α-Mannan induces Th17-mediated pulmonary graft-versus-host disease in mice

Short title: α-Mannan amplifies pulmonary GVHD

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Key Points

- α-Mannan stimulates macrophages via Dectin-2 to induce donor Th17 differentiation after allogeneic bone marrow transplantation.
- α-Mannan-induced Th17 cells accumulate in the lungs to cause pulmonary GVHD.
Abstract

Allogeneic hematopoietic stem cell transplantation (HSCT) is a curative therapy for various hematopoietic disorders. Graft-versus-host disease (GVHD) and infections are the major obstacles of HSCT, and their close relationship has been suggested. Although roles of bacterial and viral infections in the pathophysiology of GVHD are well described, impacts of fungal infection on GVHD remain to be elucidated. In mouse models of GVHD, injection of \( \alpha \)-Mannan, a major component of fungal cell wall, or heat-killed Candida albicans exacerbated GVHD, particularly in the lung. \( \alpha \)-Mannan induced donor T cell polarization towards Th17 and lung specific chemokine environment in GVHD led to accumulation of Th17 cells in the lung. The detrimental effects of \( \alpha \)-Mannan on GVHD depended on donor IL-17A production and host c-type lectin receptor Dectin-2. These results suggest a previously unrecognized link between pulmonary GVHD and fungal infection following allogeneic HSCT.
Introduction

Allogeneic hematopoietic stem cell transplantation (HSCT) is an effective therapy for hematologic malignant disorders, bone marrow failure, or inherited immunodeficiency syndromes, however, graft-versus-host disease (GVHD) and related microbial infections remain major obstacles to perform HSCT. It has been well demonstrated that both bacterial and viral infections play a significant role in amplifying GVHD by stimulating innate immune responses via pattern recognition receptors (PRRs) to produce inflammatory cytokines that upregulate expressions of MHC and costimulatory molecules and therefore enhance allogeneic T cell responses.\(^1\) Clinical data also suggest that bacterial and viral infections lead to the exacerbation of acute GVHD.\(^2,3\)

Mycosis is also a serious and frequent complication after allogeneic HSCT, however, its effects on GVHD remain to be elucidated. A previous study demonstrated that prophylactic administration of fluconazole was associated with a reduced incidence of severe acute GVHD,\(^4\) but its mechanism is yet to be defined.

C-type lectin receptors recognize polysaccharide chain on fungal cell wall, which is mainly composed of multiple layers of carbohydrates, including \(\alpha\)-Mannan (Mn) (polymers of mannose), \(\beta\)-glucan (polymers of D-glucose linked by \(\beta\)-glycosidic bonds) and chitins (polymers of N-acetyl-D-glucosamine).\(^5,6\) Mn and \(\beta\)-D-glucan are recognized by Dectin-2 and Dectin-1 receptors, respectively, on host cells to activate macrophages and dendritic cells as antigen-presenting cells, forces them to produce inflammatory cytokines and drives naïve T-cell
differentiation toward Th17 to eliminate fungi.\textsuperscript{7-10} Host defense against various fungi is dependent on the corresponding receptors to specific fungal species; recognition of Mn by Dectin-2 and subsequent Th17 responses are essential for candida elimination in mice.\textsuperscript{11}

In this study, we have evaluated whether fungal infection could affect severity of acute GVHD using Mn and heat-killed \textit{Candida albicans} (\textit{C. albicans}) in mouse models of bone marrow transplantation (BMT), and found a previously unrecognized association between antifungal immunity and pulmonary GVHD.
Materials and Methods

Mice. Female C57BL/6 (B6, H-2^b, CD45.2^+), B6-Ptprc^a (H-2^b, CD45.1^+), B6D2F1 (H-2^bd, CD45.2^+), BALB/c (H-2^d) and C3H.Sw (H-2^b) mice were purchased from Charles River Japan, KBT Oriental, or Japan SLC. IL-17A deficient (IL-17A^-/-), Dectin-2 deficient (Dectin-2^-/-) mice, and IL-17A-YFP reporter mice (Il17a^{Cre}R26R^{eYFP}) with the B6 background were generated as previously reported. All animal experiments were performed under the auspices of the Institutional Animal Care and Research Advisory Committee.

BMT. Mice were transplanted as previously described. In brief, B6D2F1 and B6 recipients were given X-ray total body irradiation (TBI) with doses of 12.5 Gy and 10.0 Gy, respectively, TBI was split into 2 doses with 4 h interval to reduce gastrointestinal toxicities, and followed by i.v. injection with 4 x 10^6 T-cell-depleted BM (TCD-BM) cells plus 4 x 10^6 splenic T cells on day 0. Isolation of T cells and T-cell depletion were performed using the Pan-T cell isolation kit and anti-CD90-MicroBeads, respectively, and the AutoMACS (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer’s instructions. Mice were i.p. injected with Mn (Sigma-Aldrich, St. Louis, MO) diluted in phosphate-buffered saline (PBS) or PBS control on day 1. Mice were maintained in specific pathogen-free condition and received normal chow and autoclaved hyperchlorinated water for the first 3 wk post-BMT and filtered water thereafter. Survival after BMT was monitored daily and the degree of clinical GVHD was assessed weekly by a scoring system, as previously described. To determine oxygen saturation levels of peripheral artery, a pulse oxygen sensor, 2000SL, was attached to the left rear paw and
monitored by 9847V monitor (Nonin Medical Inc., Plymouth, MI).  

**C. albicans culture and injection.** *C. albicans* (NBRC1385) was obtained from NITE Biological Resource Center (Chiba, Japan) and grown at 30 ºC on potato dextrose agar plates (Eiken Kizai, Tokyo, Japan). Yeast form of *C. albicans* was harvested and killed with heat (65 ºC, 2 h) as previously shown. Groups of recipients were i.p. injected with heat-killed *C. albicans* at doses from 10⁵ to 10⁷ per mouse on the day of BMT.

**Histological analysis.** Paraffin sections of the lung, liver, and small intestine were stained with H&E. Acute GVHD was assessed by detailed histopathological analysis, using a semiquantitative scoring system in a blinded fashion. Pictures from tissue sections were taken at room temperature using a digital camera (DP72; Olympus, Tokyo, Japan) mounted on a microscope (BX51; Olympus).

**Isolation of leukocytes from tissues.** Tissue-localized leukocytes were isolated after perfusion with 10ml of cold PBS from the left ventricle of the heart, as previously described. The lungs were digested with 100 U/ml collagenase D (Roche Diagnostics, Indianapolis, IN) and 1 μg/ml DNase (Sigma-Aldrich) in complete media that is consist of RPMI 1640 with 10% FCS (Gibco, Tokyo, Japan), 100 U penicillin/streptomycin (Gibco), 50 μM 2-mercaptoethanol (Sigma-Aldrich), 20 mM L-glutamine (Gibco) for 1 h at 37°C. The homogenized lungs were suspended in 4 ml of 40% Percoll (GE Healthcare, Tokyo Japan) and layered onto 4 ml of 70% Percoll, and centrifuged (2,400 rpm, 4°C, 30 min). The cells were collected from the Percoll interface.
**Cell cultures.** 5 × 10^4 T cells were cultured with 1 × 10^5 peritoneal macrophages in complete media and stimulated with 0.5 μg/ml of anti-CD3 (2C11), 10 ng/ml TGF-β (PeproTech, Rocky Hill, NJ) in the presence or absence of 100 μg/ml Mn. Ninety-six hours later, cytokine levels in culture supernatants were determined with a BD™Cytometric Bead Array (BD Pharmingen, San Diego, CA), following a manufacture’s instruction.

**Protein extraction from lung tissues.** Lung tissues were homogenized in 5 µL/mg wet tissue weight of lysis buffer, consisting of 10mM Tris-HCl (pH 8.0), 1% NP-40 (CALBIOCHEM, La Jolla, CA), 5mM EDTA, and Protease inhibitor cocktail (Sigma-Aldrich), using TissueRuptor (QIAGEN). After 1hr incubation at 4 °C, the lysates were centrifuged and the supernatants were collected. Cytokine levels in lung extracts were determined by using a BD Cytometric Bead Array according to the manufacture's instruction.

**Intracellular cytokine staining and cytokine analysis.** Lymphocytes were stimulated in vitro with 50 ng/ml of phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 100 ng/ml of ionomycin (Sigma-Aldrich) at 37°C for 3 h. Cells were then incubated with GolgiStop (BD Pharmingen) for an additional 2 h. Then, the cultured cells were stained with surface markers and intracellular cytokines. mAbs conjugated with FITC, phycoerythrin, peridinin-chlorophyll protein complexes, allophycocyanin, or allophycocyanin-Cy7 were purchased from BD Pharmingen or eBioscience (San Diego, CA). 7-Aminoactinomycin D (7AAD; BD Pharmingen) was used for the exclusion of dead cells. Cells were analyzed on a FACSCalibur and FACSCanto II flow cytometer (BD Biosciences, San Jose, CA) and FlowJo software (TreeStar, San Francisco, CA).
Ashland, OR). Cell sorting was performed using a FACS­Aria III sorter (BD Biosciences).

**Quantitative real-time PCR analysis.** Total RNA from pulmonary lymphocytes or frozen tissues was extracted using ISOGEN II (Nippon Gene, Tokyo, Japan). Reverse transcription was done with a Quanti­Tect reverse transcription kit (QIAGEN, Venlo, Netherlands) and specific primer and probe sets (Applied Biosystems, Foster, CA and Sigma-Aldrich). Quantitative real-time PCR was performed on ABI 7300 (Applied Biosystems) with TaqMan Universal PCR Master Mix (Applied Biosystems). 18S ribosomal RNA (rRNA) was separately amplified in the same plate as an internal control for variation in the amount of cDNA in PCR. The collected data were analyzed using the Sequence Detector software (Applied Biosystems). Relative amounts of individual genes’ mRNA were calculated by the comparative ΔC (t) method.

**Statistical analysis.** Mann-Whitney U tests were used to compare data, the Kaplan-Meier product limit method was used to obtain survival probability, and the log-rank test was applied to compare survival curves. Analyses were performed using JMP Pro Version 10.0.2 (SAS, Cary, NC). \( P < .05 \) was considered statistically significant.
Results

Administration of Mn increases mortality and morbidity of GVHD

To evaluate the effects of Mn administration on acute GVHD, B6D2F1 mice were lethally irradiated with 12.5 Gy TBI and i.v. injected with $4 \times 10^6$ purified T cells plus $4 \times 10^6$ TCD-BM from MHC-haploidentical C57BL/6 (B6) or syngeneic donors on day 0. Mice were i.p. injected with $1\text{mg/g BW}$ of Mn or diluent on day 1. All syngeneic mice survived, while 60% of allogeneic control mice died by 14 wk after BMT (Figure 1A). Injection of Mn neither induced mortality nor increased clinical scores in syngeneic animals. In contrast, injection of Mn significantly increased mortality and morbidity of GVHD, resulting in increased clinical GVHD scores and 100% mortality in 6 wk (Figure 1A-B). The effect of Mn was dose-dependent (Supplementary Figure 1). We also confirmed the similar deteriorating effects of Mn on GVHD in a MHC compatible but mH–mismatched C3H.Sw → B6 BMT model (Figure 1C-D) and in a MHC fully mismatched BALB/c → B6 model (Figure 1E). The effects of Mn administration were abrogated when recipient mice lacked a corresponding receptor, Dectin-2 (Figure 1E). In contrast, the recipients transplanted with bone marrow and T cells from Dectin-2$^{-/-}$ donor showed the comparable aggravation of GVHD to the recipients of WT donors after Mn-injection (Figure 1F). These results indicated the critical role of Dectin-2 signaling in host cells for Mn-induced GVHD exacerbation.

Mn and C. albicans exacerbate pulmonary GVHD pathology
Next, we performed histologic assessment of the lung, liver, and gut on days 7, 14, and 21 after BMT. In allogeneic control mice, pathology scores of all evaluated organs were significantly higher than those of syngeneic animals on days 14 and 21 after BMT. Mn administration dramatically exacerbated GVHD pathology in the lung after allogeneic BMT, whereas the pathology scores of the liver and small intestine were only modestly increased (Figure 2A-D). The pulmonary lesions were characterized by perivascular cuffing, vasculitis, peribronchiolar cuffing, and alveolar hemorrhage, which were consistent with acute pulmonary GVHD (Figure 2A).14,19,20 The peripheral oxygen levels determined by using a pulse oxygen sensor were not changed on days 7 and 14 after syngeneic or allogeneic BMT (Figure 2E). Mn administration significantly decreased them 14 d after allogeneic BMT. These results indicated that Mn administration accelerates GVHD with most prominent changes in the lung, leading to the respiratory failure. Importantly, Mn-administration did not show any detrimental effects after syngeneic transplantation. To test the clinical relevance of these findings, the recipient mice were treated with $1 \times 10^5$, $1 \times 10^6$, or $1 \times 10^7$ of heat-killed *C. albicans* after BMT. *C. albicans* significantly exaggerated lung GVHD in a dose dependent manner, suggesting a causative role of fungal infection in lung GVHD (Figure 2F-G).

**Mn and *C. albicans* promote accumulation of Th17 cells in the lung**

Next, we characterized donor cells infiltrated in the lung on day 14 after BMT. Absolute numbers of donor CD4$^+$ T cells, CD8$^+$ T cells, and neutrophils were significantly increased in
the lungs of allogeneic animals than those in syngeneic animals (Figure 3A). Mn administration further increased infiltration of these cells only in allogeneic animals (Figure 3A). CD4+ T cells were isolated from the lungs and expression levels of Ifng, Il4, Foxp3, and Il17a, the representative cytokines and transcription factor associated with Th1, Th2, Treg, and Th17, respectively, were evaluated. Expression levels of Ifng and Il4 were significantly greater, whereas Foxp3 expression was dramatically lower in allogeneic animals compared to those in syngeneic animals (Figure 3B). While expression of Il17a was not detected in allogeneic animals, administration of Mn significantly enhanced Il17a expression, but not Ifng, Il4, or Foxp3 (Figure 3B). Expression levels of Rorc and Tgfb3 were also significantly enhanced in Mn treated allogeneic animals, further confirming Th17 differentiation (Figure 3B). These Th17-related changes were not observed in Mn-treated syngeneic animals. Intracellular cytokine staining demonstrated that Mn injection increased frequencies and absolute numbers of IL-17A+ CD4+ T cells (Figure 3C-E), but not IFN-γ+ CD4 T cells in the lung (Figure 3F-G). Furthermore, similar results were obtained when C. albicans was administered after allogeneic BMT (Figure 3H-I). Altogether, fungal components of C. albicans and Mn selectively promoted accumulation of Th17 cells in the lungs of allogeneic animals.

Mn polarizes donor T cells toward Th17 via Dectin-2 signaling in macrophages

To further investigate the underlying mechanisms of the deteriorating effects of Mn on lung GVHD, we evaluated effects of Mn on T-cell responses in vitro. Given the abundant
and specific expression of Dectin-2 (Clec4n) in macrophages (Supplementary Figure 2), CD4+ CD25- T cells were cultured with peritoneal macrophages in the presence of anti-CD3 mAb and TGF-β for 5 d. Addition of Mn into the culture significantly increased the levels of IL-6, IL-17 and TNF-α in the supernatants without changing IFN-γ and IL-4 levels (Figure 4A). Intracellular cytokine staining showed that Mn dramatically increased frequency and number of IL-17 producing CD4+ T cells, confirming Mn-induced Th17 differentiation (Figure 4B-D). This Th17 differentiation was abrogated when macrophages lacked Dectin-2, indicating the critical role of Dectin-2 on macrophages for Mn-induced donor Th17-differentiation (Figure 4E-G). To further examine the role of macrophages in Th17 differentiation in vivo, the cytokine expressions of macrophages isolated from the recipients on days 7 and 14 post-transplant were analyzed. Lung macrophages were sorted according to their expression of CD11b, CD11c, CD45 and F4/80 (Figure 5A), and their expression levels of Th17-driving cytokines, such as Il6, Il1b, and Il23 were examined by a quantitative PCR method. Expression of Il6 and Il1b was significantly upregulated in Mn-treated allogeneic animals compared to allogeneic controls by day 7 after BMT, while expression of Il23 was not detected on day 7 but upregulated on day 14 (Figure 5B-D). This sequential upregulation of cytokine expression reflects roles of IL-6 and IL-1β in early phase of Th17 differentiation and role of IL-23 in later phase of Th17 induced pathology. The increased productions of Th17 related cytokines were further confirmed in the lung protein extracts; IL-6 and TNF-α were significantly increased in the lungs of allogeneic controls compared to those of syngeneic controls and Mn treatment further increased these
cytokine production in allogeneic recipients, but not in syngeneic recipients (Figure 5 E-F). Given the critical role of host Dectin-2 in Mn-induced exacerbation of GVHD (Figure 1E), host macrophages should persist after allogeneic BMT to prime donor Th17 cells. We studied kinetics of macrophage turnover by transplanting CD45.1+ B6 cells into CD45.2+ B6D2F1 recipients. As expected, most of lung macrophages are host-derived (CD45.1-CD45.2+) on day 7 after BMT irrespective of Mn-treatment, while neutrophils and monocytes were completely converted to donor type (CD45.1’CD45.2’) at this time point (Figure 5G-H and data not shown). Host macrophages still persisted 14 days after allogeneic BMT but were significantly less in Mn-treated animals compared to controls, likely reflecting exacerbated GVHD in Mn-treated recipients (24.3 ± 4.4% vs. 6.7 ± 1.1%) (Figure 5H). These data, together with the critical role of Dectin-2 on macrophages (Figure 4E-G), show that Mn administration to allogeneic recipients promotes donor Th17 differentiation by inducing the productions of Th17-related cytokines, such as IL-6, IL-1β TNF-α and IL-23, from macrophages via Dectin-2 dependent manner.

Mn administration promotes differentiation of the unique IL-17 producing CCR6+CCR4+ donor Th17 cells after allogeneic BMT

To further study the mechanisms by which Mn promoted the accumulations of donor Th17 cells in the lung, IL-17A-YFP-fate mapping mice, in which IL-17A producing cells and their progenies were labeled with YFP, were used as BMT donors.12 Mn treatment significantly
expanded YFP⁺ CD4⁺ cells in the spleens, livers, lungs, and mesenteric lymph nodes 7 d after
BMT, indicating that Mn promoted systemic Th17 differentiation early after allogeneic BMT
(Figure 6A-B). Next, donor CD4⁺ T cells isolated from the lung on day 14 were divided into 3
populations according their expression of YFP and CCR6: YFP⁺ CCR6⁺ cells, YFP⁺ CCR6⁻
cells, and YFP cells, as previously described (Figure 6C). These subpopulations were sorted
and mRNA was extracted from each population. YFP⁺ CCR6⁺ cells displayed significantly
higher expression of Il17a, Ccr4 and Ccr6 compared to other populations (Figure 6D). This
population is a mouse counterpart of human Th17 memory cells that produce large amount of
IL-17. Frequencies and absolute numbers of the YFP⁺CCR6⁺ subset in the lungs of
Mn-treated allogeneic recipients were significantly increased, whereas this population was
hardly detected in syngeneic or allogeneic controls 14 d after BMT (Figure 6E-F). Interestingly,
this subset uniformly distributed in the spleen, liver and lung of Mn-treated allogeneic recipients
7 d after BMT, but preferentially accumulated in the lung 14 d after BMT (Figure 6G-H).
Indeed, Mn treatment dramatically increased the absolute numbers of CCR6⁺YFP⁺ cells in the
lungs from allogeneic animals on day 14 compared to those on day 7 (Figure 6H). In contrast,
the numbers of these cells in the spleen and liver were unchanged between 7 d and 14 d after
BMT. The temporal change of the distribution of CCR6⁺YFP⁺ donor T cells that also express
high amount of CCR4, prompted us to examine the expression of corresponding chemokines
CCL20, CCL17, and CCL22 in GVHD target organs. In naïve mice, CCL20, the ligand for
CCR6, are highly expressed in the small intestine, while its expression was downregulated 14 d
after both syngeneic and allogeneic BMT (Figure 6I). In sharp contrast, CCL20 expression was dramatically increased in the lungs after allogeneic BMT, irrespective of Mn treatment. Similarly, expression levels of CCL17 and CCL22, both are the ligands for CCR4, were high in the lung of allogeneic recipients, irrespective of the Mn treatment (Supplementary Figure 3A-B). These results suggest that the lungs are vulnerable to Th17 infiltration in GVHD after allogeneic BMT.

**IL-17 production by donor T cells is critical for the development of lung injury in Mn-treated allogeneic recipients**

Because Mn treatment specifically induced the differentiation and the pulmonary accumulation of IL-17 producing CCR6+ Th17 cells, we hypothesize that IL-17 itself plays a critical role in Mn-induced exacerbation of lung GVHD. To investigate the role of IL-17 produced by donor T cells, lethally irradiated recipient mice were transplanted with WT TCD-BM cells plus purified WT or IL-17A-/- T cells. IL-17A-/- T cells and WT T cells caused equivalently severe GVHD without Mn treatment (Figure 7A-B). However, the Mn-induced exacerbation of GVHD was completely abrogated in the recipients of IL-17A-/- T cells. (Figure 7A-B). In contrast, the recipients of WT T cells plus either WT or IL-17A-/- TCD-BM, treated with Mn, presented comparable exacerbation of GVHD, further confirming that IL-17A production from donor T cells but not from other transplanted cells is critical for Mn-induced exacerbation of GVHD (data not shown). Histopathologic examinations of the lungs on day 21
showed Mn-treatment in allogeneic recipients of IL-17A deficient T cells failed to show any enhancement of lung GVHD pathology compared to allogeneic controls, while Mn again showed drastic exacerbation of lung GVHD in the recipients of WT T cells, confirming the critical role of IL-17A production from donor T cells for the deteriorating effects of Mn on lung GVHD pathology (Figure 7C-D).
Discussion

It has been well demonstrated that bacterial and viral infections are risks for GVHD by activating innate immunity. Experimental and clinical studies suggested that GVHD is reduced in germfree mice or by the administration of oral antibiotics,\textsuperscript{1,2,13,26} and PRR polymorphisms are also risk factors for GVHD.\textsuperscript{27} Fungi, such as Candida, Saccharomyces, Aspergillus, and Penicillium, consist of normal flora in the oropharyngeal cavity, gastrointestinal tract, and vagina of humans.\textsuperscript{28} They are capable of causing opportunistic infection following disruption of the mucocutaneous barrier and a defect in host cellular immunity. Several clinical studies suggested a link between fungal infection and GVHD. Prolonged antifungal prophylaxis resulted in a reduction of severe acute GVHD and colonization with \textit{Candida species} was associated to increased risk of acute GVHD.\textsuperscript{4,29} Interestingly, the polymorphism of \textit{dectin-1}, an innate receptor for fungi, was associated to colonization of \textit{Candida species} in the human recipients and may increase the risk of acute GVHD.\textsuperscript{29-31} While these data indicate a critical role of the fungal infection in the pathogenesis of GVHD, the mechanistic association between fungal infection and GVHD has yet to be clarified.

In vitro analysis showed that Mn polarized T cells toward Th17 in a Dectin-2 dependent manner, as has been shown.\textsuperscript{10,11} Dectin-2 is expressed most abundantly in macrophages and at lesser levels in some dendritic cell subsets. In accordance with this high
expression of Dectin-2, Mn acts on macrophages to enhance expression of Th17 related cytokines and mediates Th17 differentiation in vitro. Mn treatment induces neither expressions of IL-6 nor IL-23, and much lesser level of IL-1β expression in syngeneic recipients compared to those in allogeneic recipients, suggesting that macrophages need to be primed by allogeneic T cell responses, but not by irradiation, to secrete Th17 related cytokines in response to Mn, as allogeneic T-cell derived IFN-γ primes macrophages to enhance the responses to TLR ligands. However, it remains to be investigated if fungal infection could induce lung GVHD in BMT models following chemotherapy conditioning.

Macrophages activated by both donor T cells and Mn administration expressed high levels of IL-1β and IL-6, as early as day 7, while IL-23 production was delayed by day 14. This kinetics of cytokine expression fit the cytokine requirement for the differentiation and maintenance of pathogenic Th17 cells; IL-1β and IL-6 are critical for initial Th17 differentiation, and IL-23 is required for maintenance and induction of more pathogenic Th17 cells that is marked by TGF-β3 production. Previous reports show early stage of Th17 cells in turn promotes IL-23 secretion of macrophages.

Our results showed that host Dectin-2 is critical for Mn-mediated donor Th17 differentiation and GVHD exaggeration. Dectin-1 also induces Th17 responses. While the defense against C. albicans is dependent on Dectin-2 signaling, Dectin-1 seems to be more responsible for the defense against aspergillus and certain strains of C. albicans. It is thus possible that not only candida but also aspergillus may exaggerate GVHD. Furthermore both
Dectin-1 and Dectin-2 are known to activate Nlrp3 inflammasome. Recently, host NLRP3 is shown to be critical for GVHD maximization through caspase-1 cleavage and productions of inflammatory cytokines. It is intriguing to study the role of host inflammasome in fungus-related lung GVHD by analyzing caspase-1 cleavage in the host macrophage in future studies. These findings altogether suggest more broad interaction between GVHD and various fungal infections may exist.

It should be noted that Mn-induced GVHD exacerbation was most prominent in the lungs, while we found that Mn administration exaggerated systemic acute GVHD much lesser extent. Mn-induced lung pathology was characterized by infiltrations of donor T cells surrounding vessels and bronchioles together with parenchymal injury and consistent with acute pulmonary GVHD. Lung acute GVHD typically developed later than gut GVHD in mice models. Mn-treatment systemically induced the differentiation of CCR6+ CCR4+ IL-17A producing Th17 cells in allogeneic mice, while this population was barely detected in syngeneic recipients regardless of Mn-treatment or in allogeneic control mice. The expressions of CCL17, CCL20, and CCL22, corresponding ligands to CCR6 and CCR4, were dramatically and specifically increased in the lungs of allogeneic recipients, irrespective of Mn-treatment by day 14 after BMT. The lung-specific upregulations of these chemokines resulted in the accumulation of CCR6+ CCR4+ IL17-producing donor T cells, the population induced only in Mn-treated allogeneic animals, in the lung. These results indicate GVHD mediated
inflammatory environment in the lung may be responsible for accumulation of Th17 cells, predominantly in the lung during GVHD after allogeneic BMT. This has also clinically important implication; fungal infection in the tissues outside of the lung also induce lung specific GVHD exaggeration via Mn-dependent systemic expansion of Th17 cells and induction of Th17-attracting chemokines in the lungs during GVHD.

Emerging evidences indicate the important roles of donor Th17 cells in GVHD, particularly in the skin and lung GVHD, although there are some controversies. These experiments produced conflicting results, possible due to the difference in model systems. We didn’t find any difference of morbidity and mortality after transplantation with IL-17A deficient donor T cells and WT donor T cells. However, we found that Mn dramatically potentiate role of Th17 cells particularly in the lung. The role of Th17 cells in the lung GVHD was also demonstrated by transplanting with ex vivo generated Th17 cells. Given the fact that Th17 cells have several functions other than IL-17A secretion, such as secretion of IL-21, IL-22, TNF-α, and GM-CSF, it is remarkable that IL-17A production from donor T cells alone was solely critical for Mn-induced GVHD exacerbation. We did not inquire the mechanism of tissue damages caused by IL-17A, enhancement of antigen-specific cytotoxic T cell development and myeloid inflammatory cell recruitment may contribute to the enhancement of GVHD. Indeed, we found that significantly increased numbers of donor neutrophils infiltrated into the lungs of Mn-treated allogeneic animals compared to controls. The critical role of neutrophils in
the lung injury by producing proteases and reactive oxygen species (ROS) is well described. More importantly, recipients’ neutrophils infiltrating in the gut early after transplantation contribute to GVHD and depletion of host neutrophils early after BMT could ameliorate GVHD. Thus it is possible that neutrophils also play a role in GVHD acceleration. Based on our results indicating that IL-17A deficiency abrogates Mn induced exacerbation of GVHD, IL-17 inhibitors can be beneficial to prevent lung GVHD after fungal infection in clinical allogeneic HSCT.

Our findings uncover previously unrecognized mechanistic links between fungal infection and GVHD and may afford new insights to establish novel therapeutic strategies that can be used to prevent and treat Th17-driven GVHD. To best our knowledge, this is the first study to demonstrate distinct mechanistic association between in-vivo-induced Th17 by fungal-derivative and GVHD. This may lead to improve the clinical outcome of allogeneic BMT.
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Authorship contributions
H.U., D.H., and T.T. developed the conceptual framework of the study, designed the experiments, conducted studies, analyzed data and wrote the paper. K.K., E.H., S.M., S.T., R.O., H.I., T.M, S.S conducted experiments. Y.M., Y.I., G.H., and K.A. supervised experiments.

Disclosure of Conflicts of Interest

The authors have no conflict of interest to declare.
References


21. Heng TS, Painter MW, Immunological Genome Project C. The Immunological...


28. Cohen R, Roth FJ, Delgado E, Ahearn DG, Kalser MH. Fungal flora of the normal


36. Ghoreschi K, Laurence A, Yang XP, et al. Generation of pathogenic T(H)17 cells in...


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**Figure Legends**

**Figure 1. Administration of Mn exacerbates acute GVHD after allogeneic BMT.**

(A, B) Lethally irradiated B6D2F1 mice were transplanted with $4 \times 10^6$ TCD-BM cells plus $4 \times 10^6$ T cells from allogeneic (Allo) B6 donors or syngeneic (Syn) donors on day 0, followed by single i.p. injection of Mn (1mg/g BW) or PBS. Survival (A) and clinical GVHD scores (B, means ± SE) of Syn controls (open circles), Mn-treated Syn (open diamonds), Allo controls (closed circles), and Mn-treated Allo (closed diamonds) from three independent experiments were combined. (n = 12-15 / group). (C, D) Lethally irradiated B6 mice were transplanted from C3H.Sw donors. Survival (C) and clinical GVHD scores (D, means ± SE) of Allo controls (open circles) and Mn-treated Allo (closed circles) from three independent experiments were combined (n = 9 / group). (E) Lethally irradiated WT or Dectin-2−/− B6 mice were transplanted with cells from BALB/c donors. Survival of WT controls (open-circles), Dectin-2−/− controls (open diamonds), Mn-treated WT mice (closed circles), and Mn-treated Dectin-2−/− mice (closed diamonds) from three independent experiments were combined (n = 6-10 / group). (F) Lethally irradiated B6D2F1 mice were transplanted with BM and T cells from WT or Dectin-2−/− B6 donors. Survival of Allo controls with WT donors (open circles), Allo controls with Dectin-2−/− donors (open diamonds), Mn-treated mice with WT donors (closed circles), and Mn-treated mice with Dectin-2−/− donors (closed diamonds) from two independent experiments were combined. (n = 5-10 / group). *P < .05, **P < .01.
**Figure 2. Mn and C. albicans exaggerated GVHD pathology.**

BMT were performed as shown in Figure 1A. (A) The representative images of the H&E-stained lungs on day 21 after syngeneic (Syn) and allogeneic (Allo) BMT with or without Mn-treatment are shown (scale bars: 100 μm). Pathology scores of the lung (B), liver (C), and small intestine (D) from Syn controls (white bars), Mn-treated Syn mice (light grays), Allo controls (deep grays) and Mn-treated Allo mice (blacks) of three independent experiments were combined (n = 5 / group, means ± SE). (E) Peripheral arterial oxygen saturation levels from two independent experiments are combined and means ± SE are shown. (F, G) Allogeneic recipient mice were i.p. injected with 1 × 10^5, 1 × 10^6, or 1 × 10^7 of heat-killed C. albicans on the day of BMT. The representative images (F) of the H&E-stained lungs on day 14 after allogeneic (Allo) BMT with or without the injection of 1 × 10^7 C. albicans are shown (scale bars: 100 μm). The bar graph (G) shows pathology scores of the lung from Allo controls (deep grays) and Allo mice treated with indicated doses of C. albicans (blacks) are shown. Data from one of two independent experiments were shown. (n = 3-5 / group, means ± SE). *P < .05, **P < .01.

**Figure 3. Mn administration increases infiltration of Th17 cells in the lung.**

BMT were performed as shown in Figure 1A. (A) Numbers of donor CD4+ T cells, CD8+ T cells, and CD11b+Ly6G+ neutrophils per lung were enumerated on day 14 after after BMT and shown as means ± SE. (n = 4-5 / group). (B) CD3+ CD4+ cells were sorted and their
expression of Ifng, Il4, Foxp3, Il17a, Rorc, and Tgfb3 mRNA were determined by quantitative PCR method. (C-G) MNCs harvested on day 14 after BMT were stained for CD3, CD4, CD8 and intracellular IFN-γ and IL-17A. The gating strategies of staining (C), frequencies (D), and absolute numbers (E) of CD4⁺ IL-17A⁺ T cells, and frequencies (F) and absolute numbers (G) of IFN-γ⁺ CD4⁺ T cells are shown. Data of Syn controls (white bars), Mn-treated Syn (light grays), Allo controls (deep grays), and Mn-treated Allo (blacks) from one of 5-6 independent experiments are shown as means ± SE. (H, I) Allogeneic recipient mice were i.p. injected with 1 × 10⁷ of heat-killed C.albicans on the day of BMT. The frequencies (H) and absolute numbers (I) of IL-17A⁺ CD4⁺ T cells in Allo controls (deep grays) and C. albicans-treated Allo (blacks) mice are shown. Data from one of two independent experiments are shown as means ± SE. *P < .05, **P < .01, n.d.: not detected.

**Figure 4. Mn induces Th17 differentiation of donor T cells in a Dectin-2 dependent manner.**

(A-G) 5 × 10⁴ T cells were cultured with 1 × 10⁵ 12Gy-irradiated macrophages in the presence of 0.5 µg/ml of CD3 mAbs and 10 ng/ml of TGF-β with or without 100 µg/ml of Mn for 5 d. (A) Levels of cytokines in supernatant from one of three independent experiments are shown as means ± SE. (B) Representative dot plots of flow cytometric analysis of intracellular IL-17A staining of CD4⁺ T cells after 5-day culture are shown.
Frequencies (C) and numbers (D) of IL-17A+CD4+ Th17 cells from one of 3 independent experiments are shown as means ± SE. (E-G) Intracellular IL-17A staining of T cells cultured with WT or Dectin-2−/− macrophages were performed. Representative dot plots (E) show IL-17A production in CD4+ T cells after co-culture with WT (top panels) or Dectin2−/− (bottom panels) macrophages in the presence or absence of Mn. Frequencies (F) and numbers (G) of IL-17A+CD4+ Th17 cells from one of three independent experiments are shown as means ± SE. *P < .05.

Figure 5. Mn stimulates macrophage expression of cytokines related to Th17 differentiation.

Lung macrophages were sorted as DAPI F4/80+ CD45high CD11blow CD11chigh cells on days 7 and 14 after BMT. Sorting strategies (A) for lung macrophages are shown. Expressions of Il6 (B), Il1b (C), and Il23 (D) mRNA in macrophages sorted from Syn controls (white bars), Mn-treated Syn mice (light grays), Allo controls (deep grays) and Mn-treated Allo mice (blacks) were quantitated by real-time PCR method. Representative data from three independent experiments are shown as means ± SE. (E, F) Levels of IL-6 and TNF-α in the protein extracts from recipients’ lungs on days 7 and 14 are shown as means ± SE. (G, H) B6D2F1 mice were transplanted from B6-CD45.1+ mice. The donor (CD45.1+CD45.2+) and host (CD45.1+CD45.2+) neutrophils and macrophages from recipients’ lungs were analyzed.
on days 7 (G, H) and 14 (H) and representative dot plots (G) and host chimerism (H) are shown. (n = 3-5 / group). *: \( P < 0.05 \), n.d.: not detected.

**Figure 6. Mn administration promotes differentiation of the unique CCR6\(^+\)CCR4\(^+\) donor Th17 cells after allogeneic BMT.**

Lethally irradiated B6 or B6D2F1 mice were transplanted with \( 4 \times 10^6 \) TCD-BM plus \( 4 \times 10^6 \) T cells from B6-background IL-17A-YFP fate mapping mice. Representative figure (A) of flow cytometric analysis 7 d after BMT is shown. The frequency (B) of YFP\(^+\) cells in CD4\(^+\) T cell fraction in the spleen, lung, liver, and mesenteric lymph nodes (MLNs) from Syn controls (white bars), Mn-treated Syn mice (light grays), Allo controls (deep grays), and Mn-treated Allo mice (blacks) are shown as means ± SE. (n = 3 / group). (C) Representative dot plot shows three distinctive cell populations defined as YFP\(^+\) CCR6\(^+\) (a), YFP\(^+\)CCR6\(^-\) (b), and YFP\(^-\)CCR6\(^-\) (c), respectively. (D) mRNA expression in each cell fraction from Mn-treated allogeneic mice are shown as means ± SE. Frequencies (E) and numbers (F) of YFP\(^+\) CCR6\(^+\) in the lung tissue 14 d after BMT are shown as means ± SE. Frequencies (G) and absolute numbers (H) of CCR6\(^+\)CD4\(^+\) YFP\(^+\) cells from the spleen, liver and lung from Mn-treated allogeneic recipients on day 7 and day 14 after BMT are shown as means ± SE (n=3). (I) Expression levels of \( Ccl20 \) mRNA in different tissues 14 d after BMT are shown. Representative data from three independent experiments (means ± SE) are shown. *\( P < 0.05 \), **\( P < 0.01 \).
Figure 7. IL-17A production from donor T cells is required for the Mn-induced lung GVHD exacerbation.

Lethally irradiated B6D2F1 mice were transplanted with $4 \times 10^6$ TCD-BM plus $4 \times 10^6$ T cells from WT or IL-17A$^{-/-}$ B6 mice. Survival (A) and clinical GVHD scores (B, means ± SE) in WT controls (closed-circles), IL-17A$^{-/-}$ controls (open-circles), Mn-treated mice with WT donors (closed-diamonds), and Mn-treated mice with IL-17A$^{-/-}$ donors (open-diamonds) from three independent experiments were combined and shown ($n = 12-15$ / group). Representative H&E stained lung sections (C) and pathology scores of the lungs (D) from Allo controls with WT donors (gray bar), Allo controls with IL-17A$^{-/-}$ donors (vertical-striped bar), Mn-treated Allo with WT donors (black bar) and Mn-treated Allo with IL-17A$^{-/-}$ donors (horizontal-striped bar) were analyzed 3 wk after BMT. Data from three independent experiments were combined and shown as means ± SE. Scale bars; 100 μm, *$P < .05$, **$P < .01$. 
Figure 1

A

B

C

D

E

F
Figure 2

A

Syn | Syn-Mn | Allo | Allo-Mn

B

Lung

Pathology Scores

Day7 | Day14 | Day21

C

Liver

Pathology Scores

Day7 | Day14 | Day21

D

Small Intestine

Pathology Scores

Day7 | Day14 | Day21

E

Hemoglobin Saturation (%)

Naive | Syn | Syn-Mn | Allo | Allo-Mn | Syn | Syn-Mn | Allo | Allo-Mn

Day7 | Day14

F

Allo + PBS | Allo + C. albicans

Pathology Scores

Allo + C. albicans

G

Pathology Scores

Allogeneic | 10^5 | 10^6 | 10^7 | Allo + C. albicans

*
Figure 3

A

CD4 T cells (×10^6)

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Il4 mRNA

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Tgfb3 mRNA

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C

SSC

FSC

CD3

CD4

D

% IL-17A/CD4+ T cells

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IL-17A+CD4+ T cells (×10^6)

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% IFN-γ+CD4+ T cells

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IFN-γ+CD4+ T cells (×10^6)

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IL-17A+CD4+ T cells (×10^6)

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IL-17A+CD4+ T cells (×10^6)

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Figure 4

A

B

C

D

E

F

G
Figure 5

A

C

D

E

F

G

H

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Figure 7

A

Survival Rate (%)

Weeks post-BMT

WT

IL-17A\(^{+}\)-Mn

IL-17A\(^{-}\)

WT

Mn

**

B

Clinical GVHD Scores

Weeks post-BMT

WT-Mn


C

WT donor T cells  IL-17A\(^{-}\) donor T cells

Allo-Diluent

Allo-Mn


D

Lung Pathology Scores

Donor T cells: WT  IL17A\(^{+}\)  WT  IL17A\(^{-}\)

Treatment: Diluent  Mn

*
α-Mannan induces Th17-mediated pulmonary graft-versus-host disease in mice

Hidetaka Uryu, Daigo Hashimoto, Koji Kato, Eiko Hayase, Satomi Matsuoka, Reiki Ogasawara, Shuichiro Takahashi, Yoshinobu Maeda, Hiromi Iwasaki, Toshihiro Miyamoto, Shinobu Saijo, Yoichiro Iwakura, Geoffrey R. Hill, Koichi Akashi and Takanori Teshima