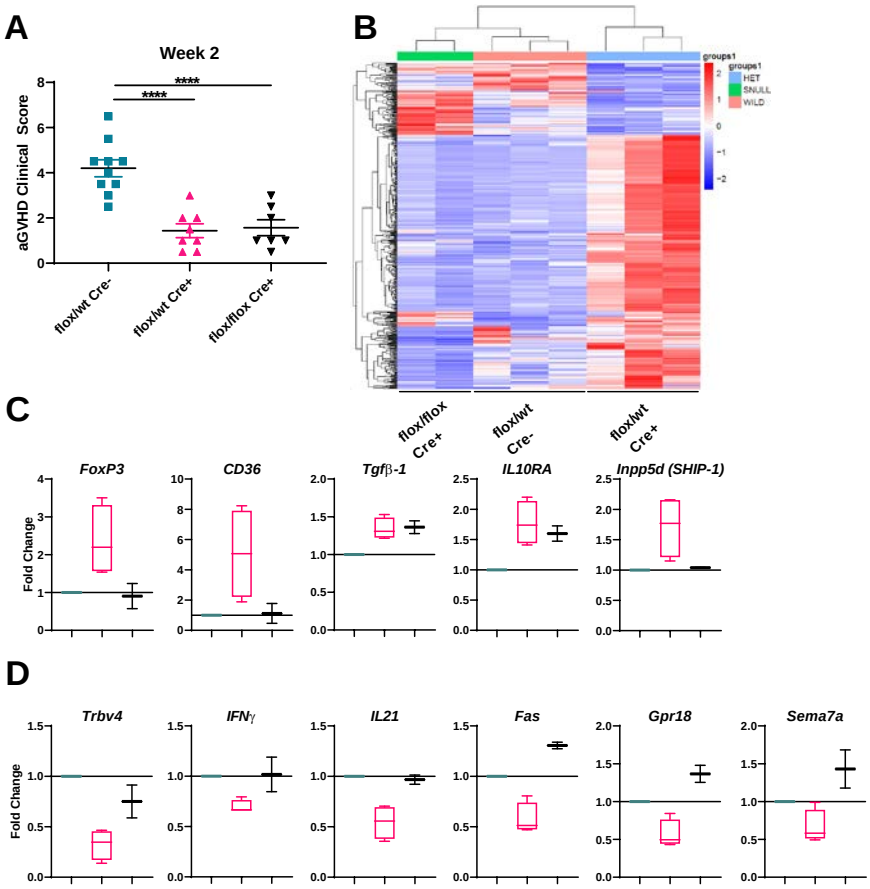

Figure 3.8 Fli-1 contributes to regulation of genes involved in Treg and effector T-cell development and function. aGVHD BMT was performed using 0.5 x 10^6 Fli-1^{WT}, Fli-$1^{flox/wt}$, or Fli-$1^{flox/flox}$ purified donor total T cells and 5 x 10^6 donor TCD-BM from CD45.1 C57BL/6 mice. Week 2 after aGVHD BMT cumulative clinical scoring data **(A)**. On day 14 after aGVHD BMT, total $CD4^+$ and $CD8^+$ cells were isolated from spleens of BMT recipients via positive selection. Total mRNA was extracted from purified T cells and subjected to RNAseq analysis. Heatmap cluster analysis of differentially expressed genes (DEG) between wild-type (Wild), Fli-1 heterozygous (Het), and Fli-1 homozygous (SNull) groups **(B)**. Five of the significantly upregulated DEG **(C)** and six of the significantly downregulated DEG **(D)** were confirmed via qRT-PCR and are shown as fold change over Fli-1^{WT}. aGVHD scores in **(A)** represent three independent experiments. RNAseq was performed on one of three sets of mice from **(A)** where sample sizes were as follows (WILD n=4; HET n=4; NULL n=2).

3.3.7 Camptothecin and etoposide target Fli-1 and ameliorate cGVHD

To determine if inhibiting Fli-1 could be a potential translational strategy for targeting aberrant T-cell activation and GVHD, we utilized a currently available pharmacological agent—camptothecin (CPT)—which has been shown previously to potently inhibit Fli-1 (112). We also verified that CPT could inhibit Fli-1 in a human transformed T-cell line (Jurkat) (Supplemental Fig. 6 A). Each cell line tested (Jurkat, EL4, and A20) had significant induction of apoptosis via CPT by culture day 3 compared to vehicle treatment (Supplemental Fig. 6 B-E and data not shown). To determine the extent of specificity of low-dose CPT for Fli-1 versus topoisomerase I inhibition, we performed a topoisomerase I enzymatic activity assay. Here, we found that low-dose CPT did not significantly hinder the ability of topoisomerase I to relax supercoiled DNA—the primary function of this enzyme—even after 48h incubation of activated T cells with low-dose CPT (Supplemental Fig. 6 F). We then tested the impact of low-dose CPT on primary murine polyclonally activated T cells *in vitro* and found that 30nM CPT reduced T-cell proliferation but preserved IFN-γ production (Supplemental Fig. 6 G and H).

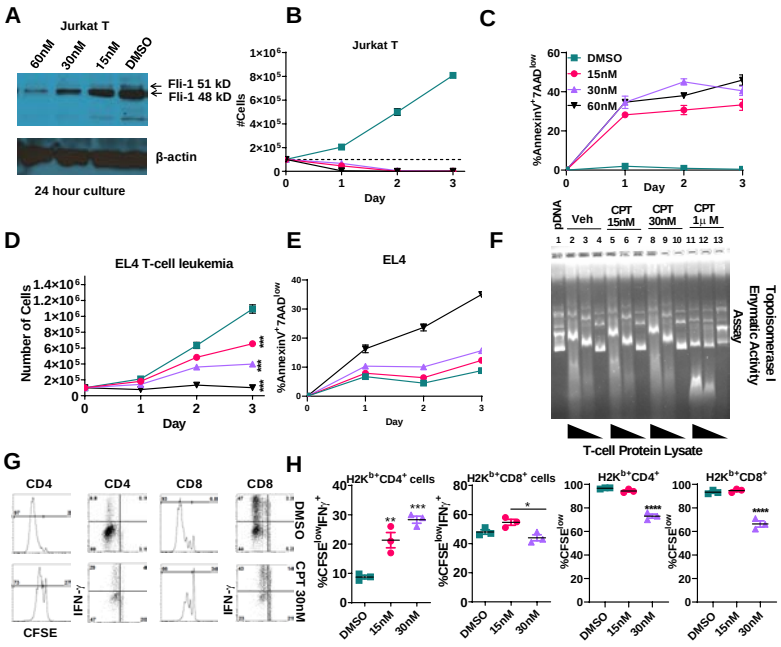

Figure 3.9 Low-dose camptothecin inhibits Fli-1, reduces tumor growth and murine T-cell proliferation. Human Jurkat cells were cultured with vehicle or 15nM to 60nM CPT for ~24 hours. At 24h, total cell lysates were obtained from Jurkat cultures and blotted for Fli-1 and β-actin **(A)**. Jurkat cell

growth **(B)** and apoptosis (Annexin V+7AAD^{low}) **(C)** was measured via flow cytometry at the indicated time points. Murine tumor line EL4 (T-cell leukemia) was also treated with vehicle or indicated doses of CPT and cell growth **(D)** and apoptosis **(E)** was quantified via flow cytometry. Topoisomerase I enzyme assay **(F)**: Primary murine wild-type T cells were activated via polyclonal stimulation in the presence of vehicle, 15nM, 30nM, or 1μM CPT for ~48h. On day 2, total cell lysates were extracted from cultured T cells and subjected to topoisomerase I assay as described in "methods" section. Gel loading order: L1= plasmid DNA; L2-4=T cells + vehicle; L5-7= T cells + 15nM CPT; L8-10= T cells + 30nM CPT; L11-13= T cells + 1μM CPT. The amount of T-cell protein lysate added into the plasmid DNA Top I enzyme reaction was titrated down from right to left (5μg, 2.5μg, 0.5μg total protein) within each condition. Murine primary T cells from wild-type B6 mice were CFSE labeled and activated *in vitro* via polyclonal stimulation for ~72h in the presence of vehicle or 15nM to 30nM CPT. Representative flow cytometry plots show cell proliferation and intracellular IFN-γ production **(G)** and frequencies of proliferated and IFN-γ producing cells **(H)**. Data represent two independent experiments. *p<0.05, **p<0.01, ***p<0.001.

We next investigated whether this strategy would be beneficial for cGVHD prevention or treatment using a murine model. CPT at low doses was able to effectively prevent cGVHD development in mice, reflected by clinical manifestations and pathological damage in GVHD target organs skin and small intestine (Fig. 4 A-E). At a late-phase of cGVHD, we found that the recipients treated with CPT mice had significantly increased number of $CD4^+CD8^+$ thymocytes, and significantly reduced $CD4^+$ T_{FH} and $CD8^+$ T_{FH}-like cells (Fig. 4 F and G). In addition to cGVHD prevention, a delayed CPT administration was able to rapidly and significantly reverse already established cGVHD severity (Fig. 4 H). To determine if another drug which could also target Fli-1 could reduce cGVHD, we then tested an alternative more clinically relevant drug to CPT (etoposide or ETO) as a potential translational strategy. We found that although it had a higher IC_{50} compared to CPT, ETO exhibited Fli-1 inhibition in Jurkat cells compared to vehicle control (Fig. 5 A). Used as a prophylactic strategy in a cGVHD mouse model, we found that 5mg/kg ETO was able to dramatically reduce cGVHD and was associated with significant increases of thymic $CD4^+CD8^+$ and splenic $B220^+$and $CD4^+FoxP3^+$ populations 60 days post-BMT (Fig. 5 B-C). There was also a significant reduction of $CD4^+$IFN-γ^+, $CD8^+$IFN-γ^+, and $CD4^+$IL-$17A^+$ T-cell frequencies in peripheral lymph nodes (Fig. 5 D). Overall, these results suggest that targeting Fli-1 using low-dose CPT and an FDA-approved alternative ETO are effective methods to prevent and or reverse cGVHD, where these effects can be attributed, at

least in part, due to Fli-1 inhibition on lymphocytes since these drugs have established Fli-1 inhibitory activity as shown here and by others (112).

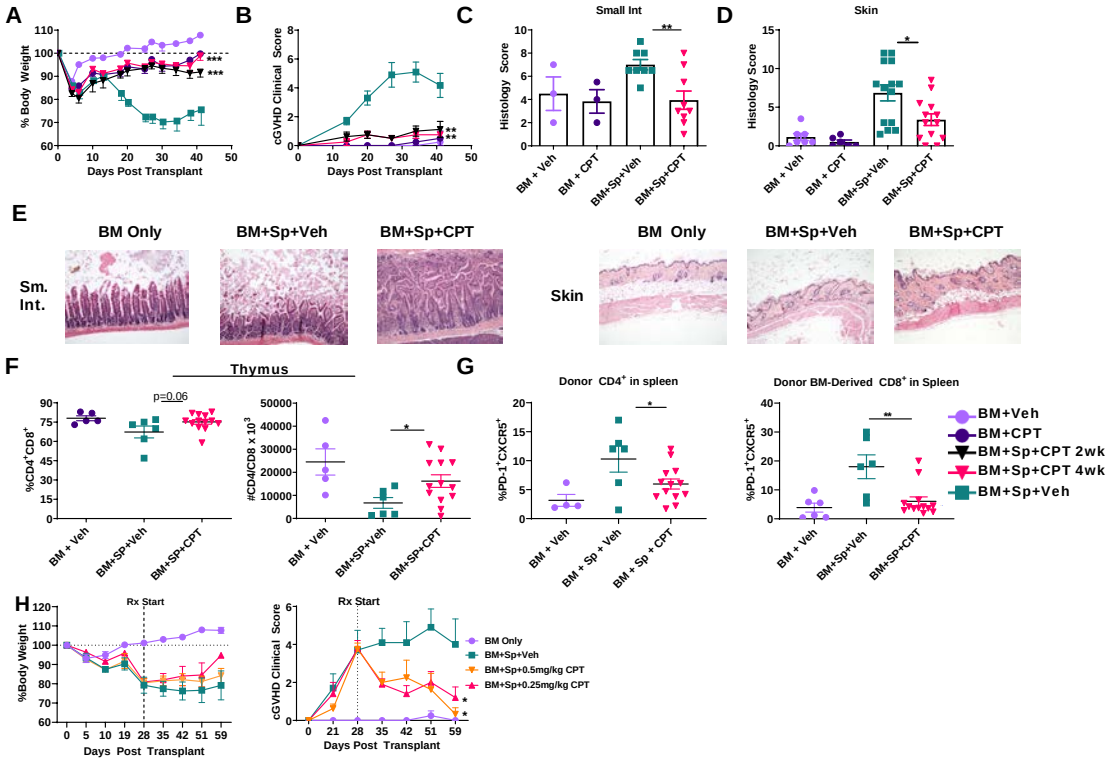

Figure 3.10 Low-dose Camptothecin prevents and reverses cGVHD. Lethally irradiated BALB/c mice were transplanted with 5 x 10^6 TCD-BM from CD45.1$^+$ or CD45.2$^+$ B6 donors supplemented with or without 0.3 to 0.5 x 10^6 total splenocytes. Shortly prior to transplant (2-4h) mice were injected i.p. with either vehicle (DMSO) or 0.25 to 0.5mg/kg camptothecin (CPT) every other day for 2 to 4 weeks. Body weight **(A)** and cGVHD clinical score **(B)** were monitored weekly after allo-BMT. Cumulative scores by an independent pathologist were obtained from histological sections and H&E staining that were performed on the indicated tissues in **(C)** and **(D)**. Representative pictomicrographs of small intestine and skin H&E sections from the indicated groups **(E)**. Day 40 cumulative analysis of the indicated thymic **(F)** and splenic **(G)** populations via flow cytometry. Similar experiments were performed except that vehicle and CPT administration was delayed until 28-30 days after BMT and body weight and cGVHD clinical score were monitored weekly following BMT **(H)**. Data in A-H represent three independent experiments. *p<0.05, **p<0.01, ***p<0.001.

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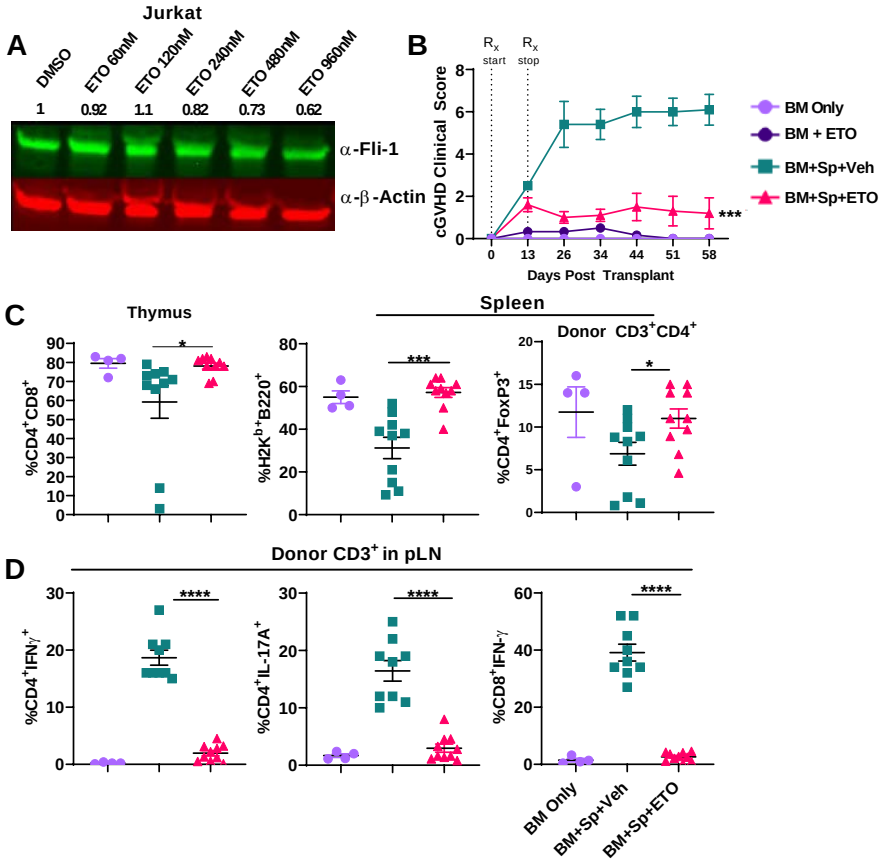

Figure 3.11 Etoposide prevents cGVHD. Jurkat cells were treated with the indicated concentrations of etoposide (ETO) for ~24h. At ~24h, total cell lysates were extracted for western blot of Fli-1 and β-actin proteins **(A)**. cGVHD BMT was performed similarly as described in figure 2, except mice were given donor BM and splenocytes from WT-B6 mice and supplemented with or without vehicle or 5mg/kg etoposide i.p. starting on day 0 and administered every other day until day 14 post-BMT. cGVHD clinical score was monitored weekly **(B)** and frequencies of thymic $CD4^+CD8^+$ and splenic $B220^+$ and $CD4^+FoxP3^+$ populations are shown in **(C)**. Peripheral lymph node (pLN) T-cell populations producing cytokines were analyzed on day ~60 post-BMT **(D)**. Data in A is from an individual experiment and data in B-D are from two independent experiments

3.3.8 Low-dose Camptothecin prevents aGVHD and preserves GVL effect

To determine the effect of low-dose CPT on the GVL effect, we employed a haploidentical murine model of aGVHD supplemented with an aggressive P815 mastocytoma. In this model, mice given TCD-BM without mature T cells succumb to P815 mediated mortality rapidly, whereas mice that receive mature T cells from allo-BMT are protected against P815 outgrowth. Here, allo-BMT recipients given TCD-BM, P815, mature T cells, and treated with vehicle developed moderate to severe aGVHD leading to significant GVHD-related mortality. In contrast, the recipients under the same conditions but treated with low-dose CPT had significantly improved GVHD survival (Fig. 6 A), no P815 tumor mortality (Fig. 6 B) and reduced aGVHD clinical score (Fig. 6 C). Mechanistically, mice treated with low-dose CPT had improved thymic $CD4^{+}CD8^{+}$ reconstitution and higher splenic donor-derived $B220^{+}$ B-cell reconstitution compared to vehicle treated mice (Fig. 6 D and E). Importantly, while mice given TCD-BM plus P815 had rapid P815 outgrowth (100% mortality by day 14), low-dose CPT treated mice that received mature allogeneic T cells had complete protection against P815 relapse (0/13 by day 80), similar to their vehicle treated counterparts. To examine the direct impacts of low-dose CPT against P815 itself, we also treated mice given TCD-BM and P815 with CPT without mature T cells, which resulted in early tumor protection, but ultimately did not directly prevent tumor relapse (100% mortality by day 30) (Fig. 6 B and F). P815 cells expressed a low amount of Fli-1 compared to Jurkat cells (Fig. 6 G). Taken together, these results indicate that low-dose CPT is an effective strategy to reduce aGVHD severity and lethality after allo-BMT, while preserving the ability of alloreactive T cells to prevent leukemia relapse, even long after treatment withdrawal.

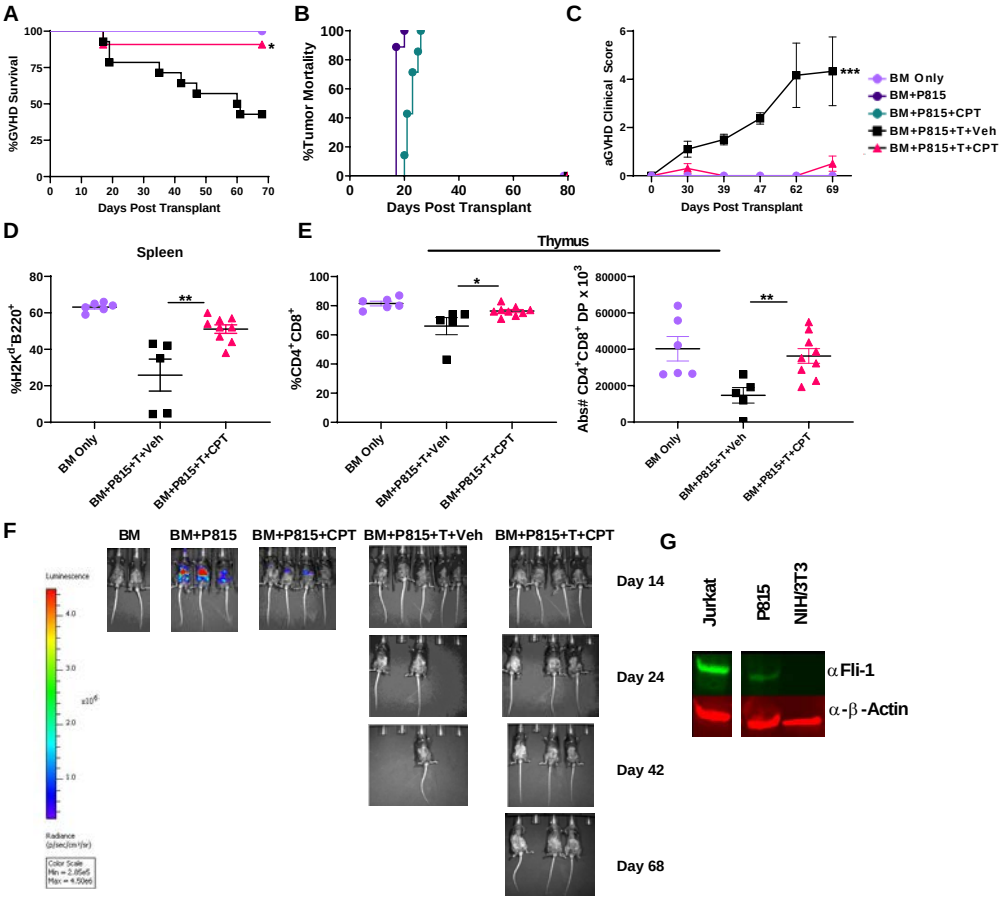

Figure 3.12 Low-dose Camptothecin prevents aGVHD and preserves the GVL effect. Lethally irradiated B6D2F1 mice were transplanted with 5 x 10^6 TCD-BM cells from CD45.1 or CD45.2 B6 donors supplemented with or without 3 x 10^6 purified total T cells from CD45.2 B6 donors. Three out of four groups of mice were also supplemented with 5,000 P815 at the time of BMT, which received vehicle, treatment, CPT 0.25mg/kg only, mature T cells plus vehicle, or mature T cells plus CPT 0.25mg/kg on day 0 administered every other day until day 28 post-BMT. Recipient GVHD survival **(A)**, tumor mortality **(B)**, and aGVHD clinical score **(C)** were monitored following BMT. At experimental endpoint (day ~80) splenic donor $H2K^d$-$B220^+$ **(D)** and thymic $CD4^+CD8^+$ populations **(E)** were analyzed via flow cytometry. IVIS200 imager was used to periodically monitor firefly-luciferase expression of transplanted P815 cells in recipient mice injected with D-luciferin substrate at each imaging time-point **(F)**. Western blot analysis of indicated proteins and tumor cell lines after 24 h in culture **(G).** Data in A-F represent three independent experiments. Data in G is from an individual western blot *p<0.05, **p<0.01, ***p<0.001.

3.3.9 Camptothecin inhibits Fli-1 on human T cells and reduces GVHD in a xenograft model

To further increase clinical relevance of our study, we tested CPT in human cells and found that CPT at a very low-dose was able to potently inhibit Fli-1 protein level in polyclonally stimulated human PBMC's *in vitro* (Fig. 7 A). To further confirm Fli-1 protein expression specifically in human T cells, we utilized an available human anti-Fli-1 flow cytometry antibody. In agreement with western blot data, we found significantly reduced expression of Fli-1 in both $CD3^+CD4^+$ and $CD3^+CD4^-$ T cells that had been treated with 15 nM CPT compared to vehicle treatment (Fig. 7 B). Low-dose CPT treatment of activated human PBMC's was able to significantly reduce T-cell proliferation measured via CFSE dilution, but was able to highly preserve T-cell IFN-γ production, especially in $CD8^+$ T cells (Fig. 7C-D). We then tested if low-dose CPT would be able to reduce GVHD in a human-to-mouse xenograft model. Here, low-dose CPT administration given prophylactically for 2 weeks led to a significantly increased survival rate compared to vehicle treated mice, and that the surviving CPT-treated mice maintained high body weight until experimental endpoint (Fig. 8 A-B). Peripheral blood taken from recipient mice on day 14 after transplant confirmed human T-cell engraftment and specificity of human flow cytometry staining by using recipients given irradiation but no human PBMC as a negative control (Figure 8 C). We examined human T-cell subsets in the recipient spleens on day 15 post-transplant, and found significant reductions in the frequency and number of both $CD3^+CD8^-$ and $CD3^+CD8^+$ populations, although their ability to produce IFN-γ^+ was not reduced on a per-cell basis (Fig. 8 D-E). We then confirmed our *in vitro* data that CPT also acts as a Fli-1 inhibitor *in vivo*, as cells extracted from splenocytes of mice treated with CPT showed obvious reduction in Fli-1 protein level compared to vehicle treated controls (Fig. 8 F). Cumulatively, these data indicate a novel ability of CPT to act as a Fli-1 inhibitor on primary human lymphocytes, both *in vitro* and *in vivo*, and that CPT can reduce human T-cell proliferation as well as improve survival of GVHD recipients in a xenograft model.

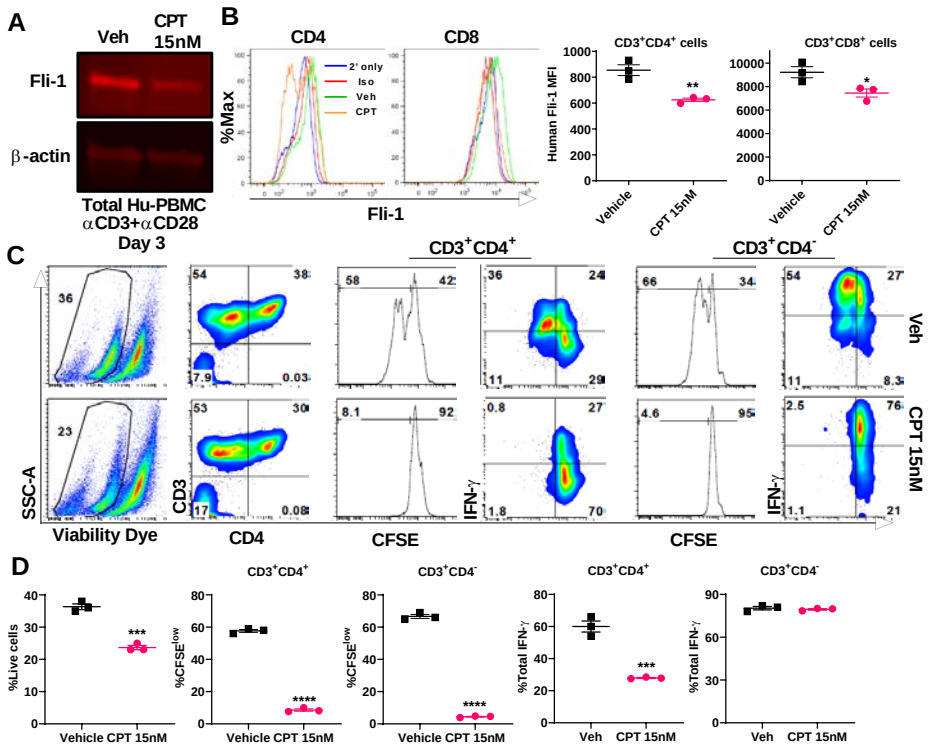

Figure 3.13 Camptothecin acts as a Fli-1 inhibitor on human T cells and reduces their proliferation *in vitro*. Total human PBMC isolated from healthy donors were CFSE labeled and activated *in vitro* via soluble αCD3/αCD28 (2μg/ml) and co-cultured with DMSO (vehicle) or 15nM camptothecin for 3 days. Cultures were harvested and lysed for western blot analysis of Fli-1 protein and β-actin loading control on day 3 **(A)**. Representative flow cytometry histograms show intracellular Fli-1 expression in $CD3^+CD4^+$ and $CD3^+CD4^-CD8^+$ gated T cells treated with vehicle or 15nM CPT (left) and representative Fli-1 MFI values (right); isotype control (red line); secondary antibody only (blue line); vehicle treated cells (green line); CPT treated cells (orange line) **(B)**. Representative flow cytometry plots show proliferation (CFSE dilution) and IFNγ cytokine production of human T cells **(C)**. Representative frequencies of live cells in culture (left), CD4 proliferation and cytokine production (middle) and CD8 proliferation and cytokine production (right) are shown **(D)**. Data in A-D represent two independent experiments. *p<0.05, **p<0.01, ***p<0.001.

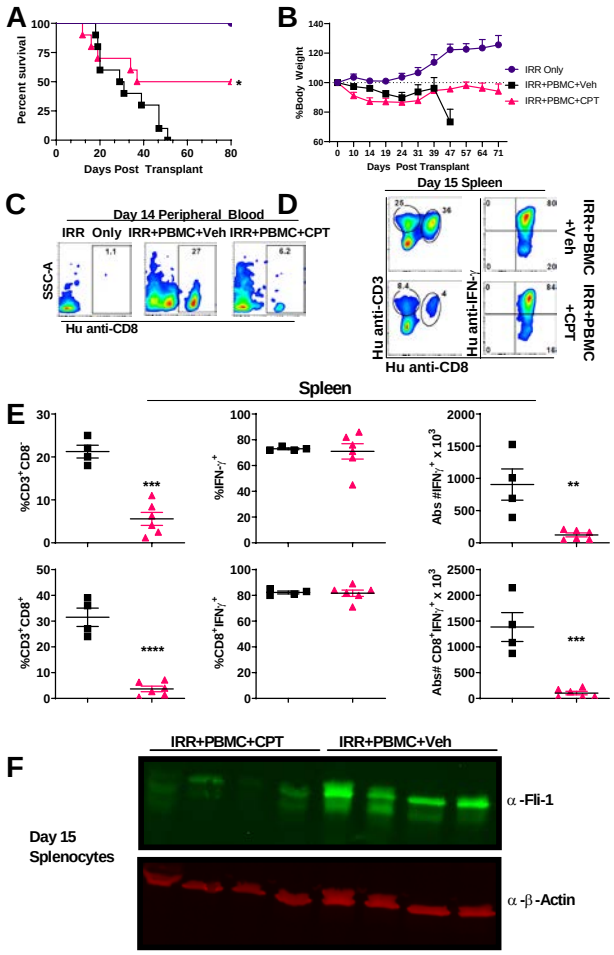

Figure 3.14 Camptothecin inhibits human Fli-1 and reduces GVHD in a xenograft model. HLA-A2$^+$ NSG mice were sub-lethally irradiated (250cGy) and transplanted with 8 to 10 x 10^6 total human PBMC from healthy donor (HLA-A2-) to induce human GVHD. Recipient survival **(A)** and body weight **(B)** was monitored up to 80 days after transplant. Peripheral blood staining of human CD8 cells on day 14 post-transplant **(C)**. Staining of human T cells from xenograft spleens on day 15 post-transplant **(D)** percentage and number of human IFN-γ producing CD3$^+$CD8$^-$ T cells (top) or human IFN-γ producing CD3$^+$CD8$^+$ T cells (bottom) **(E)**. Western blot of day 15 splenic whole-cell lysates from xenograft mice using indicated primary antibodies **(F)**. Data in A-B represent two independent experiments. Data in C-F were collected from one set of mice belonging to two independent experiments *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

3.4 Discussion

The specific role of Fli-1 in primary T cells has not been studied in depth previously, especially not in allo-HCT conditions. Combination of our findings with previous literature can allow us to posit several potential mechanisms to explain how Fli-1 is regulating the allogeneic T-cell response GVHD development.

Consistently, we observed that a PD-1^{+}CXCR5^{+} population of CD4+ T cells—resembling a T_{FH}-like phenotype—was decreased in spleens of mice given Fli-$1^{flox/wt}$ grafts. A previous report recently identified that Fli-1 was able to directly bind to the promoter of the master regulator of T_{FH}-cell differentiation BCL6 in lymphoma cells (218). In addition to its impact on T_{FH} cells, Notch-1 signaling also has been shown to promote Th17 cells and negatively regulate Treg development (207, 208, 219). Our findings that reduction of Fli-1 activity could significantly reduce *Notch1* mRNA levels in primary activated T cells suggests a potential Fli-1/Notch-1 axis that could be contributing to the ability to regulate T cells.

Particularly interesting within our study was the finding that T-cell specific homozygous reduction, but not heterozygous reduction of Fli-1 activity, led to distinct outcomes in genotype-matched spleen and bone marrow chronic GVHD transplants, but this outcome did not occur when the source of donor bone marrow was changed to wild-type marrow. When we switched Fli-$1^{flox/flox}$ bone marrow to Fli-1^{WT} marrow, this was correlated with a significant increase in the frequency of $CD4^{+}CD8^{+}$ thymocytes. Indeed, a previous group discovered that germ-line heterozygous mutation of Fli-1 resulted in normal thymus development, but homozygous mutation resulted in significantly reduced thymocyte numbers that was attributed to defects in pre-thymic T-cell progenitors (114). This report is consistent with our findings that homozygous reduction but not heterozygous reduction of Fli-1 activity on donor bone marrow-derived T cells can impair frequency of $CD4^{+}CD8^{+}$ thymic repopulation after allo-BMT.

In spleens of mice with cGVHD, we found a population of late-phase (days 40-60 after BMT) $CD8^+$ T cells, which expressed high levels of surface PD-1 in mice given wild-type allogeneic splenocytes. Similar results were observed by our group previously, and these T cells were also capable of producing high levels of IFN-γ (26). Previous reports have identified that this population ($CD8^+PD\text{-}1^+$) was a clonally expanding effector population that was not exhausted and maintained functionality in the context of a chronic inflammatory disease (39). This report is in agreement with our consistent results that reduction of cGVHD correlates with a reduction in surface PD-1 expressed on $CD8^+$ T cells in the spleen, whereas mice with moderate to severe cGVHD have high levels of PD-1 expression on $CD8^+$ T cells.

RNAseq and qRT-PCR study of T cells from mice given allo-BMT uncovered that heterozygous reduction of Fli-1 activity led to both upregulated and downregulated transcriptomic changes in activated T cells during early phase acute GVHD. Some of the most significantly upregulated genes in $Fli\text{-}1^{flox/wt}$ were related to Treg differentiation and function, whereas some of the most significantly downregulated genes were related to effector T-cell differentiation and function, suggesting together with our other *in vitro* and *in vivo* data that Fli-1 may be playing a significant role in Treg development, while also being able to mediate pathogenic effector T cells. While the precise roles of Fli-1 in global regulation of T cells need to be further studied, this is out of the scope of the current study.

There has still been relatively little progress in the field for developing a Fli-1 specific inhibitor, despite its known involvement in multiple types of malignant neoplasms. Therefore, some of the only pharmacological strategies available to date which can inhibit Fli-1 protein or Fli-1 transcriptional activity are known chemotherapy drugs such as camptothecin and etoposide. Thus, we employed two of these available drugs—CPT and ETO—in our study to determine if targeting Fli-1 pharmacologically would be beneficial in allo-HCT. We observed that CPT did not obviously impair the enzymatic activity of its only known target—topoisomerase I—at low

concentrations (15nM-30nM) in activated murine T cells, even when cultured with the drug for 48h. It was also previously reported that CPT was significantly less effective at preventing growth of malignant cell lines designed to overexpress Fli-1 (112), suggesting that CPT acts through Fli-1 inhibition as one of its primary mechanisms of action.

To attempt to translate these *in vitro* findings to an *in vivo* system, we employed low-dose CPT in both a cGVHD model and an aGVHD model using P815 to study the GVL response. We found in subsequent studies following allo-HCT experiments that P815 expressed low levels of Fli-1 compared to other cell lines such as Jurkat. This could potentially explain why there was an early benefit of CPT administration against P815, but since this cell line does not strongly express Fli-1, it may not rely on it for a survival advantage, and thus CPT treated mice who did not receive mature T cells had rapid relapse and mortality from P815. We also found that ETO was able to reduce Fli-1 expression and cGVHD development that was associated with increases in $CD4^+$ Tregs and reductions in inflammatory T-cell subsets in lymphoid tissues, which was in agreement with a previous report that ETO is able to selectively target activated T cells (220). These effects of CPT and ETO may be due, at least in part, to reduction of Fli-1 activity (112). Interestingly, ETO used in the clinic as myeloablative conditioning regimen has compared favorably to other agents such as cyclophosphamide for the ability to reduce leukemia relapse and GVHD severity (221, 222). Thus, it is worth exploring in future studies if currently employed chemotherapeutic agents such as topotecan, etoposide, and other chemically related drugs (e.g., irinotecan) could be repurposed as strategies to reduce Fli-1 activity and prevent or treat GVHD in the clinical setting.

Although we present evidence that targeting Fli-1 is beneficial towards reducing T-cell mediated inflammation and fibrosis after allo-HCT, this strategy is not without caveats. Sato et al., 2017 demonstrate that keratinocyte-specific Fli-1 deficiency was associated with sclerotic disease induction driven by promotion of Th2 and Th17 polarization and generation of

autoreactive T cells, while in skin disease Fli-1 was important for mTEC functions in the thymus (223). However, since the effects of fibrosis in this previous report were mediated largely by autoreactive T cells, we have shown in the current study that reducing Fli-1 expression directly on T cells can mitigate the damage caused by activated T cells. Thus, by reducing Fli-1 expression on alloreactive T cells, downstream effects of Fli-1 reduction on endothelial or other cell types could potentially be offset via Fli-1 inhibiting therapy such as CPT or ETO.

In conclusion, we show—for the first time to our knowledge—evidence that Fli-1 plays a critical role in the alloreactive and antigen-specific T-cell response, and based on these results we show that Fli-1 is a pathogenic factor which can promote inflammatory T-cell phenotypes and suppress regulatory T cells, both *in vitro* and *in vivo*. Thus, targeting Fli-1 using a pharmacological strategy could potentially provide two benefits in the allo-HCT setting: 1) Targeting leukemias and lymphomas which overexpress or rely on Fli-1 and 2) Targeting pathogenic alloreactive T cells which utilize Fli-1 to some extent for differentiation, survival, or cellular functions. Overall, these results suggest that utilizing strategies to reduce Fli-1 expression or transcriptional activity may be a promising area of future research for therapies which aim to reduce GVHD development without compromising the ability of T cells to mediate anti-leukemia activity.

3.5 Abstract

Allogeneic hematopoietic stem-cell transplantation (allo-HCT) remains an effective available treatment for hematological malignancies such as leukemia. However, Graft-versus-Host Disease (GVHD), manifesting in either acute (aGVHD) or chronic (cGVHD), presents significant life-threatening complications. Here, we investigated Friend Virus Leukemia Integration 1 (Fli-1) in GVHD pathogenesis. Heterozygous genetic ablation of two critical exons

of the fli-1 gene in donor-derived T cells was associated with significant reduction of disease development in allo-HCT models of cGVHD and aGVHD. Reduction of cGVHD was associated with increased frequencies of regulatory T cells (Tregs) and decreased frequencies of IFN-γ+, IL-17A+, and TFH-like T cells in lymphoid organs. Accordingly, HY-antigen-specific transgenic T cells bearing fli-1 deficiency displayed increased and decreased propensities to differentiate into iTreg and Th17 cells in vitro, respectively. We also demonstrate that low-dose camptothecin exhibits action as a potent Fli-1 inhibitor, both in vitro and in vivo against mouse and human Fli-1. Utilization of two different drugs that targeted Fli-1 was able to significantly prevent cGVHD development and even reverse established cGVHD. Camptothecin also prevented aGVHD while preserving the graft-versus-leukemia (GVL) effect against P815 mastocytoma. Further, camptothecin reduced human T-cell proliferation, yet spared CD8+ T-cell IFN-γ production both in vitro and in vivo, while also significantly reducing GVHD in a human xenograft model. Using novel T-cell specific Fli-1 mutant mouse strains, we provide the first evidence that Fli-1 plays a crucial role in alloreactive T-cell activation and differentiation, and that targeting Fli-1 may be an attractive strategy for treating GVHD without compromising the GVL effect.

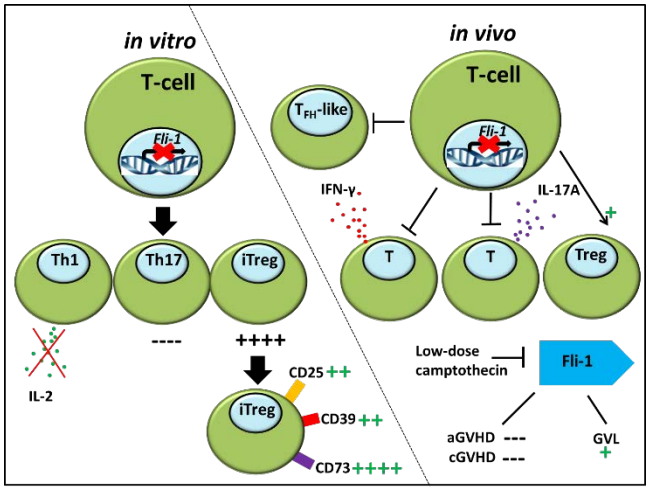

Figure 3.15 Graphical Abstract of: Fli-1: A Druggable Transcription Factor that Regulates T-cell Immunity and Experimental GVHD

Chapter 4: Conclusions and Future Directions

While it has been shown previously that Fli-1 is critically involved in the development of murine SLE, the role of Fli-1 in GVHD development has not been studied thus far, other than in the current study. Further, although several important molecules have previously been discovered to be regulated via Fli-1 in T-lymphocytes, such as IL-6 cytokine producing T cells, CXCR3, and C16-ceramide, if and how Fli-1 specifically regulates T-cell development and differentiation—especially in the context of allo-HCT—has not been characterized in depth.

One of the most intriguing findings from our Fli-1 study was that Fli-1 contributed toward the development of antigen-specific iTreg and Th17 cells, and that donor-derived $CD4^{+}FoxP3^{+}$ and $CD4^{+}IL\text{-}17A^{+}$ T cells in lymphoid organs of cGVHD murine recipients were increased and decreased—respectively—relative to wild-type controls as a consequence of reduced Fli-1 activity. Thus, how Fli-1 regulates Treg and Th17 development and or function, either by direct or indirect mechanisms, would be an interesting avenue of future investigation. One potential mechanism of how Fli-1 could regulate both iTreg and Th17 differentiation is through Notch signaling, where it has been shown that Fli-1 positively regulates Notch-1 expression in hematopoietic progenitor cells (111), and that Notch-1 itself is a critical factor involved in Treg and Th17 differentiation as well as GVHD (205-208). Previous reports have also shown that Fli-1 positively regulates the micro RNA cluster 17-92 (miR17-92) on lung cancer cells *in vitro (118).* Whether this same phenomenon of miRNA regulation via Fli-1 occurs on primary murine and human T cells, or in leukemia is unknown and could be an important area of investigation.

T cells are essential and important drivers of GVHD pathogenesis, however multiple other lymphocytes play critical roles in GVHD development and can contribute differentially based on whether GVHD is in the acute or chronic phase. B cells in particular have been recently identified to be essential for the development of the chronic phase of GVHD due to their effector functions (alloreactive antibody deposition into GVHD target tissues) and co-stimulatory functions to

further prime, activate, and differentiate alloreactive T cells. Fli-1 has previously been shown to play an essential role in B cells, as proliferation of B cells, production of IgG1 antibodies to T-cell dependent and independent antigens, and B-cell class switching to IgG1 *in vitro* were reduced in mice with blocked Fli-1 activity(132, 133). Mechanistically, it was found that reduction of Fli-1 activity resulted in reduced Pax-5, E2A, and Egr-1 mRNA expression in B cells, which are important proteins for B-cell development and function. However, the role of Fli-1 in B cells in human diseases, particularly in GVHD, has not been elucidated. Thus, studies to determine the role of Fli-1 in B cells during GVHD development—particularly during the chronic phase of GVHD—would be an interesting avenue of research based on the current studies from our laboratory. Initially, studies could utilize pre-clinical models of cGVHD using donor lymphocytes that bear reduced Fli-1 activity specifically in B cells via *CD19*Cre or *CD23*Cre. If experiments are successful in indicating that Fli-1 deficiency specifically in B cells reduces cGVHD, then determining the molecular mechanisms of how Fli-1 regulates B-cell pathogenesis during cGVHD development would be of great interest to the allo-HCT field.

In addition to T cells and B cells, NK and NKT cells also have been documented to play roles in GVHD development (224, 225). It was previously shown that reduced Fli-1 expression was associated with elevated numbers of NK cells in peripheral blood and bone marrow(135). Related to this previous finding, genes related to NK cells were also identified as differentially expressed in our RNAseq analysis of RNA extracted from $CD4^+$ and $CD8^+$ positively selected cells from spleens of mice with acute GVHD, suggesting that NKT cell populations and or functions may be altered as a consequence of reduced Fli-1 activity. Thus, if and how Fli-1 regulates NK and or NKT cell differentiation and function during GVHD pathogenesis is another potential interesting area of future investigation. Studies could be initially conducted using Fli-$1^{flox/flox}$ mice crossed to *Cd1d*-Cre mouse strain which would reduce Fli-1 activity in NKT cell populations and could be used as a donor source of pre-clinical murine GVHD studies. If successful, subsequent molecular

mechanism studies of if and how Fli-1 regulates NKT cells would provide a highly novel and intriguing contribution toward understanding of NKT cell biology, especially during GVHD development.

Not only do donor lymphocytes play a key role during GVHD development, but recipient hematopoietic antigen presenting cells such as dendritic cells and macrophage are crucial for priming of alloreactive T cells and induction and maintenance of GVHD. Previously it was identified that reduction of Fli-1 activity was associated with elevated levels of dendritic cells and macrophage in bone marrow and spleen of Fli-1 mutant mice (134). Although dendritic and macrophage populations were found to be increased via mutation of Fli-1 in the 2013 study, whether their functions are similar to wild-type dendritic cells and macrophage in the capacity to prime and activity alloreactive T cells is still unclear, thus suggesting that study of the role of Fli-1 in these two cell populations in the development of alloreactive T-cell responses and in GVHD would be a novel area of investigation. Study of Fli-1 in these two cell populations could be carried out via utilization of *CD11c*-Cre and *LysM*-Cre mouse strains bred to our *Fli-1*$^{flox/flox}$ strain. While performing a multitude of different functions, often B cells, macrophage, and dendritic cells (all performing jobs as APC's) share many similar characteristics and can have similar transcriptional programming (e.g., similar roles of IRE-1α/XBP-1 pathway are shared between macrophage, dendritic cells, and B cells). Thus, it could be hypothesized that if Fli-1 contributes toward B-cell differentiation and function, there is rationale to also study Fli-1 in macrophage and dendritic cells.

Tregs are also currently promising strategies in the clinical setting for GVHD, where these cells are induced and expanded *ex vivo* and transplanted into patients prior to allo-HCT or after transplant to attempt to ameliorate GVHD(40, 199). Thus, since the current study identified that inhibiting Fli-1 activity could enhance iTreg differentiation and iTreg functional molecules, silencing Fli-1 on Tregs *ex vivo* and determining the stability and suppressive function against

alloreactive effector T cells *in vivo* would be a potential area of future investigation. Because siRNA, shRNA, and CRISPR/Cas9 mediated silencing systems are costly and time consuming for clinical applications, drug-mediated silencing of Fli-1 for these *ex vivo* Treg studies would therefore be ideal. Thus, similar pharmacological strategies that we have demonstrated in the current study could be used to attempt to reduce Fli-1 in Tregs and enhance their stability or function such as using camptothecin, etoposide, and or more specific inhibitors like A661 and A665 (see (226)). Of particular interest is camptothecin, because in the current study it was identified that this drug is a powerful Fli-1 inhibitor in healthy human cells, and when used for *in vitro* differentiation and expansion, there is little to no concern for recipient side effects or toxicity due to drug administration of camptothecin given directly to recipients. However, additional studies implementing more specific Fli-1 inhibitors like A661 and A665 for *in vitro* Treg differentiation and expansion could be carried out to determine if they exhibit similar effects on human T cells *in vitro* compared to camptothecin or etoposide.

Because Fli-1 is a transcription factor, it has been notoriously difficult to specifically or directly target thus far, a similar phenomenon faced by attempts to target most other transcription factors. Several attempts have previously been made to identify Fli-1 specific inhibitors using large library drug screening luciferase promoter assays (112, 201, 226, 227), however a completely Fli-1 specific inhibitor has yet to be identified. While A661 and A665 exhibited direct binding to the Fli-1 protein and successful Fli-1 depletion, it was noted that A661 and A665 induced reduced proliferation in tumor cells which expressed no Fli-1, indicating that these compounds could target other non-Fli-1 proteins. However, in tumors cells designed to overexpress Fli-1, the effect of A661 and A665 on cell proliferation and apoptosis was driven primarily through the Fli-1 protein. However, novel technologies continue to emerge in regard to being able to target and degrade or deplete protein targets such as transcription factors. One example of this technology is proteolysis-targeting chimeric molecules (PROTACs). PROTACs

can be newly designed or already existing small-molecule inhibitors, which are then joined to a linker fused to a ligand for E3 ubiquitin ligase, allowing detection and transport of the protein of interest to the proteasome for degradation (228). Thus, through collaboration with biochemistry and drug discovery laboratories, it could be feasible to develop a novel small-molecule pharmacological agent that can specifically bind to Fli-1–regardless of whether it possesses Fli-1 inhibitory activity or not—and target the Fli-1 protein for proteasomal degradation via addition of a PROTAC sequence. Thus, strategies to develop a Fli-1 specific inhibitor would not only be potentially beneficial for anti-leukemia and anti-GVHD applications but could also be widely applied to many different diseases where Fli-1 is implicated in pathogenesis such as SLE, Ewing's Sarcoma, triple-negative breast cancer, among several others.

Not only is finding a specific Fli-1 inhibitor a highly desirable outcome in the scientific field, but also there are several currently known Fli-1 inhibitors, two of which we have employed in the current study, where the precise mechanism of action behind the inhibition of Fli-1 is currently still unknown (229). Thus, in order to move the field forward in both the pre-clinical and clinical study of Fli-1, the precise biochemical mechanisms of how currently available drugs such as camptothecin and etoposide reduce Fli-1 protein levels should be determined in order to provide invaluable insights into potential strategies that could be developed to specifically target and degrade Fli-1 protein levels. Drug-protein interaction studies should be carried out using techniques such as mass-spectrometry to determine if and how drugs such as camptothecin and etoposide directly bind to Fli-1 protein, and the specific sites and structures that they bind to (230). If camptothecin, etoposide, and other known Fli-1 inhibitors do indeed directly bind to Fli-1 to cause Fli-1 protein inhibition, the mechanism of Fli-1 protein depletion could indeed be studied and determined, providing invaluable insight into the biochemical regulation of Fli-1.

In addition to Fli-1, there are also several other closely related ETS transcription factors that have been documented to play roles in lymphocytes and human disease but have not yet been

fully characterized in mouse or human GVHD pathogenesis. Two of the most intriguing of these closely related transcription factors are Ets-1 and Spi1(PU.1). PU.1 has been documented to be the master transcriptional regulator of Th9 cells which can produce high levels of IL-9, and this subset was identified to play a role in promoting IL-17 production(231). From our RNAseq data, we identified that in Fli-1 heterozygous T cells, PU.1 expression was significantly increased compared to wild-type T cells, however IL-9 cytokine gene expression was not significantly altered. This data could suggest that PU.1 expression may compensate for loss of Fli-1 activity and may play a role in the anti-GVHD property of T cells lacking Fli-1 activity. Thus, further study of the connection between Fli-1 and PU.1 could provide important insights into the balance between these two transcription factors in T cells and GVHD development. In addition to PU.1, Ets-1 is an important transcription factor that has been documented to be involved as a FoxP3 co-factor and is required for Treg stability and function (232, 233) . Supplementary to its effects on Treg, Ets-1 was also recently identified as being a critical factor regulating Type 2 (Th2) T-follicular helper cells, and played a significant role in the development of experimental autoimmune encephalomyelitis (EAE) (234). Of interest would be determining if there is a reciprocal increase in Ets-1 expression following reduced Fli-1 expression in T cells, and if this increased Ets-1 expression plays a role in stabilizing and or enhancing Treg development and or function. Thus, how inhibition or blockade of PU.1 and Ets-1 affect Fli-1 expression and function in T cells and the impact on GVHD as well as vice versa would be novel areas of investigation to further understand the molecular mechanisms of ETS transcription factors in T-cell biology and GVHD pathogenesis.

Additionally, XBP-1 is an important transcription factor involved in both B- and T-lymphocyte differentiation and function that has been shown through our work to be critically involved in cGVHD development, and that a pharmaceutical strategy to target the IRE-1/XBP-1 pathway is an effective prophylaxis against cGVHD pathogenesis (26). Previous literature has

identified that XBP-1 can directly bind to the Fli-1 gene in T cells, suggesting that XBP-1 could either positively or negatively regulate the expression or function of Fli-1, although if and how XBP-1 regulates Fli-1 is unclear and deserves further study (75). Thus, using an XBP-1s antibody in a chromatin immunoprecipitation (ChIP) experiment could help to determine if XBP-1 binds to Fli-1 gene promoter or enhancer sequences during GVHD development. To then validate if XBP-1 positively regulates Fli-1 expression or activity on T cells during GVHD, transplanted T cells from XBP-1 deficient donors could be isolated from murine allo-HCT recipients and subjected to analysis of Fli-1 expression level (mRNA and protein) and function (Fli-1 binding to target gene promoters via chIP). Further, our studies on XBP-1 revealed a novel role of XBP-1 in regulating B-cell derived IL-4/5 and IL-12p40 cytokine production *in vitro* in response to LPS and IL-4 stimulation (26). The precise mechanisms by which XBP-1 promotes IL-4/5 and IL-12p40 cytokine production in B cells were not elucidated in our study, therefore how these interesting effects of XBP-1 on B-cell cytokine production are regulated remains unknown. In this case, it is possible that XBP-1 may be controlling B-cell cytokine production through either direct binding to cytokine gene promoter regions to enhance their transcription, or indirectly regulating their production through action on upstream regulators such as T-bet and GATA3. Thus, determination of the mechanisms of how XBP-1 regulates B-cell cytokine production would provide additional insight into how the XBP-1 pathway regulates B-cell biology during inflammatory responses.

Not only is the role of the IRE-1α/XBP-1 pathway important for B cells, but it is now evident that it is also critically involved in T-cell immunity. Although roles of XBP-1 in CD8+ T cells as well as Th2 and Th17 cells have now been defined, the role of XBP-1 in Treg differentiation and or function has not been fully elucidated (235). While a recent report identified that IRE-1α kinase activity played an important role in Treg suppressive capacity of effector T cells and inflammation, and that XBP-1 splicing was increased in Tregs exposed to inflammation, how specific XBP-1 deficiency regulates Treg was not investigated in this study (78). Thus, using

single IRE-1α$^{-/-}$, single XBP-1$^{-/-}$, and IRE-1α/XBP-1 double-KO mice would be able to provide novel insight into the specific role of XBP-1 in Tregs. In our XBP-1 study, inhibition of the IRE-1α/XBP-1 pathway using B-I09 was able to prevent cGVHD development, but the specific effect on Treg was not examined. Thus, determination if one of the primary mechanisms of action of B-I09 against cGVHD development is enhancement of Treg differentiation and or suppressive capacity would be an interesting area of future investigation.

To effectively translate our studies on both XBP-1 and Fli-1 into the clinic, future studies to further provide clinical evidence for the relevance of XBP-1 and Fli-1 in GVHD would be prospective collection and analysis of lymphocytes from GVHD patient peripheral blood, and analysis of XBP-1s and Fli-1 gene expression levels using qRT-PCR. To assay the tissue-specific roles of the transcription factors in lymphocyte migration to target tissues, skin biopsies could be taken from cGVHD patients and multi-plex IHC staining for T cells and Fli-1, or *in situ* hybridization in the case of XBP-1. Peripheral blood and skin biopsies from healthy control patients, autologous-HCT patients, and patients with GVHD that has gone into remission would be critical controls to determine accurate gene and or protein expression levels. If Fli-1 and or XBP-1 are increased at the transcript and or protein level in GVHD patient circulating lymphocytes, this would provide additional rationale to target these two important transcription factors in the clinical setting of allo-HCT.

In summary, allo-HCT and GVHD therapies targeting alloreactive T cells is naturally an attractive strategy toward the attempt to ameliorate GVHD which is driven primarily by this cell type, and several therapies have been proposed, studied, and tested pre-clinically as well as clinically. However, the potential caveat with many therapies which directly target T cells is that excessive inhibition of the T-cell response will impair their ability to recognize and eliminate malignant cells from the patient's primary disease such as leukemia. Importantly, this work has shown that by using prophylactic low-dose administration of a Fli-1 targeting drug—

camptothecin—was associated with reduction of both acute and chronic GVHD, and that in acute GVHD, the GVL effect of donor T cells was not impaired against P815 tumor. These results open the door for additional study of Fli-1 targeting therapies such as etoposide, topotecan, A661, A665, and others in order to prevent and or treat GVHD without necessarily compromising the GVL effect of donor T cells and to determine if Fli-1 inhibition is the significant mechanism behind the effectiveness of these drugs against activated T cells and hematopoietic malignancies such as leukemias. Further, XBP-1 was previously shown to be a critical regulator of chronic GVHD pathogenesis through its direct action on B cells, and action on T cells through ITK (25, 51). Thus, determining if Fli-1 is positively regulated by the XBP-1 transcription factor would be a novel addition to the XBP-1 pathway of action and could be exploited to treat both hematological and non-hematological malignancies that have exhibited aberrant Fli-1 activity. Further, several pharmaceutical modalities have now recently been developed to target both the IRE-1α kinase domain and the endoribonuclease domain (55, 78), thus presenting a translational opportunity to study if targeting the IRE-1α/XBP-1 pathway can be beneficial against preventing cGVHD development without impairing the GVL effect.

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Message:

IACUC has approved the protocol with the following details.

Protocol ID: IACUC-2018-00446
Principal Investigator: Yu, Xuezhong
Department: Microbiology and Immunology
Protocol Title: Prevention of Graft versus Host Disease (GVHD) while Preserving Graft-versus-Leukemia (GVL) Effect
Review Type: FULLBOARD
Approval Date: January 29, 2020

You may print an official Approval Letter from your protocol. Click on the protocol number and open your protocol in View Mode. Select "Event History" from the left-hand menu, and make sure you are viewing the year of approval under "Event History" to access "Approval Letter" from the right-hand column. Please note that "Email History" will provide different information.

IMPORTANT: If this protocol is a 3-year renewal, you must transfer your animals on campus from the old protocol to this protocol prior to the expiration date.

About the Author

Steven Douglas Schutt was born in Clearwater, Florida (1990) to Kim and Jeff Schutt. He graduated from Furman University with a Bacherlors of Science degree with a major in Neuroscience. During his undergraduate studies, he contributed to ongoing work on toluene and drug of abuse neuroscience studies being conducted by Dr. Onarae Rice at Furman University. After receiving his Bachelor's degree, he utilized his knowledge of drug and behavior murine studies to begin work as a research specialist in the lab or Dr. Ronald See at MUSC in the Neuroscience Departmnet. He then joined Dr. Xue-Zhong Yu's laboratory as a research specialist and worked as a laboratory manager and performed studies focusing on murine chronic GVHD, where he was able to publish his first primary author article on Ibrutinib in chronic GVHD. He then joined the Master in Biomedical Science program at MUSC in the Microbiology and Immunology track, continuing his work on studying the XBP-1 and Fli-1 transcription factors in GVHD. Desiring to advance his career in science, he then joined the PhD program in Biomedical Sciences at MUSC with continued focus on Microbiology and Immunology and in GVHD studies in Dr. Yu's lab, where he was able to publish his second primary author paper focusing on the role of the IRE-1α/XBP-1 pathway in chronic GVHD development. These works attracted the attention of two individual fellowship awards, the TL1 predoctoral training award and the Hollings Cancer Center Graduate Fellowship, oral presentation at the 2017 Association for Clinical and Translational Science (ACTS), and abstract achievement award at the 2019 American Society of Hematology (ASH) conference. Subsequent to meeting all the obligations and requirements of his degree, he will begin working on cancer immunometabolism as a post-doctoral fellow at the National Cancer Institute in Dr. Daniel McVicar's lab.