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Abstract

Acute graft-versus-host disease (GVHD) can affect the central nervous system (CNS). The role of microglia in CNS-GVHD remains undefined. In agreement with microglia activation, we found that profound morphological changes, MHC-II- and CD80-upregulation occurred upon GVHD induction. RNA-sequencing-based analysis of purified microglial obtained from mice with CNS-GVHD revealed TNF upregulation. Selective TNF gene deletion in microglia of Cx3cr1<sup>creER</sup>:Tnf<sup>fl/-</sup> mice reduced MHC-II-expression, decreased CNS T-cell infiltrates and VCAM-1<sup>-</sup> endothelial cells. GVHD increased microglia TGF-β-activated kinase-1 (TAK1) activation and NF-κB/p38-MAPK-signaling. Selective Tak1-deletion in microglia using Cx3cr1<sup>creER</sup>:Tak1<sup>fl/fl</sup> mice resulted in reduced TNF-production, microglial MHC-II, and improved neurocognitive-activity. Pharmacological TAK1-inhibition reduced TNF-production and MHC-II-expression by microglia, Th1 and Th17 T-cell infiltrates, VCAM-1<sup>-</sup> endothelial cells and improved neurocognitive activity, without blocking graft-versus-leukemia effects. Consistent with these findings in mice, we observed increased activation and TNF-production of microglia in the CNS of GVHD-patients.

In summary, we prove a role for microglia in CNS-GVHD, identify the TAK1/TNF/MHC-II axis as mediator of CNS-GVHD and provide a TAK1 inhibitor-based approach against GVHD-induced neurotoxicity.
**Introduction**

Acute graft-versus-host disease (GVHD) is a life-threatening complication after allogeneic hematopoietic cell transplantation (allo-HCT). About 50% of the patients with severe acute GVHD fail to respond to corticosteroids, and steroid-refractory severe GVHD has a dismal prognosis with a 1-year survival rate of less than 20% (1). GVHD was classically considered to involve only the skin, the intestinal tract and the liver, which was defined as the "tissue tropism of acute GVHD". However, there is increasing evidence that the effects of acute GVHD are not limited to the three classical target organs, but can also occur in the central nervous system (CNS). Animal studies showed that the transfer of allogeneic T cells caused CNS infiltration by effector memory T cells during GVHD and apoptosis in neurons, as well as reduced exploratory activity, spatial learning and memory in the recipient mice (2). Evidence for CNS-GVHD was not restricted solely to the murine model, as other investigators reported that CNS infiltration by CD8⁺ T cells was a key feature of GVHD in non-human primates (3). Conversely, treatment of primates with immunoprophylaxis after allo-HCT reduced the abundance of T cell infiltration into the brain (3). Consistent with findings in preclinical models, human brain analysis of female sex-mismatched bone marrow transplant recipients have identified donor (Y-chromosome⁺) derived cell infiltrates (4). In agreement with the occurrence of CNS-GVHD in patients, neurological deficits and MRI findings have been reported in patients developing GVHD (5).

The role of microglia for CNS-inflammation during GVHD has remained unclear. Here we show for the first time that GVHD caused activation of microglia cells. Based on selective genetic ablation of TGF-β-activated kinase-1 (TAK1) or tumor necrosis factor (TNF) in microglia we identify the TAK1/ TNF/MHC-II axis as a central mediator of CNS-inflammation. In a translational approach we show that TAK1 inhibition reduces GVHD-induced neurotoxicity in mice, which provides a scientific rationale for testing this approach in a phase-I trial in humans.

**Results:**

**Microglia activation and expansion are features of CNS-GVHD**

To determine if GVHD induction in our models caused T cell infiltration into the CNS as a characteristic feature of CNS-GVHD (2), we analyzed the brain of mice that underwent syngeneic (syn-HCT) or allogeneic HCT (allo-HCT). The abundance of CD3⁺ T cells increased in the cortex and the meninges of mice that underwent allo-HCT but not syn-HCT (Figure 1A-D). Allo-HCT also led to an increased frequency of CD11b⁺CD45⁺high cells (Figure
The CD11b⁺CD45^{high} cells in the CNS of mice undergoing allo-HCT included dendritic cells, macrophages and monocytes (Supp. Figure 1A). Of these myeloid cells, monocytes were most abundant with a median of 23% (range 3 to 35%) of the CD11b⁺CD45^{high} cells. To quantify the contribution of donor derived monocytes to the increased TNF production and MHC-II expression, we used donor mice lacking CCR2, which exhibit reduced monocyte migration. We observed no reduction of TNF production or MHC-II expression in the group that had received the BM graft from CCR2^{−/−} donors (Suppl. Figure 1B-C).

We hence next analyzed the morphology of microglia cells. We observed that the filament dendrite length, the number of dendrite segments, branching points and dendrite terminal points declined in mice that developed GVHD compared to mice that underwent syn-HCT or untreated mice (Figure 1G-K). Comparable morphological changes have been previously reported as features of microglia activation in autoimmune disease of the CNS (6). In aggregate these findings show that profound morphological changes indicative for microglia activation occur upon CNS-GVHD induction.

**MHC class II and CD80 expression is increased on microglia cells of mice developing GVHD**

The CNS of mice undergoing allo-HCT harbored increased numbers of Iba-1⁺ microglia cells on day 14 post allo-HCT compared to syn-HCT (Figure 2A, B). Conversely the microglia decreased on day 7 in both groups receiving total body irradiation (Suppl. Figure 1D). To characterize the transcriptional profile of microglia under GVHD conditions, we next isolated microglia based on CD11b and CD45^{low} expression from mice undergoing allo-HCT versus syn-HCT. RNAseq analysis showed close clustering of individual samples belonging to one group (Figure 2C). Microglia isolated from mice developing GVHD displayed a strong upregulation of genes involved in antigen presentation, compared to untreated mice or mice having undergone syn-HCT (Figure 2D). In line with the RNAseq results, the microglia cells (CD11b⁺CD45^{low}) expressed higher protein levels of MHC-II and CD80 on their surface, which have both been shown to be activation and maturation markers of myeloid cells (Figure 2E-H). We also observed reduced expression of CX3CR1 on microglia upon GVHD induction (Figure 2I, J) which is consistent with reports showing that this chemokine receptor declines on microglia upon activation (7).

**Microglia cells exhibit increased TNF production upon CNS-GVHD and genetic deletion of TNF in microglia reduces disease activity**
To further characterize the transcriptome of the activated microglia cells under GVHD conditions we next analyzed their cytokine expression in mice undergoing allo-HCT versus syn-HCT. RNAseq analysis showed that multiple pro-inflammatory cytokines increased in microglia cells from the allo-HCT group (Figure 3A). A major GVHD-related cytokine that can mediate local cytotoxic effects is TNF. We hypothesized that TNF could be responsible for local tissue damage in the CNS because of its cytotoxic activity. To validate the findings on the protein levels we next analyzed microglia by flow-cytometry and found increased TNF production in mice that underwent allo-HCT compared to the syn-HCT group (Figure 3B, C).

To probe if microglial TNF plays a functional role in CNS-GVHD, we next induced GVHD in Cx3cr1creER: Tnffl/− mice, that lack TNF in microglia. The Cx3cr1creER model was chosen because following tamoxifen induced gene deletion short living peripheral CX3CR1 positive monocytes and DCs are rapidly replenished within 4 weeks of tamoxifen induction and hence re-express the target gene while in contrast the long-living microglia cells maintain the gene deletion (6). We found that Cx3cr1creER: Tnffl/− mice exhibited lower frequencies of CD3+ cells in the cortex and meninges compared to littermate controls (Figure 3D-F). RNA sequencing of microglia showed a reduction of Tnf, confirming the gene ablation, as well as of Cd74 and MHC-II (H2-Eb1) in Cx3cr1creER: Tnffl/− mice compared to littermate controls (Figure 3G).

These studies identify TNF as a major pathogenic cytokine in CNS-GVHD based on unbiased RNA sequencing of microglia and functional gene deletion using the Cre/lox system.

GVHD enhances pro-inflammatory MAPK/NF-κB/TAK1 signaling in microglia cells

Since TNF, IL-6, IL-12, IL-18 and IL-1β, which we had found in the RNAseq analysis of the microglia, activate various signaling cascades in immune cells, we next aimed at understanding which signalling events were responsible for the observed microglia activation. Transcriptome analysis of microglia showed upregulation of multiple signaling molecules including Map3k7, encoding TGF-β- activated kinase 1 (TAK1) (Figure 4A). In agreement with this upregulation we found increased phosphorylation of p38 MAPK indicating the activation of MAPK/NF-κB signaling pathway, in mice developing CNS-GVHD (Figure 4B, C). This finding is consistent with our observation that TNF expression is increased in microglia and with published data showing that TAK1 / NF-κB signaling triggers TNF production (8).

TNF can be induced by NF-κB signaling but can also by itself amplify NF-κB pathway activity. Therefore, we studied the effect of TNF on NF-κB signaling in microglia in vitro by exposing the cells to increasing TNF concentrations. We observed that TNF caused activation of TAK1, JNK and p65 (Figure 4D-I). In agreement with this result IκB was increasingly
degraded as a sign for higher NF-κB activity (Figure 4J). These findings show that the TAK1/NF-κB signaling cascade is active in microglia and can be induced in vitro by TNF exposure.

**Deletion of TAK1 in microglia reduces CNS-GVHD**

It was previously shown that upon stimulation TAK1 and its adaptors, TAB2, TAB3 and NEMO, are recruited to the polyubiquitinated receptor (TNFSF)-interacting serine-threonine kinase 1 (RIPK1), in turn allowing TAK1 to phosphorylate and activate the catalytic inhibitor of IκB kinase (IKK) subunits (9). The resulting IKK activation induces expression of multiple cytokines and chemokines. In this context TAK1 was shown to be a central mediator in pro-inflammatory cytokine signaling, including TRAF6/TNF (10). Induction of GVHD in Cx3cr1creER: Tak1fl/fl mice that lack TAK1 in microglia but not peripheral macrophages (Suppl. Figure 1E-H), caused reduced CD3+ T cell frequencies in the meninges and cortex compared to littermate controls (Figure 5A-D).

In line with reduced CNS inflammation, Cx3cr1creER: Tak1fl/fl mice exhibited lower numbers of Iba-1+ cells in the meninges and cortex compared to littermate controls (Figure 5E, F). Also lower MHC-II levels were found on microglia of Cx3cr1creER: Tak1fl/fl mice compared to littermate controls (Figure 5G, H). Consistent with a concept that TAK1 promotes TNF production we observed lower TNF expression in microglia of Cx3cr1creER: Tak1fl/fl mice compared to littermate controls (Figure 5I, J).

To analyze if the CX3CR1-Cre system was specifically active after tamoxifen treatment we used Cx3cr1creER: Tak1fl/fl mice without tamoxifen treatment. There was no evidence for TAK1 deletion in Cx3cr1creER: Tak1fl/fl mice without tamoxifen treatment (Suppl. Figure 1I, J). Also there was no evidence for Cre expression in neurons of Cx3cr1creER: R26 tomato reporter mice (Suppl. Figure 1K). We observed no reduction of TNF or MHC-II in the Cx3cr1creER: Tak1fl/fl mice that were not treated with tamoxifen post allo-HCT in comparison to Tak1fl/fl mice undergoing allo-HCT (Suppl. Figure 2A, B).

The TAK1-deletion effect was restricted to the CNS as GVHD-related mortality was not different when Cx3cr1creER: Tak1fl/fl mice and littermates were compared (Figure 5K). Consistent with similar survival rates, the histopathological scoring of peripheral GVHD organs including small intestines, large intestines and liver was not different between Cx3cr1creER: Tak1fl/fl mice and littermate controls (Suppl. Figure 2C-E).

However, consistent with improved neurocognitive function, Cx3cr1creER: Tak1fl/fl mice exhibited higher exploratory activity in the Elevated Plus Maze test compared to littermate controls (Figure 5L). Additionally, the novel object recognition test showed that Cx3cr1creER: Tak1fl/fl mice had a better recognition memory compared to controls (Figure 5M). The differences in motoric function were not due to a general weakness of the mice, as the
Cx3cr1\textsuperscript{creER}:\textit{Tak1}\textsuperscript{fl/fl} mice and controls exhibited similar grip strength (Figure 5N). Also the neurocognitive differences were not due to a loss of vision as the Cx3cr1\textsuperscript{creER}:\textit{Tak1}\textsuperscript{fl/fl} mice and controls exhibited similar results in the visual cliff test (Suppl. Figure 2F).

These findings indicate that a selective deletion of TAK1 in CX3CR1 positive microglia reduces inflammatory T cell infiltration into the brain, reduces Iba-1\textsuperscript{+} microglia, MHC-II and TNF expression by microglia and promotes neurocognitive function of mice developing GVHD without blocking peripheral GVHD.

**Therapeutic TAK1 inhibition reduces inflammatory features of microglia during CNS-GVHD**

To test for a potentially translational approach we next treated mice with two different TAK1 inhibitors, Takinib and 5Z-7-Oxoezaenol (5-Oz) in comparison to vehicle and then analyzed the CNS. We observed that TAK1 inhibition reduced the expression of MHC-II and TNF compared to the vehicle-treatment (Figure 6A-D). Additionally TAK1 inhibition reduced the number of CD3\textsuperscript{+} T cells and Iba1\textsuperscript{+} microglia compared to the vehicle-group (Figure 6E-I).

In addition to the decreased accumulation of total CD3\textsuperscript{+} T cells in the CNS upon TAK1 inhibition we observed a decrease of CD4\textsuperscript{+}IFN\textgamma\textsuperscript{+} T cells (Th1 cells) and CD4\textsuperscript{+}IL-17\textsuperscript{+} T cells (Th17 cells) in the CNS of mice undergoing allo-HCT upon TAK1 inhibition compared to the vehicle group (Figure 6J-M). Other T cell subpopulations including naive T cells, central memory T cells, effector memory T cells and CD4\textsuperscript{+}IL-4 T cells (Th2 cells) were not different in the takinib group compared to the vehicle group (Suppl. Figure 3A-D). We observed a non-significant trend towards an increase of CD4\textsuperscript{+}FoxP3\textsuperscript{+} T regulatory cells (Treg) in the CNS of mice undergoing allo-HCT upon TAK1 inhibition compared to the vehicle group (Suppl. Figure 3E).

To understand why T cell infiltration was reduced in the CNS of mice that were treated with the TAK1-inhibitor or lacking TNF in CX3CR1\textsuperscript{+} cells, we studied molecules that are required for T cell extravasation. T cell adhesion to endothelial cells is mediated via VCAM-1 and ICAM-1 (11), which then allows T cells to exit the vessel and enter the CNS.

We found reduced expression of VCAM-1 and ICAM-1 on endothelial cells (CD31\textsuperscript{+} CD105\textsuperscript{+}) in the CNS of mice treated with takinib compared to mice treated with vehicle (Figure 7A-E, Suppl. Figure 4A). Also we found a reduced number of VCAM-1\textsuperscript{+} endothelial cells in the CNS of Cx3cr1\textsuperscript{creER}:\textit{Tnf}\textsuperscript{fl/fl} mice (TNF deletion) compared to \textit{Tnf}\textsuperscript{fl/fl} (no TNF deletion) mice (Figure 7F, G). These findings support the concept that the TAK1/TNF axis promotes VCAM-1 expression on endothelial cells. Genetic deletion of TNF or TAK1 inhibition reduces VCAM-1 expression during CNS-GVHD.
These findings in the CNS of TAK1 inhibitor treated mice were connected to improved neurocognitive function as the TAK1 inhibitor group exhibited more frequent entries into open arm in the Elevated Plus Maze test compared to the vehicle group (Figure 7H). The novel object recognition test showed that the TAK1-inhibitor treated group performed better compared to the vehicle treated group (Figure 7I). The differences in motoric function were not due to a general weakness of the TAK1 inhibitor-treated mice as they exhibited similar grip strength (Suppl. Figure 5A). Also the neurocognitive differences were not due to a loss of vision as the TAK1 inhibitor-treated mice and the vehicle-treated mice exhibited similar results in the visual cliff test (Suppl. Figure 5B). In order to be potentially applicable in a clinical setting, engraftment of the donor hematopoietic system is essential. We observed that the TAK1 inhibitor-treated mice and the vehicle-treated mice exhibited similar engraftment (Suppl. Figure 5C) while there was no change of GVHD scores in the small and large bowels but a reduced liver GVHD score in the TAK inhibitor-treated mice (Suppl. Figure 5D-F). TAK1 inhibitor-treated animals exhibited a lower frequency of monocyte infiltrates or activated microglia compared to the vehicle-treated mice (Suppl. Figure 5G, H).

To evaluate the impact of takinib treatment on graft-versus-leukemia (GVL) activity we used a genetic AML model (FLIT3-ITD / MLL-PTD) and a cell line based AML model (WEHI-3B) (12). The AML cells were on the same genetic background as the recipient mice to mimic the clinical situation. In both models the group that received T cells experienced an improved survival compared to the group that received AML cells and BM only, indicating the GVL effect. The treatment with takinib did not reduce the GVL effect in both leukemia models (Figure 7J, K).

Microglia cells expand and produce TNF in human CNS-GVHD

To understand if patients developing acute GVHD had comparable changes in microglia cells as we had observed in mice, we studied the brains of patients who died after allo-HCT. We observed an increased number of Iba-1⁺ microglia in patients who developed acute GVHD compared to patients who underwent allo-HCT but did not develop GVHD. The increase was found in grey and white matter of the frontal lobe (Figure 8A-D). TNF in microglia was visualized by IHC for Iba-1 and TNF. The frequency of TNF and Iba-1 double positive microglia was significantly increased in patients that had developed acute GVHD (Figure 8E, F). In agreement with CNS-GVHD these patient exhibited also higher CD3⁺ T cell frequencies Figure 8G, H). These observations made in patient samples indicate that major findings made in the mouse model may also hold true for humans.
In addition, we reviewed the literature for cases of CNS-GVHD and listed the reports in Suppl. Table 4. In the majority of the cases of CNS GVHD the diagnosis was histology proven supporting the concept that CNS GVHD is a clinically relevant complication.

To further elucidate the potential clinical relevance of CNS GVHD we analyzed the frequency of neurological symptoms that occurred at the same time when a patient developed acute GVHD grade II-IV in 503 patients undergoing allo-HCT at Freiburg University Hospital, Germany. Neurologic symptoms related to infection or vascular events were not included. The time interval included the first 2 years after allo-HCT because acute GVHD occurs in 90% of all cases within this time interval. The multivariable logistic regression analysis included presence of acute GvHD grade II-IV, donor type (MRD, MUD, MMUD, haploidentical donor), conditioning (MAC vs RIC), gender and age >40 vs <40 years as possible risk factors for the occurrence of neurologic complications. We found that development of acute GVHD grade II-IV was independently associated with increased risk of noninfectious neurologic complications with an Odds ratio of 491 and a P < .0001 (Suppl. Tables 5 - 6). Our results are consistent with a recent retrospective analysis on the incidence and risk factors of noninfectious neurologic complications in 971 consecutive patients with hematologic malignancies undergoing allo-HCT (13). The authors found in multivariable analysis, that development of acute GVHD grade II-IV was independently associated with increased risk of noninfectious neurologic complications with a hazard ratio of 3.3 and a P < .00001.

Despite these reports it is important that other diseases like progressive multifocal leukoencephalopathy or viral encephalitis (e.g. HSV, CMV or VZV) are ruled out before CNS-GVHD is diagnosed in a patient with GVHD developing neurological symptoms.

**Discussion**

The clinical picture of acute GVHD is often connected to neurological deficits in patients, morphological CNS white matter changes detectable by magnetic resonance imaging and intraparenchymal lymphocytic infiltration of the brain upon autopsy (5, 14). While previous work had shown that T cells contribute to CNS-GVHD (2, 3), the role of microglia cells had not been studied. We have previously extended the concept that GVHD is a purely T cell-mediated disease by showing that myeloid cells including neutrophils (15) and monocytes (16) contribute to GVHD development by tissue damage and MHC-II dependent antigen presentation (17). Here we show that microglia, a prototypical tissue macrophage population in the CNS, contributes significantly to the pathogenesis of CNS-GVHD.
Our first observation that microglia cells exhibit an activated phenotype during GVHD with reduced numbers of branches is consistent with findings in CNS autoimmunity models describing this phenotype when experimental autoimmune encephalomyelitis (EAE) evolves (6). Our observation that CX3CR1 expression on microglia declined during GVHD is consistent with reports showing high CX3CR1 expression on resting microglia and a decline upon microglia activation (7). The morphological microglia changes occurred in parallel to the upregulation of MHC-II and CD80, which allow for antigen presentation to donor T cells and co-stimulation of these T cells, respectively. In agreement with this concept in CNS-GVHD it was shown that also non-hematopoietic antigen presenting cells contribute to peripheral GVHD, while classical dendritic cells were dispensable (18). Comparable to our findings in CNS-GVHD, others have shown in EAE that myeloid antigen presenting cells are recruited to the brain which correlated with disease severity (19, 20) and that these cells present antigen to autoreactive T cells to mediate CNS inflammation (21).

In addition we found increased levels of TNF in microglia during CNS-GVHD. TNF has direct cytotoxic effects on cells during GVHD (22) which could be responsible for the neuronal death observed previously after allo-HCT (2). Consistent with the concept that TNF plays a major role in CNS-GVHD we found that T cell infiltration into the CNS and MHC-II expression on microglia were reduced when microglia cells were deficient for TNF.

We observed that TNF also increased in the syn-HCT group, although not to the same extend as in the allo-HCT group, which is likely due to the TBI-induced damage. The observation that CD3⁺ T cells are not increased in the syn-HCT groups indicate that a higher level of TNF may be required to attract T cells to the CNS, as it is the case in the allo-HCT group.

Our finding that Th1 and Th17 cells declined in the CNS upon TAK1 inhibition is compatible with findings in other models of CNS inflammation. Th1 and Th17 cells were shown to be pro-inflammatory and to promote the development of EAE (23, 24).

In our studies we also identify increased TAK1 activation and NF-κB/p38 MAPK signaling activity in microglia during GVHD and show a functional role for TAK1 as selective Tak1-deletion in microglia reduced TNF-production, T cell infiltrates in the CNS and MHC-II levels. Mechanistically, the TAK1/TNF axis was connected to VCAM-1 expression on endothelial cells. Genetic deletion of TNF or TAK1 inhibition reduced VCAM-1 expression during CNS-GVHD. T cell adhesion to endothelial cells is mediated via VCAM-1, which then allows T cells to exit the vessel and enter the CNS (11), supporting the concept that TAK1-inhibition reduced TNF and consecutively VCAM-1 expression, thereby decreasing donor T cell infiltration into the CNS. Consistent with a functional role for TAK1 in CNS-GVHD the neurocognitive function of mice lacking TAK1 in microglia was improved. Besides the genetic Tak1 deletion approach we also show that pharmacological TAK1-inhibition reduced TNF-
production and MHC-II expression by microglia cells and improved neurocognitive activity. Our previous work using Janus kinase inhibitors has shown the high potential of kinase inhibition to prevent or treat GVHD in mice (25) and patients (26). Our findings are potentially clinically relevant as the treatment with the TAK-1 inhibitor takinib did not affect GVL effects in two AML models. In agreement with the functional role of microglia derived TNF for CNS GVHD in mice, we observed an increase of the microglia cell number and their TNF production in the CNS of patients who developed GVHD.

In aggregate we report a role for microglia in CNS-GVHD and identify the TAK1/TNF/MHC-II axis as a central mediator of this disease. Additionally, we provide a serine-threonine kinase inhibitor-based approach against GVHD-induced neurotoxicity identified based on RNA sequencing analysis of the CNS.

**Material and Methods:**

**Mice**

C57BL/6 (H-2Kb, Thy-1.2) and BALB/c (H-2Kd, Thy-1.2) mice were purchased either from Janvier Labs (France) or from the local stock of the animal facility at University of Freiburg. \(Tak1^{fl/fl}\) and \(Cx3cr1^{creER}\cdot Tak1^{fl/fl}\) (6), \(Cx3cr1^{creER}\cdot R26-yfp\) (6), CCR2\(^{-/-}\) mice (27), and \(Cx3cr1^{creER}\cdot R26\)-Tomato reporter mice were bred in the animal facility at University of Freiburg. \(Tnf^{fl/fl}\) and \(Cx3cr1^{creER}\cdot Tnf^{fl/fl}\) (27) mice were bred at the Weizmann Institute of Science, Rehovot, Israel. Mice were used between 6 and 14 weeks of age and only female or male donor/recipient pairs were used.

**Bone marrow (BM) transplantation model and histopathology scoring**

BM transplantation experiments were performed as previously described (12, 28). Briefly, recipients were exposed to lethal irradiation of 9-11 Gy. CD4\(^+\) and CD8\(^+\) T cells were isolated from donor spleens and enriched by positive selection with the MACS cell separation system (Miltenyi Biotec, USA) according to the manufacturer’s instructions. Anti-CD4 and anti-CD8 MicroBeads were used. Alternatively, T cells were isolated from donor spleens by negative selection (Pan T Cell Isolation Kit-II), with the MACS cell separation system (Miltenyi Biotec, USA) according to the manufacturer’s instructions. CD4\(^+\)/CD8\(^+\) T cell purity was at least 90% as assessed by flow cytometry (data not shown). To induce GVHD, \(5\times 10^6\) bone marrow cells and CD4\(^+\)/CD8\(^+\) T cells from a major MHC mismatched donor (allogeneic stem cell transplantation) were given at a dosage of \(3\times 10^5\) (C57BL/6-derived into BALB/c) or \(8\times 10^5\) (BALB/c-derived into C57BL/6) i.v. on day 0. For behavioral studies, sublethal dose of \(5\times 10^5\) BALB/c-derived T cells were used. As a control, syngeneic stem cell transplantation (Syn-HCT) was performed by injecting the same amount of BM and T cells from a donor mouse.
with the same genetic background as that of the recipient. Slides of small intestine, large intestine, and liver specimens collected after allo-HCT were stained with Hematoxylin/Eosin and scored by an experienced pathologist blinded to the treatment groups. GVHD severity was determined according to a previously published histopathology scoring system (29).

All other methods can be found in the Suppl. Methods part.

**Statistical analysis**

For the sample size in the murine GVHD survival experiments a power analysis was performed. A sample size of at least n=8 per group was determined by 80% power to reach a statistical significance of 0.05 to detect an effect size of at least 1.06. Differences in animal survival (Kaplan-Meier survival curves) were analyzed by Mantel Cox test. The experiments were performed in a non-blinded fashion except for the GVHD severity scoring, the analysis of human tissue specimens and behavioural experiments with the knockout mice. To obtain unbiased data, a pathologist blinded to both the genotype and the treatment group performed the histopathological scoring of GVHD severity. Only after finalization of the quantitative GVHD severity scores the samples were allocated to their genotypes/treatment group. Analysis of human brain specimens was also performed in a blinded manner and the samples were allocated to the group after finalization of the analysis. All samples or mice were included in our analysis.

For statistical analysis of 2 groups an unpaired 2 tailed Student’s *t* test was applied. All data were tested for normality applying the Kolmogorov-Smirnov test. If the data did not meet the criteria of normality, the Mann-Whitney *U* test was applied. If more than 2 groups were analyzed we used the Kruskal-Wallis-Test if non-parametric testing was suggested and we performed a one-way ANOVA in case of normally distributed data. Statistical analysis was performed using GraphPad Prism (GraphPad Software; San Diego, CA). Data are presented as mean and s.e.m. (error bars). Differences were considered significant when the *P*-value was <0.05.

**Data and materials availability:** RNA sequencing data are deposited in the database GEO, GEO accession GSE141663.

**Study approval**

**Human tissue analysis and patients**
The study included formalin-fixed and paraffin-embedded (FFPE) tissue specimens of different brain regions from patients that died unrelated to allo-HCT, patients who died after allo-HCT without GVHD and patients who died after allo-HCT and had GVHD (grade 3-4). The study has been approved by the local ethics committee (protocol no.: 547/14; Ethic committee, Albert-Ludwigs-University, Freiburg, Germany). Patients who underwent autopsy within a time period of 16 years (2001-2017) at the Institute of Neuropathology, University Hospital Freiburg, Germany were analyzed. Patient characteristics are shown in Suppl. Tables 1-3.

A retrospective analysis of 503 patients undergoing allo-HCT at Freiburg University Medical Center with at least a follow-up of 12 months was performed. The analysis was approved by the Institutional Ethics Review Board (IRB) of the Medical Center at the University of Freiburg, Germany (Protocol numbers: 10024/13, 26/11 and 509/16). Written informed consent was obtained from each patient. All analyses of human data were carried out in compliance with the relevant ethical regulations. The analysis included neurological symptoms that occurred at the same time when a patient developed acute GVHD grade II-IV. Neurological symptoms related to infection or vascular events were not included. The observation time interval included the first 2 years after allo-HCT because acute GVHD occurs in 90% of all cases within this time interval. The multivariable logistic regression analysis included presence of acute GVHD grade II-IV, donor type (MRD, MUD, MMUD, haploidentical donor), conditioning (MAC vs RIC), gender and age >40 vs <40 years as possible risk factors for the occurrence of neurologic complications. Patients characteristics are provided in Suppl. Tables 5 - 6.

Mice
Animal protocols (Protocol numbers: G-13/045, G13-116, G-15/018, G-17/063, X-13/07J, X-15/10A) were approved by the Regierungspräsidium Freiburg, (regional council), Germany (Federal Ministry for Nature, Environment and Consumers Protection). Animals at the Weizmann Institute of Science were handled according to protocols approved by the Weizmann Institute Animal Care Committee as per international guidelines.

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**Author contributions:**

N.R.M. and J.M.V. performed the majority of the murine and cell line experiments and helped to develop the overall concept and writing of the manuscript. The authorship order of the two first authors was assigned based on the first key experiments that were performed and analyzed by N.R.M.. P.A., D.E and G.I. collected and analyzed patient samples. J-S.K performed cell sorting. S.H, K.H, N.K, D.S and T.G helped with in vivo experiments. G.A, M.B and L.C-M performed RNA sequencing and analyzed the data. T.B. helped to develop the concept and provided help with behavioral studies in mice. J.D., J.F., B.R.B., S.J., M.P., contributed to critical analysis of the data, developed concepts and provided reagents. R.Z. developed the overall concept, supervised the experiments, analyzed data and wrote the manuscript.

**References:**


Figure 1: Microglia display activated morphology and T cells infiltrate the CNS during GVHD

(A-D) Histology of brain samples immuno-stained for CD3+ T cells (brown) from untreated BALB/c mice (n=10) or BALB/c mice on day 14 after syn-HCT (n=9) or after allo-HCT (n=11)
as indicated. (A, C) Representative image from each group are shown. Scale bars, 50µm.
(B, D) The scatter plot shows the number of CD3+ T cells (per mm²) in cerebral meninges and cortex. The experiment was repeated two times and the results (mean ± s.e.m.) were pooled. The $P$-values were calculated using one-way ANOVA.
(E, F) Flow cytometry for CD45^{hi} cells from CD11b^{+} cells in the CNS from untreated BALB/c mice (n=10) or BALB/c mice on day 14 after syn-HCT (n=10) or after allo-HCT (n=11) as indicated. (E) A representative flow cytometry plot from each group is shown. (F) The scatter plot shows the quantification of CD45^{hi} cells among CD11b^{+} cells from different groups as indicated. The experiment was repeated three times and the results (mean ± s.e.m.) were pooled. The $P$-values were calculated using the one-way ANOVA.
(G) Representative images showing Imaris (Bitplane)-based 3D-reconstruction of Iba-1^{+} microglia cells from untreated BALB/c mice or BALB/c mice on day 14 after syn-HCT or allo-HCT as indicated. Scale bar, 10µm
(H-K) Scatter plots showing Imaris-based automated quantification of microglial morphology from microglia cells of untreated BALB/c mice (n=6) or BALB/c mice on day 14 after syn-HCT (n=6) or allo-HCT (n=6) as indicated. The experiment was repeated two times and the results (mean ± s.e.m.) were pooled. The $P$-values were calculated using the one-way ANOVA.
Figure 2: Microglial numbers and co-stimulatory molecules are increased during GVHD

(A) Histology of brain samples immunostained for Iba-1⁺ cells from untreated BALB/c mice or BALB/c mice on day 14 after syn-HCT or allo-HCT as indicated. Scale bar, 100µm

(B) The scatter plot shows the number of Iba-1⁺ cells (per mm²) in cerebral cortex from untreated BALB/c mice (n=10) or BALB/c mice on day 14 after syn-HCT (n=9) or allo-HCT
(n=11) as indicated. The experiment was repeated two times and the results (mean ± s.e.m.) were pooled. The $P$-values were calculated using the one-way ANOVA.

(C) Principle component (PC) analysis of RNAseq analysis of sorted microglia cells isolated from the CNS of untreated BALB/c mice (n=4) or BALB/c mice on day 14 after syn-HCT (n=4) or allo-HCT (n=4).

(D) Heat map based on RNAseq showing the top 20 genes involved in antigen processing and presentation from microglia of untreated BALB/c mice (n=4) or BALB/c mice on day 14 after syn-HCT (n=4) or allo-HCT (n=4). Colour code represents the Z-score log2 intensity.

(E-F) The scatter plot shows the quantification (fold change of MFI) and respective flow cytometry plots of MHC-II expression on microglia (CD45$^{low}$ CD11b$^+$) from brains of untreated BALB/c mice (n=10) or BALB/c mice on day 14 after syn-HCT (n=10) or allo-HCT (n=11) as indicated. (G-J) The scatter plot shows the quantification (fold change of MFI) and respective flow cytometry plots of CD80 (G, H) and CX3CR1 (I, J) expression on microglia (CD45$^{low}$ CD11b$^+$) from brains of untreated BALB/c mice (n=10) or BALB/c mice on day 14 after syn-HCT (n=17) or allo-HCT (n=18) as indicated. The experiment was repeated three times and the results (mean ± s.e.m.) were pooled. The $P$-values were calculated using the one-way ANOVA.
Figure 3: Microglia-derived TNF is essential for the infiltration of T cells into the brain

(A) Heat map based on RNAseq showing the top 20 differentially regulated cytokines from microglia of untreated BALB/c mice (n=4) or BALB/c mice on day 14 after syn-HCT (n=4) or allo-HCT (n=4). The colour code represents the Z-score log2 intensity.

(B) A representative flow cytometry plot showing intracellular TNF expression in microglia (CD45<sup>low</sup> CD11b<sup>+</sup>) from brains of untreated BALB/c mice or BALB/c mice on day 14 after allo-HCT or syn-HCT.

(C) The scatter plot shows the quantification (fold change of MFI) of intracellular TNF expression in microglia from the CNS of untreated BALB/c mice (n=13) or BALB/c mice on day 14 after syn-HCT (n=18) or allo-HCT (n=18) as indicated. The experiment was repeated three times and the results (mean ± s.e.m.) were pooled. The $P$-values were calculated using the one-way ANOVA.
(D-F) Histology of brain samples for CD3⁺ T cells from Tnf⁻/⁻ (n=9) and Cx3cr1creER Tnf⁻/⁻ (n=10) mice on day 14 after allo-HCT as indicated. (D) Representative images showing meningeal CD3⁺ T cells in each group. Scale bars, 50µm. (E, F) The scatter plot shows the number of CD3⁺ T cells (per mm²) in (E) cerebral meninges and (F) cortex of Tnf⁻/⁻ (n=9) and Cx3cr1creER Tnf⁻/⁻ (n=10) mice. The experiment was performed once. The P-values were calculated using the two-sided Student’s unpaired t-test (E) and two-sided Mann-Whitney U test (F).

(G) Volcano plot based on RNAseq showing the top differentially regulated genes in Tnf⁻/⁻ (n=9) and Cx3cr1creER Tnf⁻/⁻ (n=10) mice on day 14 after allo-HCT as indicated. Cd74, Tnf and H2-Eb are upregulated in microglia of the Tnf⁻/⁻ mice compared to the Cx3cr1creER Tnf⁻/⁻ mice.
Figure 4: Downstream targets of TAK1 signaling are elevated in microglia during GVHD

(A) Heat map based on RNAseq showing the top hits of hallmark PI3K-Akt-mTOR signaling pathway from the microglia of untreated BALB/c mice (n=4) or BALB/c mice on day 14 after allo-HCT (n=4) or syn-HCT (n=4). Colour code represents the Z-score log2 intensity.

(B) A representative flow cytometry plot showing intracellular phospho p38 MAPK in microglia (CD45^low CD11b^+) from brains of untreated BALB/c mice or BALB/c mice on day 7 after syn-HCT or allo-HCT.

(C) The scatter plot shows the quantification (fold change of MFI) of intracellular phospho p38 MAPK expression in microglia from brains of untreated BALB/c mice (n=14) or BALB/c mice on day 7 after syn-HCT (n=15) or allo-HCT (n=15) as indicated. The experiment was repeated three times and the results (mean ± s.e.m.) were pooled. The *P*-values were calculated using the one-way ANOVA.

(D-J) Western blot using protein derived from primary murine microglia treated with different concentrations of murine TNF for 24 hours as indicated. (D, F, H) Representative western
blot images showing the expression of (D) phospho-tak1 and total tak1 (F) phospho JNK and total JNK and (H) phospho NF-kB p65, total NF-kB p65 and IkB with β-actin as loading control.

(E, G, I, J) Quantification of (E) phospho tak1/total tak1 (G) phospho JNK/total JNK (I) phospho NF-kB p65/total NF-kB p65 and (J) IkB normalized to β-actin (fold change with respect to vehicle (0µM TNF) treated controls) in microglia treated as described. The experiment was repeated four times and the results (mean ± s.e.m.) were pooled, n=4 biologically independent samples per group. Each data point represents an individual sample of one independent cell culture experiment. The $P$-values were calculated using the one-way ANOVA.
Figure 5: Deficiency of TAK1 in microglia alleviate CNS-GVHD-associated pathology, cognitive and memory deficits

(A-F) Histology of brain samples immunostained for (A) meningeal and (C) cortical CD3\(^+\) T cells, (E) cortical Iba1\(^+\) cells from Tak1\(^{fl/fl}\) or Cx3cr1\(^{creER}\) Tak1\(^{fl/fl}\) (n=10 each) mice on day 14 after allo-HCT. Scale bars, 50\(\mu\)m. (B, D, F) The scatter plot shows the number of (B) meningeal, (D) cortical CD3\(^+\) T cells and (F) cortical Iba-1\(^+\) cells. The experiment was repeated thrice, the results (mean ± s.e.m.) were pooled.
(G-J) A representative flow cytometry (G, I) and scatter plot showing TNF and MHC-II expression in microglia (CD45\textsuperscript{low} CD11b\textsuperscript{+}) from brains of Tak1\textsuperscript{fl/fl} and Cx3cr1\textsuperscript{CreER} Tak1\textsuperscript{fl/fl} mice. Quantification of (H) MHC-II expression from Tak1\textsuperscript{fl/fl} (n=10) and Cx3cr1\textsuperscript{CreER} Tak1\textsuperscript{fl/fl} (n=10) and (J) TNF expression from Tak1\textsuperscript{fl/fl} (n=11) and Cx3cr1\textsuperscript{CreER} Tak1\textsuperscript{fl/fl} (n=10) on day 14 after allo-HCT are shown. The experiment was repeated thrice, the results (mean ± s.e.m.) were pooled.

(K) Percentage survival of Tak1\textsuperscript{fl/fl} (n=9) and Cx3cr1\textsuperscript{CreER} Tak1\textsuperscript{fl/fl} (n=10) mice that underwent allo-HCT. The experiment was performed thrice, the results were pooled.

(L) The scatter plot shows the percentage of open arm entries by Tak1\textsuperscript{fl/fl} and Cx3cr1\textsuperscript{CreER} Tak1\textsuperscript{fl/fl} (n=17 each) mice on day 21 after allo-HCT in an elevated plus maze test. (M) The scatter plot shows the percentage of time spent by Tak1\textsuperscript{fl/fl} (n=18) and Cx3cr1\textsuperscript{CreER} Tak1\textsuperscript{fl/fl} (n=19) mice in exploring a novel object with respect to the total time on day 19 after allo-HCT in a novel object recognition test. (N) The scatter plot shows the grip strength normalized to body weight (N) of Tak1\textsuperscript{fl/fl} and Cx3cr1\textsuperscript{CreER} Tak1\textsuperscript{fl/fl} (n=19 each) mice on day 20 after allo-HCT in a grip strength test. The experiments were repeated thrice, the results (mean ± s.e.m.) were pooled. The \( P \)-values were calculated using the Mann-Whitney \( U \) test (B, D, I), two-sided Mantel-Cox test (K) or two-sided Student's unpaired t-test (F, H, J, M, N).
Figure 6: Therapeutic TAK1 inhibition alleviates CNS-GVHD-associated pathology, cognitive and memory deficits

(A-D) Flow cytometry plots and the respective scatter plots showing the quantification (fold change of MFI of MHC-II (A, B), TNF (C, D)) expression in microglia (CD45<sup>low</sup> CD11b<sup>+</sup>) from brains of BALB/c mice treated with vehicle or takinib or (5Z)-7-Oxozaenol (5-Oz) on day 14 after allo-HCT as indicated. The experiment was repeated three times and the results (mean ± s.e.m.) were pooled. The P-values were calculated using the one-way ANOVA.
(E-H) Histology of brain samples immunostained for CD3+ T cells and Iba-1+ cells from brains of BALB/c mice treated with vehicle (n=10) or takinib (n=14) or (5Z)-7-Oxozeaenol (5-Oz) (n=12) on day 14 after allo-HCT as indicated. Representative images for (E) meningeal CD3+ T cells and (G) cortical Iba1+ cells from each group are shown. Scale bars, 50µm. The scatter plot shows the number (per mm²), of (F) meningeal CD3+ T cells, (H) cortical Iba-1+ cells and (I) cortical CD3+ T cells. The experiment was repeated three times and the results (mean ± s.e.m.) were pooled. The P-values were calculated using one-way ANOVA.

(J, K) Representative flow cytometry plots (J) and cumulative scatter plot (K) show the quantification (fold change of MFI) of IFNγ expression in CD4+ T cells isolated on day 14 after allo-HCT from the CNS of BALB/c mice treated with vehicle (n=5) or takinib (n=5). (L, M) Representative flow cytometry plots (L) and cumulative scatter plot (M) show the quantification (fold change of MFI) of IL-17 expression in CD4+ T cells isolated on day 14 after allo-HCT from the CNS of BALB/c mice treated with vehicle (n=8) or takinib (n=8). (K, M) The experiments were performed three times and the results (mean ± s.e.m.) were pooled. The P-values were calculated using the two-sided Student's unpaired t-test (K, M).
Figure 7: VCAM-1 and ICAM-1 expression after allo-HCT declines upon TAK-1 inhibition

(A-D) Representative flow cytometry plots and the respective cumulative scatter plots showing the quantification of the fold change of MFI of VCAM-1 (A, B) and ICAM-1 (C, D) expression in endothelial cells (CD31⁺, CD105⁺) from the CNS of BALB/c mice treated with vehicle (n=6) or takinib (n=7) isolated on day 14 after allo-HCT. The experiment was performed once.
(E-G) Immunofluorescence staining and Scatter plot indicating the percentage of brain CD34⁺ (endothelial cells) expressing VCAM-1 and DAPI derived from BALB/c mice treated with vehicle (n=7) or takinib (n=7) and from the (F) Tnf⁻/⁻(n=7) or Cx3cr1creER Tnf⁻/⁻ (n=6) mice respectively on day 14 after allo-HCT. (G) A representative image from Tnf⁻/⁻ and Cx3cr1creER Tnf⁻/⁻ mice respectively is shown. Scale bars, 50µm. The experiment was performed once.

(H) Scatter plot showing the percentage of open arm entries by vehicle (n=11), takinib (n=12) and (5Z)-7-Oxozeaenol (5-Oz) (n=12) mice in an elevated plus maze test. (I) Scatter plot showing the percentage of time spent by vehicle (n=13), takinib (n=12) and (5Z)-7-Oxozeaenol (5-Oz) (n=12) mice in exploring a novel object in a novel object recognition test. The experiments were performed thrice and the results (mean ± s.e.m.) were pooled.

(J) The survival rates of C57BL/6 mice transplanted with AML (FLT3-ITD/MLL-PTD) cells and BALB/c (WT) BM along with (empty and blue circles) and without Allogeneic T cells (Black circles) are shown. (K) The survival rates of BALB/c mice transplanted with AML (WEHI-3B) cells and C57BL/6 BM (WT) along with (Empty and blue circles) and without Allogeneic T cells (Black circles) are shown. The experiments were performed twice and the results were pooled.

The P-values were calculated using the two-sided Student’s unpaired t-test (B, D, F), Mann-Whitney U test (E), one-way ANOVA (H, I) or two-sided Mantel-Cox test (J, K).
Figure 8: Microglia are activated and T cells infiltrate the CNS of GVHD patients

(A-D) Histology of brain samples immunostained for Iba-1+ cells from the cortex of patients who have not undergone allo-HCT (no allo-HCT) (n=9), have undergone allo-HCT with no GVHD symptoms (allo-HCT/no GvHD) (n=8) and have undergone allo-HCT with grade III-IV GVHD symptoms (allo-HCT/GvHD) (n=9). (A, C) Representative image from each group are shown. Scale bars, 50µm. (B, D). The scatter plot shows the number of Iba-1+ cells (per...
mm$^2$) in (B) grey matter and (D) white matter. The experiment was performed once. The $P$-values were calculated using the one-way ANOVA.

(E, F) Immunofluorescence staining of brain samples for Iba-1$^+$ microglia, TNF and DAPI from no allo-HCT (n=8), allo-HCT/no GVHD (n=9) and allo-HCT/GVHD (n=9) groups of patients. (E) A representative image from no allo-HCT and allo-HCT/GVHD groups are shown. Scale bars, 50µm. (F) Scatter plot indicating the percentage of Iba-1$^+$ microglia expressing TNF from different groups is shown. The experiment was repeated once. The $P$-values were calculated using the one-way ANOVA.

(G-H) Histology of brain samples immunostained for CD3$^+$ T cells from the perivascular regions of brain from no allo-HCT (n=9), allo-HCT / no GVHD (n=8) and allo-HCT / GVHD (n=10) groups of patients. (G) Representative images from each group are shown. Scale bars, 50µm. (H) The scatter plot shows the number of CD3$^+$ T cells (per mm$^2$) in perivascular regions of brain. The $P$-values were calculated using the one-way ANOVA.