Bone Marrow Transplantation (2018) 53:949–959
https://doi.org/10.1038/s41409-017-0078-0

REVIEW ARTICLE

Selected biological issues affecting relapse after stem cell transplantation: role of T-cell impairment, NK cells and intrinsic tumor resistance

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Received: 11 September 2017 / Revised: 12 December 2017 / Accepted: 15 December 2017 / Published online: 24 January 2018

Abstract
The graft vs. leukemia (GvL) effect as a method of preventing relapse is well described after allogeneic hematopoietic cell transplantation (HCT), but the mechanisms to this effect and how tumor sometimes develops resistance to GvL are just beginning to be understood. This article reviews and expands upon data presented at the Third International Workshop on Biology, Prevention and Treatment of Relapse after Stem Cell Transplantation held in Hamburg, Germany, in November 2016. We first discuss in detail the role that T-cell impairment early after HCT plays in relapse by looking at data from T cell-depleted approaches as well as the clear role that early T-cell recovery has shown in improving outcomes. We then review key findings regarding the role of specific KIR donor/recipient pairings that contribute to relapse prevention after HCT for several tumor types. Finally, we discuss a unique mouse model following the development of tumor resistance to GvL. Detailed molecular characterization of events marking the development of tumor resistance to the immunotherapy of GvL may help in developing future strategies to overcome immune escape.

Introduction

Patients undergoing allogeneic hematopoietic cell transplantation (HCT) have generally either already developed or are at high risk of developing resistance to standard chemotherapy approaches. The therapeutic advantage of proceeding to HCT is then found in development of a graft vs. leukemia (GvL) or immunotherapeutic effect from the allogeneic cells delivered in order to decrease the risk of relapse. This effect is only beginning to be understood, and the discussion below will focus on key findings associated with (1) the importance of early immune recovery in preventing relapse, (2) the role of the immune effect of killer cell immunoglobulin-like receptor (KIR) in prevention of relapse and (3) studies of molecular changes that lead to development of escape from the GvL effect. By better understanding these effects, HCT clinicians and scientist are developing strategies aimed at enhancing and preserving the GvL effect, resulting in improved outcomes after HCT procedures.

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The role of T-cell impairment and factors influencing T-cell reconstitution in allogeneic stem cell transplantation (Lorenz Jahn, Marcel van den Brink)

Immune reconstitution, T-cell recovery and outcomes of allogeneic HCT

Allogeneic hematopoietic cell transplantation (alloHCT) is a curative treatment for hematological malignancies but is

complicated by conditioning regimen-related toxicities, infections, relapse and graft vs. host disease (GvHD). GvHD is caused by activation of donor-derived T cells contained in the graft that recognize host-specific alloantigens. The risk of developing GvHD can be significantly reduced by administering T cell-depleted (TCD) hematopoietic stem cells (HSCs) in patients of chronic myelogenous leukemia [1–4]. This benefit is, however, offset by increased risk of relapse [1–3]. It is notable that in some studies patients receiving TCD HCT can have reduced risk of developing GvHD without increases in relapse rates or non-relapse-related mortality (NRM) [5–13]. It has also been noted in studies by Devine et al. [10]. and Barba et al. [13]. that a major benefit of TCD alloHCT is improvement in GvHD-free relapse-free survival. Although patients with acute leukemia benefited from TCD alloHCT without increases in relapse rates, higher incidence of relapse was observed in patients suffering from chronic leukemia. These differences may stem from varying dependencies on the GvL effect for different diseases [14].

In patients of acute leukemia, overall survival (OS) is similar between recipients of TCD and unmodified allogeneic grafts. However, Wagner et al. [9] reported on increased mortality caused by opportunistic viral and fungal infections after TCD transplants. Allogeneic HCT is associated with significant and prolonged immunosuppression, increasing the susceptibility to opportunistic infections. Cytoreductive conditioning regimens not only eliminate malignant cells but also deplete the recipient's immune system. Several studies have demonstrated a positive correlation between early immune reconstitution and clinical outcomes after alloHCT. Absolute lymphocyte counts (ALCs) assessed within the first months post transplant are a strong predictive marker for the outcome of alloHCT [15–18]. Lower rate of relapse, decreased probability of GvHD, lower cumulative incidence of opportunistic infections and increased OS were significantly associated with higher ALCs assessed within the first month [16, 17] and at month 2 [19] following alloHCT. Similarly, lower rates of relapse, occurrence of acute GvHD and incidence of NRM with significantly better OS was observed in patients separated with ALC above the median on day 30 post transplant [15, 18].

To overcome prolonged phases of post-transplant immunodeficiency associated with TCD alloHCT while limiting risk of GvHD, additional strategies involving T cell-replete grafts have been clinically evaluated. Post-transplant high-dose cyclophosphamide in combination with T cell-replete grafts has been successful in reducing the severity of acute GvHD and the risk of developing chronic GvHD in patients suffering from myeloid and leukemic malignancies [20–22]. It is important to note that in these studies, reduction in GvHD did not lead to increases in relapse. These data suggest that even low levels of immunologic activity by donor T cells suffice to maintain GvL [20]. Depletion of T cell receptor-αβ (TCRαβ) T cells from stem cell grafts has been proposed to limit the occurrence of GvHD, while GvL was maintained through other immune cells such as natural killer (NK) cells or γδ T cells still contained in the graft [23, 24]. Faster recovery of NK and T cells was reported in recipients of TCRαβ T cell-depleted grafts [23, 24], while risk for GvHD remained comparable to other TCD graft approaches [23]. Despite faster immune recovery, viral infections posed a serious risk and were a major contributor to transplant-related mortality [24]. In contrast, in recipients of naive T cell-depleted grafts, cytomegalovirus (CMV)-specific T-cell populations were measurable early after transplant and coincided with CMV reactivation, indicating that CMV immunity can be transferred with the graft [25]. Naive T cells are believed to be the main drivers of GvHD and GvL. Nonetheless, patients suffering from acute leukemia receiving naive T cell-depleted grafts showed lower relapse rate and higher 2-year disease-free survival rate than recipients of T cell-replete grafts or historical controls of TCD alloHCT recipients [25]. Although no changes in the incidence of acute GvHD were noted, significant reduction in occurrence of chronic GvHD was observed in the naive T cell-depleted graft recipients compared to T cell-replete graft recipients.

T cells as part of the adaptive immunity significantly contribute to positive outcomes of alloHCT by mediating beneficial GvL and providing protection from opportunistic infections. Faster T-cell recovery, determined by higher CD4 T-cell counts in recipients of alloHCT, and T-cell function, assessed by in vitro stimulation, was positively associated with better OS, lower risk of GvHD, lower NRM and lower risk of opportunistic infections [26–29]. Besides absolute T-cell counts, the probability of recognizing and mounting an effective immune response against pathogens is also determined by the diversity in the TCR repertoire. Advances in next-generation sequencing allow for the monitoring of αβTCR repertoires and the tracking of individual T-cell clones over time [30]. Conditioning regimens and TCD alloHCT severely depress TCR repertoire diversity [31, 32]. Nonetheless, differences in diversity of these contracted TCR repertoires correlate with alloHCT outcome. Yew et al. [31] reported on a cohort of 21 patients where GvHD and relapse, exclusive of each other, were associated with lower TCR repertoire diversity and higher frequency of certain T-cell clones. The authors argue that diminished TCR diversity associated with GvHD may be the result of strong expansion of T-cell populations involved in GvHD pathology, whereas higher TCR diversity in the non-relapse group reflects the higher GvL effect manifesting in diverse T-cell evolution over time. In contrast, Heijst et al. [30] found increased TCR diversity in

Table 1 Strategies to enhance thymic function (adapted from Chaudhry et al. [40])

Strategy	Regenerative targets	Stage of development
IL-7	BM HSPCs thymocytes	In trials
IL-12	Thymocytes	Preclinical
IL-21	Thymocytes	Preclinical
IL-22	Thymic epithelial cells	In trials
Ftl3L (FMS-like tyrosine kinase 3 ligand)	BM HSPCs thymocytes	Preclinical
IGF-I (insulin like growth factor I)	Thymic epithelial cells	Preclinical
GH (growth hormone)/ Ghrelin	Thymocytes	In trials
KGF (keratinocyte growth factor)	Thymic epithelial cells	In trials
SCF (stem cell factor)	Thymocytes	Preclinical
Sex steroid inhibition	Thymic epithelial cells BM HSPCs thymocytes	In trials
Precursor T cells	Thymocytes	IND pending
HSPCs	Thymocytes	Preclinical
Ex vivo thymic epithelial cells	Thymic epithelial cells thymocytes	Preclinical

BM bone marrow, *HSPCs* hematopoietic stem/progenitor cells, *IND* investigational new drug

patients suffering from acute GvHD. However, discrepancies between these studies may be due to differences in stem cell sources. Of note, the γδ T-cell compartment is also severely contracted following alloHCT but reconstitutes quickly and is influenced by viral infections post transplant [33].

Parameters influencing T-cell recovery

Restoration of T-cell immunity in recipients of alloHCT is severely delayed and can take 1–2 years to reach normal levels [28, 34–38]. T-cell reconstitution in patients undergoing alloHCT is driven by (1) peripheral expansion of residual host or graft-contained T cells in the early post-transplant period and (2) de novo generation of naive T cells in the thymus at later stages. Peripheral expansion is predominately observed in the CD8 memory T-cell compartment in response to antigen-dependent and homeostatic cytokine-driven proliferation. CMV infections and CMV seropositivity of the recipient are associated with increased CD8 T-cell counts [35, 39] but diminished TCR diversity [30]. This initial wave of reconstitution results in a contracted and skewed TCR repertoire. In contrast, de novo T lymphogenesis in the thymus is the main mechanism generating a diverse TCR repertoire. Thymopoeisis early after alloHCT is compromised by thymic injury caused by conditioning regimens. Additionally, age-related involution of the thymus leads to a decline in thymic output in healthy individuals [40, 41]. Indeed, age of alloHCT recipients is inversely associated with increased T-cell recovery by the production of naive T cells [27, 34, 38, 42]. Thymic output as measured by T-cell receptor excision circles (TRECs) is significantly impaired early after alloHCT and can require 1–2 years to reach baseline levels [41, 42]. Higher TREC levels within the first 6 months after alloHCT correlated with lower risk for and less severe opportunistic infections [42]. The frequency of circulating naive CD4 T cells post alloHCT is positively correlated with increased TCR diversity [32]. While no differences in TCR diversity were found in recipients of TCD matched donor or haploidentical cord blood transplants at 100 days post transplant [31], Heijst et al. [30] reported that in cord blood-graft recipients measures of TCR diversity at 6 months post transplant are comparable to healthy individuals, whereas TCR diversity in recipients of TCD grafts was significantly lower, indicating an important role of the stem cell source in T-cell recovery. Other studies have also highlighted the impact of graft source. Faster CD4 T-cell recovery was associated with stem cell grafts from related donors [27, 34]. Kanda et al. [37] demonstrated that CD4 and CD8 T-cell recovery is slower in recipients of dual umbilical cord blood compared to recipients of T cell-replete peripheral blood stem cells from matched sibling or matched unrelated donors.

Human leukocyte antigen (HLA) disparity between donor and recipient and occurrence of chronic GvHD are associated with delayed recovery of both CD4 and CD8 T cells [35]. Additionally, chronic GvHD also correlated with low TRECs following alloHCT [42, 43]. These data as well as studies in mice suggest that the thymus can be a target of GvHD, hampering T-cell neogenesis. However, conditioning regimens using anti-thymocyte globulin (ATG) for T-cell depletion and GvHD prophylaxis also significantly delay reconstitution of the T-cell compartment. While Castillo et al. [38] reported that ATG dose inversely correlated with delayed CD4 and CD8 T-cell recovery, Goldberg et al. [28] only found a significant delay of the CD4 T-cell compartment.

Boosting T-cell recovery

Faster T-cell recovery following alloHCT is positively correlated with transplant outcomes. However, transplant modalities and biological factors strongly influence T-cell reconstitution. Therefore, developing strategies that boost posttransplant immune recovery by acting on thymic regeneration as well as peripheral T-cell immunity will likely result in improved outcome of alloHCT [44, 45]. Strategies under investigation focus on either driving homeostatic T-cell expansion in the periphery, restoration of

the thymic stromal environment or increasing thymocyte proliferation. These strategies, summarized in Table 1 and reviewed by Chaudhry et al. [46], work through the administration of cytokines and hormones, sex steroid ablation or infusion of lymphoid and T-cell precursors.

T-cell immune reconstitution: summary

Immunodeficiency post allogeneic HCT is associated with increased risk of relapse, higher NRM and decreased OS. Early immune recovery as measured by ALC within the first 2 months after HCT is correlated with lower rate of relapse, decreased probability of GvHD, lower cumulative incidence of opportunistic infections and increased OS in recipients of TCD alloHCT. High-dose post-transplant cyclophosphamide, TCRαβ- or naive T cell-depletion have emerged as additional strategies to limit occurrence of GvHD while maintaining GvL and improving immune recovery. Nonetheless, T-cell immunity is severely impacted by these approaches and requires years to reach normal levels. Several factors such as CMV seropositivity, graft source, occurrence of GvHD and conditioning regimen impact T-cell reconstitution, which is manifested in not only the quantity of T cells but also in the diversity of the TCR repertoire. Preclinical and clinical studies focusing on strategies to boost post-transplant hematopoiesis and thymic regeneration by administration of cytokine and growth factors or cell-based therapies will likely lead to faster immune recovery, possibly improving outcomes after alloHCT.

The role of NK cells and KIR genotype (Markus Uhrberg)

NK cells are early-acting cytotoxic immune cells, accounting for roughly 10% of the entire lymphocyte population in healthy individuals. Notably, NK cells represent the predominant lymphocyte population during the early reconstitution phase of HSCT, well before newly generated T and B cells appear in the periphery [47, 48]. It is in this early phase of HSCT that NK cells are believed to play a decisive role in eliminating residual tumor cells in the absence of an effective anti-leukemic T-cell response. The role of NK cells is particularly relevant in the haploidentical setting, where T-cell reconstitution is deeply compromised, but NK cell recovery can progress quickly. Notably, failure to develop mature NK cells at early time points negatively correlates with relapse control [49]. Thus, given the large genetic diversity of the NK cell receptor family KIR and the heterogeneity of NK cell effector subsets, it is of great importance to understand how NK cells develop in the setting of HSCT in order to appreciate NK cell-mediated anti-leukemic immune responses.

The KIR gene family, which is tightly clustered on chromosome 19, is exceptionally polymorphic in the human population at the gene and allele levels. A remarkable feature is their polygenicity, creating donor-to-donor variability in terms of number and kind of KIR receptors [50, 51]. A useful way to look at KIR gene polymorphism is to differentiate between extended genetic linkage groups, the KIR haplotypes. The most frequent group of haplotypes across ethnically diverse human populations are KIR-A haplotypes and are unique in that they encompass inhibitory KIR genes for the three major HLA class I-encoded epitopes C1 (KIR2DL3), C2 (KIR2DL1) and Bw4 (KIR3DL1) together with a single stimulatory KIR (KIR2DS4), which is frequently present as a deletion variant [50, 52]. Different KIR-A haplotypes share the same gene content but exhibit substantial allelic polymorphism. All haplotypes that differ from KIR-A in terms of gene content are referred to as KIR-B haplotypes and in most cases carry fewer inhibitory and more stimulatory KIR genes.

In the setting of clinical HSCT for hematological malignancies, a beneficial effect of KIR-B haplotypes on relapse control has emerged as a common theme and has been shown for myeloid leukemia [53] as well as childhood acute lymphoblastic leukemia [54], multiple myeloma [55], and non-Hodgkin lymphoma [56]. The underlying mechanism for these observations is unclear. The most common model is based on the fact that KIR-B haplotypes harbor a larger number of stimulatory KIR genes than KIR-A haplotypes. However, the beneficial effect of KIR-B haplotypes cannot be replaced by simply counting the number of stimulatory KIR genes nor is there a consensus on particular stimulatory KIR responsible for exerting the anti-leukemic response. Moreover, with the exception of the C2-specific KIR2DS1 (which was already shown to confer relapse protection in acute myeloid leukemia (AML) patients in an HLA-C-dependent manner [57]), stimulatory effects based on interaction of stimulatory KIR with HLA class I are weak [58]. The question thus emerges of how KIR-B haplotypes, representing a mixed bag of haplotypes with vastly different content of stimulatory KIR genes, are able to exert a common protecting influence on relapse control.

An alternative explanation for the observed beneficial effects of KIR-B haplotypes is based on the different ligand affinities of inhibitory KIR. In this regard, it is helpful to distinguish between the centromeric and telomeric parts of KIR haplotypes, which are separated by a recombination hotspot [59]. In the Caucasian population, the nine most frequent haplotypes are built from three conserved centromeric and telomeric linkage groups [60]. This model was first applied to the HSCT setting by Cooley et al. [53]. It appeared that the centromeric part of KIR-B haplotypes

(cenKIR-B cluster) accounted for most of the protective effect [53, 61]. A hallmark of the cenKIR-B cluster is KIR2DL2 (in tight linkage with KIR2DS2), which is in allelic relationship with the KIR-A-specific KIR2DL3. Although both are specific for the C1 ligand, KIR2DL2 has a higher affinity for C1 than KIR2DL3 [62]. The second difference pertains to the KIR2DL1 gene, which in the cenKIR-B cluster is either present as functionally attenuated allelic variant or absent altogether. In contrast, in the cenKIR-A cluster, KIR2DL1 alleles generally have strong affinity for C2-encoded HLA-C allotypes [63]. Together, the defining features of the cenKIR-B cluster are, on the one hand, presence of a high-affinity C1-specific inhibitory receptor and, on the other hand, absence of a high-affinity C2-specific inhibitory receptor. Put together, these observations make it difficult to develop a mechanistic model for an improved recognition of leukemic cells solely based on differences in receptor/ligand affinity.

Finally, besides KIR polymorphism, the C1/C2 polymorphism of HLA-C itself constitutes an important genetic factor influencing relapse control in the HSCT setting. Specifically, homozygosity for C2 emerges as a robust risk factor for relapse in myeloid and lymphoid leukemia [64–69]. In the prevailing mechanistic model, this observation is explained by the sequential acquisition mode of KIR receptors during HSCT reconstitution [65, 70]. The model is based on in vitro [65, 71] and in vivo data [49, 72–74] showing that the C1-specific KIR2DL2 and KIR2DL3 receptors are expressed on newly developed NK cells earlier and at much higher frequencies than the C2-specific KIR2DL1 receptor (Fig. 1). Importantly, this process is genetically hardwired and thus independent of the patients' C1/C2 status. The predominant development of KIR2DL2/3-expressing NK cells in a C2/C2 host leads to accumulation of unlicensed and thus hyporesponsive NK cells in this patient group. The late acquisition of KIR2DL1 creates a hole in the KIR repertoire in these patients, which impairs control leukemic HLA-C levels. Notably, it was recently shown that downregulation of HLA-C but not HLA-A and -B is a common phenomenon on leukemic blasts in ALL patients [75]. Moreover, the sequential acquisition mode of KIR might also contribute to the beneficial effect of the cenKIR-B cluster on relapse. Functional analysis revealed that the cenKIR-B-derived KIR2DL2 also has affinity for C2 and is thus much more efficiently inhibited by most C2 alleles than the cenKIR-A-derived KIR2DL3 (Fig. 1) [76, 77]. Thus, cenKIR-B donors provide KIR2DL2-expressing, licensed NK cells able to monitor HLA-C levels on leukemic cells in patients expressing C2, whereas homozygous cenKIR-A donors (encoding two copies of KIR2DL3) lack that ability during the reconstitutional phase.

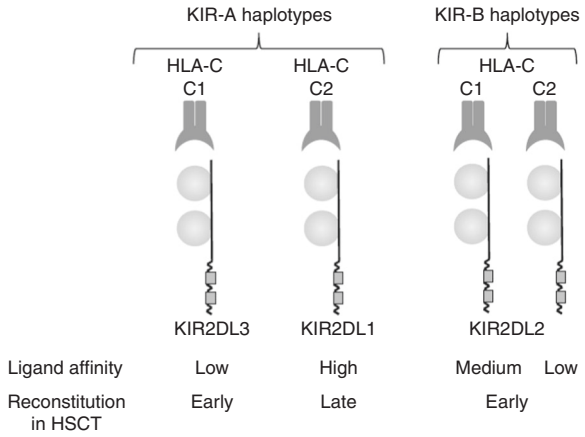

Fig. 1 Properties of HLA-C-specific inhibitory KIR. The centromeric part of the *KIR* locus encodes three HLA-C-specific inhibitory *KIR* genes that are either part of *KIR-A* haplotypes (*KIR2DL1* and *KIR2DL3*) or *KIR-B* haplotypes (*KIR2DL2*). Of note, on some *KIR-B* haplotypes *KIR2DL1* alleles with attenuated signal transduction capacities are present. The affinity of each KIR for the two mutually exclusive KIR ligands C1 and C2 as well as their kinetics of expression during NK cell reconstitution in HSCT are depicted. (Color figure online)

In summary, many studies have addressed the role of KIR polymorphisms in HSCT with heterogeneous results. This may partially be due to differences in HSCT platforms, including donor–recipient HLA matching, conditioning regimen, graft composition and post-transplantation GvHD prophylaxis. Even with this caveat, however, it is important to stress that there are two opposing genetic effects that seem to be robustly associated with relapse control in different disease settings and HSCT regimens: (1) presence of centromeric KIR-B genes in the donor (beneficial) and (2) homozygosity for HLA-C-encoded C2 ligands in the patient (detrimental). As outlined above, both effects might be at least partly related to the genetically hardwired sequential acquisition mode of HLA-C-specific KIR receptors. More detailed analysis of the role of NK cells incorporating KIR gene copy number and allelic variation will help to differentiate between the contribution of stimulatory KIR, ligand affinity differences between inhibitory KIR and the role of sequential KIR acquisition to the control of leukemia relapse after HSCT.

Role of intrinsic tumor resistance in relapse: generation and characterization of murine allogeneic T cell-resistant AML cells (John F. DiPersio)

Background

Despite initial sensitivity to chemotherapy, most adult patients with AML and acute lymphoblastic leukemia ultimately relapse and die from progressive disease. For this reason, patients in complete remission are often treated with

multiple rounds of consolidation chemotherapy, and patients at moderate to high risk of relapse often undergo consolidation with allogeneic HSCT [78–80]. This therapy is thought to provide benefit not only through the cytotoxic effects of conditioning, but also through a GvL effect in which donor immune cells react to minor histocompatibility antigens (or leukemia-specific neoantigens) from the recipient's cells to eradicate leukemia [81, 82]. While allotransplantation is a highly effective therapy that has curative potential for many patients, relapses after allo-HSCT are common, and are associated with particularly poor outcomes [78, 83]. In order to understand the biology and genomics of AML relapse after both chemotherapy and allogeneic stem cell transplantation, further study of how AML evades both chemotherapy and the GVL effect is needed.

Relapse of AML is often associated with chromosomal losses and gains and the addition and even loss of specific somatic mutations (both missense and nonsense), suggesting that they undergo clonal evolution with pressure being induced by both chemotherapy and immune modeling [84–86]. With the development of next-generation sequencing, investigators have reported that AML relapse occurring after chemotherapy often gains and loses subclones containing unique somatic variants, including putative driver mutations [87–91]. Recent studies investigating clonal evolution after HSCT relapses have focused on mutations in recurrently mutated AML genes, and while the presence of certain mutations can predict higher risk for relapse, the mechanisms by which these mutations may promote relapse remain unclear [92–94].

Several groups have reported the loss of HLA genes in AML cells at the time of relapse in a subset of HLA-mismatched HSCT [95, 96]. The HLA genes play a critical role in antigen presentation and stimulation of anti-tumor immune responses, and their loss in post-HSCT relapse represents one clearcut mechanism by which relapsing tumors may evade immune surveillance. In the case of HLA-matched transplants, however, deletion of HLA genes does not occur commonly [97]. The DiPersio lab developed a mouse model of AML in which the impact of recurrent allogeneic transplant across major histocompatibility complex (MHC) barriers can provide steady and repetitive immune pressure, resulting in the emergence of allo-resistant mouse AML cells. We will summarize this model and demonstrate that this model can be used to generate and study mouse AML cells that are resistant to allogeneic pressure and test the hypothesis that AML relapsing after HSCT may display genetic or epigenetic alterations that allow leukemic cells to escape the GvL effect, perhaps by downregulating immune pathways that identify or kill tumor cells.

We utilized a C57BL/6 (B6) PML/RARα (promyelocytic leukemia/retinoic acid receptor-α) knock-in mouse model of acute promyelocytic leukemia (APL) to examine the genetic changes that contribute to AML relapse after MHC-mismatched allo-HSCT [98, 99]. Lethally irradiated B6 recipients were reconstituted with BALB/c bone marrow (BM) alone or BM supplemented with B6 APL cells (tumor 18853 or 17857). Cohorts of mice were then left untreated or injected with allogeneic BALB/c T cells (allo-T). After demonstrating in vivo allo-T cell resistance (alloT^r), we performed whole-exome sequencing (WES) and transcriptome profiling (RNA-sequencing (RNA-seq)) on (1) the "parental" tumor, (2) the "passage-only" control tumors that underwent allo-HSCT without allo-T cells, and (3) the alloT^r tumors. The expression of immune modulating and MHC class I and class II molecules were examined in the control and alloT^r tumors by flow cytometry.

By applying selective therapeutic pressure to tumor cells by means of serial allo-HSCT we successfully generated APL tumors that are resistant to allo-T cells (Fig. 2). All mice transplanted without T cells died with a median sur-

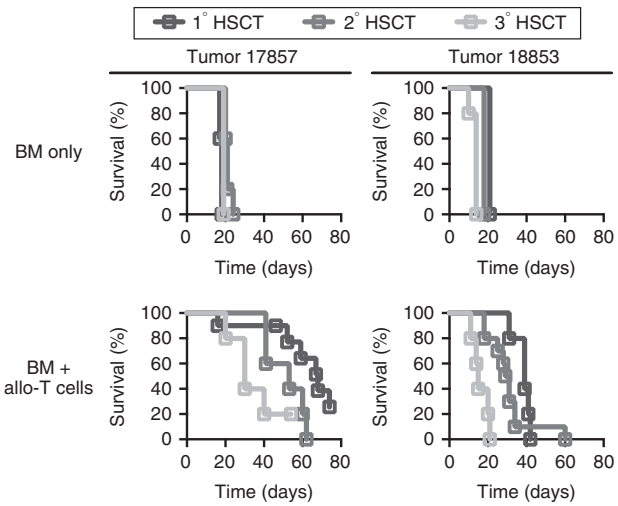

Fig. 2 APL relapses after allo-HSCT. Lethally irradiated B6 recipient mice were transplanted with syngeneic B6 APL tumors and allogeneic BALB/c BM in the presence or absence (BM only) of allogeneic wild-type T cells. APL cells were harvested from relapsing mice and re-transplanted into new B6 recipient mice. (Color figure online)

Table 2 Mutations in APL samples

Sample	No. VAFs (≥5%)	
	Tumor 18853	Tumor 17857
1°, passage, & alloT^r	39	54
Passage, & alloT^r	19	31
Passage	9	12
AlloT^r	22	19

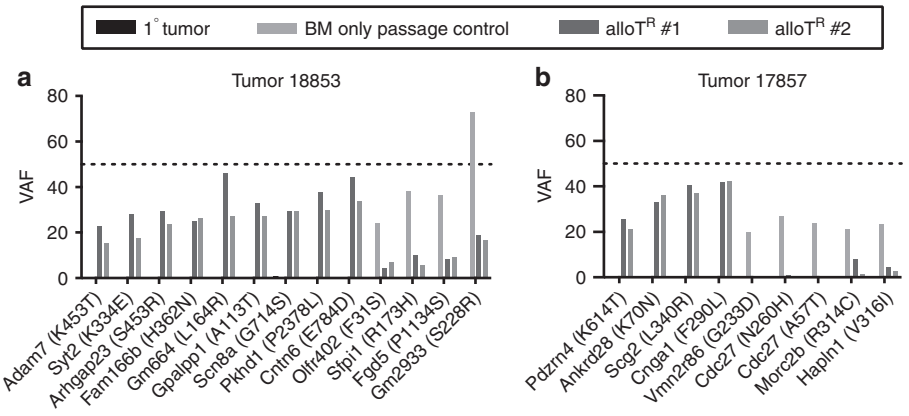

Fig. 3 Missense mutations identified by WES of alloT^r APL cells. WES was performed on primary, BM-only passage control ($n = 1$) and alloT^r ($n = 2$) APL cells derived in vivo from tumor 18853 (**a**) or 17857 (**b**). The VAF of each coding mutation is plotted. (Color figure online)

vival time (MST) between 14 and 20 days. Mice injected with APL tumor 17857 and allo-T cells displayed a significantly decreased MST from 67 days to 30 days during progression from the 1° HSCT to the 3° HSCT, respectively ($P = 0.03$). Similarly, mice injected with APL tumor 18853 and allo-T cells displayed a significantly decreased MST from 39 days to 15 days during progression from the 1° HSCT to the 3° HSCT, respectively ($P = 0.004$). Of note, nearly all of the relapsed mice treated with allo-T cells presented with hind end paralysis at the time of killing. No paralysis was observed in the passage-only controls. Histopathologic examination of the relapsed mice revealed alloT^r tumor within the BM that extended into the vertebral canal and musculature surrounding the vertebrae and ribs, and surrounding the spinal and vertebral nerves. Almost no APL cells were detected in the spleen, a primary site of relapse in mice transplanted with BM only.

Overall, 88 and 116 total mutations with variant allele frequencies (VAFs) >5% were detected in at least one of the three samples in the 18853 and 17857 alloT^r APL tumors, respectively. Approximately 45% of the VAFs (39/88 for tumor 18853 and 54/116 for tumor 17857) were detected in all three samples, indicating that the allo-HSCT tumors were related to the founding primary tumor (Table 2). Roughly 25% of the VAFs (19/88 for tumor 18853 and 31/116 for tumor 17857) were detected in both the BM-only passage control and alloT^r samples, suggesting that these mutations were enriched for and/or acquired during the serial allo-HSCT procedure (Table 2). Most relevant to our studies, 22 and 19 VAFs were specific to the alloT^r 18853 and 17857 tumors, respectively. Restricting the analysis to missense single-nucleotide variants with VAFs >5% showed that 9 missense mutations were specific to the 18853 tumors and 4 to the 17857 alloT^r tumors. Further, 4 or 5 mutations were lost in the alloT^r cells relative to the passage controls during the serial allo-HSCTs (Fig. 3).

Although our WES data suggest selection and/or generation of an APL subclone upon application of allogeneic T-cell pressure, no mutations were observed in MHC genes or in genes involved with immune function. Immunophenotypic analysis of the alloT^r cells by flow cytometry revealed a twofold decrease in expression of MHC class I (H2Db) in both alloT^r samples. The 18853 alloT^r tumor also displayed decreased expression of MHC class II (I-A) and the co-stimulatory marker OX40L, while the 17857 tumor downregulated the apoptosis mediator CD95 (Fas). There were no differences in expression of H2Kb, PD-L1, E-cadherin, galectin-9, CD40, CD80 and CD86 between the passage-only control and alloT^r tumors. A transcriptional profiling analysis of these samples by RNA-seq is currently ongoing and may provide further mechanistic insights into the genetic or epigenetic events leading to tumor immune escape.

In summary, using a serial allo-HSCT approach, we have successfully generated murine APL cells that are resistant to allogeneic T cells. Our data suggest that loss of MHC class I plus key epigenetic changes contribute to relapse in this murine APL model.

Conclusion

Early immune recovery is highly associated with better outcomes, and enhancement of immune recovery without GvHD will likely improve survival and quality of life after HCT. Understanding KIR biology and appropriate donor/recipient KIR matching can lead to decreased relapse, and better understanding of this important issue by the transplant community is vital. Finally, understanding molecular mechanisms of immune escape may help develop approaches to circumvent this issue and improve transplant outcomes.

Acknowledgements The work of MvdB was supported by the National Institutes of Health award numbers R01-HL069929 (MvdB), R01-AI101406 (MvdB), P01-CA023766 (RJ O'Reilly) and Project 4 of P01-CA023766 (MvdB). Support was also received from The Lymphoma Foundation, The Susan and Peter Solomon Divisional

Genomics Program, Cycle for Survival and P30 CA008748 MSK Cancer Center Support Grant/Core Grant. The work of MU was supported by the Deutsche Krebshilfe e.V. (project 110351) and the research commission of the Medical faculty of the Heinrich Heine University. The work of LJ was supported by the European Molecular Biology Organization (EMBO; ALTF 431-2017) and the MSK Sawiris Foundation Myeloma and Transplant Research Award. The work of MAP was supported by 2UG1HL069254-17 (NHLBI/NCI), R01 CA181050 (NCI), R34HL133384 (NHLBI) and U01AI126612 (NIAID), and by the Johnny Crisstopher Children's Charitable Foundation St. Baldrick's Consortium Grant.

Compliance with ethical standards

Conflict of interest Marcel van den Brink: current: research support: Seres; past 2 years: consultant for Jazz Pharmaceuticals, Novartis, Regeneron, Flagship Ventures, Boehringer Ingelheim, Merck and Evelo. John F DiPersio: stock/equity/founder: Magenta, Boston; honorarium: Celgene, Macrogenics; consultant for Zymeworks, Incyte and Celgene. Michael A Pulsipher: advisory board: Novartis, Adaptive, Chimerix and CLS Behring; educational meetings for Jazz, Medac and Amgen.

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