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Loss of Lymph Node Fibroblastic Reticular Cells and High Endothelial Cells Is Associated with Humoral Immunodeficiency in Mouse Graft-versus-Host Disease

Fumiko Suenaga,*†1 Satoshi Ueha,*†1 Jun Abe,*† Mizuha Kosugi-Kanaya,*†‡
Yong Wang,*† Akihiro Yokoyama,*† Yusuke Shono,*† Francis H. W. Shand,*†
Yasuyuki Morishita,† Jun Kunisawa,† Shintaro Sato,‡ Hiroshi Kiyono,* and
Kouji Matsushima*†

Graft-versus-host disease (GVHD) is a major risk factor for prolonged humoral immunodeficiency and vaccine unresponsiveness after allogeneic hematopoietic stem cell transplantation (allo-HSCT). However, the underlying mechanisms for this immunodeficiency are poorly understood. In this article, we describe previously overlooked impacts of GVHD on lymph node (LN) stromal cells involved in humoral immune responses. In major- and minor-mismatched mouse allo-HSCT models, recipients with CD8+ T cell-mediated GVHD suffered severe and irreversible damage to LN structure. These mice were susceptible to pathogenic infection and failed to mount humoral immune responses despite the presence of peripheral T and B cells. These humoral immune defects were associated with the early loss of fibroblastic reticular cells, most notably the CD157+ cell subset, as well as structural defects in high endothelial venules. The disruption to these LN stromal cells was dependent on alloantigens expressed by nonhematopoietic cells. Blockade of the Fas-FasL pathway prevented damage to CD157+ fibroblastic reticular cells and ameliorated LN GVHD. However, blockade of CD62L- or CCR7-dependent migration of CD8+ T cells to the LN was insufficient to prevent stromal cell injury. Overall, our results highlight GVHD-associated loss of functional stromal cells and LN GVHD as a possible explanation for the prolonged susceptibility to infectious disease that is experienced by allo-HSCT patients.

A llogeneic hematopoietic stem cell transplantation (allo-HSCT) is a potentially curative therapy for a variety of hematopoietic disorders (1, 2). However, relapses of primary disease and treatment-related mortalities limit the success of treatment, resulting in a 5-y survival rate of only 50%. Infection resulting from prolonged immunodeficiency is reported as the primary cause of death in 17–20% of allo-HSCT patients (3). Despite the guideline recommendation of posttransplant vaccination for reducing the risk for vaccine-preventable infections (4, 5), humoral immune responses are often limited in allo-HSCT patients.

The immunodeficiency of allo-HSCT patients is closely associated with graft-versus-host disease (GVHD) and manifests as impaired lymphocyte recovery and immune unresponsiveness (4–6). In bone marrow (BM) GVHD, donor CD4+ T cells disrupt the host BM hematopoietic niche, resulting in impaired T and B cell reconstitution. Early depletion of CD4+ T cells in allo-HSCT mice improves reconstitution of peripheral T and B cells and serum IgM levels but has less effect on serum IgG and fecal IgA recovery (7). Vaccine-unresponsiveness and low levels of IgG and IgA often persist for years in allo-HSCT patients, even after recovery of peripheral T and B cells and IgM Abs (8–11). The discordance between lymphocyte reconstitution and humoral immune competency suggests that GVHD impairs not only primary lymphoid tissues, but also secondary lymphoid tissues that are required for the induction of optimal humoral immune responses. Decreased splenic immune responses to blood-borne pathogens have been reported in a model of chronic GVHD, although the cellular mechanisms for that immunodeficiency remained to be elucidated (12).

Humoral immune responses to exogenous Ags and vaccination are mainly initiated in lymph nodes (LNs) where Ag-specific T and B cells recognize cognate Ags and interact with each other to induce protective IgG and IgA responses (13–16). These T–B interactions are supported by specialized nonhematopoietic components; high endothelial venule (HEV) cells mediate the
continuous recruitment of circulating lymphocytes, and fibroblastic reticular cells (FRCs) in the T cell–rich paracortex regulate intranodal lymphocyte migration, retention, and cellular interaction by providing an extracellular matrix network and the homeostatic chemokine CCL21 (13, 16–18). Recent studies suggest that CD8+ T cells impair FRCs in the paracortex and contribute to the loss of the humoral immune niche (19). In addition, endothelial cells are reported to be a target of allo-CD8+ T cells in allotransplantation (20, 21).

Although pathological analyses of clinical autopsy and experimental studies have shown that the LNs could be a target of GVHD (22), the cellular targets within GVHD-affected LNs and the associated impact on immune function remain poorly understood. In this study, we used several major- or minor-mismatched murine allo-HSCT models to examine the cellular and molecular mechanisms that underlie the effects of GVHD on the LNs and humoral immune responses.

Materials and Methods

Allo-HSCT and cell transfer

Mouse strains used in this study are listed in Table I. Transplantation models and histocompatibility Ag (Ha) mismatch status are summarized in Table II. T cell–depleted (TCD) BM cells were prepared by depleting Thy1.2+ mature T cells using an autoMACS system (Miltenyi Biotec). Donor CD8+ or CD4+ cells were negatively enriched from splenocytes and LN cells by autoMACS, using Abs against CD11b, B220, Ter-119, and NK.1.1, with or without CD4 or CD8 (final T cell purity: CD3+ >92%; CD8+ and CD4+ >95%). In CD8+ or CD4+ T cell transfer experiments, the frequency of contaminating CD4+ T cells in purified CD8+ T cells was <0.1% and vice versa. Recipients were lethally irradiated with 11 Gy (BDF1), 9 Gy (B6 and bm1), or 8 Gy (bm12), with radiation split into 2 doses given 3 h apart, then injected i.v. with 5 × 10^7 TCD BM cells with or without graded numbers of T cells the following day. For the blocking experiments, mice received 10^3 4-nitrophenol hapten (NP)-specific BCR knock-in B cells (B1-8 cells) 1 d before immunization. B1-8 cells were prepared from pooled splenocytes of CD45.1+ IgHa B1-8 mice by depleting CD43+, CD90.2+, and Ter-119+ cells by autoMACS. The severity of NP-specific Ab responses by ELISA, as described previously (25).

Assessment of Ag-specific Ab responses

On day 0, mice were immunized s.c. in one footpad with 50 μg NP-OVA (Biosearch Technologies) emulsified in Sigma Adjuvant System (Sigma-Aldrich). Serum was collected on day 14 after immunization for analysis of NP-specific and total IgG1 Abs by ELISA, as described previously (25).

Salmonella infection

Salmonella typhimurium was grown overnight with shaking at 180 rpm in Luria-Bertani broth at 37°C, centrifuged at 1800 × g for 20 min at 4°C, and washed in PBS before administration (1 × 10^9 CFU) (26). After challenge, survival was monitored for 3 wk.

Flow cytometry

Single-cell suspensions from the spleen, blood, and BM were prepared as described previously (27, 28). LNs were either washed through Cell Strainers (BD) for the analysis of the hematopoietic compartment or digested for 30 min at 37°C in 0.1% crude collagenase (Wako), 0.96 mg/ml Dispase II (Roche), and 20 kU/ml DNase I (Calbiochem) in DMEM supplemented with 2% FCS and 10 mM HEPES for the analysis of the stromal compartment. Single-cell suspensions from the femur, tibia, spleen, blood, and pooled inguinal and brachial LNs were incubated sequentially with anti-CD16/32 to block FcRs, then primary Abs (Table III). Data were collected using LSRII (BD Biosciences) or Galileo (Beckman Coulter) flow cytometers and analyzed using FlowJo software (TreeStar).

Coculture of T cells and LN stroma

CD8+ T cells were negatively enriched by autoMACS from spleens of naive B6 or GVHD-induced BDF1 mice on day 10 after transplantation, using Abs against CD11b, B220, Ter-119, and CD4. Single-cell suspensions from LNs were prepared by enzymatic digestion, and nonhematopoietic cells were negatively enriched from the blocked B6 or BDF1 mice using Abs against CD45 and Ter-119. Enriched CD8+ T cells and nonhematopoietic cells were cocultured at a ratio of 1:1 or 5:1 in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, and streptomycin/penicillin at 37°C for 12 h. Adherent and nonadherent cells were analyzed for intracellular cleaved caspase 3 by flow cytometry.

Immunofluorescent staining

Acetone-fixed 6-μm-thick LN sections were incubated sequentially with Blocking One (Nacalai Tesque), primary Abs, and fluorescently labeled secondary Abs. Stained sections were mounted with Prolong Gold Anti-fade Reagent (Invitrogen, Carlsbad, CA) and examined under an IX70 confocal microscope (Olympus) or a BZ-9000 fluorescent microscope (Keyence).

Statistical analysis

Statistical analysis was performed using GraphPad Prism software. A p value <0.05 was considered statistically significant.

Results

Allo-CD8+ T cells disrupt LN structure in MHC- and minor HA–mismatched allo-HSCT

To determine the involvement of T cell subsets and major or minor HA Bs (miHAs) in GVHD-associated LN impairment, we examined

<table>
<thead>
<tr>
<th>Strains</th>
<th>Haplotypes</th>
<th>Genotypes</th>
<th>Congenic Strains</th>
<th>Suppliers</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6 (B6)</td>
<td>H-2^k (K^d D^a I^A^B E^e)</td>
<td>Wt</td>
<td>CD45.2+</td>
<td>CLEA Japan</td>
</tr>
<tr>
<td>C57BL/6 × DBA/2 (F1)</td>
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<td>CD45.2+</td>
<td>CLEA Japan</td>
</tr>
<tr>
<td>C57BL/6.SIL (B6.SIL)</td>
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<td>Wt</td>
<td>CD45.2+</td>
<td>CLEA Japan</td>
</tr>
<tr>
<td>B6.C-H2^b (bm1)</td>
<td>H-2^k (K^d D^a I^A^B E^e)</td>
<td>Wt</td>
<td>CD45.1+</td>
<td>The Jackson Laboratory</td>
</tr>
<tr>
<td>B6.C-H2^b (bm12)</td>
<td>K^d (D^a I^A^B E^e)</td>
<td>Wt</td>
<td>CD45.2+</td>
<td>The Jackson Laboratory</td>
</tr>
<tr>
<td>C.B10-H2^b (BALB/B)</td>
<td>K^d (D^a I^A^B E^e)</td>
<td>Wt</td>
<td>CD45.2+</td>
<td>The Jackson Laboratory</td>
</tr>
<tr>
<td>C3H.SW-H2^b (C3H.SW)</td>
<td>K^d (D^a I^A^B E^e)</td>
<td>Wt</td>
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<td>The Jackson Laboratory</td>
</tr>
<tr>
<td>4-Hydroxy-2-nitrophenyl acetyl–specific BCR knock-in (B1-8)</td>
<td>H-2^k (K^d D^a I^A^B E^e)</td>
<td>Wt</td>
<td>CD45.1+</td>
<td>The Jackson Laboratory</td>
</tr>
<tr>
<td>B6.gld (B6gld)</td>
<td>H-2^k (K^d D^a I^A^B E^e)</td>
<td>FasL&lt;sup&gt;ld&lt;/sup&gt;</td>
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<td>SLC Japan</td>
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<tr>
<td></td>
<td></td>
<td>Igh&lt;sup&gt;Im2Cen&lt;/sup&gt;</td>
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2 LOSS OF FRCs AND HEVs UNDERLIES LN GVHD
LN cell number and pathological changes using mouse strains listed in Table I in various MHC- and miHA-mismatched GVHD models with allo-CD4⁺ or CD8⁺ T cells (Table II). In [B6 CD8→bm12] MHC class II–mismatched and [C3H.SW CD8→B6] miHA-mismatched CD8-dependent GVHD (CD8-GVHD) models, recipients with GVHD showed 300- and 30-fold decreases, respectively, in LN cell number (Fig. 1A). Recipients with CD8-GVHD also displayed severe atrophic changes on day 40 after allo-HSCT as compared with controls receiving only TCD BM (BMT) (Fig. 1B). In contrast, only mild-to-moderate reductions in LN cell number and negligible atrophy were observed in [B6 CD8→bm12] MHC class I–mismatched and [C3H.SW CD8→B6] miHA-mismatched recipients in the CD4-dependent GVHD mod-}

**Table II. Allo-CD8⁺ or CD4⁺ T cell–mediated GVHD models**

<table>
<thead>
<tr>
<th>Models</th>
<th>MHC Haplotype</th>
<th>HA Mismatch</th>
<th>T Cell Subtype</th>
</tr>
</thead>
<tbody>
<tr>
<td>[B6→BDF₁]</td>
<td>H-2b H-2bd</td>
<td>MHC class I/II</td>
<td>CD8</td>
</tr>
<tr>
<td>[B6→BDF₁]</td>
<td>H-2b H-2bd</td>
<td>MHC class I/II</td>
<td>CD8</td>
</tr>
<tr>
<td>[B6→bm12]</td>
<td>H-2b H-2bm12</td>
<td>MHC class II</td>
<td>CD4</td>
</tr>
<tr>
<td>[B6→BALB/B]</td>
<td>H-2b H-2bm1</td>
<td>miHA</td>
<td>CD4</td>
</tr>
<tr>
<td>[B6→bm1]</td>
<td>H-2b H-2bm1</td>
<td>MHC class I</td>
<td>CD8</td>
</tr>
<tr>
<td>[C2H.SW→B6]</td>
<td>H-2b H-2b</td>
<td>miHA</td>
<td>CD8</td>
</tr>
</tbody>
</table>

Collectively, these results suggest that the transfer of allo-CD4 T⁺ cells induces mild LN damage in MHC-mismatched settings only, whereas transfer of allo-CD8⁺ T cells above a certain threshold induces severe and irreversible LN damage in both MHC- and miHA-mismatched settings. Hereafter, the [B6 CD8→BDF₁] model was used unless otherwise indicated.

**Humoral immunodeficiency in CD8-GVHD–affected LNs**

The damage observed in CD8-GVHD–affected LNs motivated us to examine whether these LNs are able to support humoral immune responses. BMT or CD8-GVHD recipients were immunized s.c. with a T cell–dependent model Ag, NP-OVA, on day 40 after allo-HSCT (Fig. 2A). Although the BMT cohort mounted an NP-specific Ab response detectable in the serum by 14 d postimmunization, no NP-specific IgG1 was detectable in serum from the CD8-GVHD group (Fig. 2B). This was not due to defective B cell reconstitution because adoptive transfer of B1-8 cells 1 d before immunization augmented anti-NP IgG responses in the BMT group, but not in the CD8-GVHD group (Fig. 2B).

To evaluate mucosal immunity in recipients with CD8-GVHD–affected LNs, we immunized BMT and CD8-GVHD group mice orally with 10 µg CT on days 59, 66, and 72 after allo-HSCT, after which fecal anti-CT IgA and serum anti-CT IgG levels were measured on day 79 (Fig. 2C). Although oral immunization with CT induced detectable levels of CT-specific fecal IgA and serum IgG in mice from both the BMT and the CD8-GVHD groups, the levels of both IgGs were markedly lower in the CD8-GVHD group (Fig. 2D). Consistent with this difference in IgA levels, diarrhea induced by high-dose CT challenge was attenuated by CT immunization to a lesser extent in the CD8-GVHD group than in the...
BMT group (Fig. 2E). Defective mucosal protection was also observed in the CD8-GVHD group when the recipients were infected with a lethal dose of virulent *Salmonella typhimurium* on day 74 after allo-HSCT (Fig. 2F). Collectively, these results show that LN damage is associated with humoral immunodeficiency in recipients with CD8-GVHD. We refer to the GVHD-associated structural and functional impairments observed in the LNs as “LN GVHD.”

FRCs are eliminated from LNs early in LN GVHD

To identify the cellular target of LN GVHD, we examined the kinetics of LN stromal cell number in the [B6 CD8→BDF1] GVHD model. Flow cytometry revealed that CD45<sup>-</sup>Ter119<sup>-</sup>gp38<sup>+</sup>FRCs in the CD8 GVHD group decreased on day 14 compared with the BMT group (Fig. 3A, 3B). The decrease of FRCs was even greater within the CD157<sup>+</sup> subpopulation, which represents FRCs in the T cell area (29) (Fig. 3A, 3B). Unexpectedly, CD45<sup>-</sup>Ter119<sup>-</sup>gp38<sup>-</sup>CD31<sup>+</sup>Peripheral node addressin<sup>+</sup> (PNAd<sup>+</sup>) HEV cell numbers, which have been reported to be reduced in GVHD based on the results of autopsy studies of allo-HSCT patients (22), were equivalent between the BMT and CD8-GVHD groups on days 4, 7, and 14 (Fig. 3A, 3C). CD45<sup>-</sup>Ter119<sup>-</sup>gp38<sup>-</sup>CD31<sup>+</sup>lymphatic endothelial cells (LECs) increased slightly on day 7, and CD45<sup>-</sup>Ter119<sup>-</sup>gp38<sup>-</sup>CD31<sup>+</sup>blood endothelial cells (BECs) decreased transiently on days 4 and 7 in the CD8-GVHD group compared with the BMT group. However, no significant differences in LEC or BEC numbers were observed between the CD8-GVHD and BMT groups on day 14 (Fig. 3A, 3C). Collectively, these results demonstrate that CD157<sup>+</sup> FRCs in the LNs are a previously unreported target of GVHD that is lost early after transplantation.

To determine whether the decrease of LN FRCs in CD8-GVHD is due to the direct cytotoxic effects of allo-activated CD8+ T cells on FRCs, we cocultured CD8+ T cells and LN stromal cells for 12 h before analyzing for the presence of apoptotic cells by flow cytometry. The proportion of cleaved-caspase 3<sup>+</sup> apoptotic cells within the FRC population increased in the coculture of wild-type (Wt) allo-activated B6 CD8+ T cells and BDF1 stromal cells.
and their survival was evaluated. In two independent experiments and represent mean immunization. (100 week after the final immunization, recipients were orally challenged with adoptive transfer of 1
transplanted with TCD BM (BMT) or TCD BM + 1
duction of FRC apoptosis by activated CD8+ T cells also occurs on FRCs (Fig. 3D, 3E). Although less frequent than was observed→ the high background of apoptotic cells within these populations results were obtained for CD157+ FRCs, but not for gp38
unpublished GVHD-affected LNs from day 14 onward (Fig. 4B). These results demonstrate that both FRCs and HEVs are functionally impaired during LN GVHD, resulting in the persistent loss of the structural framework that supports adaptive immune responses.

**LN GVHD develops independently of irradiation preconditioning**

To identify the requirements for LN GVHD, we examined the effects of irradiation preconditioning, an important risk factor for severe GVHD, on the development of LN GVHD by using a [CD45.1+B6 splenocyte→CD45.2+BDF1] nonirradiated GVH reaction model. By day 40 after transfer, most of the thymocyte, splenocyte, and BM B cell populations had been repopulated by CD45.1+ donor-derived cells, and on day 50, cell numbers were comparable with those in untreated BDF1 mice (Supplemental Fig. 2A, 2B). However, LNs were severely atrophied and LN cell number was 100-fold less than in untreated BDF1 mice (Supplemental Fig. 2B, 2C). Furthermore, FRC numbers, especially the CD157+ subpopulation, were decreased as early as day 4 after transfer (Supplemental Fig. 2D), as observed in the irradiated GVHD model. These results suggest that irradiation preconditioning is not required for damage to CD157+FRCs to occur, and for the subsequent development of LN GVHD.

**LN GVHD requires nonhematopoietic alloantigens**

We next examined the requirements for alloantigens on nonhematopoietic cells. Flow cytometry revealed that FRCs constitutively expressed MHC class I and Fas, and low levels of MHC class II on day 7 after allo-HSCT (Fig. 5A). To assess whether alloantigens on nonhematopoietic cells play a role in LN GVHD, we transferred B6 CD8+ T cells to [BDF1→B6] alloantigens on nonhematopoietic cells play a role in LN GVHD, resulting in the persistent loss of the structural framework that supports adaptive immune responses.

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Depletion of CD8+ T cells and blockade of blood-borne T cell migration to LNs does not alleviate LN GVHD

Because the development of LN GVHD requires a certain number of allo-CD8+ T cells, we tested the effects of in vivo depletion of CD8+ T cells as a treatment for LN GVHD. Unexpectedly, CD8 depletion on or after day 2 of allo-HSCT eliminated CD8+ T cells in the peripheral blood, but not in the LNs on day 4 after transfer (Supplemental Fig. 4A), and failed to restore CD157+ FRCs on day 14 (Supplemental Fig. 4B). Thus, we next used a CD62L blocking Ab and Ccr72/2 CD8+ T cells to examine whether blocking the migration of CD8+ T cells to the LNs prevents LN GVHD. Blockade of CD62L reduced the accumulation of donor CD8+ T cells in peripheral LNs to 1/100 of the control at 20 h after HSCT. In contrast, Ccr72/2 CD8+ T cells accumulated in peripheral LNs at a comparable level with Wt CD8+ T cells (Supplemental Fig. 4C), but the upregulation of the early activation marker CD69 was diminished in Ccr72/2 CD8+ T cells found in the spleen and LNs (Supplemental Fig. 4D). These results suggest a division of labor between CD62L and CCR7 in which CD62L mediates early migration of donor CD8+ T cells to peripheral LNs, whereas CCR7 facilitates donor CD8+ T cell activation. Despite the efficient inhibition of donor CD8+ T cell migration and activation, CD62L blockade and loss of CCR7 had negligible effects on the number of CD157+ FRCs observed on day 14 and on LN cellularity on day 40 after allo-HSCT (Supplemental Fig. 4E, 4F).

FasL contributes to the damage of CD157+ FRCs and LN GVHD

The Fas-FasL cytotoxic pathway plays a major role in the pathogenesis of liver, intestinal, and BM GVHD (30, 31). To determine whether Fas-FasL signaling is also involved in LN GVHD, we used CD8+ T cells from FasL-mutants (gld) in the [B6 CD8→BDF1] GVHD model. The numbers of FRCs and CD157+ FRCs, respectively, were 30 and 75 times higher in the recipients of FasLgld CD8+ T cells compared with those in recipients of Wt CD8+ T cells on day 14 after allo-HSCT (Fig. 6A). Consistent with these results, gp38+CD157+ FRCs in the T cell–rich area was preserved in the recipients of FasLgld CD8+ T cells (Fig. 6B). On day 40, LN cell number was 10-fold higher in the recipients of FasLgld CD8+ T cells than in the recipients of WT CD8+ T cells (Fig. 6C). GVHD-induced LN atrophy was also visibly less severe in the recipients of FasLgld CD8+ T cells (Fig. 6D, 6E). These results demonstrate that FasL is involved in the development of LN GVHD through the damage of CD157+ FRCs.

Discussion

Current efforts to enhance immune reconstitution after allo-HSCT have mainly focused on enhancing the production of T and B cells in the primary lymphoid tissues. As such, prevention of CD4+ T cell–mediated BM GVHD promotes the recovery of peripheral T and B cells. However, mice suffering CD8+ T cell–mediated GVHD...
GVHD, which particularly impacts the LNs, also fail to mount adequate humoral immune responses. This observation highlights the importance of preventing and treating LN GVHD after allo-HSCT in addition to trying to promote T and B cell reconstitution. Our results demonstrate that allo-CD8+ T cells cause severe and irreversible damage to the LNs by inducing the early loss of FRCs and HEVs. We show that nonhematopoietic cells and FasL signaling on allo-CD8+ T cells are involved in the development of LN GVHD. Furthermore, the development of LN GVHD independently of irradiation preconditioning may explain why enhanced T and B cell reconstitution in reduced intensity conditioning HSCT patients does not necessarily translate into better immunocompetence compared with myeloablative conditioning HSCT (6). Based on our findings, we propose that the loss of LN FRCs and HEVs is an important mechanism that should be taken into account when developing strategies to overcome GVHD-associated humoral immunodeficiency and vaccine unresponsiveness (Fig. 7).

Although the clinical incidence of LN GVHD has not been investigated, some autopsy studies have demonstrated LN damage after allo-HSCT (32, 33). LNs of patients who died 15–326 d after allo-HSCT displayed reduced cellularity of the cortex and paracortex, and a loss of follicles and HEVs (22), although these pathological changes may have been influenced by several factors including the extent of immune reconstitution, primary disease, infections, and immune suppression. The establishment of non-invasive diagnostic methods for clinical LN GVHD, such as near-infrared fluorescence imaging with indocyanine green (34), may reveal the clinical incidence of LN GVHD and provide an important means of assessing the infectious risk and vaccine responsiveness of allo-HSCT patients. Given that repetitive immunization enhanced the protective humoral immune response even in mice affected by LN GVHD, early diagnosis of LN GVHD might provide a strong indication for patients to receive repetitive vaccination.

Interestingly, in contrast with the irreversible damage occurring in the LNs, GVHD-induced disruption of the splenic white pulp was reversible. Although the Fas-FasL cytotoxic pathway may serve as a therapeutic target of GVHD in LNs, as well as the liver, intestine, and BM (7, 30, 31), expression levels of Fas and MHC class I do not explain the mechanism of FRC impairment during LN GVHD in a subset- or tissue-selective manner. Sensitivity to Fas signaling and/or a molecular dependency for recovery from Fas-FasL-mediated damage may be involved in the subset selectivity. In regard to tissue selectivity, persistent loss of FRCs in LNs, but not in the spleen, may arise from functional defects in HEVs. Defective HEV function may deny access to circulating lymphoid tissue inducer cells, which could otherwise restore the

**FIGURE 4.** Structural and functional defects in CD8-GVHD–affected LNs. In the [B6 CD8→BDF1] model, LNs were collected for immunofluorescent staining with Abs against (A) gp38 (green), ER-TR7 (red), pan endothelial cell Ag (blue) or (B) PNA d (green), CCL21 (red), collagen IV (blue). Scale bars, 100 μm. Data are representative of three mice per group.

**FIGURE 5.** Alloantigens on nonhematopoietic cells are a prerequisite for LN GVHD. (A) Expression of Fas, MHC class I, and MHC class II on FRCs was analyzed by flow cytometry on day 7 after allo-HSCT in the [B6 CD8→BDF1] model. (B and C) Lethally irradiated B6 or BDF1 mice were transplanted with TCD BM from BDF1 mice to generate [BDF1→B6] or [BDF1→BDF1] BM chimeras, respectively (blood chimerism >95% in all BM chimeras). These BM chimeras were then used as recipients in the [B6 CD8→BDF1] model 8 wk after the first HSCT (see Supplementary Fig. 2A). (B) Numbers of BM B cells, thymocytes, splenocytes, and LN cells. (C) Representative H&E-stained images of LNs on day 85 after secondary allo-HSCT. *p < 0.05, ***p < 0.001 (unpaired two-tailed Student t test). Data represent mean ± SEM (n = 4–6) and are representative of two independent experiments. Scale bars, 100 μm.
T cell area stroma (19, 35). In contrast, cellular entry into the splenic white pulp is mediated mainly by an open blood system, allowing lymphoid tissue inducer cells to enter and contribute to the restoration of periarteriolar lymphoid sheath FRCs in GVHD (36, 37). Our finding that the splenic white pulp is restored over time suggests that intranodal FRC precursor cells (38) are spared, even in LN GVHD–affected mice. Approaches to preserve or restore functional HEVs, including stimulation of the lympho-toxin-β receptor signaling that is required for the function of HEVs (39), might be important for the prevention and treatment of LN GVHD.

Because depletion of CD8+ T cells in the early phase of CD8-GVHD did not ameliorate the loss of CD157+ FRCs, direct recognition and subsequent damage to these cells might take place very early in CD8-GVHD. In our experiments, blockade of CCR7 and/or CD62L was ineffective in preventing LN damage. The failure to preserve FRCs and LN structure by these strategies might be explained by the immediate recruitment of CD8+ T cells during GVHD. A relatively small number of residual LN-migrating CD8+ T cells are sufficient to trigger LN GVHD. Our findings suggest that depletion or inhibition of T cell migration into the LNs requires further consideration or an alternative strategy.

In summary, we have demonstrated that LN damage after major and minor mismatched allo-HSCT involves a drastic loss of stromal cells that is associated with prolonged humoral immunodeficiency and vaccine unresponsiveness. Limiting the number of allo-CD8+ T cells in the graft or blocking Fas-FasL signaling were effective strategies for preventing LN GVHD. Further elucidation of the molecular mechanisms of LN GVHD will likely guide the development of novel approaches to improving the immunocompetence of allo-HSCT patients.

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**Disclosures**

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