Prevention of Acute Graft-versus-Host Disease in a Xenogeneic SCID Mouse Model by the Humanized Anti-CD74 Antagonistic Antibody Milatuzumab

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ABSTRACT
Prevention and treatment of graft-versus-host disease (GVHD) remains a major challenge, given that current T-cell depletion and mainstay immunosuppressive therapies compromise preexisting T-cell immunity, often leading to severe infections and disease relapse. Thus, there is a critical need for novel anti-GVHD agents that can spare protective T-cell memory. Here we show that milatuzumab (hLL1), a humanized anti-CD74 antagonist monoclonal antibody, can moderately reduce the numbers of CD74-expressing B cells and myeloid dendritic cells, but has no effect on the survival of T cells that are CD74⁺. Consequently, milatuzumab inhibits allogeneic T-cell proliferation in mixed leukocyte reactions. In a human/mouse xenogeneic SCID mouse model in which GVHD is induced and mediated by engrafted human CD4⁺ T cells and dendritic cells, milatuzumab effectively prevents the onset and manifestations of acute GVHD, suppresses serum levels of human IFN-γ and IL-5, eliminates the infiltration of human lymphocytes into GVHD target organs (ie, lung, liver, and spleen), and significantly promotes survival (90% versus 20% for controls; P = .0012). Importantly, exposure to milatuzumab does not affect the number of cytomegalovirus-specific, IFN-γ-producing human CD8⁺ T cells in allogeneic mixed leukocyte reactions. These encouraging results warrant further exploration of milatuzumab as a possible new therapeutic agent for GVHD.

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INTRODUCTION
Graft-versus-host disease (GVHD) is the major cause of mortality and morbidity in patients undergoing allogeneic hematopoietic stem cell transplantation (allo-HSCT), a powerful and currently the sole curative therapy for many malignant and nonmalignant hematologic diseases [1]. Prevention and treatment of GVHD remains a major challenge [2], given that contemporary T-cell depletion and immunosuppressive therapies, although effective in controlling GVHD in certain patients, compromise preexisting T-cell immunity, leading to severe infections and disease relapse [3-6]. Thus, there is a critical need for novel anti-GVHD agents that spare protective T-cell memory.

Milatuzumab (hLL1) is a humanized IgG1κ mAb that reacts with human CD74 [7-9], the HLA class II-associated invariant chain [10]. Previous studies found that milatuzumab has potent cytotoxicity against CD74-expressing malignant B cells in vitro and in xenograft models [7,11], which has led to the ongoing clinical evaluation of milatuzumab in relapsed or refractory B-cell malignancies [12]. More recent preclinical studies [13] have demonstrated that milatuzumab is capable of modulating human B cell proliferation, migration, and adhesion molecule expression, suggesting the therapeutic potential of this mAb in autoimmune diseases.

As an HLA class II invariant chain molecule, CD74 is widely expressed in both hematopoietic and nonhematopoietic antigen-presenting cells (APCs), which include B cells, monocytes, macrophages, Langerhans cells, dendritic cells (DCs), and endothelial and certain epithelial cells [7,14]. Because both recipient and donor APCs, including non-hematopoietic APCs, play critical roles in the initiation of GVHD [15-24], we reasoned that milatuzumab might have therapeutic potential for GVHD by affecting recipient and/or donor APCs. The anti-GVHD potential of anti-CD74 mAb is also supported by evidence that macrophage migration inhibitory factor (MIF), the ligand of CD74 [25], is involved in the development of acute GVHD in a murine model of allogeneic stem cell transplantation [26].

In this study, we demonstrate that milatuzumab selectively reduces myeloid DCs (mDCs) and B cells, but not plasmacytoid DCs (pDCs), monocytes, or T cells, in human peripheral blood mononuclear cells (PBMCs). As a result, milatuzumab inhibits allogeneic mixed lymphocyte reactions (allo-MLRs); and in a human PBMC-transplanted SCID mouse model (hu-PBL-SCID) [27], milatuzumab effectively prevents acute GVHD, suppresses human cytokine storm, eliminates infiltration of human lymphocytes in GVHD target organs, and significantly improves survival. Unlike alemtuzumab, an anti-CD52 mAb currently used clinically for GVHD prevention, milatuzumab does not affect cytomegalovirus (CMV)-specific T-cell immunity in vitro, suggesting that it might be exploited as a novel agent for GVHD without compromising preexisting antiviral immunity.

MATERIALS AND METHODS

Antibodies and Reagents
Milatuzumab (hLL1), labetuzumab (hMN-14; a humanized anti-CEACAM5 mAb) [28], epratuzumab (hLL2; a humanized anti-CD22 mAb) [29], IMMU-T3 (a humanized anti-CD3 mAb), and hLL1-Fab-A3B3 (a fusion protein of milatuzumab Fab fragment with CEACAM5 [CD66e] A3B3 domain...
Rituximab (anti-CD20) was purchased from IDEC Pharmaceuticals (San Diego, CA). Alemtuzumab (anti-CD52) was obtained from Genzyme (Ridgefield, NJ). The mAbs used for flow cytometry were obtained from BD Pharmingen (San Jose, CA): FITC-labeled anti-CD74 (M-B741), FITC-labeled mouse IgG1, PE-labeled anti-CD25, PerCp-labeled anti-CD8, and allophycocyanin-labeled anti-CD4 and CD3; or from Miltenyi Biotec (Auburn, CA): PE-labeled mAbs to CD19 (LT19) and CD14 (TÜK4) and allophycocyanin-labeled mAbs to BDCA-1 (AD5-14H12). Human IgG was obtained from Jackson ImmunoResearch (West Grove, PA). HLA-A*0201 restricted CMV pp65 peptide (NLVPMVATV) and HIV gag peptide (SL9, SLYNTVATL) were obtained from Anaspec (Fremont, CA).

Assessment of APC Subsets in PBMCs

Buffy coats from expired whole blood were obtained from healthy donors at the Blood Center of New Jersey (East Orange, NJ), after approval by the New England Institutional Review Board. PBMCs were isolated fromuffy coats by standard density-gradient centrifugation over Ficoll-Paque. The isolated PBMCs were treated with milatuzumab or other mAbs at 37°C in 5% CO2 for 3 days. After incubation, the cells were stained with PE-labeled anti-CD14 and anti-CD19 in combination with allophycocyanin-labeled anti–BDCA-1 IgG, for analysis of mDC1, or a mixture of FITC-labeled anti–BDCA-2 and allophycocyanin-labeled anti–BDCA-3 IgG for simultaneous analysis of mDC2s and pDCs, respectively. PBMCs were gated to exclude the debris and dead cells on the basis of their forward scatter and side scatter characteristics. (A) Subpopulations of PBMCs were gated as follows: monocytes, CD14SSCmedium; B cells, CD19SSClow; T cells, CD19 CD14 SSChigh; mDC1, CD14 CD19 BDCA-1+; mDC2, CD14 CD19 BDCA-3++; pDCs, CD14 CD19 BDCA-2--; (B-E) Representative flow cytometry data for B cells (B), monocytes (B), non-B lymphocytes (B), mDC1s (C), mDC2s (D), and pDCs (E) in PBMCs after mAb treatment. (F-H) Mean percentages of live mDC1s, mDC2s, and pDCs in PBMCs after mAb treatment (n = 7 donors). Error bars indicate SD. P values were determined by the paired t test. **P < .05; ***P < .01 versus hMN-14 control.

Assessment of T-Cell Apoptosis and Survival

Human PBMCs were treated with hMN-14, milatuzumab, or alemtuzumab at different concentrations for 18 hours in the presence of phytohemagglutinin (final concentration, 2.5 μg/mL) and IL-2 (final concentration,
50 U/mL). After treatment, the cells were stained with Alexa Fluor 488–labeled annexin V, PE-labeled anti-CD25, and allophycocyanin-labeled anti-CD3. After washing, the cells were stained with 7-aminocoumarin-D (7-AAD; Life Technologies, Grand Island, NY), followed by flow cytometry analysis of early (annexin V+7-AAD+) and late apoptotic cells (annexin V+7-AAD+) in activated T cells (CD3+CD25+) and nonactivated T cells (CD3+CD25−) within PBMCs.

Two-Way Allo-MLR

Proliferation of T cells in the presence of APCs was evaluated by 2-way allo-MLR without irradiation of PBMCs from either donor [31,32]. PBMCs from different donors were labeled with 1 μM carboxyfluorescein succinimidyl ester (CFSE; Life Technologies) following the manufacturer’s instructions. After extensive washing, cells from different donors were mixed with one another at a 1:1 ratio and then cultured with milatuzumab or control mAbs at 37°C in 5% CO2 for 7–10 days. Starting on day 4, human IL-2 (final concentration 50 U/mL; Roche Applied Science, Penzburg, Germany) was added to the culture, and the cells were split every other day by adding fresh medium without mAbs. To analyze cell proliferation, cells were harvested, and the CFSElow cell frequencies were quantitated by flow cytometry. In some experiments, cells from MLRs were stained with anti-CD4 and anti-CD8 mAbs for flow cytometry analysis of the percentages of T-cell subsets in allo-MLRs.

Hu-PBL-SCID Mouse Model of GVHD

All animal procedures were approved by the Institutional Animal Care and Use Committee of the University of Medicine and Dentistry of New Jersey. The hu-PBL-SCID mouse model of GVHD was established by following the method of Wilson et al. [27]. SCID mice (CB-17-scid-129/HrTac-TcRa-Kb1, 5–7 weeks old) were purchased from Taconic (Rensselaer, NY), housed in sterile microisolation cages, and given autoclaved food and water. After a 10-day acclimation period, on study day –7, each mouse was injected i.p. with 20 μL of asialo-GM1 (Wako Chemicals, Richmond, VA) and irradiated (325 cGy). On the next day (day 0), 5 × 10⁷ fresh human PBMCs in 200 μL of serum-free therapeutic-grade AIM-V medium (Life Technologies) were transplanted into each mouse by i.p. injection. Milatuzumab or control Abs were administered by i.p. injection at 3 h before human PBMC transplantation, with doses of 350 and 700 μg/mouse, equivalent to 1.46 and 2.92 mg/kg in mice. Mice were then assessed daily using a GVHD scoring system that measures 6 items related to clinical signs of GVHD: weight loss, posture, activity, fur texture, skin integrity, and diarrhea [27]. The overall score for each mouse was the sum of the 6 individual scores (0–2 points for each). Mice with severe GVHD (overall score ≥5) or at the end of study (day 28) were euthanized, and livers, spleens, and lungs were obtained for analysis. Before euthanasia, peripheral blood was collected for serum cytokine measurements using a chromatic bead assay (CBA) kit (BD Biosciences). Livers, spleens, and lungs were fixed in 10% formalin, embedded in paraffin, and sectioned for hematoxylin and eosin histological examination of periportal lymphocytic infiltration, as well as immunohistochemical staining for human CD3+ and CD74+ cells, using the humanized mAbs IMMU-T3 and milatuzumab, respectively, as described previously [33]. In addition, blood samples collected from cardiac puncture of anesthetized animals were used for measuring circulating Th1/Th2 cytokines by CBA (BD Biosciences).

Quantitation of CMV-Specific T Cells in Allo-MLR by Assessing CMV-Specific IFN-γ Response

PBMCs from donors with HLA-A*0201–restricted, CMV-specific IFN-γ responses, as precharacterized by Cellular Technology (Shaker Heights, OH), were mixed with PBMCs from other donors, irrespective of HLA type and CMV status, in the presence of milatuzumab or control mAbs at 5 μg/mL. The mixed cells were cultured for 4 days in RPMI-1640 medium with 10% fetal bovine serum (FBS), followed by the addition of 50 U/mL of IL-2, and then cultured for another 2 days. Cells were then harvested and incubated with CMV pp65 peptide (NLVPVMATV) [27] or HIV gag peptide (SL9; SLYNTVATL) [27] at 10 μg/mL for 6 hours in the presence of brefeldin A at a final concentration of 1 μg/mL. The cells were then stained with PerCP-CD3 and FITC-CD8, followed by permeabilization and fixation using BD Cytofix/ Cytoperm solution (BD Pharmingen) and intracellular staining with allophycocyanin-IFN-γ mAb. The percentages of IFN-γ+ cells stimulated by CMV pp65 peptides in total MLR cells and CD3+CD8+ cells were determined by flow cytometry.

Statistical Analyses

Statistical significance between mAb treatment and controls was assessed using the paired t test. Animal survival data were analyzed with Prism (GraphPad Software, La Jolla, CA). Body weight changes were analyzed by analysis of variance using a generalized linear model approach, and GVHD clinical scores were analyzed using a generalized estimation equations approach [34] with SAS software (SAS Institute, Cary, NC).

RESULTS

Milatuzumab Selectively Reduces mDCs

To test the potential cytotoxicity of milatuzumab on DCs and other hematopoietic APC subsets, human PBMCs were
treated in vitro with milatuzumab or other mAbs for 3 days, followed by the enumeration of various APC subsets in PBMCs. As shown in Figure 1A–C, mDC1 cells (defined as the CD14–/CD19+ cells) in milatuzumab-treated PBMCs were reduced by 59.8% compared with control anti-carcinoembryonic antigen (CEACAM5; CD66e) antibody hMN-14 treated cells (P = .0158, n = 4 donors) (Figure 1C), and B cells were reduced by 21.1% (P = .0035, n = 4 donors), whereas in monocytes (CD14+SSC+ cell population) and non–B lymphocytes (CD14+CD19+ cells, >80% T cells [31]) were not affected (Figure 1B). In separate experiments, mDC2 cells (CD14+CD19+ BDCA-3+ cell population [31]) were reduced by 53.8% (P < .05, n = 7 donors) (Figure 1D), and pDCs (CD14–CD19–/BDCA-2+ cell population) were not affected (Figure 1E). As expected, rituximab, the chimeric anti-CD20 mAb, significantly reduced B cells (53.2% reduction; P = .0129, n = 4 donors) (Figure 1B) but had no effect on monocytes, DCs, or non-B lymphocytes (Figure 1B and E). Similar results were obtained in human whole blood, in which milatuzumab at 5 µg/mL decreased mDC1 and B cells, but not T cells, after 2 days of incubation in vitro (Supplemental Figure S1A). The differential effects of milatuzumab on different PBMC subsets may be related to the different expression levels of CD74 on these cells. As shown in Supplemental Figure S2A and B, the CD74 expression was highest on mDC2s and mDC1s and lowest on non–B lymphocytes, which is correlated with the efficacy of reduction on APC subsets by milatuzumab (Supplemental Figure S2C and D).

Compared with milatuzumab, the Fab fragment of milatuzumab (hLL1-Fab-A3B3), which is linked to an irrelevant A3B3 protein domain of CEACAM5 [30], did not exhibit any effect on all APC subsets (Figure 1F–H), indicating that either divalent binding or the Fc Ab portion is required for the action of milatuzumab. However, milatuzumab failed to kill purified mDC1s or mDC2s (Supplemental Figure S3A and B),...
Figure 3. Milatuzumab has no effect on activated or nonactivated T cells. Human PBMCs were treated with milatuzumab, control mAb hMN-14, or alemtuzumab at the indicated concentrations for 18 h in the presence of 2.5 μg/mL of phytohemagglutinin and 50 U/mL of IL-2. The cells were then stained with Alexa Fluor 488–labeled annexin V, PE-labeled anti-CD25, allophycocyanin-labeled anti-CD3, and 7-AAD for flow cytometry analysis. (A) Gating profile of early apoptotic cells, late apoptotic cells, and live cells in activated T cells (CD3+CD25+) and nonactivated T cells (CD3+CD25-). (B and C) Apoptotic and live cells in activated T cells (B) and nonactivated T cells (C) (n = 6 PBMC donors). *P < .05; **P < .01; ***P < .001 versus hMN-14 control.
suggesting that the reduction of mDC1s and mDC2s in human PBMCs may occurring through the ligation of other cell types by the Fc of milatuzumab.

**Milatuzumab Inhibits Allo-MLR**

Because DCs are the most potent APCs for stimulating T cells, we next investigated whether the reduction of mDC1s
and mDC2s in PBMCs by milatuzumab could be translated to reduced T-cell proliferation in 2-way allo-MLRs [31,32]. After 8 days of culture, allo-MLRs treated with the control anti-CEACAM5 mAb hMN-14 exhibited T-cell proliferation, characterized by 23.7% of cells with CFSE dilution. In contrast, T-cell proliferation was detected in only 8.4% of cells in allo-MLRs treated with milatuzumab (Figure 2A). Statistical analysis of 10 different stimulator–responder combinations showed a significant reduction ($P < .0001$) in T-cell proliferation in milatuzumab-treated allo-MLRs (Figure 2B). Milatuzumab also inhibited proliferation of both CD4$^+$ and CD8$^+$ T cell subsets in allo-MLRs (Figure 2C and D). The proliferation of CD4$^{high}$ T cells, a subpopulation of activated CD4$^+$ T cells [35], was inhibited as well (Figure 2D). No inhibition was found in hLL1-Fab-A3B3-treated allo-MLRs, suggesting that the inhibition of T-cell proliferation of allo-MLRs by milatuzumab also requires the Fc portion or divalent binding.

To exclude the possibility that milatuzumab might have direct cytotoxicity on activated T cells, we tested the effect of milatuzumab on apoptosis and survival of activated (CD25$^+$) and nonactivated (CD25$^-$) T cells in phytohemagglutinin-stimulated PBMCs. The results showed that milatuzumab has no effect on early and late apoptosis or survival in either activated or nonactivated T cells (Figure 2A-C). The proliferation of CD4$^{high}$ T cells, a subpopulation of activated CD4$^+$ T cells [35], was inhibited as well (Figure 2D). No inhibition was found in hLL1-Fab-A3B3-treated allo-MLRs, suggesting that the inhibition of T-cell proliferation of allo-MLRs by milatuzumab also requires the Fc portion or divalent binding.

**Milatuzumab Prevents Acute GVHD in hu-PBL-SCID Mice**

The reduction of mDCs and inhibition of allo-MLRs suggest that milatuzumab may be therapeutic for GVHD. We tested this possibility in a xenogeneic SCID mouse model in which acute GVHD is mediated by engrafted human donor CD4$^+$ T cells and DCs [27]. As shown in Figure 4A, 90% (9 of 10) of mice injected i.p. with either 350 or 700 µg of milatuzumab at 3 h before transplantation survived for the full 28-day experimental period, compared with only 20% (2 of 10) of untreated mice ($P < .0012$). All of the 8 untreated mice that did not survive died spontaneously (ie, were found dead at the daily checkup) on day 10-15 (Figure 4B). In the mice treated with milatuzumab 350 µg and 700 µg, only 1 of the 10 mice in each group did not survive the full study period (28 days). Both of these mice died spontaneously, one on day 20 (the 350 µg milatuzumab-treated mouse) and the other on day 13 (the 700 µg milatuzumab-treated mouse) (Figure 4B). Before they were found dead, the animals exhibited hunching, fur ruffling, reduced activity, and greater weight loss, but no diarrhea or skin lesions. In the same experiment, 5 µg of the clinically used anti-GVHD anti-CD52 mAb alemtuzumab protected 100% (10 of 10) of the mice at day 28.

We also performed direct temporal comparisons of GVHD clinical scores and weight loss between treatments over the full 28-day experiment. Throughout the study, GVHD scores, body weight changes, and survival were assessed on a daily basis. Mice were euthanized at a GVHD score of ≥5. Figure 4B shows daily-recorded GVHD scores in each hu-PBL-SCID mouse over the full 28-day study period, which demonstrate that both milatuzumab and alemtuzumab reduced GVHD scores in this xenogeneic animal model, and in 9 of 10 blocks of mice (Figure 4B, a-d and f-j), milatuzumab reduced the scores in a dose-dependent manner. Whereas untreated control hu-SCID mice experienced severe GVHD, mice treated with either milatuzumab (350 or 700 µg) or alemtuzumab (5 µg) had significantly lower GVHD scores (Figure 4B and C; $P < .001$) and less body weight loss (Figure 4D; $P < .001$). Similar results also were obtained from an earlier pilot animal study, showing that 2 of 2 milatuzumab-treated hu-SCID mice had much lower GVHD scores, less or no body weight loss, and no deaths throughout...
the study period, compared with severe GVHD clinical signs, body weight loss, and spontaneous death or mandatory euthanasia in both untreated mice (Supplemental Figure S4A and B). In another animal study, all (5 of 5) hu-PBL-SCID mice treated with milatuzumab 350 µg or 700 µg or alemtuzumab 5 µg survived to day 11 when the study was terminated, whereas only 2 of 5 untreated or control antibody hMN-14-treated (700 µg) hu-PBL-SCID mice survived at this time point.

**Milatuzumab Eliminates Infiltration of CD3⁺ and CD74⁺ Donor Cells in GVHD Target Organs**

At the end (day 28) of the study shown in Figure 3, all surviving mice were euthanized to collect GVHD target organs (ie, lungs, liver, and spleen) and peripheral blood for histopathological and cytokine analyses, respectively. Immunohistochemistry showed human CD3⁺ T cells infiltrating the lungs, liver, and spleen in untreated mice (Figure 5A), but not in milatuzumab-treated mice (Figure 5B) or alemtuzumab-treated mice (data not shown). These results provide histopathological evidence that acute GVHD was present in the untreated mice, and that milatuzumab and alemtuzumab effectively controlled acute GVHD in this xenogenic model.

Like human CD3⁺ cells, human CD74⁺ cells were detected in the lungs, liver, and spleen of untreated mice (Figure 5A), but not in milatuzumab-treated mice (Figure 5B) or alemtuzumab-treated mice (data not shown). Because CD74 is expressed in both APCs and a subset of activated T cells [7], the human CD74⁺ cells represent donor APCs, including mDCs, and a subset of activated T cells in this xenogenic model. The disappearance of these cells in GVHD target organs in milatuzumab-treated mice provides evidence that milatuzumab controls GVHD by a CD74-dependent mechanism. It remains to be determined whether milatuzumab, in an in vivo environment, can directly kill donor-derived APCs and activated T cells in the GVHD target organs of this model, or can simply inhibit the recruitment of these cells to these organs through the antagonistic blocking of MIF on CD74.

**Milatuzumab Suppresses Human Cytokine Storm in hu-PBL-SCID Mice**

In addition to clinical signs and pathological changes, acute GVHD is also characterized by a human “cytokine storm,” characterized by elevated serum levels of IFN-γ, TNF-α, IL-1, IL-5, IL-6, and IL-8 [27,37]. These inflammatory cytokines are responsible for or contribute to the tissue damage and clinical manifestations of acute GVHD. The anti-CD52 mAb alemtuzumab and anti-CD83 polyclonal antibody that targets mature DCs reportedly can suppress this cytokine storm in parallel to their efficacy against GVHD in hu-PBL-SCID mice [27]: thus, we examined whether milatuzumab affected serum levels of proinflammatory cytokines at the end of the animal study (ie, day 28 posttransplantation). The results demonstrate that milatuzumab at 350 or 700 µg substantially reduces the serum levels of IFN-γ (by 4–5-fold; P < .0001) and IL-5 (by 5–7-fold; P < .001), but has no significant effect on TNF-α, IL-2, IL-4, and IL-10 (Figure 5A and B). Similar results were obtained by treatment with alemtuzumab. Thus, milatuzumab is capable of suppressing the cytokine storm in acute GVHD.

**Milatuzumab Does Not Affect CMV-Specific T-Cell Immunity**

We showed that milatuzumab reduces mDC1s and mDC2s but spares CD74⁻ non-B lymphocytes (Figure 1B), which are composed mainly of T cells [31], and has no effect on activated or nonactivated T cells (Figure 1C). These results led us to postulate that milatuzumab may preserve preexisting antigen-specific memory T cells, which are essential for immunologic control of posttransplantation opportunistic infections. To verify this, we examined milatuzumab’s effects on the survival and function of CMV-specific T cells in 2-way allo-MLRs in which hPBMCs from CMV⁺, HLA-A*0201 donors (n = 5) were mixed with allogeneic PBMCs from other donors (n = 5) with uncharacterized HLA type and CMV status. The results, shown in Figure 7A and Supplemental Figure S5A and B, demonstrate that on stimulation with CMV pp65 peptide (NLVPMVATV), the frequencies of IFN-γ-producing cells in total MLR cells were no different in milatuzumab-treated (0.474 ± 0.327%), control hMN-14-treated (0.486 ± 0.336%), and rituximab-treated (0.452 ± 0.355%) MLRs. In contrast, alemtuzumab-treated MLRs had much lower frequencies of IFN-γ-producing cells than the control antibody hMN-14-treated MLRs (0.076 ± 0.032% versus 0.486 ± 0.336%; P = .044, n = 5).

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**Figure 5.** Milatuzumab eliminates human T-cell and APC infiltration in GVHD target organs. At the end of the experiment shown in Figure 3, all surviving mice were euthanized. GVHD target organs (lungs, livers, and spleens) were collected for pathological analysis. Human T cells and APCs within the GVHD target organs were identified by immunohistochemical labeling of human CD3 and CD74, respectively. Representative specimens are shown, with indications of human CD3⁺ T cells and CD74⁺ APCs in the specimens from untreated mice (A), but not in those from milatuzumab-treated mice (B). Perivascular lymphocytic infiltration is also evident in the hematoxylin and eosin—stained sections (arrows in A).
Similarly, there was no difference in IFN-γ-producing cells in gated CD3+CD8+ cells in milatuzumab-treated (1.83/6 ± 1.09%), hMN-14-treated (1.91/6 ± 1.12%), and rituximab-treated (1.54/6 ± 0.95%) MLRs (Figure 7B and Supplemental Figure S5C and D). Alemtuzumab-treated MLRs had lower frequencies of IFN-γ-producing cells in gated CD3+CD8+ cells compared with the control antibody hMN-14-treated MLRs (0.81/6 ± 0.83% versus 1.91/6 ± 1.12%; n = 5), but the difference did not reach statistical significance (P = .1169), because alemtuzumab depleted both IFN-γ-producing cells and the CD3+CD8+ cells, lessening its effect on the frequencies of IFN-γ-producing cells in CD3+CD8+ cells. These results suggest that milatuzumab maintains protective antiviral memory T-cell immunity, thus differing from the clinically used T-cell depleting mAb alemtuzumab, which causes high rates of CMV infection and reactivation [3-6].

This is the first report showing that GVHD can be controlled by an antagonist mAb against CD74. Importantly, this antibody does not affect the survival of T cells, including activated T cells, or antiviral T-cell immunity, as evidenced by the preserved CMV-specific CD8+ T cells in treated allo-MLRs, suggesting that milatuzumab may not compromise protective T-cell immunity against opportunistic infections. Because the graft-versus-leukemia (GVL) effect, the principal benefit and major therapeutic goal of allo-HSCT, is also mediated by donor T cells, the lack of effect of milatuzumab on the survival of T cells implies that milatuzumab also might not affect GVL. These promising results support further preclinical studies to examine this novel antibody for prophylactic and/or therapeutic control of GVHD.

Acute GVHD developed in hu-PBL-SCID mice is mediated by the engrafted human T cells and DCs [27]. In this animal model, the host (murine) APCs play a minor role in mediating GVHD [27]. In GVHD patients, donor APCs contribute to the development of GVHD through the pathway of "indirect presentation" by processing and presenting host antigens to donor T cells [21-23], among which mDCs are a critical subset for presenting alloantigens after experimental bone marrow transplantation [24]. Thus, the effective control of GVHD in hu-PBL-SCID mice by milatuzumab may be related, at least in part, to its ability to selectively reduce donor mDCs, thereby affecting the indirect presentation for GVHD development [17]. This is similar to RA83, a polyclonal anti-CD83 antibody that effectively controls acute GVHD in hu-PBL-SCID mice through a proposed mechanism in which RA83 selectively depletes activated donor human DCs that express CD83.

**DISCUSSION**

In this study, we have shown that milatuzumab, a humanized anti-CD74 antagonistic mAb, can reduce myeloid DCs and B cells, suppress the proliferation of allo-MLRs, and effectively prevent acute GVHD in a humanized SCID mouse model (hu-PBL-SCID). In this human/mouse xenogenic GVHD animal model, a single i.p. administration of milatuzumab before human PBMC transplantation lowered GVHD clinical scores, prevented weight loss, eliminated human lymphocyte infiltration in GVHD target organs, suppressed the cytokine storm, and significantly improved the survival of hu-PBL-SCID mice (90% versus 20% untreated controls).

This is the first report showing that GVHD can be controlled by an antagonist mAb against CD74. Importantly, this antibody does not affect the survival of T cells, including activated T cells, or antiviral T-cell immunity, as evidenced by the preserved CMV-specific CD8+ T cells in treated allo-MLRs, suggesting that milatuzumab may not compromise protective T-cell immunity against opportunistic infections. Because the graft-versus-leukemia (GVL) effect, the principal benefit and major therapeutic goal of allo-HSCT, is also mediated by donor T cells, the lack of effect of milatuzumab on the survival of T cells implies that milatuzumab also might not affect GVL. These promising results support further preclinical studies to examine this novel antibody for prophylactic and/or therapeutic control of GVHD.
CD83 is also expressed in activated T cells [38], however, and thus RA83 could deplete activated donor T cells as well, which might be an additional mechanism contributing to its therapeutic efficacy against GVHD.

In typical allo-HSCT, recipient hematopoietic and non-hematopoietic APCs play critical roles in the development of GVHD [15-21,23] via "direct presentation" [17,21]. Because of the lack of animal models, we have not evaluated the effect of milatuzumab on recipient hematopoietic and non-hematopoietic APCs. The hu-PBL-SCID mouse model only allows us to evaluate the effect of milatuzumab on donor APCs. However, because milatuzumab is cross-reactive with monkey CD74 [39], we would expect monkey GVHD models, if available, to be useful for testing the effect of milatuzumab on recipient APCs in vivo. Therapeutic evaluation of milatuzumab in monkey GVHD models may provide more relevant information for the future clinical use of this mAb in GVHD prevention.

Along with the reduction of mDCs, other possible mechanisms may be implicated in the therapeutic control of GVHD by milatuzumab. One of these mechanisms may be through MIF, the natural ligand of CD74 and a pleiotropic proinflammatory protein participating in many inflammatory diseases [40]. Patients with acute GVHD reportedly have higher serum MIF levels [41] and up-regulated local expression [42], and in a murine model of allogeneic SCT, knockout of the MIF gene in donor cells significantly prevents the onset of acute GVHD [28], suggesting that MIF/CD74 is actively involved in GVHD. MIF induces inflammation through the binding of CD74 [43]. It also binds CXCR2, CXCR4, and CD74, forming a complex to trigger chemotaxis of monocytes and T cells [44], which may promote the recruitment of leukocytes into GVHD target organs to cause tissue damage. Moreover, MIF increases leukocyte—endothelial interactions through up-regulation of adhesion molecules on epithelial cells [45], possibly via CD74. In addition, after engagement of CD74, MIF stimulates the production of IL-8 [46], a proinflammatory cytokine involved in various human inflammatory diseases [47] and a biomarker for GVHD [48]. MIF is a chemoattractant that attracts neutrophils to local sites of inflammation [47], possibly contributing to the infiltration of donor leukocytes to GVHD target organs. MIF also stimulates TNF-α production, which precedes and involves the pathogenesis of acute GVHD [49]. As a CD74 antagonist antibody, milatuzumab may target these pathways to inhibit local inflammation and prevent the recruitment of leukocytes, including T cells and APCs, into GVHD local sites.

Given MIF/CD74’s essential role in T-cell activation [36], it is also expected that milatuzumab, although having no effect on T-cell survival, may modulate the activation status of T cells via antagonistic blocking of MIF/CD74. Because
activated alloreactive T cells are the direct mediator of GVHD, modulation of the activation status of donor human T cells may be another mechanism contributing to the anti-GVHD efficacy of milatuzumab in hu-PBL-SCID mice. Furthermore, milatuzumab down-regulates the expression of adhesion molecules in human B cells, including L-selectin (CD62L) and β7-integrin [13]. It remains to be shown whether milatuzumab has a similar effect on these molecules in T cells, given that 4β7-integrin plays a central role in the homing of T cells to the gut, a major GVHD target organ, and that the absence of β7-integrin decreases the homing of activated T cells to the intestine, resulting in less GVHD [50]. In addition, it has been reported that L-selectin and integrin on donor CD4+ T cells, given that whether milatuzumab has a similar effect on these molecules modulates the activation status of donor human T cells. Activated alloreactive T cells are the direct mediator of GVHD, and the absence of CD62L decreases the homing of these cells to gut-associated organized lymphoid tissues, such as mesenteric lymph nodes and Peyer patches. Inactivation of the genes encoding these two molecules markedly reduces the accumulation of donor T cells in gut-associated organized lymphoid tissues and the severity of GVHD [51]. Thus, it would be of interest to investigate whether milatuzumab down-regulates these molecules on T cells as well.

In summary, this is the first report showing that GVHD can be controlled by an antagonist mAb targeting CD74 without impairing the protective T-cell immunity essential for antiviral and GVL effects after allo-HSCT. Our results support further investigation of CD74 as a novel therapeutic target for GVHD and of the prophylactic and/or therapeutic use of the anti-CD74 mAb milatuzumab for the control of this challenging disease.

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SUPPLEMENTARY DATA

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