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Graft-versus-host disease, but not graft-versus-leukemia immunity, is mediated by GM-CSF–licensed myeloid cells

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Abstract: Allogeneic hematopoietic cell transplantation (allo-HCT) not only is an effective treatment for several hematologic malignancies but can also result in potentially life-threatening graft-versus-host disease (GvHD). GvHD is caused by T cells within the allograft attacking nonmalignant host tissues; however, these same T cells mediate the therapeutic graft-versus-leukemia (GvL) response. Thus, there is an urgent need to understand how to mechanistically uncouple GvL from GvHD. Using preclinical models of full and partial MHC-mismatched HCT, we here show that the granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by allogeneic T cells distinguishes between the two processes. GM-CSF drives GvHD pathology by licensing donor-derived phagocytes to produce inflammatory mediators such as interleukin-1 and reactive oxygen species. In contrast, GM-CSF did not affect allogeneic T cells or their capacity to eliminate leukemic cells, retaining undiminished GvL responses. Last, tissue biopsies and peripheral blood mononuclear cells from patients with grade IV GvHD showed an elevation of GM-CSF–producing T cells, suggesting that GM-CSF neutralization has translational potential in allo-HCT.

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GM-CSF-licensed myeloid cells mediate graft-vs-host disease uncoupling immunopathology from graft-vs-leukemia

Or

GM-CSF-licensed myeloid cells mediate graft-vs-host disease but spare graft-vs-leukemia

Or

Licensed phagocytes mediate graft-vs-host disease but do not affect graft-vs-leukemia immunity

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Overline: Graft versus host disease

One sentence Summary: Myeloid cells mediate GvHD immunopathology, whereas GvL relies on T cell cytotoxicity.

Abstract

Allogeneic hematopoietic cell transplantation (allo-HCT) is an effective treatment for several hematologic malignancies, but can also result in potentially life-threatening graft-versus-host disease (GvHD). GvHD is caused by T cells within the allograft attacking non-malignant host tissues; however, these same T cells mediate the therapeutic graft-versus-leukemia (GvL) response. Thus, there is an urgent need to understand how to mechanistically uncouple GvL from GvHD. Using preclinical models of full- and partial MHC-mismatched HCT we here show that the granulocyte-macrophage colony-stimulating factor (GM-CSF) produced by allogeneic T cells distinguishes between the two processes. GM-CSF drives GvHD pathology by licensing donor-derived phagocytes to produce inflammatory mediators such as IL-1 β and ROS. In contrast, GM-CSF did not affect allogeneic T cells or their capacity to eliminate leukemic cells, retaining undiminished GvL responses. Lastly, tissue biopsies and PBMCs from patients with grade IV GvHD showed an elevation of GM-CSF-producing T cells, suggesting that GM-CSF-neutralization has translational potential in allo-HCT.

INTRODUCTION

For patients suffering from hematological malignancies, allogeneic hematopoietic cell transplantation (allo-HCT) is a potentially-curative and life-saving intervention. However, between 40 and 60% of all patients will develop clinically-significant acute or chronic graft-versus-host disease (GvHD) which together carry a mortality rate of approximately 50% (1, 2). Donor-derived allo-reactive T cells attack host malignant cells, producing the beneficial graft-versus-leukemia (GvL) effect. However, this same allo-reactivity can be targeted towards healthy tissues (typically the skin, gut and liver), leading to GvHD. Although depleting T cells from the donor material prior to allo-HCT can prevent or reduce GvHD, this comes at the cost of decreased graft-versus-leukemia (GvL) activity and increased relapse rates (3). Therefore, there is an urgent need to understand how the mechanisms of GvL and GvHD can be separated at the T cell level, and modulated for clinical benefit.

Much work has attempted to define key T cell subsets and cytokines that underpin GvHD in murine models, but to date few consistent conclusions have been drawn. While GvHD was originally proposed to be a T_H1 -mediated pathology (4), studies in mice showed that donor T cells deficient in the T_H1 -cytokine IFN γ can exacerbate the disease (5). T_H2 cells can both suppress experimental GvHD (6, 7), and induce GvHD affecting the liver and skin (8, 9). Similarly, while there is evidence that IL-17A-producing T cells can mediate immunopathology in inflammatory diseases (10), their role in GvHD remains controversial, as in murine models IL-17 seems able to either promote (11, 12) or ameliorate GvHD (9), depending on the experimental conditions. Taken together, polarized T_H cells are clearly implicated in the emergence and perpetuation of GvHD, but so far it has not been possible to identify any specific soluble mediator that has a reproducible and non-redundant function in the pathogenesis of the disease in both murine models and in humans.

One cytokine with an emerging role across a range of inflammatory disorders is GM-CSF (also known as Csf-2) (13, 14). Despite its original designation as a hematopoietic growth

factor, mice lacking GM-CSF or its receptor develop normally and possess a functional myeloid cell compartment, while exhibiting specific defects in pulmonary immuno-physiology (15), and in the homeostasis of non-lymphoid CD103⁺ and CD11b⁺ dendritic cells (DCs) in tissues (16). However, GM-CSF produced by activated T_H cells has a profound impact on the differentiation and activation of several myeloid cell subsets during pathologic tissue inflammation (13, 17, 18), and can drive myeloid cell cytokine production (19), phagocytosis (20), and oxidative burst (21). We therefore asked whether GM-CSF plays a role in the inflammatory tissue damage of GvHD following allo-HCT.

Using murine models of MHC-mismatched HCT we here show that T cell-derived GM-CSF drives GvHD without compromising allo-reactive T cell control of tumors (GvL), thus uncoupling phagocyte-mediated immunopathology from lymphocyte-mediated control of cancer cells. Therefore we propose GM-CSF as a potential therapeutic target to attenuate GvHD while maintaining GvL in patients receiving allo-HCT.

RESULTS

Tissue-infiltrating donor T cells produce GM-CSF and IFN γ following MHC-mismatched HCT

To understand the contribution of T cell-derived GM-CSF to acute GvHD, we first employed a model of MHC-mismatched HCT. We lethally-irradiated CD45.2⁺ wild-type (WT) BALB/c mice (MHC haplotype H2^d) and then intravenously injected them with T cell-depleted (TCD) bone marrow (BM) cells from CD45.1⁺ WT C57BL/6 (B6) mice (MHC haplotype H2^b), with or without CD45.1⁺ B6 splenocytes, which served as a source of mature T cells. Recipients of BM plus splenocytes developed fatal GvHD between days 3 and 6 after allo-HCT (Fig. 1a), which coincided with the reconstitution and expansion of donor CD45.1⁺ cells in the spleen, liver and skin (Fig. 1b). Within the CD45.1⁺ donor compartment, T cell populations contained high frequencies of GM-CSF- and IFN γ -producing cells, seen first in the liver (Fig. 1c) and spleen (Fig. 1d) from day 3 after allo-HCT, then in the skin from day 6 (Fig. 1d). IL-17A was barely detectable in these populations (Fig. 1c,d) but approximately half of GM-CSF producing T cells coexpressed high amounts of IFN γ (Fig. 1e). Accordingly, we found substantially higher amounts of GM-CSF and IFN γ in sera from mice receiving BM plus splenocytes compared to BM alone (Fig. 1f). We confirmed the production of GM-CSF and IFN γ during the allogeneic response *in vitro* using allogeneic mixed lymphocyte reactions (MLR) (Fig. 1g). Furthermore, T cells from B6 mice lacking GM-CSF (*Csf2*^{-/-}), IFN γ (*Ifng*^{-/-}) or IL-17A (*Il17a*^{-/-}) proliferated equally after co-culture with allogeneic DCs, excluding *a priori* differences in the proliferative potential of these cells (Fig. 1h). Taken together, GM-CSF and IFN γ , but not IL-17A, are produced by T cells infiltrating target organs during GvHD.

GM-CSF production by allogeneic T cells is essential for GvHD pathology

Given the production of GM-CSF and IFN γ by allo-reactive T_H cells, we assessed the relevance of these cytokines in determining severity of acute GvHD. As above, we lethally-

irradiated WT BALB/c mice and then compared the outcomes of injecting TCD-BM cells from WT B6 mice, with or without splenocytes from WT B6 mice, or *Csf2*^{-/-}, *Ifng*^{-/-} or *Il17a*^{-/-} mice. Mice receiving BM plus *Csf2*^{-/-} splenocytes were protected from lethal GvHD up to 20 days post allo-HCT, while all mice treated with BM plus WT splenocytes had died or reached final termination criteria by day 15 after HCT (Fig. 2a). Injecting mice with BM plus *Ifng*^{-/-} or *Il17a*^{-/-} splenocytes also significantly affected the timing of fatal GvHD, but still almost 100% of these mice died by day 20 (Fig. 2a).

As donor splenocyte preparations contain multiple different immune cell types, we then asked whether T cells were indeed the biologically-significant source of GM-CSF in our GvHD model. We injected lethally-irradiated BALB/c recipients with TCD-BM cells from WT B6 donors, with or without purified T cells isolated from spleens of WT or *Csf2*^{-/-} B6 mice. This approach confirmed that mice receiving *Csf2*^{-/-} T cells were protected from lethal GvHD up to 70 days post allo-HCT, in stark contrast to those receiving WT T cells (Fig. 2b). There was no evidence that this protective effect was related to intrinsic differences between splenic T cells from WT and *Csf2*^{-/-} mice, since both CD4 and CD8 populations exhibited comparable frequencies (Supplementary Fig. 1a), activation status (Supplementary Fig. 1b) and suppressive capacity of regulatory T cells (Tregs) (Supplementary Fig. 1c). Also after HCT, the capacity to produce GM-CSF did not affect either the ability of T cells to infiltrate GvHD target organs (Supplementary Fig. 1d, e), the frequencies of activated (CD44^{high}) T cells and Tregs in spleen or peripheral lymph nodes (LN) (Supplementary Fig. 1f), their proliferative capacity and granzyme B production (Supplementary Fig. 1g) or their ability to produce proinflammatory cytokines such as IFN γ , TNF α or IL-17A (Supplementary Fig. 1h). The transfer of non-lethal doses of T cells confirmed the protective effect in the absence of GM-CSF, leading to only a mild GvHD but a significant difference in the clinical score between the groups receiving WT or *Csf2*^{-/-} T cells (Supplementary Fig. 2a,b).

We then examined the relative contribution of GM-CSF from CD4 and CD8 T cells to GvHD pathology. For this purpose, we performed an experiment using 4 combinations of CD4 and CD8 T cells transferred at a 1:1 ratio: CD4 WT T cells + CD8 WT T cells, CD4 WT T cells + CD8 *Csf2*^{-/-} T cells, CD4 *Csf2*^{-/-} T cells + CD8 WT T cells and CD4 *Csf2*^{-/-} T cells + CD8 *Csf2*^{-/-} T cells. Only the combinations in which *Csf2* was missing from the CD4 T cells conferred protection against lethal GvHD (Supplementary Fig. 2c), supporting the notion that allo-reactive T_H cells are a prominent source of GM-CSF in GvHD pathogenesis.

The survival advantage conferred to the recipients of allo-HCT of T cells lacking GM-CSF was also evident at the level of individual GvHD target tissues. Histopathological analysis at 6 days post-allo-HCT revealed that transfer of *Csf2*^{-/-} splenocytes caused less tissue damage in skin and small intestine compared to transfer of WT splenocytes (Fig. 2c,d). The skin of mice receiving *Csf2*^{-/-} cells showed a decrease in cellular apoptosis and cellular infiltration around and within hair follicles and epidermis, whereas the small intestine showed a decrease in outright crypt destruction (Fig. 2d and Supplementary Fig. 2d,e). We also observed reduced colonic pathology upon *Csf2*^{-/-} cell transfer, as depicted by increased colon length and a decreased apoptotic index (Fig. 2e,f). A screen for subclinical signs of liver dysfunction showed decreased serum concentrations of alanine aminotransferase (ALT), alkaline phosphatase (AP) and blood urea nitrogen (BUN) in mice transferred with *Csf2*^{-/-} splenocytes in comparison to the WT counterparts (Fig. 2g). In addition, the systemic amounts of TNF α were decreased in the absence of GM-CSF, whereas the amounts of IL-6 did not change (Supplementary Fig. 2f).

As reactive oxygen species (ROS) have been implicated in GvHD tissue injury (22), we asked whether the decreased tissue damage observed in the absence of GM-CSF was associated with lower ROS production. We found that sections from liver and small intestine of mice 6 days post transfer of *Csf2*^{-/-} splenocytes contained significantly fewer cells

expressing p22phox, a critical component of the phagocyte ROS-production machinery, than did sections from mice receiving WT splenocytes (Fig. 2h).

To further confirm the functional role of GM-CSF in GvHD we treated recipient mice with anti-GM-CSF antibodies prior to MHC-mismatched HCT of BALB/c mice with WT B6 TCD-BM alone, or with BM plus WT splenocytes. Systemically blocking the action of GM-CSF successfully replicated the survival advantage of transferring *Csf2*^{-/-} splenocytes into these mice (Fig. 2i).

While murine models of fully MHC-mismatched HCT are informative, we next asked whether the role of GM-CSF in GvHD pathology was conserved in the more clinically-relevant setting of a haplo-mismatched model of acute GvHD (23). We combined WT B6 TCD-BM with WT, *Csf2*^{-/-} or *Ifng*^{-/-} splenocytes and transferred the cells into lethally-irradiated B6D2F1 mice, which are a cross between B6 and DBA mice and so bear the MHC haplotype H2b/d. The transfer of *Csf2*^{-/-} splenocytes conferred a significant survival advantage compared to mice that received WT splenocytes, protecting mice from fatal GvHD to an almost comparable level as TCD-BM alone (Fig. 3a), which was again replicated upon the transfer of purified *Csf2*^{-/-} splenic T cells (Fig. 3b). In both the splenocyte- and T cell- transfer experiments, we also noted a marked increase in mortality following transfer of *Ifng*^{-/-} cells (Fig. 3a,b), which is consistent with the known protective, immunoregulatory function of this cytokine in GvHD (5). At day 9 after HCT, the skin was the most affected organ with highest histological scores; here, we saw less tissue damage in mice receiving *Csf2*^{-/-} compared to WT splenocytes, and more tissue damage in recipients of *Ifng*^{-/-} splenocytes (Fig. 3c,d). This coincided with changes in sebaceous gland numbers, tissue organization and cellular infiltrates around and within hair follicles and epidermis (Fig. 3d and Supplementary Fig. 3a). At a later stage (28 days) after allo-HCT, the transfer of *Csf2*^{-/-} splenocytes also led to decreased liver pathology (Fig. 3e) characterized by changes in the portal tract infiltrates and bile duct disorganization (Supplementary Fig. 3b). On the cellular level we also observed signs of reduced GvHD in

the liver and small intestine of mice receiving *Csf2*^{-/-} cells, which had significantly fewer p22phox⁺ cells accompanied by decreased infiltration of F4/80⁺ myeloid cells (Fig. 3f,g) but no changes in the T cell infiltrate, similar to what we observed in the full-mismatch model (Supplementary Fig. 3c). Given the GvHD protective role of IFN γ seen in these mice, we then asked whether the absence of GM-CSF was related to amounts of this cytokine: ten days after haplo-mismatch HCT, we found significantly higher amounts of IFN γ in the serum of mice that received *csf2*^{-/-} splenocytes compared to WT splenocytes (Fig. 3h). Intriguingly, we also uncovered evidence of increased GM-CSF in sera from mice receiving *Ifng*^{-/-} splenocytes (Fig. 3h), suggesting that GM-CSF might additionally be mediating enhanced GvHD induced by the absence of IFN γ . Altogether, we conclude that T cell-derived GM-CSF has a vital role in the development of GvHD immunopathology in two murine models of allo-HCT.

GM-CSF mediates GvHD lethality through donor-derived myeloid cells

Following lethal conditioning, radio-sensitive host antigen-presenting cells (APCs) are lost within the first days after HCT and substituted by APCs from the donor. Whereas host APCs are required for the priming phase of GvHD, donor APCs play less of a role in the induction of the disease but may be involved in perpetuating tissue injury (24). To delineate whether the pathologically relevant GM-CSF-responsive cell type(s) are of donor or host origin, we used *Csf2rb*^{-/-} mice lacking the beta subunit of the GM-CSFR as donors or hosts of HCT. GM-CSFR deficiency in the recipient compartment did not influence GvHD survival (Fig. 4a). In contrast, the transfer of BM from *Csf2rb*^{-/-} mice resulted in substantially delayed mortality (Fig. 4b), phenocopying the transfer of *Csf2*^{-/-} splenocytes (Fig. 2a). We used unsupervised non-linear dimensionality reduction (t-SNE) (25) to identify and visualize the GM-CSF-responding cells within the donor graft. With this approach, we found that monocytes and neutrophils were particularly sensitive to GM-CSF, which we assessed by single cell analysis of phosphorylated STAT5, the transcription factor downstream of the

GM-CSF receptor complex (20). *Csf2rb*^{-/-} mice were used as a source of GM-CSF unresponsive cells for comparison (Fig. 4c,d and Supplementary Fig. 4a,b).

Having confirmed that the cells responding to GM-CSF are limited to the myeloid compartment, we performed a thorough characterization of myeloid cell subsets found in the inflamed target organs 5 days after allo-HCT. The transfer of *Csf2*^{-/-} splenocytes resulted in decreased frequencies of several myeloid subsets including dendritic cells (DCs), neutrophils, monocytes and monocyte-derived cells (MDCs) (Fig. 4e,f and Supplementary Fig. 4c). A decrease in neutrophils and monocyte/MDCs was also found in target organs upon anti-GM-CSF treatment, whereas the systemic numbers of these myeloid subsets remained unaltered (Supplementary Fig. 4d). We observed that donor monocytes/MDCs produced less pro-IL-1 β in the absence of GM-CSF (Fig. 4g). Importantly, IL-1 β is a signature cytokine of the pathogenic program elicited by GM-CSF (20) and was shown to play a critical role in GvHD development (26). We also investigated the expression of ROS by donor myeloid cells after allo-HCT, given their low amounts in tissue sections of mice receiving allogeneic *Csf2*^{-/-} T cells and their reported role in driving tissue damage (27). We found that the transfer of *Csf2*^{-/-} splenocytes led to a decreased ROS production by both the monocytes/MDCs and neutrophil subsets (Fig. 4h). Collectively, these findings demonstrate that GM-CSF-licensed donor-derived myeloid cells are crucial for GvHD pathology.

Efficient GvL is retained in the absence of GM-CSF

The immune responses leading to tissue damage in GvHD and tumor elimination in GvL are widely considered to be mediated through the same mechanism (28). As donor T cell-derived GM-CSF was essential for lethal GvHD, we investigated whether it also mediated GvL in the MHC-mismatch HCT model. To test this, we intravenously injected A20 B cell lymphoma cells (of BALB/c origin) co-expressing GFP and luciferase (A20-GFP-Luc) into

lethally irradiated BALB/c recipients, together with WT B6 TCD-BM cells either alone or with purified B6 WT or *Csf2*^{-/-} splenic T cells. Tumor growth was monitored by bioluminescent imaging (BLI). When A20 cells were infused with TCD-BM alone, all recipients died or reached final termination criteria from the growing tumor within 35 days (Fig. 5a-c), consistent with a critical role for T cells in the GvL effect. Accordingly, adding WT or *Csf2*^{-/-} T cells to the BM transfer resulted in efficient control of tumor growth (Fig. 5a-c), although only mice receiving *Csf2*^{-/-} T cells showed a significant improvement in survival (Fig. 5c). Whereas lymphoma-bearing mice receiving TCD-BM plus WT T cells mainly died from severe GVHD, mice receiving TCD-BM plus *Csf2*^{-/-} T cells exhibited an undiminished GvL effect, but were at the same time protected from GVHD (Fig. 5c,d). This improvement of survival in mice receiving *Csf2*^{-/-} T cells was also observed in an alternative GvL model using the monomyelocytic cell line WEHI-3 (Supplementary Fig. 5a). Also, when we increased the number of inoculated A20 lymphoma cells, *Csf2*^{-/-} T cells executed GvL even more efficiently than WT T cells, leading to a significant increase in overall survival (Supplementary Fig. 5 b,c). Consistently, *Csf2*^{-/-} and WT T cells isolated from naïve or mice receiving allo-HCT were equally capable of killing A20 lymphoma and WEHI-3 cells *ex vivo* (Supplementary Fig. 5d-f), expanded equally in spleen and LN (Supplementary Fig. 5g) and displayed similar frequencies of activated (CD44^{high}), proliferative (Ki67⁺) and granzyme B-producing cells (Supplementary Fig. 5g). To test the translatability of our findings into a clinical setting we used neutralizing mAbs against GM-CSF. Strikingly, anti-GM-CSF-treated leukemic mice receiving TCD-BM plus T cells showed a potent GvL effect and had significantly better survival than the isotype-treated control mice (Fig. 5e-h). Better survival was associated with reduced GVHD incidence (Fig. 5h). Of note, neutralization of GM-CSF did not have any impact on tumor growth in mice which did not undergo HCT.

Thus, in the absence of GM-CSF, donor T cells are effective mediators of the therapeutic GvL response in mice, without inducing lethal GVHD, even in the highly immunogenic context of an MHC-mismatched allo-HCT.

GM-CSF⁺ T cells are elevated in patients with GvHD

We next asked whether there was evidence that GM-CSF plays a parallel role in GvHD in human HCT recipients. We assessed GM-CSF gene expression and protein abundance in gastrointestinal biopsies from patients with different grades of clinically-documented acute GvHD following HCT for a range of different conditions (Supplementary Tables 1-3). At the transcriptional level we detected significantly higher expression of GM-CSF in gastrointestinal biopsies from patients with grade IV compared to grade I GvHD (Fig. 6a). Moreover, in samples from patients with grade IV GvHD, we observed strong immunoreactivity for GM-CSF in the stromal compartment; while we did not detect GM-CSF in biopsies from patients without GvHD (Fig. 6b). Importantly, the majority of cells producing GM-CSF in these samples were T cells, as indicated by the co-localization with CD3, in contrast to the minimal colocalization observed with the macrophage marker CD68 (Fig. 6c). We also analyzed the expression of GM-CSF in T cells from peripheral blood of GvHD patients (Supplementary Table 4, gating strategy shown in fig S6) and found that the frequencies of GM-CSF-producing CD4⁺ T cells were significantly increased in GvHD patients in comparison to healthy donors (Fig. 6d), supporting that activated GM-CSF producing T cells are pathogenically relevant in the human disease. We also observed a striking increase in the numbers of IFN γ -producing T cells but found no difference in IL-17-producing T cell frequencies (Fig. 6d). Together, these results implicate GM-CSF in GvHD after allo-HCT in human patients.

DISCUSSION

Although there is a large body of literature discussing the potential contributions of different T_H cell subsets and their signature cytokines to GvHD (29), the mechanisms underlying inflammation and subsequent tissue destruction are not fully understood. Here, we reveal that GM-CSF plays a critical role in driving lethal GvHD in mice, and is similarly elevated in human allo-HCT patients with severe GvHD. Moreover, GM-CSF appears to be dispensable for the therapeutic GvL effect and so may represent a promising therapeutic target in the separation of GvL from GvHD for the treatment of hematologic malignancies.

We used two different murine models of acute GvHD to show that donor T cell-derived GM-CSF directly mediates severe GvHD, which is associated with abundant myeloid cell infiltration and increased amounts of pro-IL-1 β and components of the ROS production machinery, classically used by phagocytes for host defense against pathogens. The same mechanisms are clearly detrimental in the immunopathology of GvHD, where tissue damage and leukocyte infiltration go hand-in-hand (30). The reduction of myeloid-driven oxidative stress in GvHD-susceptible organs observed here in animals receiving allo-reactive *Csf2*^{-/-} T cells supports this notion. In accordance with the reported key role of GM-CSF in chronic intestinal inflammation (31) and the recently described contribution of BATF-dependent IL-7R^{hi}GM-CSF⁺ T cells to intestinal GvHD (32), we found GM-CSF to contribute to colonic pathology. The gastrointestinal tract is not only a crucial target organ of GvHD, but also propagates the “cytokine storm” characteristic of acute GvHD (33, 34).

We uncovered evidence of a potential reciprocal regulation of GM-CSF and IFN γ during GvHD in mice. IFN γ produced by activated donor T cells has previously been shown to both promote and protect against GvHD (35, 36); here we found that mice undergoing partial MHC-mismatched HCT with IFN γ -deficient splenocytes were significantly more susceptible to lethal GvHD than those receiving WT splenocytes. Intriguingly, these mice presented elevated serum GM-CSF, while mice receiving GM-CSF-deficient splenocytes exhibited

elevated IFN γ in their sera. The ability of IFN γ to negatively regulate GM-CSF in effector T_H cells has been previously reported (17); however, GM-CSF does not directly affect T cells, and thus the mechanisms by which GM-CSF influences IFN γ production are currently not known. We also excluded a role for T-cell derived IL-17A in acute GvHD. Previous reports differ in their interpretations regarding the role of IL-17 in GvHD, which was found to be detrimental (11, 12), protective (37), or play no role in the disease (38). Although differences in the conditioning regimens may explain the discordant findings, our data is supported by the generally low frequency of IL-17-producing T cells in preclinical GvHD models. Also in peripheral blood leukocytes from GvHD patients, IL-17A frequencies were not abundant among memory T cells. Since post-transplant IL-17-producing CD8 T cells have been shown to express GM-CSF (39), we hypothesize that this cytokine might mediate the detrimental effects of CD8 T cells in GvHD pathology.

The prominent role for GM-CSF in the pathogenesis of GvHD is in line with the GM-CSF-dependency reported in various T cell-driven models of autoimmunity (13). T cells, lacking the GM-CSF receptor complex, cannot directly respond to GM-CSF, and myeloid cell subtypes were proposed to be targeted by this cytokine during disease progression. In an animal model for multiple sclerosis, for example, GM-CSF responsiveness by CCR2⁺ monocytes was shown to be essential for tissue inflammation (20). In the case of GvHD, we identified donor myeloid cells as relevant responders to GM-CSF. Although host APCs are required for the priming of acute GvHD (40), donor APCs may play a role in tissue injury. We propose that donor APCs do not largely contribute to prime allo-reactive T cells (24), and that GM-CSF licenses the myeloid population in the donor graft to produce pro-inflammatory mediators (e.g. IL-1 β and ROS) that engage and perpetuate tissue damage. Myeloid cells readily respond to GM-CSF to produce IL-1 β (20), and donor-derived APCs have been shown to be the main IL-1 β -producing myeloid subset at late GvHD stages (28). Even though ROS can directly cause tissue damage, myeloid-derived IL-1 β can also potentially act via local stimulation of alloreactive T cells. The crosstalk mechanisms

involved in GM-CSF-induced tissue damage in GvHD target organs are not yet fully elucidated.

There has been a long-standing assumption that donor T cell-driven GvHD and the therapeutic GvL effect are mediated via the same mechanisms: our data require us to question this notion. Treating mice with GM-CSF-blocking antibodies prior to allo-HCT significantly protected from the development of lethal acute GvHD, while tumor-bearing mice treated with anti-GM-CSF benefitted from effective control of tumor growth and protection from GvHD. Thus GM-CSF does not control T cell-mediated killing of malignant lymphohematopoietic cells; instead we propose that, in GvHD, GM-CSF acts as a communication conduit between allo-reactive T cells and myeloid cells licensing the latter to cause tissue destruction. While GvL likely relies on direct cytotoxicity by T cells, the GM-CSF-driven responses of myeloid cells may be more relevant for GvHD pathology, thus representing a potential mechanism to separate the GvL effect from GvHD.

Current clinical guidelines support the use of colony stimulating factors (CSFs) to mobilize peripheral blood progenitors after autologous progenitor cell transplantation. However, the same is not applicable for allogeneic progenitor cell transplantation (41) and the use of GM-CSF treatment in HTC has been associated with more days of fever and prolonged antibiotic therapy and, in addition did not decrease infection-related mortality (42). We here propose to neutralize GM-CSF in GvHD patients, given the detrimental role of this cytokine in experimental models of GvHD. We anticipate that blocking GM-CSF in allo-HCT patients could improve clinical outcome and may even permit greater incompatibility between donor and recipients while limiting the risk of fatal GvHD. The marked increase of this cytokine observed in affected tissues from severe GvHD patients further supports this idea. However, since GM-CSF is crucial for surfactant homeostasis and lung host defense (43), it will be important to closely monitor lung disease parameters when blocking GM-CSF in the clinical setting.

There is evidence in mice that GM-CSF contributes critically to IL-23-mediated immune responses (17, 44), and that anti-IL-23p19 therapy can ameliorate syngeneic GvHD-associated colitis (45). Thus the direct blockade of GM-CSF might provide a valuable complement to any IL-23-targeted clinical trials. The fact that the absence of GM-CSF can halt the development of GvHD without impairing the GvL response paves the way for testing this therapeutic strategy for the prevention and treatment of GvHD following allo-HCT. Moreover, that GM-CSF was elevated in patient samples with the severest manifestations of GvHD means that this therapeutic strategy holds particular promise for patients with the poorest predicted outcome and highest risk of fatality.

MATERIAL AND METHODS

Study design

The study was initiated to determine whether certain T cell-derived cytokines could separate GvHD from GvL and so may represent promising novel therapeutic targets for the treatment of hematologic malignancies. To achieve this aim, we used two different experimental models of GvHD, an experimental model of GvL, and samples from human subjects. For animal studies, 8- to 12-week-old mice were used. All animal experiments were approved by local authorities (Swiss Cantonal Veterinary Office) and performed under the appropriate experimental licenses (76/2012 and 052/2015). Animals were assigned randomly into the experimental groups and in-life clinical score was performed in a blinded fashion as well as image analysis processing on organ sections. Sample size and disease end time points were selected on the basis of previous studies. Flow cytometry, histopathological analysis, mixed lymphocyte reactions (MLRs), killing assays and cytokine analysis were performed to characterize the GvHD/GvL target organs. The effects of the specific GM-CSF blocking antibody on clinical score were assessed by investigators who were blinded to the treatment. To perform reliable statistical analysis, at least three independent experiments

were conducted for each data shown in the manuscript, unless differently indicated in the figure legends.

All human samples were collected after approval by the ethics committee of the Albert-Ludwigs University Freiburg, Germany (Protocol number: 267/11) following written informed consent. We performed immunohistochemistry and quantitative RT-PCR on the gut biopsies, and multiparameter flow cytometry on the PBMCs. Primary data are located in Tables S5. Also, clinical features of patients can be found in Tables S1-S4. The scoring system used for allo-HCT mice is described in Table S6 and the phenotypical analysis of human PBMCs in Table S7.

Statistical analysis

Graphs were prepared with GraphPad Prism (GraphPad Software). Survival curves were plotted by the Kaplan-Meier method and for comparison of survival curves a Log-rank (Mantel-Cox) test was used. Data are displayed as individual data points or as mean $\pm$ standard error of the mean (SEM) as depicted in the figure legends. Comparison of the means was performed using unpaired, two-tailed Student's t-tests (with Welch's correction where applicable) or one-way and two-way ANOVA with Bonferroni post-test, respectively. No statistical methods were used to predetermine sample size, but our sample sizes were similar to those generally employed in the field. No method of randomization was used.

SUPPLEMENTARY MATERIALS

Materials and Methods

Fig. S1. Comparative phenotypic analysis of T cell populations from WT and *Csf2*^{-/-} mice.

Fig. S2. GM-CSF is crucial for acute GvHD following fully MHC-mismatched allo-HCT.

Fig. S3. GM-CSF mediates GvHD pathology following partially MHC-mismatched allo-HCT.

Fig. S4. GM-CSF drives GvHD through donor derived myeloid cells.

Fig. S5. GM-CSF is dispensable for anti-tumor activity following allo-HCT.

Fig. S6. Frequency of cytokine expressing T cells in PBMCs of patients with GvHD.

Table S1. Clinico-pathological features of GvHD patient set I.

Table S2. Clinico-pathological features of GvHD patient set II.

Table S3. Characteristics of control subjects..

Table S4. Clinico-pathological features of GvHD patient PBMCs

Table S5. Primary data

Table S6. GvHD scoring system for allo-HCT mice.

Table S7. Phenotypic analysis of human PBMCs by flow cytometry.

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Data and materials availability: All data associated within this study are present in the paper or Supplemental Materials.

FIGURE LEGENDS

Figure 1. Donor T cells secrete GM-CSF and IFN γ during allogeneic responses

(A) Survival of lethally-irradiated CD45.2⁺ BALB/c mice after allo-HCT with CD45.1⁺ WT C57BL/6 TCD-BM alone or combined with 10x10⁶ CD45.1⁺ WT C57BL/6 splenocytes. Data pooled from 4 individual experiments, each with n=5/group. (B) Frequency of CD45.1⁺ cells within live singlets in spleen, liver and skin at 3 and 6 days after allo-HCT. Data pooled from 2 experiments to obtain n=8-10/group. (C) Frequency of IFN γ -, GM-CSF- and IL-17A-producing CD4⁺ and CD8⁺ T cells within the CD45.1⁺ population from spleens of mice 3 days after allo-HCT. Representative plots are shown from a total of 3 independent experiments. (D) Frequencies of IFN γ -, GM-CSF- and IL-17A- producing CD4⁺ and CD8⁺ T cells within the CD45.1⁺ populations from liver, spleen and skin 3 and 6 days after allo-HCT. Data pooled from 3 individual experiments, total n=8-10/group. (E) Frequencies of IFN γ and GM-CSF-producing CD4⁺ and CD8⁺ T cells within the CD45.1⁺ populations from liver and spleen 3 and 6 days after allo-HCT. Representative data from 1 of 3 experiment is shown, each with n=3-4/group. (F) Serum IFN γ and GM-CSF in mice 6 days after allo-HCT. Data pooled from 3 individual experiments, each with n=2-5/group. (G) IFN γ , GM-CSF and IL-17A in supernatants from co-cultures of T cells from WT C57BL/6 mice with either syngeneic (C57BL/6) or allogeneic (BALB/c) splenic CD11c⁺ DCs. Data pooled from 3 individual experiments, each with n=3-5/group. (H) Tritiated thymidine incorporation by T cells from C57BL/6 wt, *Ifng*^{-/-}, *Csf2*^{-/-} or *Il17a*^{-/-} mice co-cultured with syngeneic (C57BL/6) or allogeneic (BALB/c) splenic CD11c⁺ DCs. Data pooled from 3 individual experiments, each with n=2-5/group. For comparison of survival curves a Log-rank (Mantel-Cox) test was used in A. For comparison of the means an unpaired two-tailed t-test with Welch's correction was used in B, F and G, and one-way ANOVA with Bonferroni post-test was used in D and H, *p<0.05, **p<0.01, ***p<0.001. Data are displayed as mean +/- SEM. Abbreviations: TCD: T cell-depleted, BM: bone marrow, HCT: hematopoietic cell transfer, WT: wild type, Cpm: counts per minute.

Figure 2. GM-CSF is crucial for acute GvHD following fully MHC-mismatched allo-HCT

(A) Survival of lethally-irradiated BALB/c mice after allo-HCT with WT C57BL/6 TCD-BM alone or with 10×10^6 splenocytes from C57BL/6 WT *Csf2*^{-/-}, *Ifng*^{-/-} or *Il17a*^{-/-} mice. Data pooled from 4 individual experiments, each with n=5/group. (B) Survival of lethally-irradiated BALB/c mice after allo-HCT with WT C57BL/6 TCD-BM alone or combined with 3×10^6 T cells purified from spleens of C57BL/6 WT or *Csf2*^{-/-} mice. Data pooled from 3 individual experiments each with n=5/group. (C) Composite histo-pathological score for liver, small intestine and skin from BALB/c mice 6 days after allo-HCT with WT C57BL/6 TCD-BM combined with 10×10^6 splenocytes from either WT or *Csf2*^{-/-} C57BL/6 mice. Data pooled from 3 individual experiments, each with n=5/group. (D) Representative images of haematoxylin and eosin stained sections from skin and small intestine of mice 6 days after allo-HCT described in a, n=4-5/group (scale bar: 100 μ m). (E) Colon length in cm from BALB/c mice 6 days after allo-HCT, as described in C. Data pooled from 2 individual experiments. (F) Representative images and quantification of apoptotic cells (TUNEL staining) in the colon from BALB/c mice 6 days after allo-HCT, as described in C. Data pooled from 3 individual experiments (scale bar top 50 μ m, bottom 20 μ m). (G) Concentrations of alkaline phosphatase (AP), alanine aminotransferase (ALT), blood urea nitrogen (BUN) and albumin in serum from BALB/c mice 6 days after allo-HCT, described in C. Data are representative of 2 individual experiments, each with n=3-5/group. (H) Representative images of p22phox labeling in sections from liver, small intestine and skin (scale bar: 100 μ m) of mice 6 days after allo-HCT, described in C (left). Data were pooled from 4 independent experiments each with n=5-7/group. Quantification of mean % of total area labeled positively for p22phox per visual field (right). (I) Survival of lethally-irradiated BALB/c mice following allo-HCT with WT C57BL/6 TCD-BM alone or combined with 10×10^6 splenocytes from C57BL/6 WT mice. Mice were treated with PBS, 300 μ g of isotype control antibody, or 300 μ g of anti-GM-CSF antibody (a-GM-CSF) injected i.p. 3 times/week for the duration of the experiment, starting 2 days before HCT. Data pooled from 2 individual

experiments, each with n=5/group. For comparison of survival curves a Log-rank (Mantel-Cox) test was used in A (WT vs other groups), B (WT to *Csf2*^{-/-}) and I (a-GM-CSF vs isotype). For comparison of the means (WT vs. *Csf2*^{-/-}) an unpaired two-tailed t-test was used in C, E, F and H, *p<0.05, **p<0.01, ***p<0.001. Data are displayed as mean +/- SEM. Abbreviations: TCD: T cell-depleted, BM: bone marrow, HCT: hematopoietic cell transfer, WT: wild type.

Figure 3. GM-CSF mediates GvHD pathology following partially MHC-mismatched allo-HCT

(A) Survival of lethally-irradiated B6D2F1 mice following partially MHC-mismatched allo-HCT with WT C57BL/6 TCD-BM alone or combined with 20x10⁶ splenocytes from C57BL/6 WT, *Csf2*^{-/-} or *Ifng*^{-/-} mice. Data pooled from 5 individual experiments, each with n=5/group. (B) Survival of lethally-irradiated B6D2F1 mice following allo-HCT with WT C57BL/6 TCD-BM alone or combined with 7.5x10⁶ T cells purified from spleens of C57BL/6 WT, *Csf2*^{-/-} or *Ifng*^{-/-} mice. Data pooled from 2 individual experiments, each with n=5/group. (C) Composite histo-pathological score for liver, small intestine and skin sections from B6D2F1 mice at 11 days post allo-HCT, described in A. Data pooled from 2 individual experiments, each with n=5/group. (D) Representative images of haematoxylin and eosin stained sections from skin of mice 11 days after allo-HCT with WT C57BL/6 TCD-BM combined with 20x10⁶ splenocytes from either WT, *Ifng*^{-/-} or *Csf2*^{-/-} C57BL/6 mice, n=4-5/group (Scale bars: 100 µm). (E) Composite histo-pathological score for liver sections from B6D2F1 mice at 9 or 28 days post allo-HCT, as described in A. Data are representative of 2 independent experiments, each with n=4-6/group. (F & G) Representative images of p22phox (upper panels) and F4/80 labeling in sections from (F) liver and (G) small intestine (scale bar: 100 µm) of mice 9 days after allo-HCT with WT C57BL/6 TCD-BM combined with 20x10⁶ splenocytes from C57BL/6 WT or *Csf2*^{-/-} mice. Data were pooled from 4 independent experiments each with n=5-7/group. Quantification of % of total area labeled positively for p22phox or F4/80 per visual field. (H) Serum IFNγ and GM-CSF in mice 9 days after allo-

HCT with WT C57BL/6 TCD-BM combined with 20×10^6 splenocytes from C57BL/6 WT, *Ifng*^{-/-} or *Csf2*^{-/-} mice. Data pooled from 2-3 individual experiments, each with n=4-5/group. For comparison of survival curves (WT vs other groups) a Log-rank (Mantel-Cox) test was used in A and B. For comparison of the means (WT, *Csf2*^{-/-} and *Ifng*^{-/-}) one-way ANOVA with Bonferroni post-test was used in C. For comparison of the means (WT vs. *Csf2*^{-/-}) an unpaired two-tailed t-test was used in F and G, For comparison of the means (WT vs. *Ifng*^{-/-} or WT vs. *Csf2*^{-/-}) an unpaired two-tailed t-test with Welch's correction was used in H. *p<0.05, **p<0.01, ***p<0.001. Data are displayed as mean +/- SEM. Abbreviations: TCD: T cell-depleted, BM: bone marrow, HCT: hematopoietic cell transfer, WT: wild type.

Figure 4. GM-CSF drives GvHD through donor derived myeloid cells

(A) Survival of lethally-irradiated WT C57BL/6 and *Csf2rb*^{-/-} mice following MHC-mismatched allo-HCT with WT Balb/c TCD-BM alone or combined with 20×10^6 splenocytes from Balb/c WT mice. Data pooled from 2 individual experiments, each with n=5/group. (B) Survival of lethally-irradiated Balb/c WT mice following MHC-mismatched allo-HCT with C57BL/6 WT or and *Csf2rb*^{-/-} mice TCD-BM alone or combined with 10×10^6 splenocytes from C57BL/6 WT mice. Data pooled from 2 individual experiments, each with n=5/group. (C) Annotated t-SNE map displaying 200,000 randomly sampled cells from the bone marrow of WT C57BL/6 and *Csf2rb*^{-/-} mice showing STAT5 phosphorylation (pSTAT5, black to yellow gradient) upon GM-CSF stimulation, analyzed by flow cytometric analysis. Data represent two independent experiments, each with n=3/group. (D) Frequencies of GM-CSF-induced pSTAT5 upregulation in monocytes and neutrophils from WT C57BL/6 and *Csf2rb*^{-/-} mice as shown overlaid in B. (E) Flow cytometric analysis of different myeloid cell populations (DCs, neutrophils, monocytes and MDCs) after HCT. Example gating for the liver is shown. (F) Quantification of myeloid cell populations (from E) in the spleen (upper row) and liver (lower row). One representative from 3 individual experiments is shown, each with n=4-5/group. (G) Frequency of pro-IL-1 β -producing neutrophils and monocytes within the H2Db⁺, CD45⁺ population from spleens of mice 6 days after allo-HCT. Representative

plots are shown for neutrophils, monocytes and MDCs (gated as in E). Data represent two independent experiments, each with n=5/group. (H) Flow cytometric analysis of ROS (CellROX reagent) in mice 6 days after allo-HCT. Representative histograms of MFI for neutrophils, monocytes and MDCs (gated as in E). Data represent one experiment with n=5-7/group. For comparison of survival curves (WT vs *Csf2rb*^{-/-} recipients/donors) a Log-rank (Mantel-Cox) test was used in A and B. For comparison of the means an unpaired two-tailed t-test was used in D, F-H ***p<0.001, **p<0.01, *p<0.05. Data are displayed as mean +/- SEM. Abbreviations: TCD: T cell-depleted, BM: bone marrow, HCT: hematopoietic cell transfer, WT: wild type.

Figure 5 GM-CSF is dispensable for anti-tumor activity following allo-HCT

(A-D) Lethally-irradiated BALB/c mice were intravenously injected with 250,000 A20 tumor cells expressing GFP and luciferase, at the same time as MHC-mismatched allo-HCT with WT C57BL/6 TCD-BM alone or combined with 1x10⁵ T cells purified from spleens of C57BL/6 WT or *Csf2*^{-/-} mice. Mice treated with TCD-BM alone were used as controls. (A) Tumor growth was monitored by *in vivo* bioluminescent imaging. Images from one representative experiment of three are shown. (B) Signal intensity in the region of interest (ROI) was monitored over time. (C) Survival over time and (D) Incidence of GvHD displayed as percentage of mice in each treatment group which developed lethal GvHD over time (grey) or survivors (white). Data pooled from 3 individual experiments, each with n=5/group. (E-H) Lethally-irradiated BALB/c mice were intravenously injected with A20 tumor cells expressing GFP and luciferase, at the same time as MHC-mismatched allo-HCT with WT C57BL/6 TCD-BM alone or combined with 1x10⁵ T cells purified from spleens of C57BL/6 WT mice. Mice treated with TCD-BM alone were used as controls. Mice were treated with isotype control, or anti-GM-CSF antibody 3 times/week for the duration of the experiment, starting 2 days before HCT. (E) Tumor growth was monitored by *in vivo* bioluminescent imaging. Images from one experiment with n=6-7/group are shown. (F) Signal intensity in the region of interest (ROI) was monitored over time. (G) Survival over time and (H)

Incidence of GvHD displayed as percentage of mice in each treatment group which developed lethal GvHD over time (grey) or survivors (white). For comparison of survival curves (WT vs other groups, in C; WT vs *csf2*^{-/-} in G) a Log-rank (Mantel-Cox) test was used. *p<0.05, **p<0.01. Data displayed as mean +/- SEM. Abbreviations: TCD: T cell-depleted, BM: bone marrow, HCT: hematopoietic cell transfer, WT: wild type, BLI: bioluminescent imaging, ROI: region of interest.

Figure 6. GM-CSF⁺ T cells are elevated in patients with GvHD

(A) Relative expression of GM-CSF at the mRNA level in gastrointestinal biopsies from patients with different GvHD grades, see Supplementary Table 1. Data are displayed as mean +/- SEM. (B) Images of GM-CSF labeling in control (patients with no pathological findings) and GvHD grade IV biopsies from the small intestine of allo-HCT patients, see Supplementary Tables 2 & 3. Brown: anti-human GM-CSF, blue: Haematoxylin. Scale bars are 100 µm, 20 µm in zoom images. Representative images of 3 individual control and patient samples are shown (Supplementary Tables 2 & 3). (C) Immunofluorescence staining for CD3 (pink), CD68 (green) and GM-CSF (red) of gastrointestinal biopsies from GvHD patients grade IV (Supplementary Table 2). A representative picture of 3 individual patient samples is shown. Nuclei are depicted in blue (DAPI) (scale bar top 50 µm, bottom 20 µm). (D) Flow cytometric analysis of PBMCs collected from healthy donors (HD) and GvHD patients (GP) (Supplementary Table 4), stimulated for 4 h with PMA/ionomycin. (left) Representative plots of cytokine expressing T cells are shown. (right) GM-CSF, IFN γ , and IL-17 producing CD4⁺ and CD8⁺ T cells are presented as individual frequencies of CD4 or CD8 T cells, n=9-10/group. For comparison of the means one-way ANOVA with Bonferroni post-test was used in A and an unpaired two-tailed Mann Whitney test was used in D. *p<0.05, **p<0.01..

Supplementary information

Materials and Methods

Mice and in vivo manipulations

Mice were kept in-house in individually ventilated cages under specific pathogen-free conditions. WT C57BL/6, BALB/c and B6D2F1 mice were purchased from Janvier Laboratories, France. Congenic C57BL/6 CD45.1 mice were bred in-house. *Ifn γ ^{-/-}* and *Csf2rb^{-/-}* mice were obtained from Jackson Laboratories; *Csf2^{-/-}* mice were provided by J. Whitsett and further backcrossed to C57BL/6 using speedy congenics. *Il17a^{-/-}* mice were provided by Y. Iwakura.

Induction of GvHD

Single cell suspensions from spleen and BM of donor C57BL/6 or BALB/c mice were prepared as described below (under “Lymphocyte isolation”). T cells were depleted from the BM, using the AutoMACS Pro, Miltenyi Biotech system. For isolation of untouched T cells from splenocytes the Pan T cell isolation kit (II) from Miltenyi was used according to the manufacturers protocol. CD4 and CD8 T cells were separated using the MojoSort Mouse CD4 T cell isolation and the MojoSort Mouse CD8 T cell isolation kits according to the manufacturer’s instruction (Biolegend).

Recipient BALB/c, B6D2F1, C57BL/6 mice were lethally-irradiated with a split-dose of 850 (BALB/c), 1200 (B6D2F1) or 1100 (C57BL/6) rad. C57BL/6 recipients were injected i.v. with 5×10^6 BM cells and 10×10^6 splenocytes, 3×10^6 or 1×10^5 T cells or 1×10^5 CD4 plus CD8 T cells. Recipients were injected i.v. with 7×10^6 BM cells and 20×10^6 splenocytes per BALB/c mouse. B6D2F1 recipients were injected i.v. with 5×10^6 BM cells and 20×10^6 splenocytes or 7.5×10^6 T cells. Mice were scored daily for GvHD symptoms, adapted from (48), and shown in Supplementary Table 6. For blocking of GM-CSF, mice were treated with PBS,

300 µg isotype control (2A3) or 300 µg anti-GM-CSF antibody (MP1) (BioXCell) 3 times/week starting 2 days before HCT.

Induction of GvL

The A20-Luciferase⁺GFP⁺ B cell lymphoma cell line (A20-luc-gfp was kindly provided by E. Svensson, Department of Immunology, Genetics and Pathology, Uppsala University, Sweden). WEHI-3-luciferase⁺ cells were donated by R. Zeiser. On the day of HCT, 2.5x10⁵ A20 cells, 5x10⁵ A20 cells or 1x10⁵ WEHI-3 cells per mouse were injected intravenously, alongside 5x10⁶ WT C57BL/6 BM cells, either alone or with 1x10⁵ purified splenic T cells/mouse from either WT or *Csf2*^{-/-} C57BL/6 mice. Tumor progression was monitored by bioluminescence imaging (BLI). For blocking of GM-CSF, mice were treated as described in Induction of GvHD. From week 3 on the antibody dose was reduced to 150 µg.

Human subjects

Intestinal tissue biopsies were collected in a prospective manner from individuals with GvHD or healthy controls. The patients' characteristics, including recipient age, gender, underlying diagnosis, donor and graft type, conditioning regimen, immunosuppressive regimen and GvHD grade are detailed in Supplementary tables 1 & 3 for the GvHD patients, in Supplementary Table 2 for the control subjects, and in Supplementary Table 4 for the GvHD patients used to collect PBMCs.

Lymphocyte isolation

Mice were euthanized using CO₂ inhalation and perfused with 40 ml cold PBS. Spleen/LN: For the isolation of myeloid cells, organ pieces were incubated in 3 ml of Collagenase D (0.4 mg/ml, Roche) and 0.1 mg/ml DNase I (Sigma) in RPMI for 30 minutes at 37°C. For isolation of T cells, spleens or LNs were homogenized by mechanical disruption and filtered through a 70 µm cell strainer. BM: Femurs, tibiae and pelvis were flushed with PBS to obtain BM stem cells. Liver: organ pieces were incubated in 1.6 mg/ml Collagenase Type IV (from *Clostridium histolyticum*, Sigma) in HBSS containing 10% FCS for 45 minutes at

37°C. Cells were resuspended in 10 ml Percoll, (continuous gradient, 27%, GE) and centrifuged for 30 minutes at 1700 rpm at RT. Fat and supernatant was removed and the pellet was subjected to red blood cell lysis. Skin: organ pieces were incubated in 1 mg/ml Collagenase Type IV (from *Clostridium histolyticum*, Sigma) and 0.1 mg/ml DNase I (Sigma) in RPMI for 1.5 - 2 hours at 37°C. Small intestine: organs were separated from the mesenteric fat before luminal mucus was removed mechanically; organs were then incubated in calcium- and magnesium-free HBSS containing 2% FCS, 1 mM DTT and 1.35 mM EDTA for 15 min at 37°C. After further incubation in HBSS complemented with EDTA for 30 min at 37°C the colons were cut and digested using 0.4mg/ml collagenase IV (Sigma Aldrich) for 45 min at 37°C. The samples were then homogenized using a syringe with an 18 gauge needle and filtered through a 70 µm cell strainer.

Flow cytometry

Flow cytometric analysis was performed following standard methods, reviewed in (50). For all fluorochrome-conjugated antibodies optimal concentrations were determined using titration experiments. Antibody clones specific for mouse CD4 (GK1.5), CD8 (53-6.7), CD3 (17A2), CD45 (30F11), CD45.1 (A20), CD45.2 (104), CD44 (IM7), CD62L (MEL-14), CD69 (H1.2F3), MHC class I H2-Dd (34-2-12) and H2-Db (KH95), GM-CSF (MP1-22E9), IFN γ (TC11-18H10), IL-17A (XMG1.2), TNF α (MP6-XT22) FoxP3 (FJK-16s), IL-1 beta Pro-form (NJTEN3), CD11b (M1/70), CD11c (N418), Ly6G (1A8), Ly6C (HK1.4), CD64 (X54-5/7.1), MHC class II I-A / I-E (M5/114.15.2), CD24 (M1/69), Ki67 (16A8), Granzyme-B (GB11), F4/80 (BM8), Siglec-F (E50-2440), CD19 (1D3) and Ter119 (Ter-119), were obtained either from BD, BioLegend or eBioscience. For surface staining cells were incubated with the respective antibodies for 20 - 30 minutes at 4°C. For all experiments, dead cells were excluded from the analysis using an Aqua or Near-IR Live/Dead fixable staining reagent (Invitrogen/BioLegend); doublets were excluded by FSC-Area vs. FSC-Height gating. For intracellular cytokine staining, T cells were incubated for 4 - 5 hours at 37°C in RPMI containing 10% FCS with PMA (50 ng/ml), Ionomycin (500 ng/ml), GolgiPlug (containing

Brefeldin A, BD, 1:1000 dilution) or GolgiStop (containing Monensin, BD, 1:1000 dilution). After surface staining Cytofix/Cytoperm (BD) was used according to the manufacturer's instructions, and Perm/Wash buffer was prepared in-house (PBS containing 0.5% Saponin and 5% BSA). For intracellular staining cells were incubated with the respective antibodies for 20 - 30 minutes at 4°C. For intra-nuclear FoxP3 staining, Fixation/Permeabilization buffer (eBioscience) was used after surface staining, followed by the Perm/Wash buffer prepared in-house. In general, cells were gated based on FSC-Area and SSC-Area to exclude debris, doublets were excluded by FSC-Area vs. FSC-Height gating. Dead cells were excluded from the analysis using an Aqua or Near-IR Live/Dead fixable staining reagent (Invitrogen/BioLegend). Where applicable CD45.1 cells were gated before gating on CD4 or CD8 T cells. Flow cytometric analysis was carried out using either a FACSCanto II (BD) or a LSR II Fortessa (special order research product, BD and equipped with 405 nm, 488 nm, 561 nm and 640 nm laser lines) with FACS Diva Software. Data analysis was performed using FlowJo 10.0.x (Treestar).

Flow cytometric analysis of STAT5 phosphorylation

Single cell suspensions of bone marrow were surface stained for 20 minutes, after which GM-CSF containing medium (20 ng/ml) was added to the samples. Cells were incubated at 37°C for 30 minutes to induce phosphorylation of STAT5 before addition of 4% PFA solution (pH 7.4) to a final concentration of 2%. Cells were fixed for 20 minutes, washed and resuspended in 1ml of 4°C methanol. After 40 minutes, cells were washed twice and resuspended in 45µl of FACS buffer. 5µl of anti-pSTAT5 APC (BD Biosciences) was added to make a final staining ratio of 1:10. Cells were incubated for 45 minutes at 4°C before a final wash step and acquisition. Flow cytometric analysis was carried out using a LSR II Fortessa (special order research product, BD and equipped with 405 nm, 488 nm, 561 nm and 640 nm laser lines) with FACS Diva Software. Data analysis was performed using FlowJo 10.0.x (Treestar).

Phenotypic analysis of human PBMCs by flow cytometry

PBMCs were stained with the antibodies listed in Supplementary Table 7.

Mixed lymphocyte reaction (MLR)

C57BL/6 WT, *Csf2*^{-/-}, *Ifng*^{-/-} or *Il17a*^{-/-} responder T cells were co-cultured with DCs from BALB/c (allogeneic) or C57BL/6 (syngeneic) mice at a stimulator/responder ratio of 1/10 (2x10⁴ DCs, 2x10⁵ T cells) for 3 days. Alternatively, BALB/c WT responder T cells were co-cultured with DCs from BALB/c (syngeneic) or C57BL/6 WT and *Csf2rb*^{-/-} DCs (allogeneic). Thereafter culture supernatants were replaced by diluted 3H-thymidine (1/100, 50μCi) for 16 hours. Cells were harvested using a 96-well harvester (PerkinElmer) and scintillation was measured by a beta counter (1450 Microbeta Plus, Wallac).

Killing assays

Spleen and lymph node T cells were isolated using the Pan T Cell Isolation Kit II, mouse (MACS Miltenyi Biotec). A20-Luciferase⁺GFP⁺ or WEHI-3-Luciferase⁺ cells were stained with CellTrace Violet Cell Proliferation Kit (ThermoFisher Scientific, C34557) or PKH26 Red Fluorescent Cell Linker Mini Kit, for general cell membrane labeling (Sigma, MINI26-1KT, 110M907). Cells were incubated at an effector/target ratio of 1:1, 5:1, 10:1, 20:1 or 50:1 in the presence of soluble α-CD3 (1 ug/ml) and α-CD28 (0.5 ug/ml) in RPMI supplemented with 10% FCS for 4h at 37 °C in 5% CO₂. After removal of medium, TOPRO-3 (0.8 μM) was added to the cells and cells were acquired on a LSRII Fortessa flow cytometer (BD). Data were analyzed using FlowJo Version X (Tree Star). The percentage specific lysis was calculated as followed: [(Experimental lysis—spontaneous lysis)/(maximum lysis—spontaneous lysis)] × 100%.

Treg suppression assay

T cells were isolated from lymph nodes and spleen from C57BL/6 WT and *Csf2*^{-/-} mice, as described under “Lymphocyte isolation”. CD4 T cells were first enriched using a CD4 isolation kit (MojoSort Mouse CD4 Nanobeads), followed by staining with CD45, CD25,

CD62L and CD4. CD4⁺CD25⁻CD62L⁺ (Tconv) and CD4⁺CD25^{high} (Treg) cells were sorted by flow cytometry using BD FACS ARIA II cell sorter with a purity of 98-99%. APCs were irradiated with >3400 rads (4 min, 12.2Gy/min, 225 kV, 17.7mA) and diluted to 4x10⁶/mL (or 2x10⁶ cells/ml) in 10% clone medium. The APCs and Tconv cells were co-cultured with autologous Treg cells at varying concentrations in a 96 well round bottom plate (4x10⁴ Tconv per well). Cells were stimulated with αCD3 at a concentration of 1ug/ml at 37°C for 2 days and then incubated for 16h with 3H-Thymidine. Cells were harvested using FilterMate (PerkinElmer) and analyzed by beta counter with the program Microbeta. Treg suppression percentage was calculated among living cells as $[(\text{cpm Tconv} - \text{cpm Treg:Tconv}) / (\text{cpm Tconv alone}) * 100]$, cpm being the counts per minute, calculated as % suppression of Tconv cells.

Cytokine analysis

Serum was obtained from allo-HCT recipient mice at the times specified. Culture supernatants of MLR were collected after 3 days of co-culture. Measurement of GM-CSF, IFNγ, IL-6, TNFα and IL-17A in the serum or cell culture supernatants was performed by cytokine-specific ELISA according to the manufacturer's instructions (BD Biosciences and Biolegend).

Liver function parameters

Liver enzymes ((alanine aminotransferase (ALT), alkaline phosphatase (AP)), albumin and blood urea nitrogen (BUN) were measured in sera of mice using the Piccolo Liver Panel Plus (Abaxis).

Histology

For histo-pathological assessment, organs were fixed in HOPE (DCS, Hamburg, Germany) and embedded in paraffin. 3 - 5 μm sections were cut with a microtome (Micro HM 325, Thermo Scientific) and subsequently stained with hematoxylin and eosin. To generate the histological GvHD scores, a semi-quantitative tissue-specific scoring system was used: for

the small intestine parameters including villous blunting, crypt regeneration, crypt cell apoptosis, outright crypt destruction and lamina propria lymphocytic infiltrates were rated with the following scores: 0=normal; 0.5=focal and rare; 1=focal and mild; 2=diffuse and mild; 3=diffuse and moderate; 4=diffuse and severe, adapted from (51). For the skin the following parameters were rated: estimated hair follicle number: 0 = normal (relatively closely arranged), 0.5 = number slightly reduced (usually focally); estimated sebaceous glands number: 0 = normal (relatively large cells, small groups, at hair neck level), 1.0 = slightly reduced (number and size, usually focally), 2.0 = clearly reduced (only a few cells present); sebaceous glands apoptosis & degeneration: 0 = not present, 0.5 = rare, 1 = small number of glands involved, 2 = moderate number of glands involved; hair follicle apoptosis & degeneration: 0 = not present, 0.5 = rare, 1 = small number of follicles involved, 2 = moderate number of follicles involved, 3 = large number of follicles involved; leukocytic infiltration (extra-adnexal): 0 = low, 1 = slightly increased, 2 = moderately increased; leukocytic infiltration (adnexal): 0 = not present, 1 = slight, 2 = moderate; epidermis (apoptotic cells, leukocytic infiltration): 0 = not present, 0.5 = rare, 1 = small number, 2 = moderate number, 3 = large number; subcutaneous fat: 0 = continuous, 1 = not continuous, 2 = absent, adapted from (52). For the liver parameters including portal tract expansion and infiltrates, bile duct infiltrates, nuclear bile duct multilayering, pyknotic bile duct cells, intraepithelial bile duct cells, vascular endothelialitis, hepatocellular pan-lobular necrosis, acidophil bodies, microabscesses, mitotic figures and hepatocellular steatosis were rated with the following scores: 0=normal; 0.5=focal and rare; 1=focal and mild; 2=diffuse and mild; 3=diffuse and moderate; 4=diffuse and severe, adapted from (53). All slides were coded and read in a blinded fashion on an Olympus BX41TF microscope.

Immunohistochemistry

Immunohistochemistry was performed in fixed paraffin-embedded tissue sections using the two-step IHC staining kit EnVision + System HRP DAKO (Glostrup, Denmark) according to the manufacturer's instructions. Sections underwent heat-mediated antigen retrieval with

Dako Target Retrieval Solution (1x) for 10 min. Endogenous peroxidase activity was blocked with the DAKO endogenous peroxidase blocking kit for 30 min at RT. Primary antibodies were diluted in PBS + 5% Normal Goat Serum (NGS). Single immunostaining consisted of overnight incubation at 4°C with mouse anti-GM-CSF antibody (clone 3209.1; R&D Systems) or IgG isotype control for biopsies from human subjects and rat anti-F4/80 (clone Cl: A3-1, BioRad) and rabbit anti-p22phox (clone FL-195, Santa Cruz) for mouse tissue sections. DAB was used as the chromogen. The sections were then counterstained with hematoxylin, mounted with DPX and analyzed with a light microscope Olympus BX41 coupled to a color camera ColorViwerIIIu (Olympus) and the Fiji/ImageJ software package (GNU General Public License).

Immunofluorescence

Tissues were cryosectioned (10 µm thick) for immunohistochemistry using a Hyrax C60 cryostat (Zeiss) and stored at -80°C. Sections were fixed in 4% PFA, washed in PBS, and blocked with PBS supplemented with 0.1% Triton X-100 and 4% NGS. Subsequently, sections were incubated with the following primary antibodies: rat anti-GM-CSF antibody (BD Pharmingen, clone BVD2-21C11, 1:50), rabbit anti-CD3 (Novus, clone SP7, 1:200) and mouse anti-CD68 antibody (DAKO, clone EMB11, 1:50), diluted in blocking solution overnight at 4°C. Sections were then washed in PBS and incubated with AF647-labeled goat anti-rat, AF488-labeled goat anti-mouse and AF555-labeled donkey anti-rabbit secondary antibodies (Life Technologies, 1:500) overnight at 4°C or at room temperature for 1 h. Sections were mounted with SlowFade Gold antifade reagent with DAPI (Invitrogen). Fluorescence photomicrographs were captured with a SP5 Leica confocal laser scanning microscope (SP5; Leica, Heerbrug, Switzerland) equipped with argon and helium lasers using the 40x objective (oil immersion, NA1.25). Images were processed and merged by Imaris imaging software (Bitplane, Zurich, Switzerland).

TUNEL assay

Sections fixed in 4% PFA were processed for TUNEL Assay to detect fragmented nuclei in the colon. An ApopTag Red In Situ Apoptosis Detection Kit was used according to the manufacturer's instruction. Briefly, the slides were pretreated with H₂O₂ and incubated with the reaction mixture containing TdT and digoxigenin-conjugated dUTP for 1 h at 37 °C. Labeled DNA was visualized with AF488-conjugated secondary antibody. Fluorescence photomicrographs were captured with a SP5 Leica confocal laser scanning microscope (SP5; Leica, Heerbrug, Switzerland) equipped with argon and helium lasers using the 40x objective (oil immersion, NA1.25). Images were processed and merged by Imaris imaging software (Bitplane, Zurich, Switzerland).

RNA isolation and quantitative RT-PCR

RNA was isolated from fixed paraffin-embedded tissue sections using the RNeasy FFPE kit (Qiagen, deparaffinization using xylene) according to manufacturer's instructions. RNA concentration was estimated using a NanoDrop 1000 spectrophotometer (Thermo Scientific). For first strand cDNA synthesis MLV reverse transcriptase (Invitrogen) was used according to manufacturer's instructions. Gene expression was measured by real-time quantitative PCR analysis using the CFX 384 Real-Time detection system (Bio-Rad, Hercules, CA, USA) with SYBR Green Supermix (Bio-Rad). Sequences for PCR primers can be found in the table below. Transcript expression was normalized to the *GAPDH* housekeeping gene and represented as $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta Ct_{\text{Experimental}} - \Delta Ct_{\text{Control}}$). Primer sequence (5' to 3'): *CSF2* (Fwd: CAC TGC TGC TGA GAT GAA TGA AA; Rev: GTC TGT AGG CAG GTC GGC TC), *GAPDH* (Fwd: GAA GGT GAA GGT CGG AGT CAA C; Rev: TGA TTT TGG AGG GAT CTC GCT C).

Automated population identification in high-dimensional data analysis

Data acquired in BD FACSymphony cell analyzer were compensated, exported in FlowJo Version v10.4 (TreeStar), normalized using Cyt MATLAB (version 2017b) and unsupervised analysis were performed as described in (54, 55).

Figure S1

Supplementary Figure 1. Comparative phenotypic analysis of T cell populations from WT and *Csf2*^{-/-} mice

Flow cytometric analysis of splenic (A) T cell population frequencies, and (B) frequencies of activation marker expression in naïve C57BL/6 WT and *Csf2*^{-/-} mice. Data were pooled from 2 individual experiments with each n=3/group. (C) Suppression assay with sort purified Tregs isolated from WT and *Csf2*^{-/-} mice. One experiment with n=4 mice/group is shown. (D) Flow cytometric analysis of donor T cell infiltrates in recipient lymph nodes (LN), spleen, liver, and skin, 2 and 5 days after allo-HCT of lethally-irradiated BALB/c mice reconstituted with WT C57BL/6 TCD-BM combined with 10x10⁶ splenocytes from C57BL/6 WT or *Csf2*^{-/-} mice. Cells were gated on H2-D^b and CD45; CD4 and CD8 T cells were pre-gated on CD3. One of two individual experiments with n=4/group is shown. Data are displayed as mean +/- SEM. (E) Total count of donor T cell infiltrates in recipient lymph nodes (LN), spleen, liver, and skin, 5 days after allo-HCT of mice displayed in c. One experiment with n=4/group is shown. (F) Flow cytometric analysis of activation markers and Tregs within donor T cell infiltrates in recipient LN and spleen 5 days after allo-HCT of lethally-irradiated BALB/c mice reconstituted with WT C57BL/6 TCD-BM combined with 10x10⁶ splenocytes from C57BL/6 WT or *Csf2*^{-/-} mice. Cells were gated on H2-D^b and CD45; CD4 and CD8 T cells. One representative from two individual experiments with n=4/group is shown. (G) Flow cytometric analysis of Ki67 and granzyme B on donor T cell infiltrates in recipient spleen and liver 5 days after allo-HCT of lethally-irradiated BALB/c mice reconstituted with WT C57BL/6 TCD-BM combined with 10x10⁶ splenocytes from C57BL/6 WT or *Csf2*^{-/-} mice. Cells were gated on H2-D^b and CD45; CD3 T cells. One experiments with n=3-5/group is shown. (H) Frequency of IFN γ -, GM-CSF-, TNF α - and IL-17A- producing CD4⁺ and CD8⁺ T cells from spleens and livers of mice 5 days after allo-HCT of lethally-irradiated BALB/c mice reconstituted with WT C57BL/6 TCD-BM combined with 10x10⁶ splenocytes from C57BL/6 WT (top panel) or *Csf2*^{-/-} (bottom panel) mice. One experiments with n=3-5/group

is shown. Data are displayed as mean $\pm$ SEM. Abbreviations: TCD: T cell-depleted, BM: bone marrow, HCT: hematopoietic cell transfer, WT: wild type.

Figure S2

Supplementary Figure 2. GM-CSF is crucial for acute GvHD following fully MHC-mismatched allo-HCT

(A) Survival and (B) clinical score of lethally-irradiated BALB/c mice undergoing allo-HCT with WT C57BL/6 TCD-BM alone or combined with 1×10^5 splenocytes of C57BL/6 WT or *Csf2*^{-/-} mice. Mice treated with TCD-BM alone were used as controls. Data representative of 2 individual experiments, each with n=5/group. For comparison of survival curves a Log-rank (Mantel-Cox) test was used, ns (not significant), *p<0.05, **p<0.01, ***p<0.001. (C) Survival of lethally-irradiated BALB/c mice undergoing allo-HCT with WT C57BL/6 TCD-BM alone or combined with 1×10^5 CD4 and CD8 T cells purified from spleens of C57BL/6 WT or *Csf2*^{-/-} mice (CD4^{WT}CD8^{WT}, CD4^{WT}CD8^{*Csf2*^{-/-}}, CD4^{*Csf2*^{-/-}}CD8^{WT} and CD4^{*Csf2*^{-/-}}CD8^{*Csf2*^{-/-}}). Mice treated with TCD-BM alone were used as controls. Data representative of 2 individual experiments, each with n=5/group. For comparison of survival curves (CD4^{WT}CD8^{WT} vs other groups) a Log-rank (Mantel-Cox) test was used, ns (not significant), **p<0.01. (D and E) Individual histology scores for skin (D) and small intestine (E) from BALB/c mice 6 days after induction of GvHD as described in Fig. 2A. Data were pooled from 3 individual experiments, individual data points are displayed. For comparison of the means (WT vs. *Csf2*^{-/-}) an unpaired t-test was used. (F) Serum TNF α and IL-6 levels in mice 6 days after allo-HCT with WT C57BL/6 TCD-BM combined with 10×10^6 splenocytes from C57BL/6 WT or *Csf2*^{-/-} mice. Representative data from 2 individual experiments, each with n=4-5/group. For comparison of the means (WT vs. *Csf2*^{-/-}) an unpaired two-tailed t-test with Welch's correction was used, *p<0.05. Data are displayed as mean $\pm$ SEM. Abbreviations: WT: wild type.

Figure S3

Supplementary Figure 3. GM-CSF mediates GvHD pathology following partially MHC-mismatched allo-HCT

(A) Individual histology scores for skin from B6D2F1 mice after induction of GvHD as described in Fig. 3A. Data were pooled from 3 individual experiments, individual data points are displayed. For comparison of the means (WT, *Csf2*^{-/-}, and *Ifng*^{-/-}) one-way ANOVA with

Bonferroni post-test was used, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (B) Individual histology scores for liver from B6D2F1 mice 9 and 28 days after induction of GvHD as described in Fig. 3A. For comparison of the means (WT, *Csf2*^{-/-}) an unpaired, two-tailed t-test was used * $p < 0.05$. (C) Quantification of % of total area labeled positively for CD3 per visual field of liver and small intestine of mice 9 days after allo-HCT with WT C57BL/6 TCD-BM combined with 7.5×10^6 T cells from C57BL/6 or *Csf2*^{-/-} mice. Data was pooled from 2 independent experiments, each with $n = 5-8$ /group. Data are displayed as mean $\pm$ SEM. Abbreviations: WT: wild type.

Figure S4

Supplementary Figure 4. GM-CSF drives GvHD through donor derived myeloid cells

(A) Selected channels used in the t-SNE analysis to identify monocytes, neutrophils, eosinophils, Lin (CD3⁺, CD19⁺ and Ter119⁺) and myeloid precursors (MPs) / others (macrophages and Ly6C^{low} monocytes) are overlaid as a color-coded z-dimension. (B) Representative histograms for p-STAT5 staining in neutrophils and monocytes/MDCs. Data represent two independent experiments, each with n=6-7/group. (C) Gating strategy for the myeloid compartment characterization in the spleen, live, CD45⁺, H2Dd⁻ neutrophils (Ly6G⁺, CD11b⁺, CD24⁺), monocytes and MDCs (Ly6G⁻, Ly6C⁺, CD11b^{high}, CD64⁺, MHCII⁺). (D) Quantification of total numbers of neutrophils (Ly6G⁺, CD11b⁺) monocytes and MDCs (Ly6G⁻, Ly6C⁺, CD11b^{high}, MHCII⁺) in the blood, liver and spleen of mice treated with anti-GM-CSF antibody (a-GMCSF) or isotype control 3 times/week during 7 days, starting 2 days before HCT. Data pooled from 2 individual experiments, each with n=5-7/group. Data are displayed as mean +/- SEM. For comparison of the means an unpaired, two-tailed t-test was used *p<0.05, **p<0.01. Abbreviations: WT: wild type, MDCs: monocyte-derived cells.

A

Survival (%)

Days after HCT

○ BM only
■ BM + WEHI
● WT + WEHI
□ *Csf2*^{-/-} + WEHI

B

Survival (%)

Days after HCT

○ BM only
■ WT + A20
□ *Csf2*^{-/-} + A20
● BM + A20
○ BM + A20

C

Signal intensity ROI

Days after HCT

■ BM + A20
□ *Csf2*^{-/-} + A20
● WT + A20

D

Counts

Topro-3

1:50

A20 only A20:WT T cells A20:*Csf2*^{-/-} T cells

% Specific lysis

A20 : T cells

■ WT T cells
□ *Csf2*^{-/-} T cells

E

Counts

Topro-3

1:10

A20 only A20:WT T cells A20:*Csf2*^{-/-} T cells

% Specific lysis

A20 : T cells

□ *Csf2*^{-/-} T cells
■ WT T cells

F

Counts

Topro-3

1:50

WEHI only WEHI:WT T cells WEHI:*Csf2*^{-/-} T cells

% Specific lysis

WEHI : T cells

□ *Csf2*^{-/-} T cells
■ WT T cells

G

WT *Csf2*^{-/-}

CD62L CD44

CD3 Ki67

Granzyme

Spleen LN

Total counts

% of CD4 T cells

% of CD8 T cells

○ WT
■ *Csf2*^{-/-}

Supplementary Figure 5. GM-CSF is dispensable for anti-tumor activity following allo-HCT

(A) Survival of lethally-irradiated BALB/c mice intravenously injected with 2.5×10^5 WEHI-3 tumor cells expressing luciferase, at the same time as MHC-mismatched allo-HCT with WT C57BL/6 TCD-BM alone or combined with 1×10^5 T cells purified from spleens of C57BL/6 WT or *Csf2*^{-/-} mice. Mice treated with TCD-BM alone were used as controls. Data representative of 2 individual experiments, each with n=5/group. For comparison of survival curves (WT vs other groups) a Log-rank (Mantel-Cox) test was used, **p<0.01. (B-C) Lethally-irradiated BALB/c mice were intravenously injected with 5×10^5 A20 tumor cells expressing GFP and luciferase, at the same time as MHC-mismatched allo-HCT with WT C57BL/6 TCD-BM alone or combined with 1×10^5 T cells purified from spleens of C57BL/6 WT or *Csf2*^{-/-} mice. Mice treated with TCD-BM alone were used as controls. (b) Survival over time. Data pooled from 2 individual experiments, each with n=5/group. For comparison of survival curves (WT vs other groups) a Log-rank (Mantel-Cox) test was used, ns (not significant), **p<0.01. (D) Signal intensity in the region of interest (ROI) was monitored over time. Data pooled from 2 individual experiments, each with n=5/group. (D and E) Killing assay using the B cell lymphoma cell line A20 and T cells isolated from spleen and lymph nodes of naïve C57BL/6 WT or *Csf2*^{-/-} mice (in D) or 5 days after HCT (in E), respectively. (left) Flow cytometric analysis of dead cells by TO-PRO-3 staining, representative plots are shown. (right) Specific lysis of A20 tumor cells by indicated T cells at indicated target:effector ratios. Data are representative of two experiments, each with n=5 mice per group. (F) Killing assay using the monomyelocytic cell line WEHI-3 and T cells isolated from spleen and lymph nodes of naïve C57BL/6 WT or *Csf2*^{-/-} mice. (left) Flow cytometric analysis of dead cells by TO-PRO-3 staining, representative plots are shown. (right) Specific lysis of WEHI-3 tumor cells by indicated T cells at indicated target:effector ratios. Data are representative of two experiments, each with n=5 mice per group. (G) Flow cytometric analysis of activation markers CD44 and CD62L and Ki67 and granzyme B on

donor T cell infiltrates in recipient LN and spleen 5 days after allo-HCT described in A. Cells were gated on H2-D^b and CD45; CD3, CD4 or CD8 T cells. One experiment with n=5/group is shown. Data are displayed as mean +/- SEM. Abbreviations: TCD: T cell-depleted, BM: bone marrow, HCT: hematopoietic cell transfer, WT: wild type, BLI: bioluminescent imaging, ROI: region of interest.

Figure S6

Supplementary Figure 6. Frequency of cytokine expressing T cells in PBMCs of patients with GvHD

Gating strategy for T cells in PBMCs of patients with GvHD: FSC/SSC, single cells, live (LD⁻), CD45⁺, CD3⁺. Shown is the staining for CD4⁺ and CD8⁺ T cells. Abbreviations: LD: live/dead.

Supplementary Table 1. Clinico-pathological features of GvHD patient set I

Pat. Number	Gender	Age	Disease	Donor Type	Graft type	Conditioning	Immunosuppression	GvH D grade
1	male	70	AML	MMURD	PBSC	FLU/BCNU/MEL	CyA, Campath	4
2	male	45	AML	MRD	PBSC	FLU/BCNU/MEL	CyA, MTX	4
3	male	51	Mantle cell lymphoma	MMURD	PBSC	FLU/BCNU/MEL	CyA, Campath	4
4	male	29	Morbus Hodgkin	haploidentical sibling	PBSC	FLU/MEL/TREOS	MMF/Cyclophosphamide/Everolimus	4
5	male	43	B-ALL	MURD	PBSC	TBI/VP-16	CyA, MTX, ATG	4
6	male	62	CLL	MURD	PBSC	FLU/BCNU/MEL	CyA/Rituximab/Campath	4
7	male	-	-	-	-	-	-	4
8	Mal3	64	CLL	MURD	PBSC	FLU/BCNU/MEL	CyA/Rituximab/Campath-	4
9	male	54	CVID	MRD	PBSC	FLU/BCNU/MEL	CyA/Campath	3
10	female	60	MDS-RAEB II	MURD	PBSC	FLU/BCNU/MEL	CyA/Campath	3
11	female	61	AML	MMURD	PBSC	FLU/TT	CyA/Campath	3
12	female	59	T-NHL	MMURD	PBSC	FLU/TT	CyA/Campath	3
13	male	76	Acute erythroid leukemia	MURD	PBSC	FLU/BCNU/MEL	CyA/ATG/MMF	3
14	female	47	AML	MURD	PBSC	FLU/BU/TT	CyA/MMF/ATG	3
15	female	61	MM	MRD	PBSC	TT/BU/FLU	CyA/MMF/ATG	3
16	male	61	Morbus Hodgkin	MURD	PBSC	FLU/TT	CyA/MMF/ATG	3
17	male	52	AML	MURD	PBSC	BU/CY	CyA/Campath	1
18	male	76	MDS-RAEB	MURD	PBSC	FLU/BCNU/MEL	CyA/Campath	1
19	female	58	AML	MMURD	PBSC	FLU/TT	CyA/MMF/ATG	1
20	female	48	AML	MMURD	PBSC	BU/CY	CyA/MTX/MMF/ATG	1
21	female	71	sAML/MPN	MMURD	PBSC	FLU/TT/MEL	CyA/MMF/ATG	1
22	female	59	MM	MRD	PBSC	FLU/BU/TT	CyA/MMF	1
23	female	51	AML	MMURD	PBSC	BU/CY	CyA/MTX/MMF/ATG	1
24	female	59	AML	MRD	PBSC	BU/CY	CyA/MTX	1
25	male	47	AML	MMURD	PBSC	FU/TT	CyA/MTX	1
26	male	74	AML	MRD	PBSC	FLU/BCNU/MEL	CyA/MMF/ATG	1
27	female	70	B-NHL	MURD	PBSC	FLU/BCNU/MEL	CyA/Campath	1

AML: acute myeloid leukemia; B-ALL: B cell-acute lymphoid leukemia; CVID: common variable immunodeficiency, MDS: myelodysplastic syndrome; RAEB: refractory anemia with excess blasts; T-NHL: T cell-non-Hodgkin lymphoma; B-NHL: B cell-non-Hodgkin lymphoma, sAML: secondary acute myeloid leukemia; MPN: myeloproliferative neoplasms CLL: chronic lymphocytic leukemia; MM: multiple myeloma; MMURD: mismatched unrelated donor; MRD: matched related donor; MURD: matched unrelated donor; PBSC: peripheral blood stem cells; FLU: fludarabine; BCNU: bis-chloroethylnitrosourea; MEL: melphalan; TREOS: treosulphan; TBI: total body irradiation; VP16: etoposide; TT: thiotepa; BU: busulphan; CY: cyclophosphamide; CyA: cyclosporine A; MTX: methotrexate; MMF: mycophenolate mofetil; ATG: antithymocyte globuline.

Supplementary Table 2. Clinico-pathological features of GvHD patient set II

Pat. Number	Gender	Age	Disease	Donor Type	Graft type	Conditioning	Immunosuppression	GvHD grade
1	Male	44	CVID	MRD	PBSC	FLU/BCNU/MEL	CyA/Campath	3
2	Male	57	sAML	MMURD	PBSC	FLU/BCNU/MEL	CyA/Campath/MMF	4
3	Female	21	T-ALL	MURD	PBSC	TBI/VP16	CyA/Campath	4
4	Male	69	sAML	MURD	PBSC	FLU/BCNU/MEL	CyA/Campath	4
5	Male	6	MDS	MURD	BM	TT/FLU/ATG	CyA/MTX	3

CVID: common variable immunodeficiency; sAML: secondary acute myeloid leukemia; T-ALL: T cell-acute lymphoid leukemia; MDS: myelodysplastic syndrome; MRD: matched related donor; MMURD: mismatched unrelated donor; MURD: matched unrelated donor; PBSC: peripheral blood stem cells; BM: bone marrow; FLU: fludarabine; BCNU: bis-chloroethylnitrosourea; MEL: melphalan; TBI: total body irradiation; VP16: etoposide; TT: thiotepa; ATG: antithymocyte globulin; CyA: cyclosporine A; MMF: mycophenolate mofetil; MTX: methotrexate.

Supplementary Table 3. Characteristics of control subjects

Pat. Number	Gender	Age	Biopsy
1	male	59	No pathological finding
2	male	60	No pathological finding
3	male	46	No pathological finding

Supplementary Table 4. Clinico-pathological features of GvHD patient PBMCs

Patient	Gender	Age (years)	Disease	Graft Type	Conditioning	GvHD prophylaxis	GvHD grade
1	M	55	AML	PBSC	FLU/BCNU/MEL	CyA, MMF, ATG	Ileum IV, colon IV
2	M	64	Mantle cell lymphoma	PBSC	FLU/BU/TT	CyA, MMF, ATG	Gut III-IV, Skin III
3	F	65	AML	PBSC	FLU/MEL/TT	Everolimus, MMF	Skin IV, Gut III
4	M	37	AML	PBSC	FLU/MEL/TT	Everolimus, MMF	Liver IV, Gut IV,

							Skin III, Eyes
5	M	60	B-NHL	PBSC	FLU/MEL/TT	Tacrolimus, MTX, KRP	Skin III
6	F	73	AML	PBSC	FLU/MEL/TT	CyA, MMF, ATG	Gut IV
7	F	71	Myelofibrosis	PBSC	FLU/MEL/TT	CyA, MMF, ATG	Skin III, Gut III
8	M	31	AML	PBSC	FLU/BU	CyA, MMF	Skin III, Gut III
9	M	44	Myelofibrosis	PBSC	FLU/MEL/TT	CyA, MMF, Cyclophosphamide	Skin I, Gut II

AML: acute myeloid leukemia; B-NHL: B cell-non-Hodgkin lymphoma; PBSC: peripheral blood stem cells; FLU: fludarabine; BCNU: bis-chloroethylnitrosourea; MEL: melphalan; TT: thiotepe; BU: busulphan; CyA: cyclosporine A; MTX: methotrexate; MMF: mycophenolate mofetil; ATG: antithymocyte globuline.

Supplementary Table 5. Primary data

See attached Excel file.

Supplementary Table 6. GvHD scoring system for allo-HCT mice

GvHD criteria	Grade 0	Grade 1	Grade 2	Grade 3
Weight loss	< 10%	10 – 20%	20 – 25%	> 25%
Posture	Normal	Hunching noted only at rest	Moderate hunching also at movement	Severe hunching impairs movement
Activity	Normal	Mild to moderately decreased	Decreased	Severely decreased
Fur texture	Normal	Mild to moderately ruffling	Severe ruffling / poor grooming	Fur loss
Skin integrity	Normal	Patchy scaling of skin and/or genital oedema	Moderate scaling and inflammation of skin	Diffuse ulcerative skin lesions and severe inflammation

Supplementary Table 7. Phenotypic analysis of human PBMCs by flow cytometry

Antigens	Clones	Manufacturers
CD45	HI-30	BD Pharmingen
CD3	UCHT1	BD Pharmingen
CD8	RPT-T8	BioLegend
CD4	RPA-T4	BioLegend
INTRACELLULAR		
Antigens	Clones	Manufacturers
IL-17A	BL168	BioLegend
IFN γ	4S.B3	BioLegend
GM-CSF	BVD2-21C11	BD Pharmingen