Donor bone marrow-derived macrophage MHC II drives neuroinflammation and altered behaviour during chronic GVHD in mice

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Abstract:
Graft-versus-host disease (GVHD) remains the leading cause of non-relapse mortality after allogeneic stem cell transplantation for haematological malignancies. Manifestations of GVHD in the central nervous system (CNS) present as neurocognitive dysfunction in up to 60% of patients, however, the mechanisms driving chronic GVHD in the CNS are yet to be elucidated. Our studies of murine chronic GVHD revealed behavioural deficits associated with broad neuroinflammation and persistent Ifng upregulation. By flow cytometry, we observed a proportional shift in the donor-derived T-cell population in the chronic GVHD brain from early CD8 dominance to later CD4 sequestration. RNA sequencing of the hippocampus identified perturbations to structural and functional synapse-related gene expression, together with the upregulation of genes associated with IFN-γ responses and antigen presentation. Neuroinflammation in the cortex of mice and humans during acute GVHD was recently shown to be mediated by resident microglia-derived TNF. In contrast, infiltration of pro-inflammatory MHC class II+ donor bone marrow-derived macrophages (BMDM) was identified as a distinguishing feature of chronic CNS GVHD. Donor BMDM, which comprised up to 50% of the CNS myeloid population, exhibited a transcriptional signature distinct from resident microglia. Recipients of MHC class II knockout bone marrow grafts exhibited attenuated neuroinflammation and behaviour comparable to controls, suggestive of a critical role of donor BMDM MHC class II expression in CNS chronic GVHD. Our identification of disease mediators distinct from those in the acute phase indicates the necessity to pursue alternative therapeutic targets for late-stage neurological manifestations.

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Running Title: Donor macrophage MHC II drives CNS cGVHD

Key Points:

➢ Pre-clinical mouse modelling of chronic GVHD (cGVHD) demonstrates persistent alterations in behaviour
➢ CNS cGVHD presents with immunological features distinct from CNS acute GVHD, indicative of divergent mechanisms.

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Abstract

Graft-versus-host disease (GVHD) remains the leading cause of non-relapse mortality after allogeneic stem cell transplantation for haematological malignancies. Manifestations of GVHD in the central nervous system (CNS) present as neurocognitive dysfunction in up to 60% of patients, however, the mechanisms driving chronic GVHD in the CNS are yet to be elucidated. Our studies of murine chronic GVHD revealed behavioural deficits associated with broad neuroinflammation and persistent Ifng upregulation. By flow cytometry, we observed a proportional shift in the donor-derived T-cell population in the chronic GVHD brain from early CD8 dominance to later CD4 sequestration. RNA sequencing of the hippocampus identified perturbations to structural and functional synapse-related gene expression, together with the upregulation of genes associated with IFN-γ responses and antigen presentation. Neuroinflammation in the cortex of mice and humans during acute GVHD was recently shown to be mediated by resident microglia-derived TNF. In contrast, infiltration of pro-inflammatory MHC class II+ donor bone marrow-derived macrophages (BMDM) was identified as a distinguishing feature of chronic CNS GVHD. Donor BMDM, which comprised up to 50% of the CNS myeloid population, exhibited a transcriptional signature distinct from resident microglia. Recipients of MHC class II knockout bone marrow grafts exhibited attenuated neuroinflammation and behaviour comparable to controls, suggestive of a critical role of donor BMDM MHC class II expression in CNS chronic GVHD. Our identification of disease mediators distinct from those in the acute phase indicates the necessity to pursue alternative therapeutic targets for late-stage neurological manifestations.
Introduction

Chronic graft-versus-host disease (cGVHD) is the leading cause of non-relapse mortality after otherwise curative allogeneic stem cell transplantation (SCT) for haematological malignancies. cGVHD can be inflammatory and/or fibrotic with organ-specific or multi-organ symptomatology, and complex and protean presentations make diagnosis challenging. Manifestations of cGVHD in the central nervous system (CNS) have recently been recognised as a clinical entity independent of complications associated with pre-transplant conditioning and immunotherapy. Up to 60% of adult SCT survivors experience neurocognitive dysfunction, evidenced by impaired learning and memory, concentration deficits, and emotional changes including anxiety and depression, with reports of underlying cerebral vascular pathologies and encephalitis. Although recent studies have begun to elucidate the pathophysiology of CNS acute GVHD (aGVHD), we report here the first pre-clinical evidence of CNS cGVHD. GVHD is considered the primary determinant of post-transplant quality of life, thus understanding the effects of complex immunopathology on brain function serves to identify targets for potential preventative and therapeutic treatments.

Consistent with alloreactive donor T-cells driving peripheral GVHD pathology, murine and non-human primate CNS aGVHD models showed alloreactive T-cells to infiltrate the CNS and induce neuronal damage. Post-mortem brain samples and experimental disease models, such as in Alzheimer’s disease and multiple sclerosis/experimental autoimmune encephalitis, suggest that CNS infiltration of pathogenic T-cells with associated proinflammatory cytokine production are common features of chronic neuroinflammation. CNS damage and disease typically involve activation of tissue-resident microglia. As long-lived macrophages, microglia conduct dynamic immune
surveillance among other functions, including provision of trophic support and regulation of synaptic plasticity.\textsuperscript{14,15} Inflammatory stimuli induce a reactive phenotype characterised by morphological changes and cytokine release. Microglia may restore a quiescent state following activation or contribute to ongoing inflammation,\textsuperscript{16} exemplified by the recent description of microglial TNF production as a critical mediator of acute CNS GVHD.\textsuperscript{7} Additionally, disease models demonstrate a detrimental contribution of CNS infiltrating bone marrow-derived macrophages (BMDM),\textsuperscript{17} similar to cutaneous cGVHD induction by infiltrating donor-derived macrophages.\textsuperscript{18} Although CNS-infiltrating macrophages adopt microglial morphology and identifying surface marker (e.g. ionised calcium binding adaptor molecule-1 (Iba1)) expression, widespread profiling suggests a functionally and transcriptionally distinct population.\textsuperscript{19,20} Elucidating resident microglia phenotype and function, and the temporal contribution of donor BMDM will be instrumental for understanding biological disease mechanisms and informing effective therapeutic strategies late post-transplant.

The necessity for a controlled immunological environment to maintain optimal brain function underlies the hypothesis that GVHD-induced inflammatory changes are modulating behaviour. Here, we aimed to characterise cGVHD CNS and provide the first evidence of prolonged behavioural deficits dependent on sustained inflammation. Overall, we confirm the brain as a novel cGVHD target organ where neuroinflammation is characterized by dysregulated cytokine production and infiltration donor-derived MHC II-expressing macrophages.
Materials and Methods

Mice
Female mice were used between 8 to 12 weeks old, housed in sterilized microisolator cages with acidified autoclaved water (pH 2.5) and food available ad libitum. Strains used are listed in Supplemental Table 1. Experiments were approved by and performed in accordance with the QIMR Berghofer Animal Ethics Committee.

Bone marrow transplantation
On day 0, recipients received 1100- (B6D2F1) or 1000-cGy (C57BL/6) split-dose total-body irradiation (cesium-137 source) and 5x10⁶ (B6D2F1) or 10x10⁶ (C57BL/6) T-cell depleted (TCD) bone marrow (BM) alone (non-GVHD controls) or with 0.5x10⁶ C57BL/6 (B6D2F1) or 5x10⁶ BALB/c (C57BL/6) splenic T-cells enriched by BioMag (Qiagen) bead depletion of non-T-cells to induce GVHD. MHCII KO donor grafts contained TCD BM only. Mice were monitored daily to evaluate clinical GVHD scores as previously published.

Behavioural Testing
The Rotarod grip strength, active place avoidance task (APA), Forced swim, Open Field, Elevated Plus Maze, and Novel Object Recognition tests began on either day 14, 35 or 70 post-transplant. Detailed methodology is included in the Supplemental Materials and Methods.

Flow Cytometry and Cell Sorting
Surface staining of single cell suspensions of digested brain was performed in FACS buffer (PBS, 2% FCS, 5mM EDTA) at room temperature for 15 min in darkness. The antibodies used are listed in Supplemental Table 2. Flow cytometry acquisition was performed with an LSRFortessa cytometer (BD Biosciences), and data analysed using FlowJo software (version 10). Cell sorting was performed using a FACS ARIA III Cell Sorter (BD Biosciences).
Statistics

GraphPad Prism software (version 7.0) was used to conduct all statistical analyses. An unpaired two-tailed Student’s t test was used for comparison of two groups or a one-way ANOVA with Tukey’s multiple comparisons for GVHD groups across three time points. Longitudinal behaviour data was analysed with repeated measures two-way ANOVA with Bonferroni’s post hoc comparison, and clinical scores with a two-way ANOVA with Bonferroni’s multiple comparisons test. All data are presented as mean ± SEM, with significance at P<0.05.

RNA-Seq data available through NCBI’s Gene Expression Omnibus (GEO) database with accession number GSE179441.

Details of behavioural tests, devices used, standard procedures including tissue procurement and processing, flow cytometry, immunofluorescence (IF) and imaging acquisition, cell quantification, quantitative real-time PCR (oligonucleotide sequences listed in Supplemental Table 3), and RNA sequencing (RNA-seq) are in Supplemental Materials and Methods.

Results

cGVHD induces persistent behavioural deficits.

We employed established MHC mismatched cGVHD models\(^{10}\) (Figures 1 A, B) where transfer of a low T-cell dose induces non-lethal disease with subsequent development of sclerodermatous skin pathology post-transplant (Supplemental Figures 1A-D). Preceded by low-grade aGVHD, chronic GVHD develops in both models by day (D) 35, with persistent pathology evident in the later stages of disease (D70-100). To measure neurological manifestations of cGVHD, we first employed a battery of behavioural tests in the B6 into
B6D2F1 model. cGVHD mice showed normal grip strength (Supplemental Figure 1E) and exploratory behaviour (Supplemental Figure 1F) in the Rotarod and Open Field tests, respectively, with no evidence of anxiety-like behaviour in the Elevated Plus Maze (Supplemental Figure 1G). In support of recent findings, GVHD mice demonstrated poor recognition memory in the Novel Object Recognition test at D14, however this did not persist into the chronic stages of disease (Supplemental Figures 1H). However, using the Forced Swim test (FST) to assess the behavioural response of cGVHD mice to an aversive situation, we confirmed in our model the previous report that aGVHD mice (D14) exhibit increased mobility as a dysfunctional response in the FST (Supplemental Figures 1I). Moreover, testing at both early and late phases of cGVHD (D35 and D70, respectively) demonstrated significantly increased mobility in cGVHD mice compared to TCD controls (Figure 1C). Extending these findings we confirmed dysfunctional responses in the FST during the acute phase (D14, Supplemental Figure 1J) and at both early and late stage cGVHD using the BALB/c into B6 model (Figure 1D). We next employed the active place avoidance (APA) paradigm to assess spatial learning and memory, which has been extensively shown to depend on the integrity of the hippocampus. This task requires mice to integrate visuospatial room cues to avoid a concealed shock zone on a rotating platform. We first confirmed that TCD mice effectively complete this task in a manner similar to naïve age-matched controls, therefore indicating intact working spatial memory (Supplemental Figure 1K). Compared to TCD controls however, cGVHD mice exhibited learning deficits evidenced by increased entries into the shock zone and less time spent avoiding the shock zone (Figure 1E, F). Improvement over the 5-day testing period beginning at D70 (Figure 1F) was significantly reduced in cGVHD mice compared to TCD controls. Conclusively, these novel data indicate that cGVHD mice exhibit pronounced alterations in behaviour persisting late post-transplant.
cGVHD is associated with neuroinflammation, local proinflammatory cytokine production and T-cell infiltration.

Pro-inflammatory cytokine mediation of effector pathways is critical to the development of systemic cGVHD, and cytokine dysregulation is also a common feature of neuroinflammatory diseases. Analysis of the brain inflammatory milieu by qRT-PCR at D35 demonstrated *Ifny*, *Il1b*, *Tnf* and *Ccl2* upregulation in cGVHD mice compared to TCD (Figure 2A). Having observed deficits in hippocampal-dependent spatial learning, we additionally profiled the isolated hippocampus and observed similar trends (Figure 2B). At D70, cGVHD brains demonstrated elevated *Ifng* and *Ccl2*, with *Ifng* also upregulated in isolated hippocampi (Figures 2A, B), findings which were recapitulated in the Balb/c → B6 model (Supplemental Figure 2A, B). Notably, *Tnf* expression was attenuated at D70, indicating a restricted contribution to acute and early chronic GVHD (D35) pathology.

Recipients of NZ-eGFP BM grafts supplemented with B6.RFP+ T-cells were used for flow cytometric profiling of the CNS inflammatory infiltrate (Figure 2C). Digestion of GVHD brains at D14, 35, and 70 post-transplant revealed significant T-cell infiltration that comprised approximately 60% of CD45+ cells at D14 and gradually decreased overtime (Figure 2E, F). Consistent with reports in aGVHD, CD8+ T-cells were predominant at D14 (Figure 2G), however overtime, a gradual increase in the proportion and absolute number of CD4 T-cells was noted (Figure 2G). Initial brain-infiltrating CD8+ T-cells originated primarily from the donor graft T-cell compartment (Figure 2H). By D70 however, 20% of the CD4+ T compartment were derived from BM, suggestive of prolonged, low-grade infiltration (Figure 2I). To further identify parenchymal brain regions of interest and possible cellular routes of infiltration, five brain structures were investigated for the presence of RFP+ donor T-graft cells (Figure 3A). Both the meninges and choroid plexus represent points of access.
for inflammatory infiltrate\textsuperscript{37,38} and at D14, significant T cell infiltrate was observed in both of these sites (Figure 3B and C). While T cell numbers markedly declined in the meninges by D35, infiltration in the choroid plexus persisted out to D70. Parenchymal localisation peaked at D35 in the cortex (Figure 3D) and hippocampus (dentate gyrus region, Figure 3E) with reductions evident in both regions by D70. In addition, we observed a similar trend when examining the habenula nuclei, which are diversely involved in many neuromodulatory systems, regulates cognitive and motivational processes, and known to be dysfunctional in depression\textsuperscript{39}. Collectively, these data suggest a persistent inflammatory milieu in the cGVHD CNS with features distinct from those in aGVHD.

\textit{Microglia activation and donor-derived MHC II\textsuperscript{+} macrophage infiltration are key mediators of CNS cGVHD.}

Inflammatory insults to the brain induce activation of microglia as resident immune effector cells, commonly accompanied by BMDM infiltration\textsuperscript{40,41}. Having observed prolonged Ccl2 upregulation and CD4\textsuperscript{+} T-cell skewing by D70, we sought to establish perturbations to, and contributions of, macrophage populations in CNS cGVHD. Using flow cytometry, we examined the temporal composition of the CD45\textsuperscript{dim}CD11b\textsuperscript{+}Ly6G\textsuperscript{neg} myeloid population in brains from of B6.Csf1r\textsuperscript{-mApple}\textsuperscript{42} $\rightarrow$ Csf1r\textsuperscript{-eGFPxDBA2 F1 (MacGreen-F1\textsuperscript{43})} transplant recipients (Figure 4A, B). Infiltrating donor (mApple) BMDM were notable by D35 and by D70 comprised approximately 50\% of the CD45\textsuperscript{dim}CD11b\textsuperscript{+}Ly6G\textsuperscript{neg} cells (Figure 4C). \textit{In situ} staining for Iba-1 at D35 revealed a reactive phenotype of cortical and hippocampal host microglia (Supplemental Figures 3A-D), evidenced by an amoeboid soma and thickened, bushy dendrites\textsuperscript{44}, in comparison to the ramified morphology of TCD microglia. However, FACS analysis of MHC II expression demonstrated that host microglial activation was transient. In contrast, donor BMDM retained high levels of MHC II expression, and co-
expressed increased levels of co-stimulatory markers CD80, CD86, CD40, and the phagocytosis marker CD68 (Supplemental Figures 4A-E). Confocal imaging confirmed that by D70, in all regions examined (Figure 4F, G (hippocampus) and Supplemental Figure 5 (cortex, habenula, and choroid plexus)), MHC II expression was restricted to donor BMDM and host microglia had returned to a homeostatic phenotype. Importantly, complementary analysis at D100 demonstrated long-term persistence of altered microglial/macrophage populations as indicated by increases in total of Iba-1⁺ cell numbers, and donor BMDM in the choroid plexus, hippocampus (including the CA1 and CA3 regions) and habenula of cGVHD mice in both models (Figure 5 and Supplemental Figure 6 and 7). Comparatively, donor BMDM infiltration through the meninges dissipated after D35, and donor BMDM infiltration into the cortex was not significant at either time point, suggesting the early increase in Iba1⁺ cells likely reflects an activated-induced expansion of the host microglia.

Donor-derived macrophages and host microglia exhibit differential transcriptional profiles in the GVHD brain.

Identification of a heterogeneous brain myeloid population with differential MHC II expression in cGVHD mice warranted further investigation to understand functional properties of each subset, with the intent of identifying appropriate markers or pathways for therapeutic targeting. Again, using B6.Csf1r-mApple donors and MacGreen-F1 recipients at D70 post-transplant, donor (mApple⁺GFPneg) and host (mApplenegGFP⁺) CD45dimCD11b⁻Ly6Gneg cells were sorted from brains of cGVHD and TCD BM control mice for bulk RNA-seq (Figure 6A). Consistent with previous reports characterising resident microglia and infiltrating macrophages, GVHD donor BMDM maintained a transcriptional signature distinct from GVHD host microglia, differentially expressing 5356 genes, with 2647 transcripts upregulated, and 2709 downregulated (FDR<0.05) (Figure 6B).
A hallmark feature of microglia activation is the downregulation of signature genes. However, a comparison host microglia subsets from GVHD and TCD BM controls showed comparable expression of transcripts including Cx3cr1, P2ry12, Sall1, Tmem11, and Siglech (Figure 6C), providing strong evidence that host microglia return to a more homeostatic state late post-transplant, in line with markedly reduced MHC II expression. In contrast, these signature genes were lowly expressed or completely absent in donor BMDM, with differential expression of CX3CR1 and P2RY12 validated by flow cytometry (Figure 6D). Additionally, donor BMDM exhibited a distinct profile with transcripts for Apoe, Ccr2, and Cd38, and an overlapping perivascular macrophage signature based on Cd163 and Lyve1 expression. Gene ontology enrichment analysis indicated a strong gene profile related to cell adhesion and migration, chemotaxis, and inflammatory responses in GVHD BMDM compared to GVHD host microglia (Figure 6E). In line with this, GVHD donor BMDM showed notably high expression of markers related to extracellular matrix (ECM) degradation, including members of the MMP and Adam families (Figure 6F). The expression of a class of stimulatory CD300 molecules, involved in leukocyte response regulation, was almost exclusive to BMDM (Figure 6F). This family shows similar functions to members of the Triggering Receptor Expressed on Myeloid (TREM) cell family, such as Trem1, also found to be upregulated in GVHD donor BMDM and previously identified as a marker of pathogenic brain-infiltrating macrophages. Given our earlier findings of prolonged Ifng upregulation, we further probed this signature and found a striking upregulation of genes induced or regulated by IFN-γ signalling in the donor BMDM (Figure 6G). Additionally, GVHD BMDM and microglia differentially expressed genes for various cytokines including Il1b and Il6 (Figure 6H). Coinciding with recent reports, resident microglia showed no in vivo Il10 expression. Differential expression of Tnf in GVHD and TCD host microglia compared to GVHD donor BMDM is likely attributable to
homeostatic glial TNF production for synaptic scaling. Collectively, these results delineate donor BMDM and resident microglia as transcriptionally distinct populations, where persistent BMDM infiltration and activation marks a unique signature in the cGVHD brain.

RNA-seq reveals molecular synaptic changes in the cGVHD hippocampus and defines a critical role for donor MHC-II.

To identify targetable pathways related to cellular infiltration and inflammation that may contribute to altered behaviour in cGVHD mice, we conducted bulk RNA-seq on the hippocampi of GVHD and TCD mice at D80 post-transplant. After removal of outliers based on principal component analysis (PCA), downstream differential gene expression analysis identified 381 significantly differentially expressed genes (FDR < 0.05) (Figure 7A), with 147 genes found to be upregulated in GVHD, and 207 genes downregulated. Gene set enrichment analysis (GSEA) found that the ten most significantly upregulated gene sets, as identified with the Gene Ontology (GO) database, were related to general immune responses (Supplemental Table 4). Confirming our previous findings of a strong IFN-γ response during cGVHD, the hallmark gene set within GSEA revealed significant upregulation of the interferon-induced proteins Ifi44, Ifit3, and Ifit1 in GVHD hippocampi (Figure 7B). Ingenuity pathway analysis (IPA) also showed upregulation in the GVHD hippocampus of molecules related to antigen presentation (Figure 7C, Supplemental Table 5), including subunits of both MHC-I (Hs-T23, H2-K1, H2-Q7) and MHC II (H2-Abl, H2-Dmb1), as well as the MHC class II transactivator CIITA, and Ctss, required for degradation of antigenic proteins to peptides for MHC II presentation.

As a molecular correlate for altered behaviour, changes to synaptic structure and function are widely reported in various neurodegenerative and inflammatory CNS conditions. The GO database identified an ontology related to synapse structure and function containing 15 genes...
downregulated in the GVHD hippocampus (Figure 7D). Perturbations to the expression of genes transcribing trafficking proteins (Arhgap33)$^{52}$, components of the neurotransmitter release machinery (Prrt2)$^{53}$, cell adhesion molecules (Nrxn2, Nlgn2)$^{54}$, and post-synaptic receptor subunits (Grin2D)$^{55}$ indicate the stability, activity, and number of functional synapses may be compromised. To further investigate the premise of synapse disruption in vivo, we quantified synaptophysin (Figure 7E) and PSD-95 (data not shown) expression in the hippocampus as indicators of the pre- and post-synapse, respectively. Whilst no differences were evident in PSD-95 protein expression, by D35, the GVHD hippocampus showed a trend towards reduced synaptophysin puncta density compared to TCD BM controls, which was significantly reduced at D70. This is suggestive of a preferential disruption to the pre-synaptic compartment, suggesting that alterations to hippocampal synaptic structure and transmission may contribute the observed behavioural phenotype in cGVHD.

Given upregulation of genes related to antigen presentation in the hippocampus of cGVHD mice, alongside BMDM macrophage infiltration, we investigated whether donor MHC II expression was critical for driving the robust cGVHD neuroinflammatory profile. Allogeneic MHC II KO BM transfer (Figure 7F) induced spontaneous late cGVHD (Figure 7G) characterised by scleroderma (Figure 7H), attributable to a failure of peripheral regulatory T-cells (Treg)$^{10}$. Strikingly, recipients of MHC II KO TCD BM showed no increase in mobile behaviour in the FST compared to recipients of WT TCD BM (Figure 7I). Microglia density and phenotype in all regions of the brain (Figures 7J, K, Supplemental Figure 8A-D) and pro-inflammatory cytokine mRNA levels (Figure 7L, M) were similar between recipients of MHC II KO vs WT TCD BM. Furthermore, recipients of MHC II KO BM supplemented with WT T cells (Supplemental Figures 8E, F) demonstrated improved behaviour in the FST.
at D35 (Supplemental Figure 8G) compared to recipients of WT BM + T. Notably, MHC II deficiency did not diminish brain Ifng mRNA levels (Supplemental Figure 8H), indicating that IFN-γ signalling functions upstream of, and may be required for MHC II expression by donor BMDM to promote CNS cGVHD behavioural perturbations.

**Discussion**

Chronic GVHD represents a complicated clinical entity, with neurological manifestations remaining critically understudied despite known impact on patient quality of life. Difficulties in proper diagnosis and management of CNS cGVHD are perpetuated by an absence of pre-clinical investigation into the mechanisms underpinning cerebral pathologies and neurocognitive dysfunction observed in patients. Here, we describe the immune landscape in CNS cGVHD and begin to elucidate disease mediators that may represent viable therapeutic targets.

The primary clinical predictor of cGVHD is preceding aGVHD, with important distinctions in underlying pathology determining heterogeneous target organ effects. Understanding temporal CNS disease changes will be critical for informing therapeutic strategies. Recently, several studies have investigated aGVHD effects on the brain D7-21 post-transplant, highlighting T-cell infiltration, pro-inflammatory cytokine production, and microglia activation as immune mediators of cognitive deficits. Employing our low dose T-cell model allowed evaluation of subtle aGVHD characteristics followed by cGVHD pathology late post-transplant. The main features and divergences of acute and chronic GVHD in the CNS are summarised and compared in **Table 1**. We observed peak CD8+ T-cell infiltration into the brain at D14, supporting reported findings in murine and non-human primate models. The progressive decline in T-cell numbers by D70 and proportional shift to a CD4+ T-cell phenotype suggests two potential phenomena. Firstly, early immune cell...
infiltration may be sufficient to initiate CNS disease but prolonged inflammation may only require low-grade infiltration. Secondly, disease mechanisms driven by donor antigen presenting cells (APCs) are prominent in the brain only in cGVHD, paralleling peripheral mechanisms and indicating a clear divergence from aGVHD pathology.58

The persistent elevation of IFN-γ in the brains of cGVHD mice raises an interesting prospect given dichotomous roles for IFN-γ roles in transplantation and GVHD biology.21 High IFN-γ expression was maintained at D70 despite reduced T-cell infiltration, likely indicative of an alternative source and supportive of a role for IFN-γ in controlling donor T-cell expansion in allogeneic SCT settings.59 However, in the CNS niche, IFN-γ impairs hippocampal neurogenesis and plasticity, mediated by direct action on microglia, leading to cognitive deficits.60 Spatial learning and memory, a process intimately dependent on hippocampal neurogenesis32 and homeostatic microglia function61, was profoundly altered in cGVHD mice, particularly at D70. This suggests that the attenuation of IFN-γ overexpression by receptor blockade, cell-specific knockout, or inhibition of downstream JAK/STAT may offer benefit for reducing inflammation and consequently improving cognitive function. Selective inhibition of JAK1/2 (Ruxolitinib) has improved patient responses compared to standard therapy in steroid-refractory chronic GVHD.56 Whilst exact mechanisms of action are still being studied, our data suggests the reported reductions in IFN-γ after ruxolitinib treatment would likely result in attenuated downstream MHC II expression and thus improve CNS cGVHD outcomes.

In parallel with clinical reports,63 our findings indicate long-term behavioural changes in cGVHD mice, in conjunction with early microglia activation, donor BMDM infiltration, and sustained molecular disruption of the synapse. Microglia are responsible for the cytokine-
dependent maintenance of the CNS microenvironment and response to brain insults, but can perpetuate inflammation during disease. TNF production by activated microglia drives aGVHD primarily in the cortex, and elevated TNF expression in our D35 cGVHD likely reflects a continuation of this acute pathology. Although Mathew et al. demonstrated improved behavioural outcomes with microglial TNF reduction in aGVHD mice, inherent attenuation of TNF expression and a resting microglia phenotype by D70 in cGVHD brains with maintained behavioural deficits insists upon additional and/or alternative inflammatory mediators. Striking changes to the myeloid proportions in the brain by D70 with increased donor BMDM, coinciding with hippocampal transcriptional signatures of IFN-γ responses and antigen presentation, with the resultant reduction in resident microglia, identifies this population as a therapeutic target. BMDM in the brain parenchyma have been reported in various CNS pathologies and are known to express a distinct transcriptional signature to resident microglia, although their functional contribution to disease remains controversial. It still remains unclear if and how perturbations to the CNS myeloid compartment contribute to the observed behavioural changes in cGVHD mice, given maintained higher cognitive functioning in both the absence of resident microglia and presence of infiltrating macrophage. However, neuroinflammation is known to contribute to persistent changes in neuronal function, leading to cognitive deficits, and the role for donor APCs in perpetuating systemic cGVHD is well defined. RNA-Seq data revealed upregulation of genes related to ECM degradation in donor BMDM, which are known to compromise blood brain barrier integrity through vascular damage and disrupt neuronal signalling by altering cell-ECM interactions and modulating neurotransmitter receptor activity. cGVHD BMDM gene clusters aligning with those previously associated with neurodegenerative diseases such as Alzheimer’s suggest this population likely differs functionally to resident microglia and their disruption to the brain macrophage pool may be detrimental. Dependence of microglia
and macrophages on CSF-1/CSF-1R signalling\textsuperscript{18,71} offers a targetable axis for pharmaceutical depletion. Antibody blockade of CSF-1R signalling improved sclerodermatous cGVHD in pre-clinical mouse models\textsuperscript{18} leading to a clinical trial to test efficacy in patients (NCT03604692). Importantly, timing of delivery to deplete BMDM monocyte precursors and the integrity of the blood brain barrier to determine the availability of a blocking antibody in the brain are important considerations, additionally warranting comparison with small-molecule inhibitors of CSF-1R that can access the brain parenchyma. Investigating BMDM responses to the brain microenvironment and immune challenges will prove invaluable for modulating their function and residence in the CNS, with potential implications for disease settings beyond GVHD.

In summary, this study is the first to define features of the CNS immune landscape in cGVHD, instigated by early T-cell infiltration and late effects associated with broad infiltration of MHC II\textsuperscript{+} donor BMDM, suggestive of pathological features distinguishable from aGVHD. Whilst we have investigated these features in multiple brain regions, further studies would benefit from examining other possible mechanisms, such as a role for humoral disturbances in the hypothalamic-pituitary axis in CNS inflammation and behaviour\textsuperscript{72,73}, and expanding into the peripheral nervous system to investigate neuropathic manifestations. However, given increasing emergence of clinical reports documenting neurocognitive dysfunction in cGVHD patients, our findings have major implications for understanding CNS pathology late post-transplant, and identify novel targets, distinct from those in acute GVHD, to pursue as therapeutic strategies.
Table 1. Summarised comparison of features associated with acute and chronic GVHD in the brain.

| Features                        | Acute GVHD                                                                 | Chronic GVHD                                                             |
|---------------------------------|---------------------------------------------------------------------------|--------------------------------------------------------------------------|
| **Time of assessment post-transplant** | Days 7-21<sup>6,7,11,30</sup>                                              | Days 35 and 70-100                                                       |
| **Behavioural modifications**   | Normal grip strength and motor coordination<sup>6</sup> (Rotarod)           | Normal grip strength and motor coordination (Rotarod)                    |
|                                 | Increased mobility in FST (D7 and 14)<sup>30</sup>                          | Increased mobility in FST (D35 and 70)                                    |
|                                 | Impaired spatial learning and memory (Morris water maze task, D21)<sup>6</sup> | Impaired spatial learning and memory (active place avoidance task, D35 and 70) |
|                                 | Impaired recognition memory<sup>7</sup>                                     | Intact recognition memory                                                |
|                                 | Increased anxiety (elevated plus maze, D14)                                | Normal performance on elevated plus maze                                |
|                                 | Reduced exploratory behaviour (D21)<sup>19</sup>                           | Normal exploratory behaviour                                             |
| **Cytokine mediators**          | TNF, IL-6<sup>7,30</sup>                                                   | IFN-γ, IL-1β, CCL2                                                       |
| **T-cell infiltration dynamics**| T-cell infiltration across days 7-14, high numbers of CD8<sup>+</sup> with primarily effector memory phenotype<sup>6,7,11</sup> | T-cell infiltration peak at D14 with proportional skew towards CD4 phenotype by D70 |
| **Microglia/macrophages**       | Resident microglia activation (D14): morphological change, increased Iba1<sup>+</sup> cell count, | Resident microglia activation (D35): morphological change, increased total Iba1<sup>+</sup> cell |
| Gene expression | Increased expression of MHC II | Increased donor-derived macrophage infiltration, highest at D70 with loss of CD45 expression overtime; defined transcriptionally as distinct from resident microglia |
|-----------------|--------------------------------|----------------------------------------------------------------------------------------------------------------------------------|
| Resident microglia upregulate genes involved in antigen presentation (D14) | Antigen presentation upregulated in hippocampus (D70) |
| D14: GVHD microglia downregulate CX3CR1 (lineage marker) indicating activation | D70: GVHD resident microglia return to phenotype resembling TCD microglia, with expression of CX3CR1 and P2RY12. Donor-derived macrophages maintain activation status |
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Authorship

Contribution: RCA designed and performed experiments, analysed data, prepared figures, and wrote the manuscript; DCC designed and performed experiments, analysed data and edited the manuscript; SNS designed and performed experiments and analysed data; GTL performed experiments and approved the manuscript; RLJ analysed data and edited the manuscript; GQR and GB provided intellectual input and approved the manuscript; LTK analysed data and approved the manuscript; AM provided reagents and edited the manuscript; BRB interpreted data and edited the manuscript. KPAM and JV together conceptualized the study. JV provided intellectual input and approved the manuscript; and KPAM designed, led, and coordinated the project, performed experiments, analysed data, and prepared and edited the manuscript.
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Figure Legends

Figure 1. Chronic GVHD induces prolonged behavioural deficits.

(A) Schematic of transplant regime. Lethally irradiated B6D2F1 (H2<sup>b/d</sup>) recipient mice received 5x10<sup>6</sup> TCD BM with no T-cells or with 0.5x10<sup>6</sup> CD3<sup>+</sup> T-cells from C57Bl/6 (H2<sup>b</sup>) donors to induce low-grade, non-lethal chronic GVHD.

(B) Schematic of transplant regime. 10x10<sup>6</sup> bone marrow with or without 5x10<sup>6</sup> CD3<sup>+</sup> T cells from a BALB/c (H2<sup>d</sup>) donor were transplanted into lethally irradiated C57Bl6 (H2<sup>b</sup>) recipients.

(C) Time B6D2F1 recipients spent swimming (mobile) in the forced swim test at day (D)35 and D70 post-transplant (D35: n = 6-8 mice/group, representative of 2 independent experiments; D70: n = 9-10 mice/group).

(D) Time C57Bl6 recipients spent swimming (mobile) in the forced swim test at day D35 and D70 post-transplant (n = 8-9 mice/group).

Performance of chronic GVHD and TCD mice in the active place avoidance task for assessment of spatial learning in 20-minute sessions across 5 days beginning at D35 (E) or D70 (F) post-transplant (n = 9-10 mice/group). Recorded parameters include improvement from day 1 to 5 expressed as a percentage (calculated based on the difference in the number of entries into the shock zone), total number of entries into the shock zone per day, and maximum time spent avoiding the shock zone per day.

Data presented as mean ± SEM. Statistics: unpaired Student’s t test for differences between GVHD and TCD mice (B, D, E & F: improvement) and repeated-measures two-way ANOVA followed by Bonferroni’s post hoc comparison (E, F: entries, avoidance time). *p<0.05, **p<0.01, ****P<0.0001. Abbreviations: BM, bone marrow; TCD, T-cell-depleted; GVHD, Graft-versus-Host Disease.
Figure 2. Chronic GVHD induces T-cell infiltration and a proinflammatory cytokine profile in the brain.

mRNA expression of selected proinflammatory cytokines detected by RT-qPCR in the (A) thick coronal section and (B) hippocampus of GVHD and TCD mice at days 35 and 70 post-transplant. Data pooled from two independent experiments, n = 6-15 mice/group. Expression calculated relative to the Hprt gene and reported as a fold change of the mean of the TCD group.

(C) Schematic of transplant regime. Lethally irradiated B6D2F1 recipient mice received 5x10⁶ BM from C57Bl/6 donors ubiquitously expressing GFP and 0.5x10⁶ sort-purified CD90.2⁺ T cells from C57Bl/6 donors ubiquitously expressing RFP.

(D) Representative dot plots indicating gating strategy for identifying live CD90.2⁺ CD4 and CD8 T-cell subsets within the CD45hiCD11b⁻neg population, gated on forward and side scatter. Cells were isolated from digested coronal brain segments of transplant recipients at days 14, 35, and 70 post-transplant.

(E-I) Quantification of lymphocyte populations in dissociated GVHD brains at days 14, 35, and 70 post-transplant.

(E) CD90.2⁺ proportion of the CD45hiCD11b⁻neg population. D14: n = 6 (data pooled from two independent experiments); D35: n = 3 (data from one independent experiment); D70: n = 17 (data pooled from four independent experiments).

(F) Absolute number of CD90.2⁺ T-cells within the CD45hiCD11b⁻neg population. D14: n = 8 (data pooled from three independent experiments); D35: n = 6 (data pooled from two independent experiments); D70: n= 16, (data pooled from four independent experiments).

(G) Proportions of CD4⁺ and CD8⁺ T-cells within the CD90.2⁺ population. D14: n = 5 (data pooled from two independent experiments); D35: n = 7 (data pooled from two independent experiments); D70: n = 16 (data pooled from four independent experiments).

(H) Comparison of the proportions of CD8⁺ T-cells derived from the T-cell (red) and bone marrow (green) grafts. D14 and D35: n = 3 mice/time point; D70: n = 7 (data pooled from three independent experiments).

(I) Comparison of the proportions of CD4⁺ T-cells derived from the T-cell (red) and bone marrow (green) grafts. D14 and D35: n = 3 mice/time point; D70: n =7 (data pooled from three independent experiments).

Data are presented as mean ± SEM. Significant differences calculated using Student’s unpaired t test (A-B) or one-way ANOVA with Tukey’s multiple comparisons test (E-H). *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.
Figure 3. Temporal and regional changes in donor-derived T cell infiltration. 

(A) Schematic of coronal brain section (based on Paxinos and Franklin Atlas, 2001) indicating the regions examined with immunofluorescence: hippocampus, habenula, meninges, cortex, and choroid plexus (located in the lateral ventricular spaces). Blue shaded region of the hippocampus identifies the dentate gyrus (DG) containing the granule cell layer (GCL). Surrounding regions include the cornu ammonis 1 (CA1) and 3 (CA3).

(B-F) Representative images of donor RFP+ T cell infiltrate in brain regions of interest (B: meninges, C: choroid plexus (in lateral ventricle outlined by white dashes), D: cortex, E: hippocampus, F: habenula) with respective quantification at days 14, 35, and 70 post-transplant. D14: n = 4-7 (data pooled from two independent experiments); D35: n = 6-7 (data pooled from two independent experiments); D70: n = 9-11 (data pooled from two independent experiments).

100X magnification, scale bars 50µm. Data are presented as mean ± SEM. Significant differences calculated with one-way ANOVA with Tukey’s multiple comparisons test. **p<0.01, ***p<0.001.
Figure 4. Differential expression of MHC class II by donor-derived macrophages and host microglia late post-transplant.

(A) Schematic of transplant regime for the identification of donor macrophages and host microglia. Lethally irradiated DBA2xF1.Csf1r-eGFP (H2\textsuperscript{bd}) mice received 5x10\textsuperscript{6} TCD BM with no T-cells or with 0.5x10\textsuperscript{6} CD3\textsuperscript{+} T-cells from C57Bl/6.Csf1r-mApple (H2\textsuperscript{b}) donors to induce low-grade, non-lethal chronic GVHD.

(B) Representative flow cytometry dot plots for the identification of donor macrophages (mApple) and host microglia (MacGreen) within the CD45\textsuperscript{dim}CD11b\textsuperscript{+} population from digested coronal sections of the brains of cGVHD mice at day 70 post-transplant. Gated on forward and side scatter to identify live (Sytox Blue\textsuperscript{neg}) CD45\textsuperscript{+}Ly6G\textsuperscript{neg} cells.

(C) Proportions of host microglia and donor BMDM within the CD45\textsuperscript{dim}CD11b\textsuperscript{+} population in cGVHD brains. D14: n = 3; D35: n = 7, (data pooled from two independent experiments); D70: n = 13, (data pooled from four independent experiments).

(D) Representative histograms of MHC class II expression on TCD microglia compared to GVHD host microglia and donor BMDM at days 35 and 70 post-transplant.

(E) Fold change of MHC class II mean fluorescence intensity (MFI) on GVHD host microglia and donor BMDM relative to TCD host microglia at days 35 and 70 post-transplant. D35: n = seven (data pooled from two independent experiments); D70: n = 9 (data pooled from two independent experiments).

(F) Representative 100X magnification confocal images demonstrating differential expression of MHC class II by donor macrophages (green arrows) and host microglia (white arrows) in situ in the hippocampus of GVHD and TCD mice 70 days post-transplant. B6.Csf1r-eGFP donors used to facilitate identification of donor-derived macrophages (GFP\textsuperscript{+}/Iba1\textsuperscript{+}) compared to resident microglia (GFP\textsuperscript{-}/Iba1\textsuperscript{+}). Nuclei counterstained with DAPI. 100X magnification, scale bar 10\textmu m.

(G) Findings replicated in a second model of chronic GVHD (D70 post-transplant) where BALB/c donor BM + CD3\textsuperscript{+} T-cells were transplanted into B6.Csf1r-eGFP recipients. MHC class II expression restricted to donor Iba1\textsuperscript{+}/GFP\textsuperscript{+} BMDM (green arrows) and absent from host Iba1\textsuperscript{+}/GFP\textsuperscript{-} microglia (white arrows). 100X magnification, scale bar 10\textmu m.

Data presented as mean ± SEM. Statistical significance calculated by one way ANOVA with multiple comparisons (C) or Student’s unpaired t test (E). **p<0.01, ***p<0.001.
Figure 5. Chronic CNS GVHD is associated with resident microglia proliferation and activation, and donor-derived macrophage infiltration.

(A) Allogeneic transplant model for the delivery of $5 \times 10^6$ TCD BM from C57Bl/6 (H2$^b$) donors expressing eGFP under the Csf1r promoter (B6.Csf1r-eGFP) with no T-cells or with $0.5 \times 10^6$ CD3$^+$ T-cells into lethally irradiated B6D2F1 (H2$^{bd}$) recipients.

(B-F) Representative confocal images of parenchymal brain regions of interest in GVHD and TCD mice, showing Iba1$^+$ microglia/macrophages, GFP$^+$ donor BMDM, and merged images counterstained with DAPI (cell nuclei). Green arrows identify donor-derived (GFP$^+$) macrophages and white arrows indicate resident (GFP$^-$) microglia. Stereological quantification of each region shows the total Iba1$^+$ cell population alongside the number of GFP$^+$ donor BMDM.

(B) Meninges at D35 and D100 post-transplant. 40X magnification, scale bar 30µm.

(C) Choroid plexus at D35 and D100 post-transplant. 40X magnification, scale bar 30µm. Dashed lines outline choroid plexus in the lateral ventricle adjacent to surrounding tissue.

(D) Cortex at D35 and D100 post-transplant. 20X magnification, scale bar 50µm.

(E) Hippocampal dentate gyrus at D35 and D100 post-transplant. 20X magnification, scale bar 50µm.

(F) Habenula at D35 and D100 post-transplant. 40X magnification, scale bar 30µm.

Data presented as mean ± SEM, n = 4-5 mice/group. Statistical differences calculated with Student’s unpaired t test, *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.
Figure 6. Donor-derived macrophages remain a transcriptionally and functionally distinct cell type in the CNS.

(A) Schematic of transplant regime for the identification of donor macrophages and host microglia. Lethally irradiated DBA2xF1.\(Csfr1\)-eGFP (H2\(^{bd}\)) mice received \(5 \times 10^6\) TCD BM with no T-cells or with \(0.5 \times 10^6\) CD3\(^+\) T-cells from C57Bl/6.\(Csfr1\)-mApple (H2\(^{b}\)) donors to induce low-grade, non-lethal chronic GVHD.

(B) Heatmap of log2 counts-per-million (logCPM) values for differentially expressed genes (FDR <0.05) from bulk RNA-seq analysis of sort-purified infiltrating donor BMDM compared to host microglia from GVHD brains at day 80 post-transplant. Expression across each gene have been scaled so that mean expression is zero and standard deviation is one. Plotted alongside is the z-score of the same genes from TCD host microglia (GVHD donor n = 6, GVHD host n = 4, TCD host n = 3).

(C) Heatmap of logCPM values from RNA-seq analysis data showing expression of genes related to characteristic cellular markers of GVHD donor BMDM, GVHD host microglia, and TCD host microglia. Colour key also applicable to (E) and (G).

(D) Representative histograms and enumeration of the MFI of characteristic markers CX3CR1 and P2RY12 on donor BMDM and host microglia at D70 (n = 6 mice/group).

(E) Dot plot of GO terms from the Biological Process ontology enriched in GVHD donor BMDM compared to GVHD host microglia.

(F) Heatmap based on RNA-seq analysis data showing differentially expressed genes related to extracellular matrix degradation and the CD300 family in GVHD donor BMDM, GVHD host microglia, and TCD host microglia.

(G) Differentially expressed genes related to IFN-\(\gamma\) signaling, response, and induction in GVHD donor BMDM, and host microglia from GVHD and TCD mice.

(H) logCPM values for selected cytokines from isolated cell populations in (B). Data presented as mean ± SEM. Statistical analysis performed with Student’s unpaired t test (D) or using the glmLRT() function in R for all possible group comparisons (H), ***p<0.001, ****p<0.0001.
Figure 7. Transcriptional changes in the hippocampus in chronic GVHD and attenuation of neuroinflammation by donor MHC II KO.

(A) Heatmap representing logCPM values of 381 differentially expressed genes (FDR <0.05) based on RNA-seq analysis of isolated hippocampi from TCD and GVHD mice 70 days post-transplant (n = 3 per group). Expression across each gene have been scaled so that mean expression is zero and standard deviation is one.

(B and C) Heatmap of logCPM values from TCD and GVHD mouse hippocampi for genes related to (B) the interferon-gamma response hallmark gene set and (C) antigen presentation as identified by IPA analysis of RNA-seq data (n =3 per group).

(D) Heatmap of logCPM values for genes involved in synaptic signalling as identified by GO analysis (GO: 0045202). Enrichment plot from GSEA demonstrating downregulation of genes associated with the neuron-to-neuron synapse in GVHD compared to TCD. NES = normalised enrichment score, FDR = false discovery rate.

(E) Quantification of synaptophysin and PSD95 puncta expression in the hippocampus at day 35 (n = 8 mice/group, pooled from two independent experiments) and 70 (n = 6-11 mice/group) post-transplant.

(F) Schematic for the transplant of wild type (PTPxC57Bl/6) or MHC II KO.B6 (H2b) bone marrow depleted of T-cells into irradiated DBA2xF1 (H2Dd) recipients.

(G) Clinical scores of transplant recipients. Data pooled from two independent experiments, n = 11-12 mice/group).

(H) Representative images of haematoxylin and eosin staining of skin from recipient mice at 70 days post-transplant showing dermal thickening and loss of subcutaneous fat as evidence of scleroderma. Original magnification, x 10, scale bar 300µm.

(I) Time spent floating (immobile) and swimming (mobile) in the forced swim test (n = 4 mice/group) at D70.

(J) Representative confocal images of Iba1+ cells in the hippocampus at day 70 post-transplant. Resident microglia express GFP and are indicated with white arrows. 20X magnification, scale bar 50µm.

(K) Representative confocal images demonstrating morphological similarities in the resident microglia phenotype of recipients of MHC II KO TCD BM and WT TCD BM. 100X magnification, scale bar 10 µm.

(L and M) mRNA expression of selected genes detected by RT-qPCR in the (L) coronal brain section and (M) hippocampus of transplant recipients 70 days post-transplant. Reported
as the fold change of the expression from the WT TCD BM group, calculated relative to the 
Hpri gene. n = 3-6 mice/group. 
Data are presented as mean ± SEM. Significant differences calculated using two-way 
ANOVA (G) or Student’s unpaired t test (E, M), *p<0.05, **p<0.001.
