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## **Immune landscape after allo-HSCT: TIGIT and CD161-expressing CD4 T cells are associated with subsequent leukemia relapse**

Tracking no: BLD-2022-015522R1

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### **Abstract:**

Allogeneic hematopoietic stem-cell transplantation (allo-HSCT) is the most effective treatment for selected patients with acute myeloid leukemia (AML) and relies on a "graft-versus-leukemia" effect (GVL) where donor T lymphocytes mediate control of malignant cell growth. However, relapse remains the major cause of death after allo-HSCT. In various malignancies, several immunoregulatory mechanisms have been shown to restrain antitumor immunity, including ligand-mediated engagement of inhibitory receptors on effector cells, and induction of immunosuppressive cell-subsets such as regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs). While relapse after HSCT remains a major therapeutic challenge, immunoregulatory mechanisms involved in restraining the GVL effect need to be better deciphered in humans. We used mass cytometry to comprehensively characterize circulating leukocytes in two cohorts of patients after allo-HSCT. We first longitudinally assessed various immunoregulatory parameters highlighting specific trends, such as opposite dynamics between MDSCs and Tregs. More generally, the immune landscape was rather stable from month-3 to 6, while many variations occurred from month-6 to 12 post-HSCT. Comparison with healthy individuals revealed that profound alterations in the immune equilibrium persisted 1-year post-HSCT. Importantly, we found that high levels of TIGIT and CD161 expression on CD4 T cells at month 3 post-HSCT were distinct features significantly associated with subsequent AML relapse in a second cross sectional cohort. Altogether, these data provide global insights into the immunoregulatory landscape reconstitution following HSCT, and highlight non-canonical inhibitory receptors associated with relapse, which could open the path towards new prognostic tools or therapeutic targets to restore subverted anti-AML immunity.

**Conflict of interest:** No COI declared

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**Clinical trial registration information (if any):**

# Immune landscape after allo-HSCT: TIGIT and CD161-expressing CD4 T cells are associated with subsequent leukemia relapse

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**Running title:** Immune regulation of the GvL effect

This revised manuscript includes:

- Figures and Tables: 5 Figures and 2 Tables
- Supplemental content: 12 suppl. Figures, 3 suppl. Tables, 1 suppl. Methods

## KEY POINTS

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→ Immunoregulatory cell compartments show distinct dynamics and substantial persistent alterations at one year following allogeneic HSCT

→ High levels of TIGIT and CD161 expression on CD4 T cells early after allo-HSCT are associated with subsequent relapse in patients with AML

## 1 ABSTRACT

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2  
3 Allogeneic hematopoietic stem-cell transplantation (allo-HSCT) is the most effective treatment for  
4 selected patients with acute myeloid leukemia (AML) and relies on a “graft-versus-leukemia” effect  
5 (GVL) where donor T lymphocytes mediate control of malignant cell growth. However, relapse remains  
6 the major cause of death after allo-HSCT. In various malignancies, several immunoregulatory  
7 mechanisms have been shown to restrain antitumor immunity, including ligand-mediated engagement  
8 of inhibitory receptors on effector cells, and induction of immunosuppressive cell-subsets such as  
9 regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs). While relapse after HSCT  
10 remains a major therapeutic challenge, immunoregulatory mechanisms involved in restraining the GVL  
11 effect need to be better deciphered in humans. We used mass cytometry to comprehensively  
12 characterize circulating leukocytes in two cohorts of patients after allo-HSCT. We first longitudinally  
13 assessed various immunoregulatory parameters highlighting specific trends, such as opposite  
14 dynamics between MDSCs and Tregs. More generally, the immune landscape was rather stable from  
15 month-3 to 6, while many variations occurred from month-6 to 12 post-HSCT. Comparison with  
16 healthy individuals revealed that profound alterations in the immune equilibrium persisted 1-year  
17 post-HSCT. Importantly, we found that high levels of TIGIT and CD161 expression on CD4 T cells at  
18 month 3 post-HSCT were distinct features significantly associated with subsequent AML relapse in a  
19 second cross sectional cohort. Altogether, these data provide global insights into the  
20 immunoregulatory landscape reconstitution following HSCT, and highlight non-canonical inhibitory  
21 receptors associated with relapse, which could open the path towards new prognostic tools or  
22 therapeutic targets to restore subverted anti-AML immunity.

## 1 INTRODUCTION

---

2  
3 Allogeneic hematopoietic stem cells transplantation (allo-HSCT) is the most effective consolidation  
4 treatment for patients with intermediate to high-risk acute myeloid leukemia (AML). The efficacy of allo-  
5 HSCT mostly relies on the ability of donor T cells to eliminate tumor cells, a process referred to as the  
6 graft-versus-leukemia (GVL) effect.<sup>1-3</sup> However, relapse still occurs in around 40% of patients and  
7 remains the main cause of mortality after allo-HSCT.<sup>4-6</sup> Reduced antigen presentation via HLA class-II and  
8 overexpression of ligands for inhibitory receptors (IRs) have been recently showed on relapsing AML  
9 blasts, with concomitant upregulation of IRs on T cells.<sup>7-9</sup> However, while research efforts have largely  
10 focused on graft-versus-host disease (GVHD), immunological mechanisms behind effectiveness of – or  
11 escape from – the GVL responses still need closer scrutiny in humans.<sup>3,10</sup>

12 Immunoregulatory mechanisms hindering efficient antitumor immune responses mainly fall into two  
13 categories: (i) cell-intrinsic, via ligand-mediated engagement of IRs expressed on effector cells (PD-1,  
14 CTLA-4, TIGIT, Tim-3, Lag-3, BTLA...), and (ii) cell-extrinsic, via immunosuppressive cell subsets, such as  
15 regulatory T cells (Tregs) or myeloid-derived suppressor cells (MDSCs).<sup>11-13</sup> In contrast to Tregs, a well-  
16 defined cell type described in health and diseases, MDSCs represent a more heterogeneous population  
17 (monocytic or granulocytic) known to play a major role in restraining anti-tumor T-cell responses,<sup>12</sup> but  
18 also possibly in limiting GVHD.<sup>14</sup> Other cell subsets may also exert regulatory functions in cancer  
19 contexts, such as type-2 innate lymphoid cells (ILC2).<sup>15</sup> Indeed, we previously identified a protumor  
20 ILC2—MDSC axis in humans<sup>16,17</sup> that was also associated to tolerance induction in a mouse model of  
21 GVHD.<sup>18</sup>

22 The process of immune reconstitution after allo-HSCT is slow, complex, and yet incompletely  
23 understood (reviewed in<sup>19,20</sup>). In contrast to early recovery of innate cells (mostly neutrophils and NK  
24 cells), the reconstitution of the adaptative system is much slower and influenced by multiple patient-  
25 disease-, and transplant-related cofactors. Main transplant- and post-transplant cofactors influencing T-  
26 and B-cells reconstitution include recipients' age, graft source, the use of T-cell depleting agents and  
27 GVHD.<sup>20,21</sup> Immune reconstitution of all main immune cell subsets had been studied individually for  
28 many years by flow-cytometry, but systems-level analyses by high dimensional single cell technologies  
29 such as mass cytometry provide a wider and less biased picture of the overall process of innate and  
30 adaptative immune reconstitution.<sup>22-24</sup>

31 In this work, our aims were twofold: i) to study globally and longitudinally the immunoregulatory  
32 landscape, and ii) to identify whether and which immune cell subsets or immunoregulatory features  
33 could be associated with the emergence of subsequent relapse after allo-HSCT. As immune regulatory  
34 mechanisms involve a complex and inter-connected network of diverse immunosuppressive cells  
35 subsets and inhibitory receptors,<sup>25</sup> mass-cytometry was used to study circulating leukocytes following  
36 allo-HSCT in i) a longitudinal cohort up to 1 year post-HSCT, and ii) a cross-sectional cohort at month 3  
37 post-transplant including two groups – matched for most relevant clinical variables – with patients  
38 either showing subsequent relapse or being in long-term persistent remission.

## METHODS

---

### *Human biological samples*

Two cohorts of HSCT recipients were studied and detailed in supplemental methods section. The first cohort included patients with AML or myelodysplastic syndrome (MDS), with pre-specified sampling at 3, 6 and 12 months post-HSCT in the absence of hematological relapse,<sup>26,27</sup> allowed to analyze the global longitudinal changes in the immunoregulatory landscape. The second cohort included AML patients, in which we retrospectively selected samples at 3 months post-HSCT from 20 patients who subsequently relapsed and 20 patients without relapse, matched for age at transplantation, donor type, AML cytogenetic and molecular risk.<sup>28</sup> Ethics approval were obtained according to French regulation (as detailed in the supplemental methods). Blood specimens were also collected from healthy volunteers (median 36 years, IQR 29-51) through the French blood bank.

### *Mass cytometry panel design and staining*

We designed a 44 metal-labelled antibody panel as reported in Suppl. Table 1. Protocol is detailed in the supplemental methods section.

### *Mass cytometry data analyses and statistics*

Cell events were acquired on the CyTOF Helios Mass Cytometer (Fluidigm) and data were normalized as detailed in the supplemental methods section. Data were analyzed by classical gating, as well as using the unsupervised clustering algorithms FlowSOM and UMAP for dimensional reduction.<sup>29,30</sup> The analyses were performed using FlowJo (v10.6), the Cytobank platform (Beckman Coulter), as well as R (v4.0.2), as detailed in the supplemental methods section. Non parametric (Mann-Whitney, Wilcoxon and log-rank) tests were used as detailed in the supplemental methods section.

## RESULTS

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### *Immunoregulatory landscape dynamics following allo-HSCT*

We first aimed to comprehensively analyze longitudinal changes in peripheral immune cell subsets abundance and functional phenotype following allo-HSCT. We included 37 AML/MDS patients from the first longitudinal cohort, in whom PBMCs were collected at months 3 (M3), M6 and M12 after transplantation, as well as 20 healthy donors (**Fig. 1A**). Patients' characteristics are described in **Table 1** and **Suppl. Table 2**. Twelve patients had relapse at a later time after sampling (particularly late with a median of 15 [IQR 8–69] months post-HSCT). Most patients with AML had intermediate or adverse factors according to the ELN classification. Relapsed patients were statistically more likely to have received a reduced intensity conditioning and not to be in complete remission at the time of transplantation. All other characteristics were similar between patients who did or did not relapse. As expected, relapsed patients had worse survival (**Table 1**).

We designed a mass-cytometry panel to assess the distribution of all main immune cell types, with a particular focus on immunoregulatory cells and receptors (i.e. Tregs, MDSCs, and a range of inhibitory

1 receptors). Unsupervised clustering analysis using FlowSOM (Fig. 1B and suppl. Fig. 1A-B) allowed  
2 identification of 121 cell clusters classified into 18 relevant metaclusters (MC) of various relative  
3 abundance (suppl. Fig. 1A). Principal component analysis revealed very distinct immune profiles in  
4 patients as compared to healthy donors, while time point after allo-HSCT did not segregate patients'  
5 samples (Fig. 1C).

6  
7 We next tested variations of each cell cluster in paired samples at the different time points and found  
8 very few changes between M3 and M6, while frequencies of several cell clusters significantly varied  
9 from M6 to M12 (Fig. 1D) (detailed in suppl. Fig. 1C). At the metacluster level, we found that proportion  
10 of MC1 (hematopoietic stem/progenitor cells, HSPCs), MC2 (monocytes), MC16 (NK cells) and MC20  
11 (CD117<sup>+</sup> ILCs) were decreasing (Fig. 1E), while that of MC4 (CD4 T cells), MC5 (Tregs), MC9 (Th2/Tc2  
12 cells) and MC15 (B cells) increased over time (Fig. 1F). Analyses using classical manual gating strategies  
13 (suppl. Fig. 2) confirmed that only few significant changes occurred between M3 and M6 (Fig. 1G), while  
14 several immune parameters varied between M6 and M12 (Fig. 1H). Regarding immunoregulatory cell  
15 populations, all three MDSCs subsets (monocytic, granulocytic, and early-stage MDSCs<sup>31</sup>) were elevated  
16 in patients at M3, as compared to healthy donors (suppl. Fig. 3). Of note, the frequency of e- and M-  
17 MDSCs subsets decreased from M6 to M12 (Fig. 1H). In contrast, Tregs frequencies among PBMCs  
18 increased, as a reflect of increased total CD4 T cells. More specifically, only memory (CD45RA<sup>neg</sup>) – both  
19 "activated" (HLA-DR<sup>+</sup>) and "cytokine-secreting" (HLA-DR<sup>neg</sup>)<sup>32,33</sup> – but not naïve/resting Tregs significantly  
20 increased at M12 (Fig. 1H). Besides, the proportion of cells expressing the TIGIT inhibitory receptor  
21 significantly increased from M6 to M12, not only among CD8 T<sub>CM</sub> cells, but also among  $\gamma\delta$ -T and NK cells  
22 (Fig. 1H). There was also an increase in BTLA positivity on T cell subsets at M12 when compared to M3  
23 (suppl. Fig. 4). Expression of the other inhibitory receptors (PD-1, LAG-3, Tim-3 and CTLA-4) did not  
24 significantly change over time.

25  
26 Although limited by patient numbers, subgroup analyses with clinical parameters suggest an impact of  
27 ATG serotherapy in the longitudinal study of the CD4 T cell compartment (lower frequencies of CD4 T  
28 cells up to M12 in patients with ATG infusion) (suppl. Fig. 5) and of CMV reactivation on CD8 T and B cell  
29 relative abundance (suppl. Fig. 6).

### 30 31 32 *Persistent alterations at 1 year after allo-HSCT*

33 We then investigated whether and how the circulating immunoregulatory landscape remained altered  
34 one year after transplantation, when compared to the immune equilibrium at homeostasis. Hence, we  
35 first identified cell metaclusters with significantly different relative abundance between patients at M12  
36 and healthy donors (Fig. 2A-B). This showed reduced relative levels of MC1 (HSPCs), MC4 (CD4 T cells),  
37 MC6 (DCs), MC13 (MAIT and other TCR  $\alpha\gamma 7.2^+$  cells), MC14 (pDCs) and MC20 (CD117<sup>+</sup> ILCs), and  
38 elevated relative levels of MC16 (NK cells) in patients compared to healthy donors. We then generated a  
39 global atlas of each immune parameters found to be reduced (Fig. 2C, left panel) or elevated (Fig. 2C,  
40 right panel) in patients at M12. Of 203 parameters, 77 (38%) were increased and 45 (22%) decreased in  
41 transplant recipients (as shown in Fig. 2C) thus emphasizing profound and diverse persistent alterations  
42 in the immune equilibrium in transplant recipients. As summarized in Fig. 2D, these analyses confirmed  
43 long-term delayed relative reconstitution of CD4 T cells, MAIT cells, pDCs, non-classical monocytes and  
44 of the three subsets of innate lymphoid cells (ILC1, ILC2, and ILC3/P), in contrast to the immature  
45 (CD56<sup>bright</sup>CD16<sup>neg</sup>) NK cells that were overrepresented in patients. Moreover, the T-cell compartment



1 was biased toward the effector memory (EM) and terminally differentiated (TEMRA) phenotype,  
2 mirrored by a persistent lower frequency of naïve and central memory (CM) T cells. Of note, the  
3 intracellular cytotoxic molecule Granzyme B was expressed at higher levels in most T cell subsets, as well  
4 as in NK cells. Regarding immunoregulatory cell populations, MDSCs only partly normalized with  
5 persistently elevated relative levels of cells with phenotype of e- and G-MDSCs, while M-MDSC levels  
6 were similar to those of healthy donors. Total Treg frequency was also similar in patients and healthy  
7 donors, but the Treg compartment was biased toward activated (CD45RA<sup>neg</sup>HLA-DR<sup>+</sup>) Tregs. Importantly,  
8 most inhibitory receptors (TIGIT, PD-1, Tim-3, LAG-3, CTLA-4) as well as the immunoregulatory  
9 ectoenzyme CD39 were expressed on a higher proportion of CD8 and/or CD4 T cells and NK cells from  
10 patients (Fig. 2B-C). Surprisingly, the opposite was observed for the BTLA inhibitory receptor, that was  
11 expressed at lower frequencies on T cells from patients. This may reflect the above-mentioned  
12 deficiency in naïve T cells in patients, as BTLA downregulation is a known feature of T-cell  
13 differentiation.<sup>34</sup>

### 15 *Identification of an immune profile associated with subsequent late relapse*

16 In this longitudinal cohort, late AML/MDS relapse occurred in 12 patients (median 15 months post-  
17 HSCT). We investigated whether the immunoregulatory profile showed particular features in these  
18 patients with late relapse compared to those in long-term persistent remission (median follow-up 9.8  
19 [IQR 8.3–9.9] years). When assessed at M3, frequencies of naïve/CM CD8 T cells and  $\gamma\delta$  T cells were  
20 found at higher levels in patients with persistent remission (with only 9 patients of the relapsing group  
21 available at this time point) (suppl. Fig. 7A). We then compared immune parameters at the M12 (or last  
22 available) time point, and found a slight segregation between patients with or without subsequent  
23 tumor relapse in a principal component analysis (PCA) (Fig. 3A). Most metaclusters were found at similar  
24 relative levels between both groups (suppl. Fig. 7B). However, frequencies of MC18 ( $\gamma\delta$  T cells) and  
25 MC13 (MAIT cells) were significantly decreased in patients with subsequent relapse as compared to  
26 those with sustained remission (Fig. 3B). Among all single immune parameters differing between both  
27 patient groups (Fig. 3C), we identified that increased proportions of all innate-like T cell subsets (MAIT,  
28  $\gamma\delta$  T and NKT cells) and of naïve CD8 T cells were associated with long-term remission. Notably,  
29 frequencies of TIGIT-expressing cells were increased among various T-cell subsets in patients with  
30 subsequent relapse (Fig. 3C).

### 32 *Cross-sectional cohort study reveals T-cell subsets associated to subsequent AML relapse*

33 The first cohort had substantial limitations to study immune parameters associated to relapse (few and  
34 only late relapses, disease heterogeneity, no matching for confounding risk factors). We thus next  
35 investigated a homogenous retrospective cohort including 40 patients at M3 post-transplant (Fig. 4A).  
36 Patients' characteristics are described in Table 2 and Suppl. Table 2. Twenty patients with AML relapse  
37 (after M3, median 8.7 months) and 20 patients without documented relapse (median follow-up 32 [IQR  
38 17–52] months) were matched according to known disease relapse risks, as described in the method  
39 section. All other parameters were similar between both groups, with only non-significant higher risk of  
40 subsequent chronic GVHD, and, as expected, worse survival in relapsing patients (Table 2). We first  
41 performed a FlowSOM analysis of all PBMCs from all patients and including 28 subsets-defining  
42 phenotypic markers (suppl. Fig. 8). In this well-matched cohort, we found no difference between both  
43 patient groups in the frequency of any FlowSOM-generated cell clusters (data not shown). We next

1 focused on the T-cell compartment and performed an ad hoc FlowSOM analysis of pre-gated total T-  
2 cells (Fig. 4B), with all functional markers included for the cluster generation (suppl. Table 1). While  
3 none of the clusters was enriched in non-relapsing patients, three CD4 T-cell clusters (C25, C26, C22)  
4 and one rare CD8 T-cell cluster (C79) were found at increased frequencies in patients with subsequent  
5 relapse (Fig. 4C-D). In comparison to bulk CD4 or CD8 T cells, all of these cell clusters expressed higher  
6 surface levels of several inhibitory receptors, and higher CD39 expression (for C25 and C26) (Fig. 4E).

### 8 *CD161 and/or TIGIT are peculiar features of T-cell clusters associated to subsequent relapse*

9 We then further deciphered what distinguished relapse-associated T-cell clusters from bulk CD4 or CD8  
10 T cells. All of the identified clusters expressed PD-1, as well as at least one of the following receptors:  
11 TIGIT (C25, C26, C79), BTLA (C25, C26), and/or CD161 (C26, C22) (Fig. 4F-G). To highlight specific  
12 features, we measured the enrichment in frequency or density of inhibitory receptors expression on the  
13 clusters versus bulk CD4 (Fig. 4H) or CD8 (Fig. 4I) T cells. Notably, while PD-1 was expressed on all cells  
14 of the four identified cell subsets, it was only poorly enriched due to substantial PD-1 expression on  
15 other T cells not associated to tumor relapse. In contrast, cell clusters had stronger enrichment in either  
16 TIGIT (C25, C26) and/or CD161 (C26, C22) expression, both when measured as frequency of positive cells  
17 or as expression density (Fig. 4H-I). Thus, TIGIT and/or CD161 expression appeared the most  
18 distinguishable features of T-cell clusters (non-MAIT) enriched in relapsing patients. Accordingly, higher  
19 levels of TIGIT<sup>+</sup> (C25+26), as well of CD161<sup>+</sup> (C26+22) CD4 T-cell clusters at M3 post-transplant were  
20 associated with shorter AML relapse-free survival (Fig. 4J).

### 23 *CD4 T cells expressing the non-canonical CD161 inhibitory receptor differ from cells with classical 24 exhaustion phenotype and are strongly associated with poor relapse-free survival*

26 Interestingly, CD161 was recently described as a novel inhibitory receptor restraining anti-tumor T cells  
27 in the context of malignant glioma where tumor cells expressed the CD161 ligand CLEC2D.<sup>35</sup> Notably, in  
28 an ad-hoc transcriptomic analysis using the *TCGA Pan-Cancer Atlas Studies* on cBioPortal,<sup>36</sup> we found  
29 that AML ranked third out of the 35 cancer types with regard to CLEC2D expression levels (suppl. Fig. 9).

30 Based on the results from the unsupervised clustering analysis described above, we next sought to  
31 further characterize total CD161<sup>+</sup> but also TIGIT<sup>+</sup> cells by classical gating in non-Treg CD4 T cells from  
32 healthy donors and patients of the cross-sectional cohort (Fig. 5A). PD-1<sup>+</sup> CD4 T cells were also studied  
33 as comparison. Albeit CD161 is highly expressed on MAIT cells, the analysis of CD161<sup>+</sup> CD4 T cells did not  
34 include MAITs which are almost all CD4<sup>neg</sup> (CD8<sup>+</sup> or double-negative).

35 First, we found in a Boolean analysis that frequencies of CD4 T cells in patients at M3 post-HSCT  
36 expressing i) CD161<sup>+</sup>, ii) TIGIT<sup>+</sup>, as well as iii) at least TIGIT and/or CD161, were significantly elevated in  
37 subsequently relapsing patients (Fig. 5B), while only a trend was observed for PD-1<sup>+</sup> CD4 T cells. Of note,  
38 comparison to healthy donors showed that CD161 and TIGIT expression were upregulated in patients  
39 (Fig. 5B) and remained stably elevated throughout the 1-year follow-up in the first longitudinal cohort  
40 (suppl. Fig. 10). However, CD161 upregulation (median 1.3-fold increase vs healthy donors) was modest  
41 in comparison to the more markedly elevated expression of TIGIT (2.2-fold increase) and PD-1 (2.5-fold  
42 increase). Interestingly, UMAP embedding of CD4 T cells showed that CD161<sup>+</sup> T cells poorly overlapped  
43 with TIGIT<sup>+</sup> cells, that rather clustered within PD-1-expressing cells (Fig. 5C). Accordingly, TIGIT<sup>+</sup> cells  
44 expressed higher levels of the other inhibitory receptors PD-1 and BTLA (Fig. 5D), and even higher PD-1

1 density than total PD-1<sup>+</sup> cells themselves. Furthermore, TIGIT<sup>+</sup> but not CD161<sup>+</sup> cells were significantly  
2 enriched in dividing cells (intranuclear Ki-67 positivity) and in cells expressing HLA-DR, a marker of  
3 chronic T-cell activation (Fig. 5E). Altogether, these data suggest that CD161<sup>+</sup> cells – in contrast to TIGIT<sup>+</sup>  
4 cells – exhibit a profile that is rather distinct from the classical exhaustion phenotype.

5 We then determined whether levels of CD161<sup>+</sup> or TIGIT<sup>+</sup> CD4 T cells may vary depending on various  
6 clinical parameters, including conditioning regimen, donor type, GVHD and CMV reactivation. Notably,  
7 no significant difference was observed for most parameters, except for increased frequencies of TIGIT<sup>+</sup>  
8 cells in patients transplanted from an unrelated donor and lower levels of CD161<sup>+</sup> CD4 T cells in patients  
9 with acute GVHD (Fig. 5F), and more specifically  $\geq$  grade 3 aGVHD (suppl. Fig. 11).

10 As TIGIT expression on not only CD4 but also CD8 T cells were associated to late relapse in the  
11 longitudinal cohort, we asked whether TIGIT<sup>+</sup> T cells were associated to relapse-free survival in the  
12 cross-sectional cohort. Notably, high TIGIT expression on CD4 T cells and on naïve – but not bulk – CD8 T  
13 cells were significantly associated with poor relapse-free survival (suppl. Fig. 12).

14 Finally, in light of these results raising interest in CD161-expressing CD4 T cells, we further deciphered  
15 our previously-mentioned FlowSOM clustering with phenotypical markers on bulk PBMCs (suppl. Fig.  
16 8A) since CD161 was included as a marker for MAIT cell identification. Interestingly, the cluster C34  
17 corresponded to the total CD161<sup>+</sup> (non-MAIT, non- $\gamma\delta$  TCR<sup>+</sup>, non-Treg) CD4 T cell population (rather than  
18 a smaller FlowSOM subcluster as identified in Figure 4). Albeit frequency among total PBMCs was not  
19 found to be related to relapse, we normalized its level to the CD4 compartment. Strikingly, high C34  
20 cluster frequency among CD4 T cells was strongly associated to poor AML relapse-free survival (Fig. 5G),  
21 thus confirming the clinical relevance of CD161 expression within CD4 T cells with regard to subsequent  
22 AML relapse.

## 23 24 25 **DISCUSSION**

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26  
27 In this study, high dimensional mass cytometry allowed to comprehensively analyze the global immune  
28 reconstitution – focusing on the immunoregulatory landscape – after allo-HSCT in a total of 77 patients  
29 with myeloid malignancies. We highlighted the overall relative dynamics and persistent alterations of a  
30 large range of immunoregulatory parameters. Importantly, a second specifically designed cohort study  
31 revealed, by both classical and unsupervised clustering analysis, that TIGIT and CD161 expression on  
32 CD4 T cells were distinct early parameters strongly associated to subsequent relapse.

33  
34 The first cohort allowed to globally assess the longitudinal variations in distribution and phenotypic  
35 functional states of the main immune cell compartments during the first year post-HSCT. Although there  
36 was substantial interindividual variability, the immune landscape was surprisingly stable between M3  
37 and M6 while many significant changes occurred at M12. As previously demonstrated in studies focusing  
38 on individual cell subsets, proportion of B cells and CD4 T cells were increasing at 1-year, at the expense  
39 of monocytes and NK cells frequencies, reflecting the well-described reconstitution kinetics of these  
40 subsets.<sup>19,20</sup> At 1-year, there was a persistent proportional lack of naïve T cells, MAITs, ILCs (all subsets)  
41 and pDCs. Conversely, the NK cell compartment was disproportionate due to increased frequencies of  
42 cells with an immature CD56<sup>bright</sup>CD16<sup>neg</sup> profile. Besides, nearly all T and NK cell subsets had higher  
43 expression of Granzyme B and activation/exhaustion markers. This global approach thus highlighted that

1 the overall immune landscape was still largely imbalanced even 1-year post-transplant. Of note, data  
2 highlight a persistent general immune activation state, which may have implication for long-term risk of  
3 cardiovascular comorbidities after HSCT,<sup>37</sup> as well described for residual chronic immune activation in  
4 treated HIV-infection.<sup>38</sup>

5  
6 Regarding regulatory cell subsets, we found opposite patterns in Tregs and MDSCs dynamics. As other  
7 CD4 T cells, Tregs frequencies increased and were biased toward the activated phenotype at 1-year  
8 post-HSCT. In line with previous studies, this may reflect the followings: i) thymic generation of Tregs,  
9 compared to other CD4 T cells, is markedly impaired after HSCT, so that Tregs in this context mostly  
10 expand by lymphopenia-driven homeostatic proliferation,<sup>39</sup> and ii) naïve Tregs have a strong potential to  
11 proliferate and convert to activated Tregs.<sup>32</sup> This may thus account for the delayed increase in total  
12 Tregs and shrinkage of the naïve Treg pool. In sharp contrast to Tregs, relative levels of monocytic,  
13 granulocytic and early MDSCs subsets were all abnormally elevated at M3 and decreased over time.  
14 Only sparse data are available about human MDSCs subsets after allo-HSCT but, in line with our results,  
15 increased M-MDSCs levels were reported in this context.<sup>40</sup> Of note, we found that only M-MDSCs levels  
16 normalized at 1-year, while levels of G- and e-MDSC remained significantly elevated. The inflammatory  
17 context and severe immunosuppression following HSCT conditioning may be the driving forces for  
18 MDSCs expansion favored by inflammatory cytokines (e.g. IL-6 or IL-1 $\beta$ ) and emergency  
19 myelopoiesis.<sup>41,42</sup> Of note, MDSCs may also originate directly from the graft as these cells are enriched in  
20 peripheral blood grafts following G-CSF stem cell mobilization.<sup>43</sup> The strong and persistent lack of ILC  
21 reconstitution following allo-HSCT nearly rules out involvement of the ILC2-MDSC axis in this context.

22  
23 We aimed to investigate whether immunoregulatory features after allo-HSCT may be associated with  
24 subsequent tumor relapse. In the first cohort including late relapse events, cell subsets associated with  
25 persistent remission were interestingly mostly belonging to the innate-like T cell compartment (MAIT,  $\gamma\delta$   
26 T and NKT cells) or conventional naïve T cells. These cells mainly arise from de novo production in the  
27 thymus, which may stress the importance of the thymic function in the GVL effect. However, they all  
28 show different reconstitution dynamics and innate-like T cells may also substantially depend on the  
29 commensal microbiota.<sup>44</sup> These unconventional T cell subsets with (semi-)invariant TCRs may exert  
30 antitumor functions, albeit protumor roles have also been described (reviewed in<sup>45</sup>). Their role in the  
31 context of HSCT is currently emerging, although studies were limited by disease heterogeneity and low  
32 patient number.<sup>44</sup> The first cohort in the present work also showed important limitations to study  
33 disease relapse (few and only late relapse events).

34  
35 We thus specifically designed a homogeneous and balanced cohort matched for important confounding  
36 risk factors. Thereby, we identified via both classical and unsupervised clustering strategies, that early  
37 TIGIT and CD161 expression on CD4 T cells are associated to subsequent leukemia relapse. Higher PD-1  
38 expression on CD8 T cells has been reported at the time of relapse after HSCT,<sup>7,8</sup> which could be a cause  
39 but most probably a consequence of tumor development. However, exhausted PD-1<sup>+</sup>Eomes<sup>+</sup>T-bet<sup>neg</sup>  
40 CD8 T<sub>SCM</sub> cells in the bone marrow early after transplant – as well as tumor-specific PD-1<sup>+</sup> CD8 T cells –  
41 have been described in patients prone to relapse.<sup>8</sup> Our work confirmed that PD-1-expressing T cell  
42 clusters were associated to subsequent relapse. Nevertheless, we found that a more specific feature of  
43 T cells associated to relapse was expression of a less commonly studied (TIGIT) and a newly identified  
44 (CD161) inhibitory receptor.<sup>35</sup> Elevated TIGIT expression on CD8 T cells was previously reported in AML  
45 patients and correlated to poor clinical outcome.<sup>46</sup> Importantly, the TIGIT ligands CD155/PVR and

1 CD112/PVRL2 are expressed on AML blasts,<sup>7,46,47</sup> and percentage and/or expression density of these  
2 receptors are even increased on AML blasts at relapse post-HSCT compared to those at diagnosis.  
3 Together with our results, data argue for the involvement of TIGIT-mediated immune escape in this  
4 context.

5 Of note, DNAM-1 (CD226) share the same nectin(-like) ligands as TIGIT, but is conversely an activating  
6 receptor involved in T- and NK cell-mediated cytotoxicity. Interestingly, contrary to TIGIT, DNAM-1  
7 expression is decreased on T and NK cells from AML patients.<sup>46,48</sup> One could hypothesize that  
8 concomitant loss of DNAM1 together with TIGIT upregulation is an exhaustion phenotype that may play  
9 a role in AML relapse, as shown in a murine model of myeloma escape after autologous HSCT.<sup>49</sup>

10  
11 While CD161 is a classical marker for MAIT cells, unsupervised analyses unexpectedly pinpointed the  
12 clinical relevance of CD161 expression on CD4 T cells that were neither MAIT, nor Treg or  $\gamma\delta$ -T cells. Of  
13 note, CD161-expressing CD4 T cells may encompass Th17 cells may but these are rare compared to total  
14 CD161<sup>+</sup> CD4 T cells. Interestingly, CD161 was very recently described as a novel inhibitory receptor  
15 restraining anti-tumor CD8 and CD4 T cells in glioma.<sup>35</sup> Of note, CD161 and TIGIT expression poorly  
16 overlapped and CD161<sup>+</sup> CD4 T cells were more distinct from a classical exhaustion phenotype than  
17 TIGIT<sup>+</sup> CD4 T cells. As high level of CD161<sup>+</sup> CD4 T cells was strongly associated with poor relapse-free  
18 survival – and also with absence of severe acute GVHD –, the functional role of this molecule will  
19 deserve further investigation in this context. Importantly, the CD161 ligand CLEC2D (LLT1) showed one  
20 of the highest expression levels in AML when comparing to a large range of cancer types (including  
21 glioblastoma).

22  
23 Interestingly, we found association between relapse and IR expression mostly within the CD4 T cell  
24 compartment. Notably, it was previously demonstrated that (i) MHC class-II (rather than class-I)  
25 molecules are downregulated on AML blasts at relapse versus at diagnosis,<sup>7,9</sup> (ii) HLA-DP mismatches  
26 reduce the risk of leukemia relapse<sup>50</sup> and (iii) CD4 T cells are required – and possibly sufficient – for GVL  
27 responses in murine models of donor lymphocyte infusion.<sup>51,52</sup> Altogether, these data suggest a crucial  
28 role for effective CD4 T cells in the GVL effect, either as cytokine-secreting helper cells but also as direct  
29 cytotoxic lymphocytes.<sup>53</sup>

30  
31 In the present study, neither Tregs, nor any MDSCs subsets, were associated to subsequent relapse. This  
32 is of interest for strategies aiming at increasing their number to prevent or treat GVHD while preserving  
33 the GVL effect. Indeed, besides ongoing effort on Treg cell therapies, MDSCs were also found to inhibit  
34 GVHD in preclinical models,<sup>54</sup> and ILC2 infusion is also raising interest to induce tolerance via MDSCs  
35 induction.<sup>18,55</sup>

36  
37 Limitations of this work include the lack of data regarding the bone marrow immunoregulatory  
38 landscape or regarding TCR repertoire diversity and T-cell metabolism that may also be of interest with  
39 regard to the GVL effect.<sup>56-58</sup> Moreover, this study was limited to HLA-matched transplantation to obtain  
40 a homogeneous cohort thus limiting confounding factors, but more recent approaches such as  
41 haploidentical HSCT may warrant further investigations. Notably, in the latter setting, impaired NK cell  
42 recovery after posttransplant cyclophosphamide was found to predict relapse.<sup>59</sup> Finally, it remains an  
43 open question what factor(s) may impact TIGIT and CD161 expression levels, which were highly  
44 heterogenous between patients but rather stable over the months. It would be of interest to assess  
45 whether these are correlated to the levels found in the corresponding graft donors.

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Overall, there is now a growing body of evidence arguing for IR-mediated AML immune escape following allo-HSCT. Yet PD-1 blockade is the most commonly used immunotherapy to date, one could speculate that TIGIT and CD161 may represent more relevant targets in this context. The use of immune checkpoints-blocking antibodies may raise concerns about exacerbating GVHD, so that innovative therapeutic strategies could be required to selectively target and reinvigorate tumor- but not healthy tissue-specific alloreactive T cells.

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### **Authorship Contributions**

Contribution: M.F.C conceived the study, analyzed the data, and wrote the manuscript; V.G, V.P., N.V and K.V conducted the experiments and analyzed data; A.C, J.B, M.L, D.M, participated in experimental data, methodology, and editing; V.G, R.PdL and G.S provided patient samples and data; V.G, S.C-Z and G.S discussed the results and edited the manuscript.

### **Conflict of interest statement**

The authors declare no competing financial interests related to this work.

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## FIGURE LEGENDS

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### Figure 1. Longitudinal changes in the immunoregulatory landscape after allo-HSCT

(A) Thirty-seven patients with AML/MDS were included in the longitudinal cohort. PBMCs were collected at months 3, M6 and M12 after transplantation and analyzed by mass-cytometry. Twenty healthy donors were also analyzed. (B) Unsupervised cell clustering analysis by FlowSOM, with 18 indicated relevant metaclusters. (C) Principal component analysis (using FlowSOM data) of all tested samples from patients at indicated time points (M3, n=34; M6, n=31; M12, n=33) and from healthy donors (HD). (D) Highlight of cell clusters showing significant longitudinal variations between indicated time points. (E-F) Frequencies among PBMCs of cell metaclusters decreasing (E) or increasing (F) over time. (G-H) Volcano plots showing changes between M3 and M6 (G) and between M6 and M12 (H) in immune parameters assessed by classical manual gating. Significant parameters with  $|\text{percent change}| > 15\%$  are annotated (% cell population is among total CD45<sup>+</sup> live cells, or among a parent cell subset when indicated by “/subset”).

P-values correspond to Wilcoxon signed-rank tests. \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001. MC: metacluster.

### Figure 2. Persistent alterations in the immunoregulatory landscape at M12 after allo-HSCT

(A-B) Frequencies of cell metaclusters (FlowSOM) among PBMCs from patients at month 12 (M12) after transplantation were compared to those of healthy donors. Six out of 18 metaclusters were significantly decreased (A) and one was increased (B). Mann-Whitney tests: \*p < 0.05; \*\*p < 0.01; \*\*\*p < 0.001; \*\*\*\*p < 0.0001. (C) Each immune cell subsets (% cell subset is among total CD45<sup>+</sup> live cells, or among a parent cell subset when indicated by “/subset”) assessed by classical manual gating was also compared between patients at M12 and healthy donors. Significant (Mann-Whitney tests) parameters are annotated. (D) Altered expression of immunoregulatory molecules in indicated T and NK cell subsets. Color circles indicate significantly increased (red) or decreased (gray) median expression of the regulatory molecules in patients at M12 post-HSCT compared to healthy donors.

### Figure 3. Immune parameters associated to late relapse in the longitudinal cohort

(A) Principal component analysis (using FlowSOM data) of last available samples from patients with (n=12) or without (n=25) subsequent tumor relapse, as well as of healthy donors. (B) Frequencies of cell metaclusters (FlowSOM) in last available samples were compared between patients with or without subsequent tumor relapse. Two out of 18 metaclusters were significantly decreased in patients with subsequent relapse, and one metacluster tended to be increased. These 2 indicated metaclusters are shown here, while other metaclusters (similar in both groups) are shown in suppl. Fig. 3B. \*p < 0.05 (Mann-Whitney test). (C) Volcano plots showing differences between patients with and without relapse in immune parameters at last available time point assessed by classical manual gating. P-values correspond to Mann-Whitney tests. Parameters significantly different between both groups are annotated (% cell population is among total CD45<sup>+</sup> live cells, or among a parent cell subset when indicated by “/subset”).

**Figure 4. Inhibitory receptors expression on T cells associate with subsequent AML tumor relapse in the cross-sectional cohort**

(A) PBMCs from 40 AML patients with (n=20) and without (n=20) documented subsequent tumor relapse were collected at month 3 after transplantation and analyzed by mass cytometry. (B) Unsupervised cell clustering of the T-cell compartment was performed using FlowSOM. (C) Volcano plots showing differences between patients with and without relapse in frequencies of FlowSOM-generated T-cell clusters. P-values correspond to Mann-Whitney tests. Clusters significantly different between both groups are annotated (% cell cluster is among total CD3<sup>+</sup> T cells, or among CD8 or CD4 T cells when indicated). (D) Frequencies of identified cell clusters among CD4 or CD8 T cells in relapsing (R) and non-relapsing (NR) patients. (E) Heat map showing expression levels of indicated immunoregulatory molecules in relapse-associated cell clusters. (F) Expression of CD161, PD-1, TIGIT and BTLA on total and relapse-associated CD4 T-cell clusters. (G) Expression of PD-1 and TIGIT on total and relapse-associated CD8 T-cell cluster. (H-I) Enrichment in indicated inhibitory receptors positivity or expression density (median metal intensity, MMI) in relapse-associated clusters relative to total CD4 (H) or CD8 (I) T cells. (J) Frequencies of combined "TIGIT clusters" (C25+C26) or "CD161 clusters" (C22+C26) in relapsing (R) and non-relapsing (NR) patients. Relapse-free survival (RFS) assessed using the Kaplan-Meier approach in patients with high (> median) versus low (< median) frequencies of indicated cell clusters are shown in right panels. P-values corresponding to Mann-Whitney or to log-rank tests are indicated to compare cluster frequencies and RFS, accordingly.

**Figure 5. Characterization and clinical relevance of CD161<sup>+</sup> and TIGIT<sup>+</sup> CD4 T cells**

(A) Representative gating of CD161<sup>+</sup>, TIGIT<sup>+</sup> or PD-1<sup>+</sup> CD4 T cells (after exclusion of Treg and MAIT cells). (B) Percentage of cells (non-Treg/non-MAIT) expressing CD161, TIGIT, at least CD161 or TIGIT (Boolean gating), or PD-1, among total CD4 T cells from healthy donors (n=20), or from patients (at month 3 after allo-HSCT) with persistent remission (n=20) or with subsequent tumor relapse (n=20). (C) Uniform Manifold Approximation and Projection (UMAP) plots showing total CD4 T cells clustering. Indicated cell subsets (from manual gating) are colored while other ungated CD4 T cells are shown in grey (left panel); and expression levels of CD161, TIGIT and PD-1 are depicted (right panels), showing poor overlap between CD161<sup>+</sup> and TIGIT<sup>+</sup> cells, but strong overlap between TIGIT<sup>+</sup> and PD-1<sup>high</sup> cells. (D) Frequencies of cells expressing various inhibitory receptors among the indicated CD4 T-cell subsets. PD-1 cell surface density is also shown (expressed as median metal intensity, MMI) (lower right panel). (E) Frequencies of HLA-DR<sup>+</sup> or intranuclear Ki-67<sup>+</sup> cells among CD161- and TIGIT-expressing CD4 T cells compared to those that do not express CD161 and TIGIT as control (ctrl). (F) Frequencies of CD161<sup>+</sup> as well as TIGIT<sup>+</sup> CD4 T cells were compared with regard to sex and various clinical parameters, i.e. conditioning regimen, donor type, CMV reactivation and acute GVHD. RIC: reduced intensity conditioning; MAC: myeloablative conditioning; MRD: matched related donor; MUD: matched unrelated donor. (G) FlowSOM clustering of PBMCs from the 40 patients at M3 post-HSCT was performed based on lineage-defining markers (suppl. Fig. 4). Left panel: frequencies of cluster 34 (corresponding to CD161<sup>+</sup> CD4 T cells) among CD4 T cells, in non-relapsing and relapsing patients. Right panel: relapse-free survival (RFS) assessed using the Kaplan-Meier approach in patients with high (> 22%) versus low (< 22%) frequencies of C34 in CD4 T cells. Censored patients are represented by symbols. P-values from Mann-Whitney (panels B, F, G), Wilcoxon (panel E) or log-rank (panel G) tests are indicated: \*p <0.05; \*\*p<0.01; \*\*\*p<0.001; \*\*\*\*p<0.0001.

**Table 1: Patients' characteristics in the longitudinal cohort**

Patient Characteristics	All patients. N=37 Median [IQR] or N (%)	No relapse. N=25 Median [IQR] or N (%)	Relapse. N=12 Median [IQR] or N (%)	p-value
Age at transplant, years	51.5 [35.4-61.2]	47.0 [33.9-60.0]	54.0 [37.7-62.8]	0.40
Sex				1
Female	17 (46)	12 (48)	5 (42)	
Male	20 (54)	13 (52)	7 (58)	
Diagnosis				0.12
AML	26 (70)	20 (80)	6 (50)	
MDS	11 (30)	5 (20)	6 (50)	
ELN classification (AML)				0.74
Favorable	7 (27)	6 (30)	1 (17)	
Intermediate	10 (38)	8 (40)	2 (33)	
Adverse	9 (35)	6 (30)	3 (50)	
Number of pre-transplant treatment				0.73
0	2 (5)	2 (8)	0 (0)	
1	23 (62)	14 (56)	9 (75)	
≥ 2	12 (32)	9 (36)	3 (25)	
Disease status at transplant				0.11
CR1	17 (46)	13 (52)	4 (33)	
CR≥2	10 (27)	8 (32)	2 (17)	
No CR	10 (27)	4 (16)	6 (50)	
Conditioning				0.01
Myeloablative	14 (38)	13 (52)	1 (8)	
Reduced intensity	22 (59)	12 (48)	10 (83)	
Sequential	1 (3)	0 (0)	1 (8)	
Serotherapy	20 (54)	12 (48)	8 (67)	0.32
Donor				0.58
Related	18 (49)	11 (44)	7 (58)	
Matched unrelated	16 (43)	11 (44)	5 (42)	
Mismatched unrelated	3 (8)	3 (12)	0 (0)	
Stem cells source				0.11
Bone marrow	1 (3)	1 (4)	0 (0)	
Peripheral blood	34 (92)	24 (96)	10 (83)	
Umbilical cord blood	2 (5)	0 (0)	2 (17)	
GVHD prophylaxis				0.12
CSA	1 (3)	1 (4)	0 (0)	
CSA + MMF	19 (51)	10 (40)	9 (75)	
CSA + MTX	17 (46)	14 (56)	3 (25)	
Acute GVHD all grades	18 (49)	15 (60)	3 (25)	0.08
Acute GVHD grade III/IV	3 (8)	3 (12)	0 (0)	0.54
Chronic GVHD	10 (27)	9 (36)	1 (8)	0.12
None	3 (30)	3 (33)	0 (0)	
moderate	5 (50)	4 (44)	1 (100)	
extensive	2 (20)	2 (22)	0 (0)	
CMV reactivation	6 (16)	4 (16)	2 (17)	1
Death	8 (22)	1 (4)	7 (58)	0.0005
Cause of death				
GVHD	1 (13)	1 (4)	0 (0)	0.0005
Relapse	7(87)	0 (0)	7 (58)	

Abbreviations: AML: acute myeloid leukemia ; CMV: cytomegalovirus ; CSA: cyclosporine ; ELN: European LeukemiaNet ; GVHD: graft versus host disease ; MMF: mycophenolate mofetil ; MTX: methotrexate ; CR: Complete response.

**Table 2: Patients' characteristics in the cross-sectional cohort**

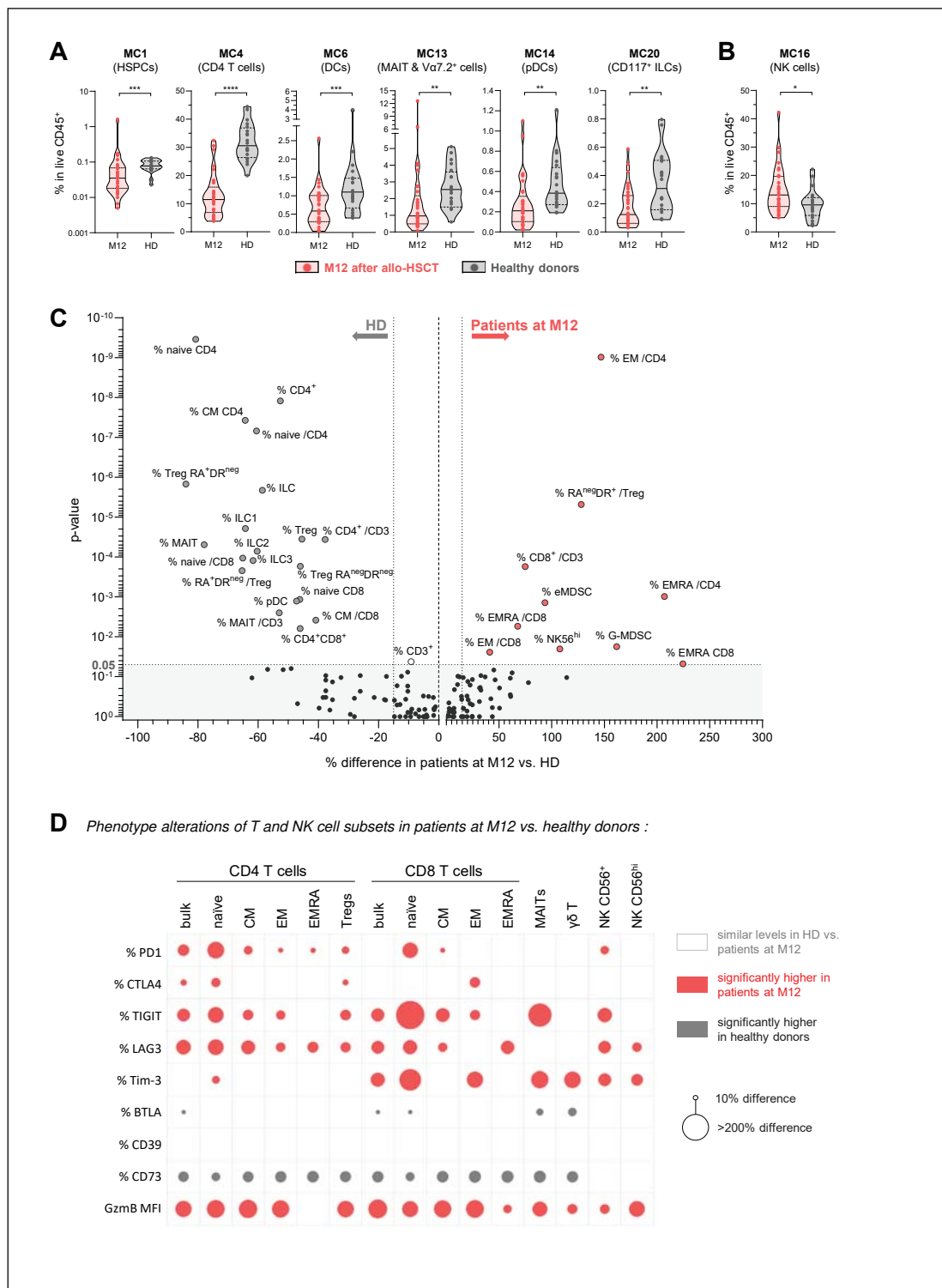
Patient Characteristics	All patients. N=40 Median [IQR] or N (%)	No relapse. N=20 Median [IQR] or N (%)	Relapse. N=20 Median [IQR] or N (%)	p-value
Age at transplant, years	52.5 [43-59]	52.5 [45.5-60.5]	52.5 [38.7-58.5]	0.51
Sex				1
Female	21 (52)	11 (55)	10 (50)	
Male	19 (48)	9 (45)	10 (50)	
ELN classification				0.65
Favorable	5 (12,5)	2 (10)	3 (15)	
Intermediate	18 (45)	10 (50)	8 (40)	
Adverse	17 (42.5)	8 (40)	9 (45)	
Number of pre-transplant treatment				0.16
1	29 (72)	17 (85)	12 (60)	
≥ 2	11 (28)	3 (15)	8 (40)	
Disease status at transplant				0.10
CR1	27 (67)	17 (85)	10 (50)	
CR≥2	8 (20)	2 (10)	6 (30)	
No CR	5 (13)	1 (5)	4 (20)	
Conditioning				0.46
Myeloablative	9 (22,5)	5 (25)	4 (20)	
Non myeloablative	3 (7.5)	2 (10)	1 (5)	
Reduced intensity	25 (62,5)	13 (65)	12 (60)	
Sequential	3 (7.5)	0 (0)	3 (15)	
Serotherapy	32 (80)	16 (80)	16 (80)	1
Donor				0.79
Related	19 (47.5)	10 (50)	9 (45)	
Matched unrelated	18 (45)	8 (40)	10 (50)	
Mismatched unrelated	3 (7.5)	2 (10)	1 (5)	
Stem cells source				0.22
Bone marrow	2 (5)	0 (0)	2 (10)	
Peripheral blood	37 (92,5)	20 (100)	17 (85)	
Umbilical cord blood	1 (2.5)	0 (0)	1 (5)	
GVHD prophylaxis				0.17
CSA	2 (5)	0 (0)	2 (10)	
CSA + MMF	28 (70)	13 (65)	15 (75)	
CSA + MTX	10 (25)	7 (35)	3 (15)	
Acute GVHD all grades	14 (35)	8 (40)	6 (30)	0.74
Acute GVHD grade III/IV	7 (17.5)	4 (20)	3 (15)	1
Chronic GVHD	12 (30)	9 (45)	3 (15)	0.08
None	2 (5)	2 (22)	0 (0)	
moderate	4 (10)	3 (15)	1 (5)	
extensive	6 (15)	4 (20)	2 (10)	
CMV reactivation	7 (17.5)	3 (15)	4 (20)	1
Death	22 (55)	3 (15)	19 (95)	<b>4.10<sup>-7</sup></b>
Cause of death				
GVHD	1 (2.5)	1 (5)	0 (0)	
Relapse	18 (45)	0 (0)	18 (90)	
Secondary cancer	1 (2.5)	1 (5)	0 (0)	
Toxicity	1 (2.5)	1 (5)	0 (0)	

**Abbreviations:** AML: acute myeloid leukemia ; CMV: cytomegalovirus ; CSA: cyclosporine ; ELN: European LeukemiaNet ; GVHD: graft versus host disease ; MMF: mycophenolate mofetil ; MTX: methotrexate ; CR: Complete response.

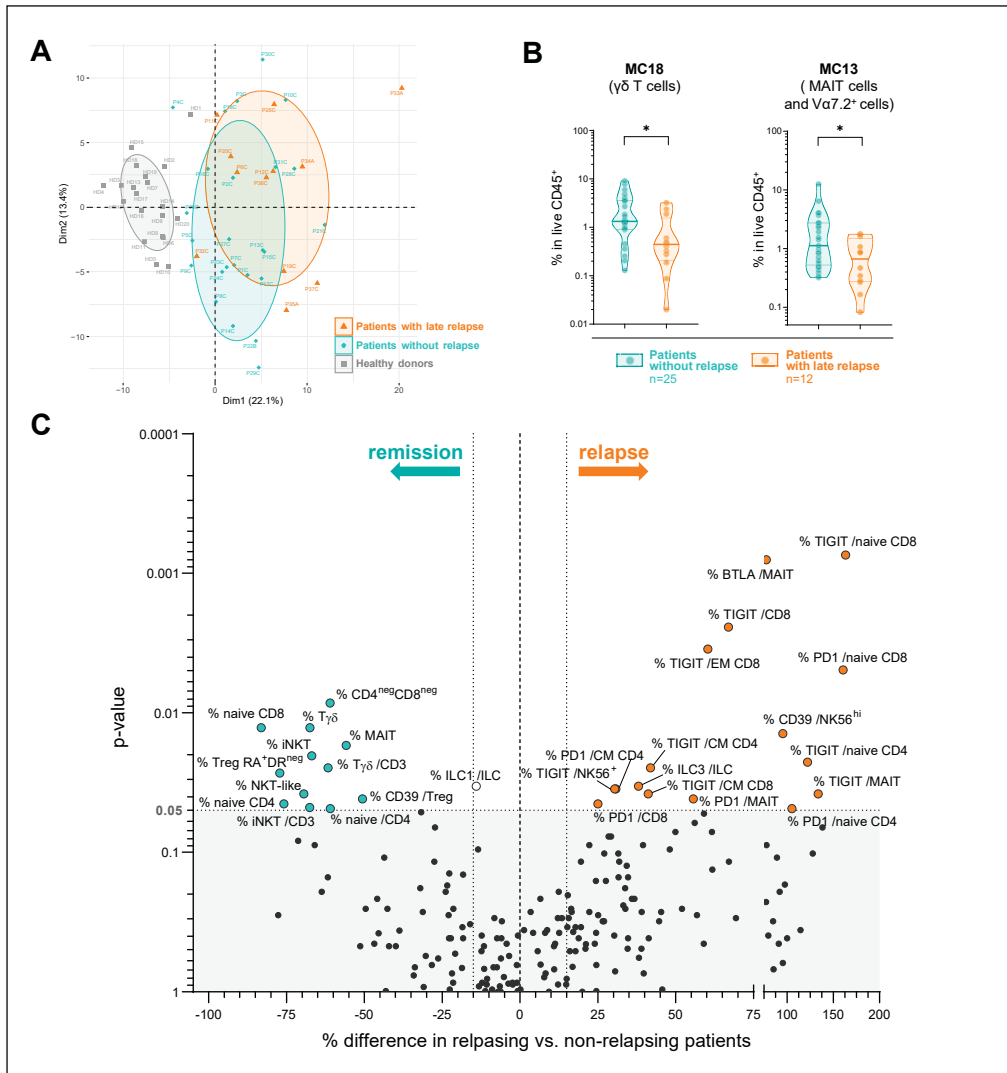
**Figure 1 – Longitudinal changes in the immunoregulatory landscape after allo-HSCT**



**Figure 2 – Persistent alterations in the immunoregulatory landscape at M12 after allo-HSCT**

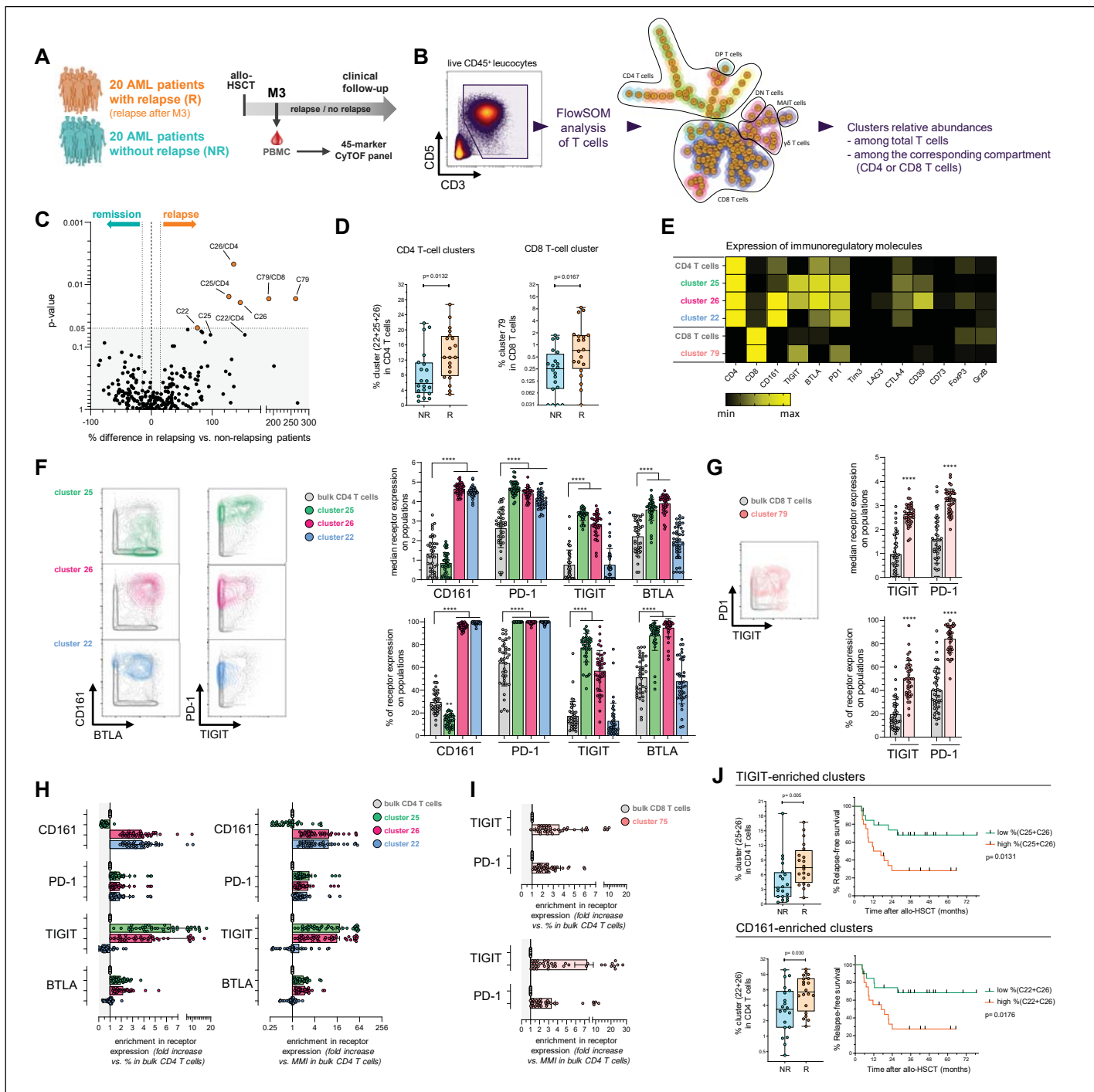


**Figure 3 – Immune parameters associated to late relapse in the longitudinal cohort**





**Figure 4 – Inhibitory receptors expression on T cells associate with subsequent AML tumor relapse in the cross-sectional cohort**



**Figure 5 – Characterization and clinical relevance of CD161<sup>+</sup> and TIGIT<sup>+</sup> CD4 T cells**

