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Oncogene induced TIM-3 ligand expression dictates susceptibility to anti-TIM-3 therapy in mice

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Abstract

Leukemia relapse is a major cause of death after allogeneic hematopoietic cell transplantation (allo-HCT). We tested the potential of targeting TIM-3 for improving graft-versus-leukemia (GVL) effects. We observed differential expression of TIM-3 ligands when hematopoietic stem cells overexpressed certain oncogenic-driver mutations. Anti-TIM-3 Ab-treatment improved survival of mice bearing leukemia with oncogene-induced TIM-3 ligand expression. Conversely, leukemia cells with low ligand expression were anti-TIM-3 treatment-resistant. In vitro, TIM-3 blockade or genetic deletion in CD8+ T cells (Tc) enhanced Tc activation, proliferation and IFN-γ production while enhancing GVL effects, preventing Tc exhaustion and improving Tc cytotoxicity and glycolysis in vivo. Conversely, TIM-3 deletion in myeloid cells did not affect allogeneic Tc proliferation and activation in vitro, suggesting that anti-TIM-3-treatment-mediated GVL effects are Tc-induced. In contrast to anti-PD-1 and anti-CTLA-4-treatment, anti-TIM-3-treatment did not enhance acute graft-versus-host-disease (aGVHD). TIM-3 and its ligands were frequently expressed in acute myeloid leukemia (AML) cells of patients with post-allo-HCT relapse.

We deciphered the connection between oncogenic mutations found in AML and TIM-3 ligands expression and identify anti-TIM-3-treatment as a strategy to enhance GVL effects via metabolic and transcriptional Tc-reprogramming, without exacerbation of aGVHD. Our findings support clinical testing of anti-TIM-3 Abs in patients with AML relapse post-allo-HCT.
Introduction

Acute myeloid leukemia (AML) relapse is the main cause of death after allogeneic hematopoietic cell transplantation (allo-HCT) (1). Mechanisms promoting relapse include MHC class II downregulation (2, 3), loss of mismatched HLA (4), immune checkpoint ligand upregulation (3), reduced IL-15 production (5) and leukemia-derived lactic acid release (6) among others (reviewed in (7)). A longstanding approach to address relapse is treatment with cellular therapy such as donor lymphocyte infusions (DLI), either alone or with hypomethylating agents (HMA) (8, 9). A retrospective analysis on response to DLI reported complete response (CR) rates of 17% for patients with AML (10) and another large retrospective, multicenter study reported a 2-year overall survival (OS) upon DLI treatment of 20% versus 9% upon chemotherapy-only treatment (9). These reports indicate that donor T cells (Tc) can exert graft-versus-leukemia (GVL) effects, but also demonstrate the high unmet medical need for therapies that enhance GVL effects. Current pharmacological approaches for prevention and treatment of AML relapse include the use of FMS-like tyrosine kinase 3 (FLT3)-kinase inhibitors (5, 11, 12), immune checkpoint inhibitors (ICIs) (13-17), HMA (8, 18), B-cell lymphoma 2 (BCL-2) inhibitors (19), mouse double minute 2 (MDM2) inhibition (20) and others (21).

T cell immunoglobulin and mucin-containing molecule 3 (TIM-3) has recently emerged as an inhibitory receptor (22, 23), and more broadly, as a marker for Tc dysfunction in cancer (24). It was recently described that TIM-3 may also play an important role in regulating immune responses in solid tumors as evidenced by recent data from mouse models of melanoma (23). In addition to its role in immune cells, TIM-3 is a promising target in AML because it is highly expressed on leukemic stem cells (LSC) (25). Functionally, TIM-3 and its ligand, galectin-9 (Gal-9), induce an autocrine loop that is essential for LSC self-renewal and AML development (26). Additionally, refractoriness to treatment correlated with TIM-3 expression in AML (27).

Early clinical trials testing TIM-3 blockade using the humanized IgG4 anti-TIM-3 Ab (sabatolimab, MBG453, Novartis) in combination with decitabine in 60 evaluable patients with AML outside of the allo-HCT setting reported CR rates of 35.3% (28). The contribution of TIM-3 in LSCs contrary to Tc for clinical activity of anti-TIM-3 Abs remains unclear.

Using an anti-TIM-3 blocking Ab, we observed that TIM-3 inhibition enhanced the survival of leukemia-bearing mice post allo-HCT and reduced exhausted TIM-3+PD-1+ Tc frequencies. Importantly, we report that cell specific TIM-3 deletion in CD8+ Tc (Havcr2fl/fl;E8i cre/+ ) caused enhanced Tc proliferation, activation, effector phenotype and anti-leukemia immunity.
Results
Differential TIM-3 ligand expression in hematopoietic stem cells (HSCs) upon oncogene activation confers susceptibility to anti-TIM-3 Ab therapy in multiple AML models

We previously reported that the JAK2-V617F oncogenic mutation enhances the expression of the immunosuppressive programmed death-ligand 1 (PD-L1) (29) and sought to investigate connections between oncogenes found in AML and the expression of TIM-3 (encoded by the hepatitis A virus cellular receptor 2 (Havcr2) gene) and its ligands. We observed that the TIM-3 ligands Gal-9 (encoded by Lgals9) and carcinoembryonic antigen-related cell adhesion molecule 1 (Ceacam1) were differentially expressed when compared to empty vector control depending on the oncogene that was introduced into HSCs of BALB/c mice, with FLT3-ITD showing the greatest upregulation of both ligands. c-KIT-D816V oncogenic-driver mutation led to a significant increase of Lgals9 in HSCs (Figure 1A, B). Conversely, we found no association between oncogenes and TIM-3 or TIM-3 ligand high–mobility group box 1 (Hmgb1) expression (Suppl. Figure 1A, B). Consistent with the findings in primary HSCs cells, the transduction of the myeloblast-like murine cell line 32D with FLT3-ITD caused enhanced secretion of Gal-9, while less Gal-9 was secreted upon overexpression of FIP1L1-PDGFRα (Suppl. Figure 1C). We then transferred HSCs previously transduced with different oncogenes in mice undergoing allo-HCT and allogeneic Tc transfer and compared the efficacy of anti-TIM-3 treatment after FLT3-ITD or FIP1L1-PDGFRα transgenic (Tg) HSCs injection. Mice were treated i.p. every 3 days from day 7 to day 28 with 150μg of anti-TIM-3 or isotype Ab. Anti-TIM-3 treatment was efficient in mice undergoing allo-HCT carrying FLT3-ITD Tg HSCs or c-KIT-D816V Tg HSCs but not in mice carrying FIP1L1-PDGFRα Tg HSCs (Figure 1C-E). We observed a higher expression of TIM-3 ligands in FLT3-ITD and c-KIT-D816V Tg HSCs compared to FIP1L1-PDGFRα Tg HSCs, which is in agreement with the concept that anti-TIM-3 therapy sensitivity is associated with the expression of TIM-3 ligands.

TIM-3, Gal-9 and CEACAM1 expression levels were increased on CD8+ Tc at days 17 and 24 in mice injected with FLT3-ITD MLL-PTD AML cells and undergoing allo-HCT with allogeneic Tc transfer compared to untreated control mice (Suppl. Figure 1D). This increase likely reflects the activation of allo-reactive T cells. Conversely, gene expression levels of Havcr2 and Ceacam1 did not differ on leukemia cells at different time points after allo-HCT as compared to untreated controls. Lgals9 gene expression was only increased on day 10 after allo-HCT (Suppl. Figure 1E).

We further tested the effect of anti-TIM-3 treatment on the GVL effect in additional models using WEHI-3B AML and the previously described FLT3-ITD MLL-PTD driven AML model (5). In all models, donor Tc are infused two days after allo-HCT to reproduce clinical conditions where DLI is administered during low leukemia burden, as shown previously (5, 20). FLT3-ITD MLL-PTD driven AML cells exhibited higher TIM-3 and ligand expression compared to WEHI-
3B AML cells (Figure F-I). Anti-TIM-3 treatment improved the survival in both models, with a more prominent effect observed in FLT3-ITD MLL-PTD driven AML, consistent with previous observations (Figure 1J, K). Notably, mice treated with anti-TIM-3 Ab without allogeneic Tc transfer were not protected from FLT3-ITD MLL-PTD driven AML (Figure 1J) indicating that anti-TIM-3 Ab requires allo-HCT to exert its effects. Anti-TIM-3 treatment also reduced the leukemia burden in FLT3-ITD MLL-PTD driven AML, as evidenced by decreased AML cell frequency in the bone marrow (BM) at day 23 post allo-HCT (Figure 1L). Additionally, mice treated with anti-TIM-3 Ab in the absence of allo-HCT were not protected from FLT3-ITD MLL-PTD driven AML (Suppl. Figure 2A), supporting the hypothesis that anti-TIM-3 Ab has no direct cytotoxic effect against AML cells but requires allo-HCT. These results highlight that oncogene-driven expression of TIM-3 and its ligands contributes to disease progression and targeting TIM-3 in the presence of donor allogeneic T cells enhances the GVL effect.

Anti-TIM-3 Ab treatment after allo-HCT reduces Tc exhaustion, increases glycolytic capacity of Tc and enhances immune responses

Since Ab dependent cell-mediated cytotoxicity (ADCC) of the anti-TIM-3 Ab on AML cells was unlikely based on the lack of efficacy against AML cells in vivo in the absence of Tc (Figure 1J), we investigated the effect of anti-TIM-3 Ab treatment on Tc phenotype in both WEHI-3B and FLT3-ITD MLL-PTD AML models. Tc exhaustion, characterized by the co-expression of PD-1 and TIM-3, has been observed in AML patients relapsing after allo-HCT (30). Using WEHI-3B cells with allo-HCT and Tc transfer followed by anti-TIM-3 or isotype Ab treatment, we found a lower frequency of exhausted TIM-3+PD-1+ donor Tc in CD4+ and CD8+ Tc subsets, in the spleen and in the BM isolated on day 23 post allo-HCT, upon anti-TIM-3 treatment compared to isotype Ab treatment (Figure 2A, B, Suppl. Figure 3A-C). Importantly, this reduction in TIM-3+ Tc was not due to steric hindrance by the anti-TIM-3 blocking Ab, as the antibodies used for flow cytometry (FC) detection recognize different epitopes, and TIM-3 staining was unaffected by anti-TIM-3 Ab treatment (Suppl. Fig. 3D, E). To extend our analysis of Tc exhaustion, we conducted high-resolution spectral FC-based analysis on Tc isolated from spleens at day 23 post allo-HCT in FLT3-ITD MLL-PTD AML-bearing mice. The analysis revealed several CD4+ and CD8+ Tc subsets (Figure 2C, Suppl. Figure 4A). By investigating both phenotypic and functional markers, we observed that anti-TIM-3 Ab treatment profoundly remodeled both CD4+ and CD8+ Tc subsets when compared to isotype Ab treatment. Notably, we found an increased expression of granzyme B in CD8+ effector Tc subset upon anti-TIM-3 Ab, as well as an increased expression of CD38 and the ectonucleoside triphosphate diphosphohydrolase-1 (CD39) in precursor exhausted CD8+ Tc (Figure 2D, E), which have been described as tumor-specific CD8+ Tc that exhibit potent anti-tumor activity against different solid cancers (31-34). Following our previous work showing that reduced glycolytic
activity of Tc is associated to impaired GVL effects (6), we next utilized a single-cell FC-based assay to measure the metabolic response in donor Tc by quantifying protein synthesis levels across different immune cells (35). Tc were isolated from spleens of FLT3-ITD MLL-PTD AML-bearing mice post allo-HCT and Tc transfer, because of the improved survival of anti-TIM-3 Ab-treated mice in this model (Figure 1J). We observed a higher glycolytic capacity of CD4+ and CD8+ Tc upon anti-TIM-3 Ab treatment compared to isotype Ab treatment (Figure 2F, G). Additionally, non-targeted metabolomics analysis using liquid chromatography–mass spectrometry (LC-MS) revealed elevated levels of all identified members of the Kyoto Encyclopedia of Genes and Genomes (KEGG) module "glycolysis" in Tc from anti-TIM-3 Ab-treated mice (Figure 2H). The analysis of the 10 most significantly enriched pathways revealed an enrichment of the pathway "glycolysis and gluconeogenesis" in Tc isolated from anti-TIM-3 Ab compared to isotype-treated mice (Suppl. Figure 4A, B). Taken together, these findings indicate that anti-TIM-3 treatment enhances glycolytic activity in Tc following allo-HCT in leukemia-bearing mice.

Using high-dimensional spectral FC, we next examined the impact of anti-TIM-3 Ab treatment on the myeloid lineage phenotype in FLT3-ITD MLL-PTD AML-bearing mice that had undergone allo-HCT and Tc transfer on day 2 after allo-HCT (Figure 2I). Analysis of the spleen at day 23 post allo-HCT revealed a decreased frequency of leukemic blasts, in line with a more potent GVL effect, and a higher frequency of neutrophils upon anti-TIM-3 treatment (Figure 2K, L). Consistent with a potential role for neutrophils in ICI-mediated GVL effects, recent evidence has suggested that neutrophils contribute to ICI-induced anti-tumor immune responses (36, 37). Additionally, we found an increased frequency of type-1 conventional DCs (cDC1s) and macrophages upon anti-TIM-3 compared to the isotype Ab treatment (Figure 2M, N). cDC1s have been described to provide an essential niche to promote memory precursor T cell maintenance and differentiation in the context of anti-PDL-1 therapy (38). Furthermore, we found that the expression of TIM-3 and Gal-9 was significantly enriched in distinct myeloid cell types upon anti-TIM-3 treatment. Notably, expression of TIM-3 was increased in cDC1s and macrophages, while monocytes and macrophages were enriched for Gal-9 (Suppl. Figure 4B).

Consistent with the effects of anti-TIM-3 treatment observed in vivo, we observed similar effects following in vitro anti-CD3/CD28-mediated Tc activation. When the isotype Ab was added to the culture, more than 30% of CD4+ and CD8+ Tc subsets displayed a phenotype of exhaustion (based on the co-expression of PD-1 and TIM-3 on Tc) after 14 days of culture (Figure 3A). Consistent with our in vivo studies, addition of anti-TIM-3 Ab in vitro resulted in reduced frequencies of exhausted Tc in both CD4+ and CD8+ subsets (Figure 3A-E). Additionally, we observed a decreased expression of the exhaustion-associated transcription
factor thymocyte selection-associated HMG BOX (TOX) in CD4+ and CD8+ Tc when the anti-TIM-3 Ab was added to the culture (Figure 3F-I).

These findings indicate that anti-TIM-3 Ab treatment induces several changes in both Tc and myeloid cell populations. We found phenotypic features of Tc exhaustion to be reduced in vitro and in vivo. Accordingly, we observed increased effector functions in different Tc subsets in vivo, which is in line with a more effective elimination of AML blasts.

**Genetic TIM-3 deletion in CD8+ Tc enhances Tc activation, proliferation and IFN-γ production as well as GVL effects**

Since the anti-TIM-3 Ab will block TIM-3 on multiple cell types, we next used TIM-3 conditional knockout mice (Havcr2Δ/Δ) generated previously via Cre/lox based gene deletion (23), to identify the cell types relevant for the anti-TIM-3 Ab-mediated effects. To test the role of TIM-3 for allo-antigen driven Tc activation, Tc were exposed in vitro to allogeneic stimulating Tc-depleted PBMCs. We observed that specific TIM-3 deletion in CD8+ Tc (Havcr2Δ/Δ;E8pre+) caused increased proliferation compared to Havcr2Δ/Δ Tc (Figure 4A). Conversely, TIM-3 deletion in all DCs (Havcr2Δ/Δ;Cd11ccre+) or conventional DC (Havcr2Δ/Δ;Zbtb46cre+) subsets used for allogeneic stimulation did not affect Tc proliferation (Suppl. Figure 6A, B).

Additionally, upon co-culture with allogeneic stimulating cells, Havcr2Δ/Δ;E8pre+ Tc exhibited elevated expression of activation markers CD25 and CD69 compared to Havcr2Δ/Δ Tc (Figure 4B-E), which is consistent with the increased proliferation of CD8+ Tc. In contrast, TIM-3 deficiency in the stimulating cells did not induce CD25 and CD69 upregulation in Tc (Suppl. Figure 6C-F).

To analyze the contribution of recipient DCs, we pre-treated the mice with anti-TIM-3 Ab at day -4 and day -1 before allo-HCT. The Ab would bind to the recipient’s APCs and therefore induce blockade of TIM-3 on these. However, we found no survival advantage in mice pre-treated with anti-TIM-3 Ab before allo-HCT (Suppl. Figure 6G), confirming our hypothesis that anti-TIM-3 Ab treatment acts on allogeneic Tc to reinvigorate CD8+ Tc and thus reduce Tc exhaustion.

Consistent with enhanced activation, IFN-γ production was increased in Havcr2Δ/Δ;E8pre+ Tc compared to Havcr2Δ/Δ Tc upon stimulation with allogeneic APC in vitro for 2 days (Figure 4F) or 4 days (Figure 4G). Next, we aimed to investigate Tc function in vivo by injecting both TIM-3 conditional knockout (Havcr2Δ/Δ, C57BL/6) BM and Tc into WEHI-3B-bearing allogeneic BALB/c recipient mice. We observed that transfer of Tc caused improved survival of AML-bearing mice after allo-HCT when TIM-3 was deleted in CD8+ Tc compared to Havcr2Δ/Δ Tc (Figure 4H). Moreover, using Havcr2Δ/Δ;Cd4cre+ mice as donors for allogeneic BM and Tc led to improved survival of AML-bearing mice after allo-HCT compared to Havcr2Δ/Δ Tc (Figure 4I).

Supporting our findings in vivo, Havcr2Δ/Δ;Cd4cre+ Tc exhibited higher cytotoxic capacity against WEHI-3B cells compared to Havcr2Δ/Δ Tc in vitro (Figure 4J).
We analyzed the phenotype of Tc from the spleens of Havcr2\textsuperscript{cko} mice under steady state conditions and observed no difference with respect to CD4\textsuperscript{+} and CD8\textsuperscript{+} Tc frequencies (Suppl. Figure 7A-B) and phenotype (Suppl. Figure 7C) between the three different genotypes. We assessed the development of acute graft-versus-host-disease (aGVHD) by histological scoring of the liver, small intestines (SI) and colon and found no significant difference between mice that had received Havcr2\textsuperscript{fl/fl};E8i\textsuperscript{cre/+} or Havcr2\textsuperscript{fl/fl};Cd4\textsuperscript{cre/+} compared to Havcr2\textsuperscript{fl/fl} Tc (Suppl. Figure 8A-C). Additionally, we found a reduction of the leukemia burden in allo-HCT mice that had received Havcr2\textsuperscript{fl/fl};E8i\textsuperscript{cre/+} or Havcr2\textsuperscript{fl/fl};Cd4\textsuperscript{cre/+} Tc compared to Havcr2\textsuperscript{fl/fl} Tc (Suppl. Figure 8D). We also observed a reduced frequency of TOX\textsuperscript{+} cells and an increased frequency of the transcription factor 7 (TCF-7\textsuperscript{+}) cells among CD8\textsuperscript{+} Tc in allo-HCT mice that had received Tc isolated from Havcr2\textsuperscript{fl/fl};E8i\textsuperscript{cre/+} or Havcr2\textsuperscript{fl/fl};Cd4\textsuperscript{cre/+} Tc compared to Havcr2\textsuperscript{fl/fl} Tc (Suppl. Figure 8E-G). TCF-7 is a transcription factor known to be critical for the generation of the CD8\textsuperscript{+} Tc memory response (39). Accordingly, we observed a significant expansion of phenotypic CD8\textsuperscript{+} effector memory Tc, as seen by the increased frequency of IFN-\gamma \textsuperscript{+} TNF-\alpha\textsuperscript{+} cells among TCF-7\textsuperscript{+} CD8\textsuperscript{+} Tc in allo-HCT mice that had received Tc isolated from Havcr2\textsuperscript{fl/fl};E8i\textsuperscript{cre/+} or Havcr2\textsuperscript{fl/fl};Cd4\textsuperscript{cre/+} Tc compared to Havcr2\textsuperscript{fl/fl} Tc (Suppl. Figure 8H). These results suggest a more efficient and sustainable CD8\textsuperscript{+} Tc immune response, which could explain the benefit in the survival outcomes observed when TIM-3 is deleted in all donor Tc compared to the deletion of TIM-3 in CD8\textsuperscript{+} Tc only.

Overall, deleting TIM-3 in both CD4\textsuperscript{+} and CD8\textsuperscript{+} Tc has an additive effect compared to deletion in CD8\textsuperscript{+} Tc alone, potentially because CD4\textsuperscript{+} Tc support CD8\textsuperscript{+} Tc function by producing IFN-\gamma. TIM-3 may inhibit CD4\textsuperscript{+} Tc activity. Conversely, deletion of TIM-3 in CD4\textsuperscript{+} Tc may lead to their consecutive activation, which may further enhance CD8\textsuperscript{+} Tc effector function against AML cells.

Taken together, these results indicate that genetic deletion of TIM-3 in CD8\textsuperscript{+} Tc enhances Tc activation, proliferation and IFN-\gamma production in vitro. In vivo deletion of TIM-3 in CD8\textsuperscript{+} Tc or all Tc improves GVL effects. Conversely, TIM-3 deletion in DCs did not affect allogeneic Tc responses in vitro.

**Single-cell RNA sequencing (scRNA-seq) reveals that genetic TIM-3 deletion in CD8\textsuperscript{+} Tc expands CD8\textsuperscript{+} stem-like Tc and promotes features of effector Tc**

To decipher the mechanism underlying the enhanced GVL effect seen when deleting TIM-3 in CD8\textsuperscript{+} Tc, we performed scRNA-seq on donor Havcr2\textsuperscript{fl/fl} and Havcr2\textsuperscript{fl/fl};E8i\textsuperscript{cre/+} Tc isolated from the spleen of WEHI-3B-bearing BALB/c recipient mice. We prepared scRNA-seq libraries from
CD3⁺ Tc for 4 samples (n=2 Havcr2⁺/+ and n=2 Havcr2⁺/+;E8pre⁺). Data integration revealed 21 distinct clusters (Suppl. Figure 9A) before exclusion of non-CD3⁺ cells (Suppl. Figure 9B). The analysis was then sub-clustered into CD4⁺ and CD8⁺ Tc (Suppl. Figure 9C). Clustering analysis of CD8⁺ Tc revealed 10 populations of Tc, including 2 clusters of progenitor cells (cluster 4 and cluster 5), 5 subsets of effector Tc (cluster 0, 1, 2, 3, 8 and 9) and 3 populations of exhausted Tc (clusters 2, 6 and 7) (Figure 5A). The top 6 genes in each cluster of CD8⁺ Tc is shown in Suppl. Figure 10A. All clusters of exhausted Tc expressed characteristic genes, including those encoding for TOX (40), PD-1 and Lymphocyte-activation gene 3 (LAG-3). However, in contrast to cluster 6 and cluster 7, cells in cluster 2 also expressed high levels of the genes encoding for the transcription factors Eomesodermin (Eomes) and nuclear receptor 4A2 (Nr4a2), which have been reported to be strongly associated with exhaustion (41). Moreover, cluster 6 and cluster 7 were the only clusters to express Tcf7 and the signaling lymphocytic activation molecule family member 6 (Slamf6), allowing us to identify cluster 6 and 7 as precursor exhausted (Tₚₑₓ) and cluster 2 as terminally exhausted (Tₜₑₓ) CD8⁺ Tc (Figure 5B; Suppl. Figure 10A). Tₚₑₓ are non-fully functional CD8⁺ Tc displaying a stem-like profile that persist long-term and differentiate into Tₜₑₓ subset. Consequently, Tₚₑₓ have been described to better control tumor growth than Tₜₑₓ in several solid cancers. Additionally, it has been shown that Tcf7 and Slamf6 expressing CD8⁺ Tc contribute to ICI-mediated anti-tumor immune responses (42-44). All clusters were represented in both Havcr2⁺/+ and Havcr2⁺/+;E8pre⁺ conditions, albeit in different proportions (Figure 5C, Suppl. Figure 10B-C) and the number of cells that are present in each CD8⁺ Tc cluster is shown in Suppl. Table 1. In particular, cluster 6 containing Tₚₑₓ cells was more abundant in Havcr2⁺/+;E8pre⁺ Tc compared to Havcr2⁺/+ Tc. In contrast cluster 2, which represents Tₜₑₓ cells, was less abundant in Havcr2⁺/+;E8pre⁺ Tc. Additionally, cluster 8 and 9, identified as short-term effector CD8⁺ Tc, were less abundant in Havcr2⁺/+ Tc. (Figure 5C). Moreover, Tc in cluster 6 expressed higher levels of Tcf7 and Slamf6 in Havcr2⁺/+;E8pre⁺ Tc (Figure 5D) and cluster 7 was strongly enriched for a transcriptomic signature of memory precursor Tc (Figure 5E), indicating an enriched Tₚₑₓ signature for these 2 clusters of CD8⁺ Tc in Havcr2⁺/+;E8pre⁺ Tc. Additionally, we performed gene-set-enrichment in CD8⁺ Tc clusters, revealing significant transcriptional differences between Havcr2⁺/+;E8pre⁺ and Havcr2⁺/+ groups (Suppl. Figure 10D). In particular, Tₚₑₓ subsets in Havcr2⁺/+;E8pre⁺ group showed enrichment in the “IL-2/Stat5 signaling pathway” and “inflammatory response”. Notably, cluster 7 (Tₚₑₓ subset) was enriched for the “Wnt/β-catenin signaling” pathway, crucial for Tc differentiation, effector functions, and migration (45). Furthermore, several studies have described the importance of Wnt/β-catenin signaling in the activation and maintenance of Tₚₑₓ, which display indispensable antitumor capacities (46, 47), confirming an enriched signature for memory Tc in the Havcr2⁺/+;E8pre⁺ group. These results
demonstrate that TIM-3 deletion in CD8+ Tc promotes the expansion of polyfunctional T_{PEX} and effector Tc, contributing to the enhanced GVL effects observed in mice receiving Havcr2^{fl/fl};E8^{pre/} Tc compared to those receiving Havcr2^{fl/fl} Tc.

Within CD4+ Tc subclusters, we identified 6 distinct cell types, including a Treg cluster (cluster 5), and 5 clusters of effector/memory CD4+ Tc (Suppl. Figure 11A, B). Clusters 0 and 2, containing effector memory/effector Tc (Tem/Teff), were more abundant in TIM-3-deficient CD8+ Tc. Cluster 4, identified as resident-memory-like CD4+ Tc, was more abundant in Havcr2^{fl/fl} Tc (Suppl. Figure 11C). Cluster 1, characterized by high transcription Factor 7 (Tcf7) and Slamf6 expression, defined precursor effector memory Th1 cells, required for long-term and heightened anti-tumor immunity (48), including in the context of allo-HCT (49). We found that cells in cluster 1 exhibited an enriched transcriptomic signature for memory precursor Tc in the Havcr2^{fl/fl};E8^{pre/} group (Suppl. Figure 11D). Additionally, cluster 3 was strongly enriched for a transcriptomic signature of early activated cytotoxic CD4+ Tc in Havcr2^{fl/fl};E8^{pre/} Tc (Suppl. Figure 11E). The top 6 genes in each cluster of CD4+ Tc is shown in Suppl. Figure 12A. All clusters were represented in both Havcr2^{fl/fl} and Havcr2^{fl/fl};Cd4^{cre/+} Tc (Suppl. Figure 11C, Suppl. Figure 12B-C) and the number of cells that are present in each CD4+ Tc cluster is shown in Suppl. Table 1. Additionally, gene-set enrichment analysis in CD4+ Tc clusters revealed enriched signatures for interferon responses and an enriched "inflammatory response" pathway in the Havcr2^{fl/fl};E8^{pre/} group, indicating a more efficient immune response. (Suppl. Figure 12D). These results demonstrate that TIM-3 deletion in CD8+ Tc contributes to the expansion of precursor effector memory Th1-like and early activated cytotoxic CD4+ Tc subsets, highlighting TIM-3's complex role in Tc subpopulations.

**Anti-TIM-3 treatment results in immune recall against AML cells without enhancing acute GVHD (aGVHD)**

We assessed the potential of TIM-3 Ab treatment to induce immune memory against AML cells by isolating donor Tc from AML-bearing mice post allo-HCT treated with anti-TIM-3 or isotype Ab. These Tc were transferred into secondary AML-bearing mice post allo-HCT, resulting in improved survival in mice that received Tc from anti-TIM-3-treated recipients compared to isotype-treated mice (Figure 6A).

To evaluate the impact of anti-TIM-3 treatment on aGVHD, we examined SI, colon and liver tissues from mice treated with anti-PD-1, anti-cytotoxic T-lymphocyte-associated Protein 4 (anti-CTLA-4) or anti-TIM-3 Abs after allo-HCT. All Abs (150μg) were given daily, from day 1 to day 5. Histopathological analysis revealed increased aGVHD severity in all organs from mice treated with anti-PD-1 or anti-CTLA-4 compared to their respective isotype Abs treatment.
Conversely, anti-TIM-3 treatment did not exacerbate aGVHD severity in these organs compared to the isotype Ab (Figure 6D).

The clinical grade humanized anti-TIM-3 Ab sabatolimab enhances the GVL effect

To further explore the potential of TIM-3 blockade in improving GVL effects against human leukemia, we used a humanized mouse model. Immunodeficient Rag2⁻⁻/Il2rg⁻⁻ mice were injected with the human FLT3-ITD mutant AML cell line MOLM-13uc⁻ and allogeneic human Tc (18). These Rag2⁻⁻/Il2rg⁻⁻ mutant mice lack T, B and NK cells and are therefore suitable hosts for transplanted human immune cells. Mice were treated i.p. with 150μg of the humanized anti-TIM-3 Ab (MBG453) or vehicle every 3 days from day 7 to day 28. Anti-TIM-3 Ab treatment resulted in improved survival (Figure 7A) and reduced leukemia burden, as assessed on day 21 post allo-HCT by luciferase imaging (Figure 7B, C) compared to vehicle-treated mice. Additionally, Rag2⁻⁻/Il2rg⁻⁻ mice receiving CD3-depleted human primary AML blasts and donor Tc from HLA mismatched healthy donors and treated with sabatolimab showed improved survival (Figure 7D, E). We also investigated if FLT3-ITD mutational status affects TIM-3. We treated human-derived MOLM-13 cells with different FLT3 inhibitors (Crenolanib, Quinzartinib and Tandutinib) to prevent FLT3 signaling and observed a decreased expression of Gal-9, CEACAM1 and TIM-3 in FLT3-ITD mutant AML cells, but not in Kasumi-1 cells (FLT3 WT) (Figure 7F-I), indicating that oncogenic FLT3-ITD signaling upregulates TIM-3 and its ligands in human AML.

Expression of TIM-3 and its ligands in different cell populations isolated from patients with AML relapse after allo-HCT.

To clarify if our results in mice could be related to the human context, we evaluated the expression of TIM-3 (HAVCR2) and its ligands (CEACAM1, HMGB1, LGALS9 and PTDSS1) in primary patient samples. First, we interrogated published scRNA-seq datasets of human BM samples in the setting of newly diagnosed AML (50, 51), transplant-naïve (52, 53) or post-transplant AML relapse (53), and physiologic hematopoiesis (50-52, 54). To enable comparison across these datasets, we annotated hematopoietic and immune cells based on a healthy BM reference (Methods) (55) (Figure 8A). In normal hematopoiesis and in AML, HAVCR2 expression was detectable in myeloid progenitor cells, Tc and NK cells (56), and all four TIM-3 ligands were expressed by HSC- and progenitor-populations (Figure 8B, Suppl. Figure 13A). We found high HAVCR2 expression in NK cells, in agreement with previous reports (56) which might be particularly relevant for the early post-transplant context, where NK cells are more abundant than in transplant-naïve AML (Figure 8C). Additionally, it has been shown that TIM-3 is highly expressed by NK cells in patients with AML, correlating with enhanced effector functions and NK cell cytotoxicity and improved clinical outcome in AML.
patients (57). Compared to healthy myelopoiesis, we observed higher expression of HAVCR2 and LGALS9 but not HMGB1 in newly diagnosed AML (Figure 8D, Suppl. Figure 13A). Expression of CEACAM1 was found below the detection limit and only detected in 0.4% of the cells (data not shown) (50). Second, we evaluated the expression of TIM-3 and its ligands in human primary AML cells isolated from patients that experienced a relapse after allo-HCT at multiple transplant centers (Suppl. Table 2) using bulk RNA-seq. We observed heterogeneous expression of HAVCR2 and its ligands LGALS9, CEACAM1 and HMGB1 (Figure 8E) as well as variable counts of TIM-3⁺ Tc (cells/μL) in peripheral blood (PB) of patients 1, 2 or 3 months after allo-HCT (Suppl. Figure 13B). These results may suggest differential therapeutic sensitivity to TIM-3 inhibition.

To understand whether TIM-3 expression impacts survival, we analyzed the probability of survival depending on the level of expression of HAVCR2 (mean) in bulk RNA-sequencing data derived from published databases on human cells. We defined HAVCR2 gene expression more than one standard deviation above mean as high expression. We observed that patients with high HAVCR2 expression in BM cells at diagnosis showed significantly improved survival compared to patients with low HAVCR2 expression when analyzing the Target-AML cohort (58), comprising 1630 low and 276 high TIM-3 expressing BM patient samples (Figure 8F). This pattern was also observed in peripheral blood (Suppl. Figure 14A). The improved survival with higher TIM-3 expression may be linked to a high count of TIM-3⁺ NK cells, which have the highest TIM-3 levels.
Discussion
Since relapse of the underlying malignancy is the major cause of death beyond day 100 after allo-HCT (59), novel strategies to prevent or treat relapse are urgently needed. Tc exhaustion and increased immune checkpoint ligand expression have been reported in patients with AML relapse after allo-HCT (3, 30), which supports the use of approaches that target exhausted Tc populations. Prior studies have shown that targeting immune checkpoint molecules using anti-PD-1 and anti-CTLA-4 Abs have clinical activity in a fraction of AML patients after allo-HCT, however aGVHD has limited the use of these antibodies in the patients (13, 14, 60).
In this study, we deciphered the potential of targeting TIM-3 to overcome immune escape and reinvigorate immune cells to enhance anti-leukemia immunity after allo-HCT in AML.
We observed that anti-TIM-3 Ab treatment reduced features of Tc exhaustion and enhanced Tc effector functions, leading to an improved GVL activity in multiple murine and humanized AML models. Specific TIM-3 deletion on CD8+ Tc allowed us to dissect the mechanism underlying the improvement of GVL effects. We identified two subpopulations of CD8+ exhausted Tc and found that polyfunctional stem-like TPEX cells expanded when TIM-3 was deleted in CD8+ Tc while cytotoxic but short-lived TTEX population remained unchanged, leading to a more effective, robust and durable Tc-mediated immune response. TIM-3 deletion in the CD8+ Tc subset also affected CD4+ Tc subpopulations, in particular causing expansion of Th1-like memory CD4+ Tc, recently described to display improved survival and persistence functions in the context of alloimmunity (49).
TIM-3 is also highly expressed on NK cells. However, its role in modulating NK function remains unclear, particularly in human diseases. The expression of TIM-3 on NK cells has been reported to be associated with both NK activation (61, 62) and dysfunction/exhaustion (63), depending on the cytokine stimulation (64). We observed an increased frequency of TIM-3-expressing NK cells in early post-transplant AML patients, consistent with other studies reporting that high TIM-3 expression on NK cells from AML patients correlates with an enhanced NK cell cytotoxicity and effector functions, and an ultimately improved clinical outcome in AML patients (57).
We also observed a higher frequency of neutrophils within immune cells isolated from leukemia-infiltrated spleens of AML-bearing mice upon anti-TIM-3 Ab treatment, consistent with several recent independent studies showing that neutrophils may contribute to tumor control (36, 37). The authors reported that ICI-therapy exposed neutrophils acquired an IFN-γ gene signature suggesting their contribution to the anti-tumor immune responses. Hirschhorn et al. identified a distinct anti-tumorigenic neutrophil subset that occurred in ICI-treated mice (36). They described the interaction between Tc mediating the initial anti-tumor immune response and neutrophils that eliminate tumor antigen loss variants (36).
Our study revealed that enhanced anti-tumor immunity upon anti-TIM-3 Ab therapy involves additional indirect immune mechanisms, recently described upon ICI therapy in solid cancers (36, 37).

A major concern when using ICI after allo-HCT is the development of severe aGVHD reported with anti-PD-1 and anti-CTLA-4 therapy (13-16). Consistently, we found that anti-PD-1 and anti-CTLA-4 treatment induced aGVHD in mice. Conversely, we observed no GVHD enhancement when using anti-TIM-3 Ab treatment. This might be because TIM-3 expression is restricted to terminally differentiated Tc (65, 66), which may contribute less to GVHD. However, the absence of GVHD development may be strictly dependent on the specific anti-TIM-3 Ab and the administration schedule. A previous preclinical study by Veenstra et al. (67) using a TIM-3-Ig fusion protein demonstrated that TIM-3 blockade exacerbated aGVHD in mice. Conversely, we treated the mice with an anti-TIM-3 Ab (monoclonal Ab, clone 5D12). Further, the group showed that TIM-3−/− mice used as donors led to enhanced aGVHD, whereas we used mice carrying a conditional specific TIM-3 deletion in selected immune subpopulations. Additionally, the number of donor Tc used to induce GVHD was 10 times higher (3x10⁶ of purified CD25+ Tc) compared to our model (3x10⁵ pan-Tc). These studies show that usage of TIM-3 Abs requires caution with respect to aGVHD development.

Previous studies suggested that targeting TIM-3 may directly eliminate LSCs via ADCC due to high TIM-3 expression on AML blasts and interruption of the Gal-9 feedback loop by the Ab (25, 26). However, we found that anti-TIM-3 treatment's effectiveness depended on the presence of alloreactive T cells, with no direct effect on AML cells.

Furthermore, our data suggest that specific oncogenic mutations may influence the effectiveness of ICIs against AML post allo-HCT, with TIM-3 blockade potentially benefiting patients with specific mutations and high TIM-3 ligand expression. Following our previous observation that certain oncogenic mutations lead to enhanced expression of inhibitory checkpoint ligands (29), we found that TIM-3 ligands Gal-9 and CEACAM1 were overexpressed in HSCs carrying FLT3-ITD and c-kit-D816V mutation. Treating mice with mutated AML cells using an anti-TIM-3 Ab resulted in a robust response upon anti-TIM-3 treatment, confirming our hypothesis. This highlights the potential for personalized TIM-3 blockade therapy in AML patients, emphasizing the need for innovative clinical study designs that consider leukemia genetics. Taken together, our findings demonstrate that TIM-3 and its ligands contribute to immune evasion, complementing research showing higher Gal9/Tim-3 expression in AML patients who fail chemotherapy (27).

We conducted allo-HCT with donor Tc that recognize foreign MHC on AML cells, mimicking the GVL effect observed in patients. However, besides alloantigen specific immune responses also tumor antigen specific immune responses that are MHC independent may play a role.
Previous studies have demonstrated the potential to target leukemia cells using leukemia reactive T cell lines (68).

Our preclinical work in part motivated a phase Ib/II, open-label, multicenter clinical trial using sabatolimab monotherapy for patients with molecular relapse after allo-HCT (EUDRACT number 2020-000869-17, ClinicalTrials.gov Identifier: NCT04623216) which no GVHD was observed in the 21 patients enrolled in the trial to date (69).

In summary, our study elucidates the link between oncogenic mutations in AML and TIM-3 ligand expression and identify anti-TIM-3 treatment as a strategy to enhance the GVL effect through metabolic and transcriptional reprogramming of Tc after allo-HCT. These findings support the ongoing clinical evaluation of sabatolimab for AML patients following allo-HCT.
Methods

Sex as a biological variable

Our study examined male and female animals, and similar findings are reported for both sexes.

Study design

The objective of this study was to target TIM-3 to enhance anti-leukemia immune responses after allo-HCT. We employed well-established allogeneic and xenogeneic GVL and aGVHD murine models to investigate the role of TIM-3 and its ligands. We utilized mouse anti-TIM-3 Ab (clone 5D12) or isotype Ab (mlgG1), clinical grade anti-human TIM-3 Ab MBG453 Sabatolimab) or vehicle (5% glucose solution) or conditional knock-out (Havcr2cko) mice. Anti-TIM-3 Abs (5D12 and MBG453) and isotype Ab were provided by Novartis Institutes of Biomedical Research (Cambridge, US).

All Abs used for FC are summarized in Suppl. Table 3 and 4.

Animal models

C57BL/6 (H-2Kb) and BALB/c (H-2Kd) mice were purchased from Janvier Labs (France). 

\[ \text{Rag2}^{-/-}\text{Il2rg}^{-/-} \] mice were obtained from the local stock at the animal facility of Freiburg University Medical Center. C57BL/6 Havcr2cko mice (\( \text{Havcr2}^{fl/fl}; \text{E8cre/}^{+} \), \( \text{Havcr2}^{fl/fl};\text{Cd4cre/}^{+} \), \( \text{Havcr2}^{fl/fl};\text{Cd11ccre/}^{+} \) and \( \text{Havcr2}^{fl/fl};\text{Zbtb46cre/}^{+} \)) were provided by Vijay K. Kuchroo (Boston, MA, US) and were described previously (23). 7 to 12 weeks old mice and female or male donor/recipient pairs were used.

Cell lines

WEHI-3B cells (ACC 26), MOLM-13 cells (ACC 554) and 32D cells (ACC 411) were obtained from DSMZ. MOLM-13 were then transduced in our lab to express luciferase. Kasumi-1 cells were provided by Prof. Michael Lübbert (Freiburg, Germany). Cell lines were routinely tested for Mycoplasma contamination and authenticated by STR profiling. FLT3-ITD MLL-PTD splenocytes were provided by Bruce R. Blazar (Minnesota, US).

Statistics

The comparison between quantitative and qualitative variables was done using a 2-tailed Student’s t test (parametric) or the Mann-Whitney U test (nonparametric). Variance analysis was done using a 1-way ANOVA (parametric) or Kruskal-Wallis (nonparametric) test. A Cox model was applied for survival analysis. For spectral FC analysis, differentially expressed proteins were tested with moderated t-test of limma. Concerning bar diagrams representing the frequency of the different Tc clusters, adjusted P-values were calculated using Fisher’s test. Patients were stratified based on high versus low expression of HAVCR2 gene.
expression in PB. Gene expression more than one standard deviation above the mean was considered as high expression. Statistical significance was set at a P-value of less than 0.05. All statistical analyses used are described in the corresponding figure legends.

**Study approval**

**Human samples**

Human PB and/or BM samples were derived from AML patients treated at the Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, Massachusetts, USA, after prior informed consent. Human PB was collected in sterile EDTA tubes and PBMC were isolated using gradient centrifugation (Pancoll Human). Written informed consent was obtained from each patient and analysis of human data was carried out in compliance with relevant ethical regulations. The characteristics of patients are listed in the Suppl. Table 2.

**Mice**

Animal protocols were approved by the animal ethics committee Regierungspräsidium Freiburg, Freiburg, Germany (protocol numbers: G23/049, G20/103, G20/75 and G20/096).

**Data availability**

The scRNA-seq data are deposited in the database GEO repository under the GEO accession number GSE242334.

The procedures used for the analysis of human scRNA sequencing datasets are described on https://github.com/petervangalen/reanalyze-aml2019/blob/main/230830_Heatmaps_Zeiser.R. All raw data are provided in the Supporting Data Values file.

**Additional experimental procedures**

All other materials and methods are described in the Supplementary Methods.

**Conflict of interest statement:**

R.Z. reports honoraria from Novartis, MNK, Sanofi, Incyte, and VectivBio outside the submitted work. J.R. received research funding from Kite/Gilead, Novartis and Oncentral Therapeutics and serves on advisory boards for Akron Biotech, Clade Therapeutics, Garuda Therapeutics, LifeVault Bio, Novartis, Smart Immune and TScan Therapeutics. B.R.B. receives remuneration as an advisor to Magenta Therapeutics, BlueRock Therapeutics, Incyte, GentiBio, Legend Biotech; research funding from BlueRock Therapeutics, Rheos Medicines, Equilivre Biopharmaceuticals, and Carisma Therapeutics, Inc.; and is a cofounder of Tmunity Therapeutics. V.K.K. has an ownership interest in and is a member of the scientific advisory board for Tizona Therapeutics, Bicara Therapeutics, Compass Therapeutics, Larkspur
Biosciences and Trishula Therapeutics. K.O.D and V.K.K. are named inventors on patents related to TIM-3. H.D.M. reports employment with Novartis AG, Basel, Switzerland. Novartis is the owner of the investigational compound sabatolimab that has been studied in context of the work described in this article. No disclosures were reported by the other authors. All other authors have declared no conflict of interest.

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Author contributions:
N.T-B. and L.M.B. developed the overall concept of the study, performed the experiments, analyzed all data, generated figures and wrote the manuscript. N.T-B and L.M.B made important contributions, and first authorship order was based on the amount of work each first author contributed to the study. K.O.D. and V.K.K. provided the Havcr2CKO mice and helped to analyze the scRNA-seq results. M.Z. helped to perform scRNA-seq experiment. A.H helped to prepare histology slides. T.W. and L.R. generated and analyzed spectral FC data. L.P., M.A., N.S. and P. V.G. provided and analyzed human scRNA-seq datasets. G.A., M.B., N.K. analyzed
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References:


**Figure legends**

**Figure 1: TIM-3 inhibition enhances GVL effects in different AML models**

Murine primary HSCs (C57BL/6) were transduced with the indicated oncogenes or gene fusions. RNA expression of (A) Lgals9 and (B) Ceacam1 was determined by qPCR. Gene expression was normalized to Hprt. Fold change was calculated in comparison to empty vector (dotted line). Results represent mean ± SEM from 3 independent experiments. P-values were calculated using Kruskal-Wallis One-Way ANOVA multiple comparisons test. (C-E) Kaplan-Meier plots showing mouse survival in the indicated groups. BALB/c recipient mice were injected i.v. with 7x10^4 oncogene-transduced HSCs 5x10^6 allogeneic BM and/or Tc when indicated. All mice were treated i.p. with isotype Ab or anti-TIM-3 Ab as indicated. P-values were calculated using 2-sided Mantel-Cox test. (F-I) RNA expression of (F) Havcr2, (G) Lgals9 (H) Ceacam1 and (I) Hmgb1 in WEHI-3B (n=13) and in FLT3-ITD MLL-PTD cells (n=6) was analyzed by qPCR. Gene expression is shown as percent of Hprt. P-values were calculated using an unpaired Student’s t-test. (J-K) Kaplan-Meier plot showing mouse survival in the indicated groups. Recipient mice were injected i.v. with FLT3-ITD MLL-PTD (J) or WEHI-3B cells (K) and 5x10^5 allogeneic BM and/or Tc and treated with either isotype Ab or anti-TIM-3 Ab, as indicated. P-value was calculated using 2-sided Mantel-Cox test. (K) Kaplan-Meier plots showing mouse survival in the indicated groups. BALB/c recipient mice were injected with WEHI-3B cells and 5x10^6 allogeneic BM and Tc and treated with isotype Ab (n=10) or anti-TIM-3 Ab (n=10). Results represent two independent experiments. P-value was calculated using 2-sided Mantel-Cox test. (L) Results show mean ± SEM of CD45^+ H-2Kb^+ cells frequency in the BM at day 23 after allo-HCT. C57BL/6 recipient mice were injected with FLT3-ITD MLL-PTD cells and allogeneic BM and/or Tc, and treated with isotype Ab (n=10) or anti-TIM-3 Ab (n=10). P-values were calculated using one-way Anova followed by Tukey’s multiple comparisons test.

**Figure 2:**

Anti-TIM-3 Ab treatment after allo-HCT reduces Tc exhaustion, increases glycolytic capacity of Tc and induces changes in myeloid subsets

(A-B) BALB/c recipient mice were injected with WEHI-3B cells, 5x10^6 allogeneic BM and Tc and treated with isotype (n=12) or anti-TIM-3 Ab (n=13). The proportion of TIM-3^+PD-1^+ cells within CD4^+ (A) or CD8^+ Tc (B) in the indicated organ was determined by FC at d23. Results represent mean ± SEM and P-values were calculated using Mann-Whitney U test. (C-N) C57BL/6 recipient mice were injected with FLT3-ITD MLL-PTD cells, 5x10^6 allogeneic BM and Tc and treated with isotype Ab or anti-TIM-3 Ab. Donor Tc were isolated from the spleen at day 23 post-allo-HCT. (C) Analysis using high-resolution spectral-FC allows UMAP visualization of the immune landscape. (D-E) Scaled expression of 25 phenotypic or functional
markers using the FlowSOM algorithm among 6 CD4+ or 4 CD8+ Tc subsets. Log fold change of isotype Ab (n=6) compared to anti-TIM-3 Ab (n=8) treatment is shown (blue color depicts higher expression in isotype, red color higher expression in anti-TIM-3). Differentially expressed proteins with P-value < 0.05 tested with moderated t-test of limma are presented. (F-G) Glycolytic capacity of Tc subsets at day 23 assessed by FC-based single-cell metabolism. Results show mean ± SEM of n=9 biological replicates for each condition. P-values were calculated using Mann-Whitney U test. (H) Volcano plot of 1249 metabolic features from non-targeted LC-MS analysis. Features that were identified as members of the KEGG module “glycolysis" were highlighted. Results show n=5 isotype and n=8 anti-TIM-3-treated mice. (DHAP: dihydroxyacetonephosphate, PGA: phosphoglyceric acid, PEP: phosphoenolpyruvate, G6P: glucose-6-phosphate, F6P: fructose-6-phosphate, FBP: fructose-bisphosphate). (I) Analysis using high-resolution spectral-FC allows UMAP visualization of immune cells. (J) Proportion of each cell subset among total cells in the respective condition. Proportion of leukemia blasts (K), neutrophils (L), cDC1s (M), and macrophages (N) among total cells (%). P-values were calculated using unpaired Student’s t-test.

Figure 3: Anti-TIM-3 Ab treatment reduces Tc exhaustion in vitro
(A-G) Tc isolated from spleens (C57BL/6 mice) were continuously exposed to TCR stimulation (αCD3/CD28 beads) in vitro for 14 days in RPMI medium supplemented with 10% FBS, 1% penicillin / streptomycin and 30U/mL of mIL-2 to obtain highly activated/exhausted Tc. Cells were treated every second day with 10 µg/mL of isotype or anti-TIM-3 Ab. (A) Representative plots showing the proportion of TIM-3+PD-1+ cells among viable CD4+ and CD8+ Tc treated with isotype or anti-TIM-3 was determined by FC. Relative TIM-3 protein expression in CD4+ Tc (B) and in CD8+ Tc (C) based on MFI for n=6 replicates for each condition. P-values were calculated using Wilcoxon signed-rank test. (D-E) Proportion (%) of TIM-3+PD-1+ within all CD4+ Tc (D) and within all CD8+ Tc (E). Results represent mean ± SEM for isotype Ab (n=6) and anti-TIM-3 Ab (n=6) are shown. P-values were calculated using Mann-Whitney U test. (F) Relative TOX protein expression as relative MFI determined by FC in CD4+ Tc and (G) Representative FC staining of TOX expression in CD4+ Tc for both isotype Ab (blue line) and anti-TIM-3 Ab treatment (red line). (H) Relative TOX protein expression as relative MFI determined by FC in CD8+ Tc and (I) Representative staining of TOX expression in CD8+ Tc for both isotype Ab (blue line) and anti-TIM-3 Ab treatment (red line). Results represent mean ± SEM of n=5 isotype treatment or n=5 anti-TIM-3 treatment. P-values were calculated using Wilcoxon signed-rank test.

Figure 4: Genetic TIM-3 deletion in CD8+ Tc enhances Tc activation, proliferation and IFN-γ production as well as GVL effects

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(A-E) Tc labeled with CTV ("responders", C57BL/6) were co-cultured for 6 days with allogeneic non-CD3 cells ("stimulators", BALB/c). Responder cells were isolated from mice carrying TIM-3 deletion in CD8+ Tc (Havcr2<sup>ff/fl</sup>;E8<sup>cre/+</sup>) as described in (23). (A) Proportion of proliferative cells was quantified at day 6 as the percentage of CTV<sup>low</sup> cells in Havcr2<sup>ff/fl</sup>;E8<sup>cre/+</sup> CD3<sup>+</sup> responder Tc. (B) CD25 expression in proliferative CD3<sup>+</sup> responder cells were quantified at day 6 by FC. (C) Representative staining of CD25 expression in Havcr2<sup>ff/fl</sup> (blue line) and in Havcr2<sup>ff/fl</sup>;E8<sup>cre/+</sup> cells (red line). (D) CD69 expression in proliferative CD3<sup>+</sup> responder cells were quantified at day 6 by FC. (E) Representative staining of CD69 expression in Havcr2<sup>ff/fl</sup> (blue line) and in Havcr2<sup>ff/fl</sup>;E8<sup>cre/+</sup> cells (red line). (F-G) Production of IFN-γ in the supernatant of the culture analyzed at the indicated time points by ELISA. P-values were calculated using an unpaired Student's t-test. (H-I) Kaplan-Meier plots showing survival of mice in the indicated groups. BALB/c recipient mice were injected i.v. with WEHI-3B AML cells (BALB/c background) and (H) allogeneic Havcr2<sup>ff/fl</sup> (n=10) or Havcr2<sup>ff/fl</sup>;E8<sup>cre/+</sup> (n=10) BM and Tc or (I) allogeneic Havcr2<sup>ff/fl</sup> (n=10) or Havcr2<sup>ff/fl</sup>;Cd4<sup>cre/+</sup> (n=10) BM and Tc. Data were pooled from two independent experiments and P-values were calculated using the 2-sided Mantel-Cox test. (J) Percentage of specific lysis of αCD3/CD28 activated Tc isolated from Havcr2<sup>cko</sup> mice in contact with WEHI-3B cells. E:T (effector [Tc] to target [WEHI-3B cell]) ratio was titrated between 5:1 and 1:1 as indicated. Individual values are shown and mean ± SD of Havcr2<sup>ff/fl</sup> (n=6) or Havcr2<sup>ff/fl</sup>;Cd4<sup>cre/+</sup> (n=6). P-values were calculated using a two-way Anova followed by Sidak’s multiple comparisons test.

Figure 5: Deletion of TIM-3 in CD8<sup>+</sup> Tc leads to T<sub>Pex</sub> expansion

(A-E) BALB/c recipient mice were injected i.v. with WEHI-3B AML cells (BALB/c background) and 5x10<sup>6</sup> allogeneic Havcr2<sup>ff/fl</sup> (n=2) and Havcr2<sup>ff/fl</sup>;E8<sup>cre/+</sup> (n=2) BM and Tc. Tc were isolated at day 23 and stained with an oligo-tagged H-2Kb (donor) Ab allowing scRNA-seq analysis of FACS sorted donor Tc. (A) UMAP visualization of 10 clusters of CD8<sup>+</sup> Tc. (B) Feature plots showing the expression level of different marker genes relevant for the characterization of the respective cluster. (C) Bar diagram representing the frequency of the different CD8<sup>+</sup> Tc clusters. Adjusted P-values were calculated using Fisher’s test. (D) Expression level of key genes differentially expressed in Tc from AML-bearing mice receiving Havcr2<sup>ff/fl</sup> (left) and Havcr2<sup>ff/fl</sup>;E8<sup>cre/+</sup> (right) BM/Tc in cluster 6. (E) Score of the functional signature enriched in Tc from AML-bearing mice receiving Havcr2<sup>ff/fl</sup>;E8<sup>cre/+</sup> BM/Tc compared to Havcr2<sup>ff/fl</sup> BM/Tc in cluster 7. Gene expression analysis of genes within the “memory precursor” signature. (Tem: effector memory Tc; Teff: effector Tc).

Figure 6: Anti-TIM-3 treatment leads to recall immunity against AML cells without inducing aGVHD
(A) Kaplan-Meier plot showing mouse survival in the indicated groups. C57BL/6 recipient mice were injected with FLT3-ITD MLL-PTD AML cells and 5x10⁶ allogeneic BM. At day 2, mice received adoptive transfer of 3x10⁵ allogeneic donor Tc. Allogeneic Tc were isolated on day 15 from FLT3-ITD MLL-PTD AML-bearing mice, which received allogeneic BM/Tc and were treated with isotype (n=14) or anti-TIM-3 (n=14) Ab. (B-D) The scatter plots show the histopathological aGVHD severity in the indicated groups. BALB/c recipient mice were injected i.v. with 5x10⁶ allogeneic BM and 4x10⁵ allogeneic Tc. From day 1 to day 5, mice were treated (150µg, i.p.) with anti-TIM-3 / isotype, anti-PD-1 / isotype or anti-CTLA-4 / isotype. aGVHD histological scores were determined on liver, SI, and colon at day 7 for the different groups. Results show mean ± SEM from 2 independent experiments. P-values were calculated using an unpaired Student’s t-test.

Figure 7: Anti-human TIM-3 Ab sabatolimab enhances the GVL effect

(A) Kaplan-Meier plots showing mouse survival in the indicated groups. Rag2⁻/⁻Il2rg⁻/⁻ recipient mice were injected i.v. with human-derived MOLM-13⁰ LUC+ AML cells and human CD3⁺ Tc (from HLA non-matched healthy donors), and treated with vehicle (n=10) or human anti-TIM-3 (n=10). Results show 3 independent experiments and P-value was calculated using 2-sided Mantel-Cox test. (B) Representative images of Bioluminescence imaging (BLI) of MOLM-13⁰ AML-bearing Rag2⁻/⁻Il2rg⁻/⁻ recipient mice 21 days after injection of AML cells, following human Tc injection and vehicle or human anti-TIM-3 Ab treatment. (C) BLI signal quantification shows the expansion of AML cells over time. Results show mean ± SEM from 3 independent experiments using 3 different healthy T cell donors. P-values were calculated using 2-sided Mann-Whitney U test. (D-E) Kaplan-Meier plots showing mouse survival in the indicated groups. Rag2⁻/⁻Il2rg⁻/⁻ recipient mice were injected i.v. with CD3 depleted primary AML cells (from PB at primary diagnosis) and treated with vehicle (n=3) or human anti-TIM-3 (n=3). Each survival curve represents one individual AML donor patient. P-values were calculated using 2-sided Mantel-Cox test. (F) Representative Western blots showing the inhibition of phospho-FLT3 (Tyr589/591) upon treatment with 3 different FLT3 inhibitors and loading control (β-actin) in MOLM-13 cells (FLT3-ITD). (G) Gal-9 (H) CEACAM1 and (I) TIM-3 protein expression as relative MFI was determined by FC upon the treatment with the indicated FLT3 inhibitors, in FLT3-ITD-positive human cell line (MOLM-13) or FLT3 WT human cell line (Kasumi-1). Fold change is calculated in comparison to the treatment using DMSO (control treatment). Results represent mean ± SEM from 8 independent experiments using MOLM-13 cells and n=5 independent experiments using Kasumi-1 cells. P-values were calculated using ordinary one-way Anova.
Figure 8: Expression of TIM-3 and its ligands in human primary samples

(A) Overview of UMAP embedding colored by 27 projected cell types of 574,502 scRNA-seq profiles from van Galen et al., (50) (n=20,362), Abbas et al., (52) (n=127,027), Huo et al., (54) (n=20,385), Beneyto-Calabuig et al., (51) (n=101,767) and Penter et al., (17) (n=304,961). Major cell types are highlighted. (B) Scaled gene expression of HAVCR2, CEACAM1, HMGB1, LGALS9 and PTDSS1 across AML BM scRNA-seq profiles. AML BM cases include newly diagnosed and relapsed disease with or without allo-HCT. (C) Proportion of TIM-3+ cells of all NK cells across normal and AML BM datasets. (D) Heatmap generated from scRNA-seq profiles from van Galen et al., (50) showing expression of HAVCR2, LGALS9 and HMGB1 in normal hematopoiesis and AML cell types of patients at diagnosis of AML. (E) Expression of HAVCR2, LGALS9, CEACAM1 and HMGB1 in human AML cells determined by bulk RNA-seq. AML cells were isolated from patients with AML relapse after allo-HCT at multiple transplant centers. (F) Probability of survival stratified according to “High” versus “Low” HAVCR2 gene expression in AML BM at diagnosis within the Target-AML cohort (58). RNAseq data was derived from the GenomicDataCommons (GDC) library. Gene expression more than one standard deviation above mean was defined as high expression.
Figure 1

A

\[ P = 0.01 \]  
\[ P = 0.04 \]  
\[ P = 0.02 \]  
\[ P = 0.02 \]

B

\[ P = 0.01 \]  
\[ P = 0.004 \]  
\[ P = 0.0004 \]  
\[ P = 0.03 \]  
\[ P = 0.0006 \]

C

\[ P < 0.0001 \]

D

\[ P = 0.005 \]

E

\[ P = 0.0006 \]

F

\[ P < 0.001 \]

G

\[ P = 0.001 \]

H

\[ P < 0.001 \]

I

\[ P = 0.009 \]

J

\[ P = 0.004 \]

K

\[ P = 0.04 \]

L

\[ P < 0.0001 \]  
\[ P = 0.0001 \]  
\[ P = 0.02 \]

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1. **Legend**:  
   - AML (FLT3-ITD) + allo-HCT + isotype Ab (n=15)  
   - AML (FLT3-ITD) + allo-HCT + anti-TIM-3 Ab (n=21)  
   - AML (FIP1L1-PDGFRα) + allo-HCT + isotype Ab (n=15)  
   - AML (FIP1L1-PDGFRα) + allo-HCT + anti-TIM-3 Ab (n=15)  
   - AML (FLT3-ITD) + allo-HCT/Tc + isotype Ab (n=15)  
   - AML (FLT3-ITD) + allo-HCT/Tc + anti-TIM-3 Ab (n=15)  
   - AML (FLT3-ITD) + allo-HCT/Tc + anti-TIM-3 Ab (n=10)  

2. **Graphs**:

   - **A**: Bar chart showing the fold change in Lgals9/Hprt mRNA with error bars.  
   - **B**: Bar chart showing the fold change in Ceacam1/Hprt mRNA with error bars.  
   - **C**: Graph showing percent survival over days after allo-HCT for different subtypes.  
   - **D**: Graph showing percent survival over days after allo-HCT for different subtypes.  
   - **E**: Graph showing percent survival over days after allo-HCT for different subtypes.  
   - **F**: Graph showing Havcr2 (% of Hprt) for different cell lines.  
   - **G**: Graph showing Lgals9 (% of Hprt) for different cell lines.  
   - **H**: Graph showing Ceacam1 (% of Hprt) for different cell lines.  
   - **I**: Graph showing HmgB1 (% of Hprt) for different cell lines.  
   - **J**: Graph showing percent survival over days after allo-HCT for different subtypes.  
   - **K**: Graph showing percent survival over days after allo-HCT for different subtypes.  
   - **L**: Graph showing percent survival over days after allo-HCT for different subtypes.
Figure 3

A

CD4+ Tc

TIM-3

CD8+ Tc

PD-1

B

CD4+ Tc

TIM-3 on CD4+ T cells (rel. MFI)

P=0.0009

isotype Ab (n=6)

anti-TIM-3 Ab (n=6)

C

CD8+ Tc

TIM-3 on CD8+ T cells (rel. MFI)

P<0.0001

isotype Ab (n=6)

anti-TIM-3 Ab (n=6)

D

CD4+ Tc

TIM-3+PD-1+ of all CD4+ T cells (%)

P=0.008

isotype Ab (n=6)

anti-TIM-3 Ab (n=6)

E

CD8+ Tc

TIM-3+PD-1+ of all CD8+ T cells (%)

P=0.04

isotype Ab (n=6)

anti-TIM-3 Ab (n=6)

F

CD4+ Tc

TOX in CD4+ T cells (rel. MFI)

P=0.005

isotype Ab (n=5)

anti-TIM-3 Ab (n=5)

G

CD8+ Tc

TOX in CD8+ T cells (rel. MFI)

P=0.003

isotype Ab (n=5)

anti-TIM-3 Ab (n=5)
Figure 4

A. Proliferating cells on all CD3+ T cells (%)
- HAVCR2fl/fl (n=12)
- HAVCR2fl/fl;E8iCre/+ (n=11)

B. CD25 expression on CD3+ T cells (%)
- HAVCR2fl/fl (n=9)
- HAVCR2fl/fl;E8iCre/+ (n=8)

C. Counts
- HAVCR2fl/fl (n=9)
- HAVCR2fl/fl;E8iCre/+ (n=8)

D. CD69 expression on CD3+ T cells (%)
- HAVCR2fl/fl (n=6)
- HAVCR2fl/fl;E8iCre/+ (n=6)

E. Counts
- HAVCR2fl/fl (n=6)
- HAVCR2fl/fl;E8iCre/+ (n=6)

F. IFN-γ (pg/mL) on day 2
- HAVCR2fl/fl (n=6)
- HAVCR2fl/fl;E8iCre/+ (n=6)

G. IFN-γ (ng/mL) on day 4
- HAVCR2fl/fl (n=6)
- HAVCR2fl/fl;E8iCre/+ (n=6)

H. Percent survival
- AML (WEHI-3B) + allo-HCT/Tc (HAVCR2fl/fl) (n=10)
- AML (WEHI-3B) + allo-HCT/Tc (HAVCR2fl/fl;E8iCre+) (n=10)

I. Percent survival
- AML (WEHI-3B) + allo-HCT/Tc (HAVCR2fl/fl) (n=10)
- AML (WEHI-3B) + allo-HCT/Tc (HAVCR2fl/fl;Cd4Cre+) (n=10)

J. % of specific lysis
- HAVCR2fl/fl Tc (n=6)
- HAVCR2fl/fl;Cd4Cre/+ Tc (n=6)

P-values:
- 0.0004
- 0.0005
- 0.0001
- 0.0006
- 0.02
- 0.0003
- 0.02
- 0.002
- 0.023
- 0.0001
- 0.0005
- 0.0006
Figure 5

A

CD8+ Tc sub-clustering

0  Tem
1  Teff
2  TTEX
3  early Teff
4  Precursor cells
5
6  TPEX 1
7  TPEX 2
8  short-term Teff
9

B

Havcr2fl/fl;E8i cre/+;cl6 – TPEX 1

TPEX 2

Slamf6

Tcf7

C

Cluster absolute frequency (%)

P = 0.001
P = 0.04
P < 0.0001
P = 0.0002
P = 0.04

D

cl6 – TPEX 1

E

cl7 – TPEX 2

Memory precursor

Bcl2

Igl1

Percent Expressed

30
50
70

Average Expression

1.5
1.0
0.5

Sh3bp5

Tcf7

Tck

xb20

Havcr2fl/fl;E8i cre/+;cl6

Havcr2fl/fl;E8i cre/+;cl7
Figure 6

A

![Graph showing percent survival over days after allo-HCT]

- AML (FLT3-ITD MLL-PTD) + allo-HCT + Tc from isotype-treated BM/Tc mice (n=14)
- AML (FLT3-ITD MLL-PTD) + allo-HCT + Tc from anti-TIM-3-treated BM/Tc mice (n=14)

B

![Histological score comparison for liver, SI, and Colon]

- Liver
  - isotype Ab (n=10)
  - anti-CTLA-4 Ab (n=7)

- SI
  - isotype Ab (n=9)
  - anti-PD-1 Ab (n=8)

- Colon
  - isotype Ab (n=10)
  - anti-TIM-3 Ab (n=7)

C

D

- liver
- SI
- Colon

- n.s

- P<0.0001
Figure 7

A. Percent survival over days after transplant for MOLM-13\textsuperscript{luc\textsuperscript{+}} + huTc cells with vehicle (n=10) and MOLM-13\textsuperscript{luc\textsuperscript{+}} + huTc cells with sabatolimab (n=10).

B. Luminescence images showing MOLM-13\textsuperscript{luc\textsuperscript{+}} + huTc cells with vehicle and MOLM-13\textsuperscript{luc\textsuperscript{+}} + huTc cells with sabatolimab.

C. Photons s\textsuperscript{-1}cm\textsuperscript{-1} over days after transplant for MOLM-13\textsuperscript{luc\textsuperscript{+}} + huTc cells with vehicle (n=10) and MOLM-13\textsuperscript{luc\textsuperscript{+}} + huTc cells with sabatolimab (n=10).

D. Percent survival for AML from patient #1 with human primary AML cells + huTc cells with vehicle (n=3) and human primary AML cells + huTc cells with sabatolimab (n=3).

E. Percent survival for AML from patient #2 with human primary AML cells + huTc cells with vehicle (n=3) and human primary AML cells + huTc cells with sabatolimab (n=3).

F. Western blot analysis showing Gal-9, Ceacam-1, and TIM-3 expression in MOLM-13 cells treated with DMSO, Crenolanib 0.02nM, Quizartinib 10nM, and Tandutinib 0.2nM with FLT3-ITD positive and negative cell lines.

G. Gal-9 expression fold change with DMSO, Crenolanib 0.02nM, Quizartinib 10nM, and Tandutinib 0.2nM in FLT3-ITD positive and negative MOLM-13 and Kasumi-1 cells.

H. Ceacam-1 expression fold change with DMSO, Crenolanib 0.02nM, Quizartinib 10nM, and Tandutinib 0.2nM in FLT3-ITD positive and negative MOLM-13 and Kasumi-1 cells.

I. TIM-3 expression fold change with DMSO, Crenolanib 0.02nM, Quizartinib 10nM, and Tandutinib 0.2nM in FLT3-ITD positive and negative MOLM-13 and Kasumi-1 cells.
Figure 8

A

B

C

D

E

F

Bone marrow

HAVCR2 expression low high

P=0.0001

P=0.002

P=0.002

HAVCR2
LGALS9
CEACAM1
HMGB1

HAVCR2

1360  770  367  62  2
275  182  69  7  0

0 1000 2000 3000 4000

3000 4000