Donor B-cell alloantibody deposition and germinal center formation are required for the development of murine chronic GVHD and bronchiolitis obliterans.

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Short title: Chronic GVHD suppression by GC disruption

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Abstract:

Chronic GVHD (cGVHD) poses a significant risk for hematopoietic stem cell transplantation (HSCT) patients. Preclinical development of new therapeutic modalities has been hindered by models with pathological findings that may not simulate the development of human cGVHD. Previously we have demonstrated that cGVHD induced by allogeneic HSCT following a conditioning regimen of cyclophosphamide and total body radiation results in pulmonary dysfunction and airway obliteration leading to bronchiolitis obliterans (BO), pathognomonic for cGVHD. We now report cGVHD manifestations in a wide-spectrum of target organs, including those with mucosal surfaces. Fibrosis was demonstrated in the lung and liver, associated with CD4+ T-cells and B220+ B-cell infiltration and alloantibody deposition. Robust germinal center (GC) reactions were present at the time of cGVHD disease initiation. Blockade of GC formation using a lymphotoxin-beta receptor–immunoglobulin (LTβR-Ig) fusion protein suppressed cGVHD and BO. Donor bone marrow obtained from mice incapable of secreting IgG alloantibody resulted in less BO and cGVHD. We conclude that cGVHD is caused in part by alloantibody secretion, which is associated with fibrosis and cGVHD manifestations including BO and that LTβR-Ig treatment could be beneficial for cGVHD prevention and therapy.
Introduction

Chronic GVHD (cGVHD) is a significant complication of allogeneic hematopoietic stem cell transplantation (HSCT)\(^1\). Progress in developing interventional strategies to counter cGVHD has been hampered by variable onset and pathological manifestations of cGVHD, now better defined by the NIH consensus conference\(^2\), and a dearth of robust preclinical venues that closely mimic conditions in which cGVHD is generated and manifested\(^3\).

Although the exact causes of cGVHD are unknown, higher antibody levels have been associated with autoimmunity and implicated in cGVHD\(^4,5\). Studies of newly diagnosed patients with extensive cGVHD, had elevated soluble BAFF levels and anti-ds-DNA antibodies\(^6,7\). Increased soluble BAFF in cGVHD was associated with higher circulating levels of pre-germinal center (GC) B-cells and post-GC plasmablasts\(^8\). B-cells from cGVHD patients were hyper-responsive to TLR-9 signaling and have upregulated CD86 levels\(^9\); suggesting an important participatory role for B-cells in establishing cGVHD, emphasizing the need for further investigation into the immunological role of B-cells in cGVHD pathogenesis.

Existing murine cGVHD models simulate one or more of the pathological manifestations such as increased serum antibodies (typically anti-DNA antibodies), scleroderma and fibrosis of skin and liver, and the less common immune complex deposition in kidneys and glomerulonephritis\(^10-12\). The type of multi-organ involvement and alloantibodies seen in cGVHD patients often has not been well-represented in these preclinical models.
Moreover, some models do not involve conditioning regimens, whereas others relay upon radiation alone. Previously, our laboratory has studied pulmonary dysfunction and chronic GVHD (cGVHD) target organ pathology in animals conditioned with high dose cyclophosphamide (Cy) and lethal total body irradiation (TBI) rescued with allogeneic bone marrow (BM) and splenocytes\textsuperscript{13}. The functional, physiological, and pathological assays demonstrated that Cy and TBI conditioned recipients of low numbers of allogeneic T-cells developed bronchiolitis obliterans (BO)\textsuperscript{14,15}. BO, characterized by airway blockade, peri-bronchiolar fibroproliferation, and obliteration of bronchioles, is a late stage complication of GVHD, prevalent in 2-3\% of HSCT patients and up to 6\% of patients who develop GVHD\textsuperscript{16}. Patients diagnosed with BO have a 5 year survival rate of only 10\% vs. 40\% in patients without BO\textsuperscript{14}. According to the NIH consensus criteria\textsuperscript{2}, BO is the only single pathopneumonic manifestation of cGVHD; therefore, this is a bona fide cGVHD murine model.

In the present study, we identified the presence of CD4\textsuperscript{+} T helper cells and B220\textsuperscript{+} B-cells in the airways of mice that had BO, tissue-specific antibodies from sera, and alloantibody deposition in the lung and liver of cGVHD recipients. Through studies using wild-type, knockout (KO) and transgenic (Tg) donor cells, we conclusively demonstrate that donor alloantibody secretion is essential for BO generation, providing a preclinical model in which to test interventional and prophylactic approaches for cGVHD.

The mainstay of treating cGVHD and BO is anti-inflammatory therapy. Corticosteroids are contained in many of the current regimens but are still associated with a high rate of
progressive airway obliteration and subsequent mortality. Treatment of steroid refractory cGVHD patients with rituximab, a B-cell depleting anti-CD20 monoclonal antibody (mAb), has shown a beneficial role in resolution of the autoimmune disorders such as SLE and rheumatoid arthritis and cGVHD. Aggregate analysis of 6 trials of steroid-refractory cGVHD showed overall response rates of 29-36% for oral, hepatic, gastrointestinal and lung cGVHD, and 60% for cutaneous cGVHD.

Additional prevention and treatment strategies clearly are needed. We focused on GC formation, critical for efficient class switching and antibody secretion by mature B-cells and plasma cells. Lymphotoxin-beta (LT)-LTβR interactions are essential for GC formation and maintenance, ensuring proper anchoring of the GC B-cells within the network of follicular DCs and correct GC formation. Inhibiting the GC reaction and subsequent antibody secretion has proved to be efficacious in some systemic lupus and other autoimmune models where B-cells and antibody secretion have been implicated in pathology. LTβR-Ig is a novel fusion protein that binds circulating lymphotoxin and inhibits LT-LTβR signaling. We show that LTβR-Ig treatment of transplanted animals susceptible to establishment of cGVHD and BO showed improved lung function along with lower tissue-specific antibody levels in the sera and fibrosis of the lung and liver. LTβR-Ig represents a potential new therapy for cGVHD prevention and treatment.
Methods and Materials

Mice

C57Bl/6 (H2b) mice were purchased from the National Cancer Institute. B10.BR (H2b), BALB/c (H2d) and B6.129S2-Igh-6tm1Cgn/J on a C57Bl/6 background (referred to as μMT mice) were purchased from Jackson Laboratories. BALB/c (m+s)IgMxJhD Tg mice were bred at the University of Minnesota animal facility. Mice were housed in a specific pathogen-free facility and used with the approval of the University of Minnesota institutional animal care.

BMT

B10.BR recipients were conditioned with Cy on days -3 and -2 (120mg/kg/day i.p.). On day-1, recipients received TBI by x-ray (7.5Gy). Donor bone marrow (BM) was T-cell depleted with anti-Thy1.2 mAb followed by rabbit complement. T-cells were purified from lymph nodes by incubation with phycoerythrin-labeled anti-CD19 (eBioscience), followed by anti-PE beads and depletion with magnetic column (Miltenyi-Biotec). On day 0, recipients received 10x10^6 BM cells with or without allogeneic spleen cells (0.75-1x10^6) or purified T-cells (0.33x10^6), as indicated. Weights of individual mice recorded twice weekly. Where indicated, recipients in cGVHD groups were given murine LTβR-Ig or control murine MOPC21 provided by Biogen-Idec at 200 μg/dose i.p. every 3 days from days 28-52.
Pulmonary function tests (PFTs)

PFTs were performed as described\textsuperscript{13}. Anesthetized mice were weighed and lung function assessed by whole body plethysmography using the Flexivent system (Scireq) and analyzed using the Flexivent software version\textsuperscript{5.1}.

Frozen tissue preparation

All organs harvested were embedded in Optimal Cutting Temperature compound (OCT), snap frozen in liquid nitrogen, and stored in -80\degree C. Lungs were inflated by infusing 1 ml of OCT: PBS (3:1) intratracheally prior to harvest.

Histology and trichrome staining

6 \textmu m cryosections were fixed for 5 minutes in acetone and stained with hematoxylin and eosin to determine pathology and with the Masson’s trichrome staining kit (Sigma) for detection of collagen deposition. Histopathology scores were assigned as described\textsuperscript{26}. Collagen deposition was quantified on trichrome stained sections as a ratio of area of blue staining to area of total staining using the Adobe Photoshop CS3 analysis tool.

Immunohistochemistry

Acetone fixed 6 \textmu m cryosections were immunoperoxidase stained using biotinylated mAbs for CD4, CD8, and B220 (BDPharmingen)\textsuperscript{26}. Stained sections examined under 200x magnification and images captured using an Olympus BX51 microscope. Antibody-binding positive cells were quantified by counting a 100mm\textsuperscript{2} area for liver, or obtaining a ratio of positive to negative cells in 100mm\textsuperscript{2} area for lung.
**Immunofluorescence**

For Ig deposition, 6 µm cryosections were fixed with acetone blocked and stained with FITC labeled anti-mouse-Ig (BDPharmingen) or goat-anti-mouse-IgM, goat-anti-mouse-IgG1, goat-anti-mouse-IgG2b, goat-anti-mouse-IgG2c, goat-anti-mouse-IgG3, followed by FITC-Donkey-anti-Goat Ig (Jackson ImmunoResearch). For detection of tissue specific serum antibodies, fixed cryosections of organs from a naïve B10.BR mouse, were incubated with sera from the BMT recipients. Sections were incubated with FITC-labeled anti-mouse-Ig. Confocal images were acquired on an Olympus FluoView500 Confocal Laser Scanning Microscope at 200X, analyzed using FluoView3.2 software (Olympus), and processed with Adobe Photoshop CS3, version 9.0.2. Antibody deposition was quantified by scoring sections using a scale of 1-3.

**GC detection**

Fixed 6µm spleen cryosections were stained with rat-anti-mouse-VCAM1 followed by goat-anti-rat-Cy3, IgM-FITC and biotinylated-PNA (Vector Laboratories) followed by Streptavidin-Cy5. Confocal images obtained as above.

**Statistics**

Survival data were analyzed by life-table methods, and actuarial survival rates are shown. Group comparisons were made by log-rank test statistics. Group comparisons of cell counts and flow cytometry data were analyzed by Student $t$-test.
Results

Multi-organ pathology with fibrosis in Cy/TBI recipients experiencing cGVHD. B10.BR mice were conditioned with Cy/TBI and received either BM alone (BMT control group) or BM with low dose splenocytes to induce a chronic alloresponse not associated with early post-BMT mortality. Weight curves (Figure 1A) and 2 month survival rates were ≥ 90% and comparable between groups (Figure 1B). On day 60, PFTs in the cGVHD group (Figure 1C) demonstrated lung dysfunction consistent with BO. The PFTs showed an increase in airway resistance, correlating to lung constriction, increased elastance and decreased compliance signifying increased stiffness or rigidity of the lungs. These data simulate BO manifestations in cGVHD patients. A decrease in pulmonary function and increased pathology was noticed as early as 28 days post transplantation (Figure 1D and Supplemental Figure 2). Therefore this system is a bona fide model of cGVHD based upon extrapolation of NIH consensus criteria for cGVHD diagnosis in patients.

The cGVHD group displayed multi-organ pathophysiology consistent with cGVHD (Figure 2A, B). Lung histology showed airway obstruction, peri-bronchiolar infiltration and cuffing denoting the onset of obliteration of the bronchioles (Figure 2A). Many animals displayed distal pathology in the lungs including protein exudation, macrophage infiltration and start of tissue remodeling (Supplemental Figure 1). Lung pathology scores were significantly higher in the cGVHD group compared with BM controls (Figure 2B). In the liver, peri-vasculature infiltration was observed surrounding the bile duct and extending into the parenchyma (Figure 2A), resulting in a significant increase in the
pathology score in the cGVHD vs. the BM control animals (Figure 2B). Mice with higher pathology scores also had necrotic foci of cells in the parenchyma proper.

Since patients with cGVHD may also have oral mucosal involvement, histopathology of the tongue and salivary glands was examined. Areas of infiltration, inflammation and abscess formation were observed on the tongue near the taste receptors (Figure 2A). The pathology score was significantly higher, albeit modest in magnitude, in the cGVHD group compared with the BM only controls (Figure 2B). Salivary glands displayed perivascular infiltration; however this infiltration was also seen in the BM controls suggesting changes resulting from the conditioning (Figure 2A).

The thymus, known to be involved in human cGVHD27, displayed necrotic foci, and destruction of stromal matrix, resulting in a significantly higher cGVHD pathology score. The spleen had abnormal architecture similar to the thymus, albeit only modest differences were seen in the pathology score. The colon displayed moderate pathology in the cGVHD cohorts, but not as extensive or invasive as in aGVHD models. The ileum displayed mild pathology seen in both the cGVHD and the BM group, suggesting the role of pre-transplant conditioning.

cGVHD has been characterized as a fibroproliferative disease, therefore collagen levels were quantified by trichrome staining. The lung and liver (Figures 2C and D) both displayed a significant increase in collagen deposition around bronchioles and blood vessels. The organization of the collagen surrounding the bronchioles and blood vessels
is highlighted (Figure 2C) demonstrating the increase of collagen localized to specific structures and not uniformly distributed throughout the lungs and liver. Fibrosis patterns in tongue and salivary glands were somewhat more variable between individual mice within a group and trichrome staining was not significantly increased as compared with the BM controls (data not shown).

Overall, Cy/TBI conditioning followed by transfer of a number of low splenocyte infusion reproduced the pathology of cGVHD. It was distinguished from aGVHD by the absence of significant weight loss early and overall mild losses later after transplant, BO, involvement of the oral mucosa, and fibrosis in cGVHD target organs. Whereas pathology and fibrosis in the lung has been observed previously \(^ {13} \); documented reports of pathology in the other organs have not been evaluated.

**CD4\(^+\) T-cell and B-cell infiltration and alloantibody deposition in cGVHD target organs.**

We sought to determine the potential associated etiopathogenic mechanisms associated with cGVHD. We focused on the lung and liver as the two more severely affected cGVHD organs denoted by obstructive PFTs and the presence of fibrosis. CD4\(^+\) T-cell infiltration was observed in peribronchiolar areas in the cGVHD group but not in BM only controls (Figure 3A). The liver displayed substantial infiltration in the perivascular areas (Figure 3A) and for some recipients, into the parenchyma (not shown). Due to the degree of lung inflation influencing the distribution of cells, percentage of lymphocytes are quantified in the lung versus total number like with the liver. The number CD4\(^+\) T-
cells in the lung (Figure 3B) and the liver (Figure 3C) was significantly higher in the cGVHD group compared with the BM only control, consistent with other models in which CD4+ T-cells could be found in cGVHD organs10. There was only minimal infiltration of CD8+ T-cells in both groups (data not shown). B220+ B-cell infiltration in the lung and liver (Figure 3D) was seen in a pattern similar to CD4+ T-cells. A significant increase in total B220+ B-cells in the lung and liver was seen in the cGVHD group compared with the BM controls (Figures 3E, F, respectively). Salivary glands and the tongue had only minimal B220+ B-cell infiltration (data not shown). Taken together, the pathology in the lung and liver is associated with CD4+ T-cell and B-cell infiltration.

We hypothesized that B-cell secreted antibodies might play a role in cGVHD pathology and determined whether antibody deposition could be observed in cGVHD organs on day 60 post-transplantation. The lung and liver had deposits of mouse Ig within surrounding areas of infiltration and pathologic effects (Figure 4A). There were peribronchiolar deposits and perivascular deposits in both organs. Quantification of Ig deposition in the lung and liver (Figure 4B) revealed higher levels in the cGVHD group than the BM control. The predominant Ig types were IgG2c in the lung and liver (Supplemental Figure 3 and data not shown).

To determine if circulating anti-host, tissue-reactive antibodies were present, sera from transplanted animals was incubated with tissues from a naïve B10.BR mouse and then probed with fluorochrome-labeled anti-mouse Ig (Figure 4C). Fluorescence was evident in the perivascular areas in lung and liver, and peribronchiolar area in lung. These results
indicate that circulating host tissue-reactive antibodies may contribute to cGVHD pathology.

cGVHD pathogenesis requires the production and secretion of anti-host reactive antibodies. To further test our hypothesis that B-cells and secreted alloantibodies play a causative role in establishment of cGVHD, we used donor μMT mice that do not have membrane-bound IgM and are deficient in mature B cells (Figure 5). To limit the contribution of donor B-cells and avoid any issues related to defective regulatory T-cell (Treg) dysfunction reported in μMT mice, Cy/TBI conditioned recipients received WT or μMT BM with or without supplemental WT T-cells (0.33x 10^6). Recipients of WT BM plus supplemental WT T-cells versus low dose splenocytes had comparable survival and weight and significant increases in resistance and elastance with a lowering of compliance characteristic of BO. When recipients of B-cell deficient BM and WT T-cells were compared with mice with cGVHD that received WT BM with WT T-cells, PFTs demonstrated lower resistance, elastance, and higher compliance (Figure 5A). Control recipients of WT compared with μMT BM had comparable parameters. Recipients of B-cell deficient BM with WT T-cells had resistance and elastance comparable to recipients of B-cell deficient BM without supplemental T-cells. Thus, donor allogeneic T-cells and BM-derived mature B-cells are required for BO generation.

In contrast to recipients of WT BM and T-cells vs BM alone, there were no significant differences in the histopathology scores of lung and liver between μMT BM plus T-cells compared with WT BM (Supplemental Figure 4). Of note, although lymphocyte
infiltration and inflammation was observed in μMT BM and T-cells, there was no
destruction of the bronchioles or bile ducts and the degree of fibrosis mirrored the extent
of pathological injury (Figure 5B). Moreover, Ig was not deposited in the lung or liver of
mice that received μMT BM and T-cells (Figure 5C), findings which correlated with
PFTs.

We cannot exclude the possibility that antibody deposition is a bystander in the disease
process, and that the critical pathogenic mechanism is the APC function rather than Ig
capacity of activated B-cells. To distinguish between these two fundamental mechanisms,
we utilized (m+s)IgMxJhD BALB/c donors. These mice are capable of generating
membrane bound IgM and secreting IgM, but produce and secrete ≥100-fold less antigen-
specific IgG than similarly immunized WT controls. They also do not appear to have a
Tregs defect like the μMT mice (Supplemental Figure 5). Recipients were given WT BM
or Tg BM alone or with WT splenocytes. Day 60 PFTs in conditioned B10.BR recipients
of (m+s)IgMxJhD BM and WT splenocytes had significantly lower lung resistance and
elastance and higher compliance compared to recipients of WT BM with splenocytes
(Figure 6A). These data indicate that the lack of IgG alloantibody secretion by donor
BM-derived B-cells precluded the pulmonary dysfunction associated with BO. This was
confirmed by staining tissues for total Ig in the lung and liver (Figure 6D). The
(m+s)IgMxJhD BM only and (m+s)IgMxJhD BM with splenocytes both had decreased
deposition of Ig surrounding bronchioles and bile ducts compared to mice transplanted
with WT BM and splenocytes. Trichrome incorporation was significantly reduced in
mice receiving (m+s)IgMxJhD BM with splenocytes compared to the WT BM and
spleenocytes (Figure 6B). However, infiltrating CD4+ cells were still observed in the lung and liver of both WT BM and splenocytes and (m+s)IgMxJhD BM and splenocytes (Figure 6C). These data suggest that inflammation is not regulated by Ig deposition per se and therefore other mechanisms (e.g. chemokines) not as affected by Ig deposition. The pathogenicity of Ig deposition in adversely affecting PFTs may not be the result of changes in inflammation but rather situ injury from complement binding to deposited alloAb or the Ig facilitated activation of inflammatory or resident cells in situ.

**Blockade of GC formation is sufficient to prevent BO and hence cGVHD generation**

Since BM-derived B-cell production and secretion of anti-host reactive antibody appears to be critical for cGVHD generation, we sought to prevent antibody production by interrupting GC formation. GCs are sites where mature B-cells rapidly proliferate, differentiate, and undergo somatic hypermutation resulting in the production of class switched antibody. LTβ, produced by T- and B- cells and LTβR signaling is required for proper establishment of the follicular dendritic cell (DC) network and the organizational network of functional GCs in lymphoid tissues. Given the role of LTβR signaling and our finding that the dominant Ig isotypes deposited in cGVHD target organs were IgG2c, we sought to determine whether disruption of Lt:LTβR signaling by infusion of the known blocking reagent, mouse LTβR-Ig fusion protein, would reduce cGVHD pathology. Recipients were treated with LTβR-Ig or control fusion protein from days 28-52 post-transplant (Figure 7). Treatment began on day 28 because of decreases in PFTs (Figure 1D) and increased GC size and frequency (Supplemental Figure 8) demonstrating early initiation of cGVHD. To determine the effect of LTβR-Ig on GC formation in
cGVHD vs. BM recipients, spleens were analyzed by immunofluorescence (Figure 7A); PNA and IgM staining detects GC B-cells, while VCAM-1 stains follicular DCs. Untreated and control-mAb treated mice with cGVHD pathology displayed prominent GC structures with a well-organized follicular DC network, signifying a robust GC reaction. Mice treated with LTβR-Ig showed disrupted GC structures with an ill-formed follicular DC network. A decrease in size and frequency of GC was seen in LTβR-Ig treated mice (Figure 7B and C).

Consistent with the finding that disruption of GC formation would diminish BO and cGVHD generation, day 60 PFTs in the cGVHD cohorts indicated that LTβR-Ig but not control protein treated recipients displayed lower resistance and elastance and higher compliance (Figure 7D). Both liver and pulmonary fibrosis was reduced by LTβR-Ig (Figure 7E). Thus, LTβR-Ig treatment given post-transplantation ameliorates the development of BO and cGVHD. The pathological scores shows that BM+spleen+MOPC21 mean lung scores are ~5 fold higher than BM+spleen+LTβRig (Supplemental Figure 7), although these differences did not reach statistical significance. For the liver, recipients given BM+spleen+MOPC21 had a reduction in mean scores compared to BM+spleen and no Ig control, thereby making it difficult to assess the extent to which LTβR-Ig was superior to MOPC21, although we can state that BM+spleen had higher scores than BM alone which were compared to BM+spleen+LTβR-Ig, indicating that the LTβR-Ig did indeed reduce the severity of pathological injury. These data are supportive of our findings that LTβR-Ig reduces cGVHD as measured by pulmonary
function tests, fibrosis, Ig deposition and GC formation, all of which show a marked reduction in BO and chronic GVHD manifestations.

To test our hypothesis that LTβR-Ig treatment works by inhibiting antibody secretion by class-switched B-cells, we assayed for the presence of host tissue reactive antibodies in the sera of transplanted animals using a serum-binding assay. As compared to control mAb treated recipients, sera from mice that were treated with LTβR-Ig had reduced fluorescence in the peribronchiolar area of the lung and perivascular area of the liver (Figure 7F), indicating that LTβR-Ig but not control protein leads to a decrease in the production of anti-host reactive antibody, consistent with the improvement in PFTs.

These results suggest that B-cells via their antibody secretory capability contribute to pulmonary dysfunction and liver pathology in animals that suffer from chronic GVHD after transplantation, and interventional strategies that inhibit pathways involved in B-cell function and antibody secretion maybe useful in the prevention or treatment of cGVHD.
Discussion

We report that Cy/TBI conditioned allogeneic recipients given low splenocyte or T-cell doses developed multi-organ system pathology with fibrosis and BO pathognomonic of cGVHD. cGVHD development was associated with IgG2c deposition in the lung and liver and IgG2b in the liver, which was abrogated if the donor BM was deficient in mature B-cells or incapable of producing anti-host reactive IgG. Robust GC formation was seen in mice with cGVHD. By disrupting GC formation using LTβR-Ig, BO was reduced. Together, these data indicate that GC formation and IgG secretion by donor BM-derived mature B-cells are necessary for cGVHD pathology.

By using Cy, which exacerbates the TBI-induced glutathione redox reactions in the lung along with subleathal doses of donor T-cells (avoiding aGVHD), the generation of cGVHD was favored. The histological changes in this model were similar to the findings in human cGVHD. The lungs showed peribronchiolar and perivascular cuffing and infiltration of the airway epithelium. The pathology appeared predominantly bronchiolar with some alveolar involvement. Completely occluded bronchioles were observed in 20% of the animals. The pattern of inflammation along with increased collagen deposition surrounding non-obliterated bronchioles was similar to the pathology that defines occluded bronchioles in humans. The liver had inflammation and lymphocytic infiltration, along with collagen deposition in the vascular epithelium and around the bile ducts and apoptotic foci in the parenchyma. Salivary gland pathology is an important clinical manifestation of cGVHD; patients suffer from xerostomia and histolopathological analysis show mononuclear infiltration and fibrosis and salivary gland injury is a known finding in a minor mismatch mouse model of cGVHD. The
parotid and submandibular salivary glands displayed lymphocytic infiltrates in both the BM and cGVHD groups, likely due to transplantation conditioning. In the tongue, there was a quantifiable difference in the histology; however, the extent of inflammation and infiltration was mild to moderate and isolated to small regions of the epithelium. Similar profiles of fibrosis was seen in the tongue and salivary glands for both control and cGVHD groups, suggesting that the oral mucosa did not manifest cGVHD or that the kinetics are delayed compared to the liver and lung. The absence of any inflammatory or fibrotic changes in the skin differs from some other models, where the predominant feature is scleroderma [reviewed in 8]. This systemic histologic profile reinforces the observation that in mice as in humans, the pathological manifestations of cGVHD are heterogeneous.

The role of CD4+ T-cells in both aGVHD and cGVHD has been well chronicled. We also observed the presence of CD4+ T-cells in the zones of inflammation in both lung and liver of affected animals. There were a significantly higher number of B220+ B cells that were found in clustering with the CD4+ T cells, setting up a situation in which CD4+ T-cell help for B-cell alloantibody production might occur in situ in cGVHD target organs. Although donor CD8+ T-cell involvement has been reported to play a causative role in airway obliteration after BMT\textsuperscript{31}, we have not seen a higher incidence of CD8+ T-cells in the inflamed and affected regions of the lungs or livers (data not shown). We detected antibody deposition in the target areas of lung and liver, confirming the involvement of B-cells. Examining sera from these animals, we detected the presence of lung and liver tissue-specific antibodies. Further characterization is required to identify if these
antibodies are donor against host alloreactive or auto-reactive due to a breakdown in the tolerance processes after BMT. Thus, it appears that the tissue modifications and fibrosis stems from a coordinated CD4\(^+\) T-cell and B-cell response.

Alleviation of physiological and histological symptoms in animals that received B-cell deficient bone marrow compared to wild type BM, confirms the requirement of B-cells for lung dysfunction and inflammation and fibrosis in the lung and liver. These animals displayed a less restrictive pulmonary physiology, and a lower incidence of occluded and fibrotic airways, signifying healthier lungs. The liver displayed less intensive inflammation and fibrosis, consistent with a study that reported attenuated liver fibrosis after toxin injury in B-cell deficient mice\(^35\). The use of (m+s)Ig\(x\)JhD mice that can only secrete IgM and not IgG\(^29\) as donors excluded the capacity of donor B-cells to facilitate cGVHD manifestations due to enhanced pathogenic antibody production as a product of class switching and affinity maturation. B-cells and follicular DCs have intact APC function, although the B-cell receptor repertoire is restricted due to the use of a fixed IgM-Vh region\(^36\). On day 60 post-transplant, the lung physiology showed a significantly healthy phenotype when compared to cGVHD group. The lack of secreted antibody other than IgM alleviated the functional consequences of cGVHD in the lung. These data indicate that either IgG secretion is necessary for pathogenicity, and/or that a full B-cell receptor repertoire is required to promote disease. The repertoire in turn could control the quality and specificity of the alloreactive antibodies secreted, as well as any required APC function of specific B-cells for pathogenic CD4\(^+\) T-cells. Further work will be required to dissect these two related possibilities.
Given a role for IgG antibodies, we propose that alloantibody binding to the cGVHD organs could enable tissue destruction and modifications via donor cell mediated Ab-dependent cellular cytotoxicity or antibody-dependent phagocytosis. Alternatively, the pathology could be defined by the specific function of these secreted antibodies. For example, elevated titers of anti-PDGFR antibody have been detected in sera of patients with extensive cGVHD\textsuperscript{37}. This antibody has been implicated in a signal transduction pathway that ultimately leads to the induction of type 1 collagen production and fibrosis\textsuperscript{5}. Pathogenic antibody production therefore is likely to be an important inducer of cGVHD and targeting this specific function of the B-cells is an attractive strategy to prevent or treat early manifestations of cGVHD.

Treatment of cGVHD using the anti-CD20, B-cell depleting antibody, rituximab ameliorates some manifestations of cGVHD, but it rarely results in complete remission of cGVHD\textsuperscript{5}. In studies using mice and monkeys it has been shown that rituximab treatment is more successful in removal of B cells from the blood compared to the spleen and lymph nodes\textsuperscript{38,39}. GC B-cells display lower susceptibility to rituximab-mediated clearance, probably because they reside in a non optimal environment for antibody based depletion\textsuperscript{18}. Our observation that GC B-cells are critical to the development of cGVHD suggests that agents that are more effective at disrupting the GC might be more clinically useful. We are unaware of the use of rituximab in a systemic fashion to determine the effects on BO in cGVHD patients. Such studies would require patients with the same duration of onset and severity of BO. To our knowledge, the relative number and
frequency of infiltrated B-cells has not been evaluated in the secondary lymphoid organs
of patients with cGVHD, though high GC formation could occur due to increased BAFF
levels\(^7, 43\).

GCs are specialized regions found in secondary lymphoid organs, where activated B-cells
undergo diversification and affinity maturation resulting in differentiation into both
memory B-cells and long-lived plasma cells that produce high affinity antigen-specific
class switched antibodies. GCs are required for a sustained humoral immune reaction to
alloreactive antigens, but also could result in the production of autoreactive antibodies
and a dysregulated autoimmune response, which might be relevant in patients suffering
cGVHD symptoms\(^40\). Follicular B-cells express LT-\(\alpha\beta\), while the follicular DCs express
the LT\(\beta\)R. One of the important consequences of the LT-LT\(\beta\)R interaction between these
cells is the proper positioning of GC B-cells and establishing GC architecture. LT-LT\(\beta\)R
signaling also enhances the FDC-B cell interactions by upregulating expression of
adhesion factors such as VCAM-1 on the FDCs. Inhibition of this interaction by using the
LT\(\beta\)R-Ig fusion protein prevents GC formation\(^41\).

Treatment with LT\(\beta\)R-Ig had a direct effect on the symptoms of cGVHD, at least in part
by blocking GC formation. These effects included an alleviation of pulmonary
dysfunction. There was also a decrease in tissue-specific Ig levels in the sera. This
confirms that inhibiting antibody secretion from post GC B-cells and plasmablasts elicits
a positive response in animals suffering from pulmonary GVHD. The presence of IgG2c
deposits in lung and liver further supports the notion that successful GC reactions that
result in class switching is an important parameter in establishing lung and liver pathology. The fusion protein could work as a potential therapeutic agent for GVHD by causing a breakdown in GC architecture, resultant antibody production, and conversion of B-cells into memory cells and plasmablasts.

LTβR-Ig blocks both LTαβ and LIGHT, which is expressed on activated T-cells and on B-cells. LIGHT has been demonstrated to have a role in the fibrosis, smooth muscle hyperplasia and airway hyperresponsiveness associated with two murine models of chronic asthma. Pharmacological inhibition of LIGHT resulted in better lung function, and this was linked to the reduced production of TGF-β and IL-13, cytokines involved in airway remodeling after injury. The efficacy of LTβR-Ig as a cGVHD interventional strategy may be the result of multiple mechanisms of action including disruption of GCs and antibody production.

In summary, we have observed a requirement for B-cells in development of cGVHD symptoms in lung and liver in a myeloablative conditioning model of allogeneic HCST. B-cells, presumably via pathogenic antibody secretion by products of the GC reaction, including long-lived antibody forming cells, play a causal role in modulating the physiology of the lung and pulmonary dysfunction. Our studies have therefore identified important immunological processes that are involved in cGVHD and that LTβR-Ig could be a potential clinical interventional strategy for prevention and therapy of cGVHD.
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Author contributions. MS designed and performed experiments, analyzed data, and wrote the paper. RF performed experiments, analyzed data, and edited the paper. AP performed experiments and analyzed data. AR, JLB, and, MJS provided reagents, designed experiments, and edited the paper. PAT designed and performed experiments, analyzed data, and edited the paper. JR, JHA, WJM, and LL discussed experimental design and analysis and edited the paper.

Conflict of interest. AR and JLB are employees of Biogen-Idec.
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Figure Legends:

**Figure 1: Allogeneic transfer of BM and low concentrations of splenic cells in hosts treated with Cy and TBI caused cGVHD and BO.** B10.BR mice treated with 120mg/kg/day Cy (day -3, -2) and lethally irradiated (day -1) were transplanted with BM alone, or BM + 0.75-1x10^6 splenocytes from C57Bl/6 mice. n=10 mice/group. (A) Weights of the animals and (B) survival were tracked up to 60 days post transplant. Pulmonary function tests (PFTs) were performed on anesthetized animals at (C) day 60 and (D) day 28 post transplants. Animals were artificially ventilated, and resistance, elastance and compliance were measured as parameters of distress in lung function in animals receiving low dose splenocytes or T cells. Representative data from 3 individual experiments. **P< 0.01

**Figure 2: Multiorgan disease in mice with cGVHD; immune infiltration and collagen deposition.** (A) Tissues (lung, liver, tongue, salivary glands, thymus, spleen, ileum and colon) were harvested at day 60 after transplant, and stained with Hematoxylin and Eosin to determine pathology. Brightfield images were captured at 100x magnification using an Olympus BX51 microscope. (B) Inflammation, immune infiltration and parenchymal changes were scored, using a cumulative scoring system used previously. (C) Collagen deposition was determined using the Masson’s trichrome staining kit. Blue represents collagen deposition. (D) Collagen deposition is quantified as a ratio of blue area to total area of tissue. Quantification was done using the analysis tool for personal use only.
in Photoshop CS3. Data from two individual experiments were pooled to obtain pathology scores, n=12. * P<0.05; ** P<0.01; *** P<0.005

**Figure 3: CD4+ T-cell and B220+ B-cell infiltration is seen in lungs and livers of transplanted animals.** Liver and lung tissues were harvested at day 60 post transplants from animals receiving BM and BM+spleen and analyzed by immunohistochemistry and methyl blue counterstaining. Representative images from 3 individual experiments are shown for (A) CD4 and (D) B220. Images were captured using a brightfield microscope at 200x magnification. For lung, infiltration was quantified by obtaining a ratio of (B) CD4 or (E) B220 positive cells to total cells in a 100 mm² field of view under the microscope. Shown is an average of the count from four representative fields. For liver, (C) CD4 and (F) B220 cell infiltration was quantified by counting the number of antibody binding positive cells in a 100mm² field of view and obtaining an average of counts from four representative fields. n=6. * P<0.05; ** P<0.01; *** P<0.005

**Figure 4: Antibody deposition detected in target areas of lung and liver in diseased animals.** 6μm sections of frozen lung and liver tissues harvested at day 60 post transplant were analyzed by immunofluorescence. Tissues were incubated with FITC conjugated anti-mouse Ig. (A) Representative images for lung and liver from three individual experiments. n=8. (B) Ig Deposition was quantified by on a 0 to 3 scale to determine the amount of antibody in the tissues. (C) Serum from BM only and BM+spleen animals was collected at day 60 post transplant and incubated with healthy B10.BR lung and liver tissue followed by FITC conjugated anti-mouse Ig to detect the presence tissue specific
antibodies in diseased animals’ serum. White arrows depict areas of Ig deposition. Fluorescence was detected using an Olympus FluoView 500 Confocal Laser Scanning Microscope at a magnification of 200x.

**Figure 5: Animals receiving B-cell deficient BM show a decrease in pathology.**

B10.BR recipients were transplanted with WT BM or BM from μMT KO mice ± WT spleen. (A) At 60 days post transplant, mice were anesthetized and artificially ventilated to measure pulmonary function parameters. (B) Collagen deposition is quantified from Trichrome stained samples as a ratio of blue area to total area of tissue. Quantification was done using the analysis tool in Photoshop CS3. (C) Lung and liver tissues were harvested and 6um frozen sections were stained with FITC conjugated anti-mouse Ig for antibody deposition within the tissues. White arrows denote areas of Ig deposition. Images shown are representative images of three individual experiments, n=4. *P<0.05; ***P<0.005; p=0.4066 for lung BM (μMT) only versus BM (μMT)+T cells; p=0.2860 for liver BM (μMT) only versus BM(μMT)+T cells.

**Figure 6: Secreted antibody is required for pulmonary dysfunction in animals with cGVHD.** (A) B10.BR mice were transplanted with BM ± spleen from WT or (m+s)IgMxJhD BALB/c mice and anesthetized at day 60 post transplant for pulmonary function tests. Resistance, compliance and elastance were measured, n=8. (B) Collagen deposition is quantified from Trichrome stained samples as a ratio of blue area to total area of tissue. Quantification was done using the analysis tool in Photoshop CS3. (C) Infiltration of CD4 positive cells in the lung and the liver of transplanted mice. (D) Lung
and liver tissues were harvested and 6um frozen sections were stained with FITC conjugated anti-mouse Ig for antibody deposition within the tissues. White arrows denote areas of Ig deposition. Representative image from two independent experiments n=8.

*P<0.05; ** P<0.01; *** P<0.005

**Figure 7: Disruption of GC formation by LTβR-Ig treatment reduces lung dysfunction and cGVHD organ tissue fibrosis.** B10.BR recipients were transplanted as per Figure 1. A cohort of animals receiving BM+spleen was treated with 200µg murine LTβR-Ig or the control Ab, murine MOPC21 every 3 days starting day 28 post-transplant to day 52. (A) Spleen tissue harvested from these animals at day 60 was analyzed by immunofluorescence for GC structures. GCs were detected by colocalization of IgM (green), VCAM-1 (blue), and PNA (red); merged images show overlap (white) to discriminate GC. White arrows highlight GC. Images were captures using an Olympus FluoView 500 Confocal Laser Scanning Microscope at 100x magnification, n=5. (B) The size of the GC was quantified by measuring the area of PNA staining in Photoshop C3. (C) Frequency of the GC was quantified by counting the number of GCs in 1 mm² of spleen section. (D) Pulmonary function tests were performed on anesthetized animals on day 60 post-transplant to measure lung function. (E) Animals treated with LTβR-Ig and MOPC21 were examined for fibrosis in the lung and liver. (F) Presence of deposited lung and liver tissue specific antibodies in animals treated with LTβR-Ig and MOPC21 were determined by immunofluorescence by staining with FITC conjugated anti-mouse Ig. White arrows depict Ig deposition. Images were captured at 200x magnification and are representative of 2 individual experiments, n=5. * P<0.05; ** P<0.01.
Figure 1

A. Graph showing weight in grams over days post transplant for BM only and BM+spleen conditions.

B. Graph showing percent survival over days post transplant for BM only and BM+spleen conditions.

C. Bar graphs comparing resistance, elastance, and compliance for BM only and BM+spleen conditions.

D. Bar graphs comparing resistance, elastance, and compliance for BM only and BM+spleen conditions.
Figure 4

A

Lung
Liver

BM only

BM+spleen

C

Lung
Liver

BM only

BM+spleen

B

Ig Deposition

Lung
Liver

BM Only
BM+spleen

*
Donor B-cell alloantibody deposition and germinal center formation are required for the development of murine chronic GVHD and bronchiolitis obliterans

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