Functional and molecular analyses reveal impaired HSPCs in Multiple Myeloma patients post-induction

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Brief Report

Functional and molecular analyses reveal impaired HSPCs in Multiple Myeloma patients post-induction

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Abstract

High-dose chemotherapy and consecutive autologous stem cell transplantation (ASCT) remain the backbone of treatment for transplant-eligible patients of Multiple Myeloma (MM). However, patients are still at high risk of relapse or treatment-related complications. Hence, by understanding the function of hematopoietic stem and progenitor cells (HSPCs) from MM patients in more detail, transplant outcomes in MM patients might be further improved. We combine in our study functional analyses of the potential of HSPCs from newly diagnosed (NDMM) and chemotherapy treated MM patients in a xenotransplant model system with in depth single cells sequencing analysis to provide novel data that might inform clinical routine to improve the outcome of ASCT in MM. Our data demonstrate that (i) HSPCs from treated MM patients are indeed significantly impaired in their overall reconstitution potential and provide a reduced level of B-cells in comparison to HSPCs from age-matched healthy donors and NDMM patients. (ii) We further demonstrate that CD34$^+$ HSPCs acquire a high-risk MM expression profile signature upon induction treatment, which likely adds to the risk of relapse. This high-risk MM expression profile signature relies within CD34$^+$ HSPCs primarily in granulocyte/macrophage progenitors (GMPs), megakaryocyte Erythroid Progenitors (MEPs) and monocytes, while hematopoietic stem cells (HSCs) stay unaffected by transcriptional changes. These data suggest that the elimination of myeloid progenitors and more mature monocytes (likely by purification for HSCs) in HSPCs harvests from treated MM patients for subsequent ASCT might improve transplant outcomes by avoiding re-infusion of cells with a dysregulated and disease-linked transcriptional program.

Key words: autologous stem cell transplantation; hematopoietic stem cells; multiple myeloma; single cells sequencing; xenotransplant model.

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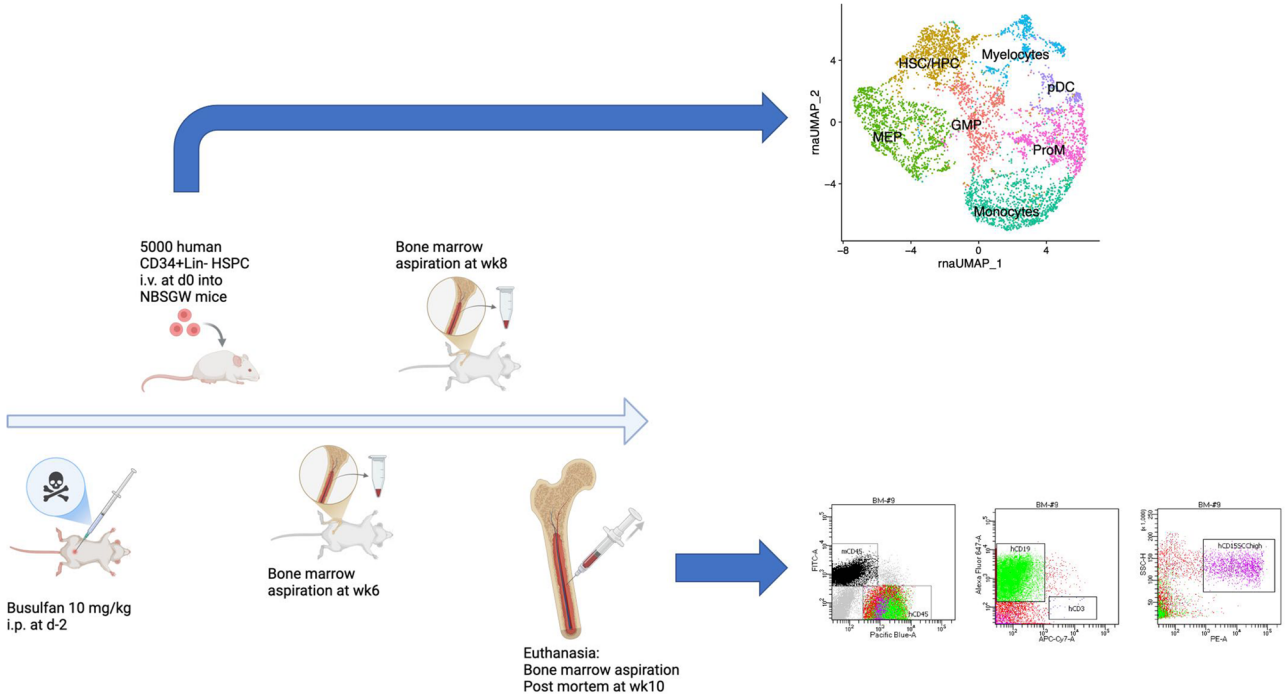
Graphical abstract



Introduction

MM is a malignant plasma cell disorder.[1] Despite the development of novel agents, an induction regimen followed by a high-dose chemotherapy and consecutive ASCT remain the backbone of treatment for transplant-eligible patients.[2,3] However, patients are still at high risk of relapse or treatment-related complications. Hence, by understanding the function of HSPCs from MM patients in more detail, outcomes in MM patients might be further improved. By providing an in-depth analysis of the function and the molecular signature of $CD34^+$ bone marrow (BM) HSPCs from NDMM and treated MM patients we are able to suggest novel approaches that might improve the outcome of ASCT in MM.

Materials and methods

BM samples

BM samples were collected at the University Hospital of Ulm. For clinical data, see Supplementary Table S1. All patients provided informed consent. Sample collection and investigation were approved by the Internal Review Board (Ethikkommission) at Ulm University (307/08, 392/16).

Flow cytometric analysis and cell sorting

MNCs were thawed and stained in PBS supplemented with 3% FBS with human specific antibodies. Antibodies used were: Fitc-conjugated antihuman lineage cocktail ((# 22-7778-72: anti-CD2 (RPA2.10), anti-CD3 (OKT3), anti-CD14 (61D3), anti-CD16 (CB16), anti-CD19 (HIB19), anti-CD56 (TULY56), anti-CD235a (HIR2)) from eBioscience, APC-conjugated antihuman CD34 (581 # 555824) from BD Pharmingen.

Xenotransplantation

For *in vivo* analyses NBSGW mice ($n = 5$ per donor) were injected with busulfan 10 mg/kg body (Busulfex® Otsuka Pharmaceutical Co) weight intraperitoneally and transplanted with 5000 $CD34^+Lin^-$ HSPC from either healthy donor ($n = 4$), NDMM ($n = 8$), or treated ($n = 8$) MM patients via tail vein two days after. BM aspirates were drawn in week 6, 8, and 10 post-xenotransplantation from the femora. Human cells were identified using these antibodies: VioBlue-conjugated antihuman CD45 (5B1 # 130-113-684) and APC-Vio 770-conjugated antihuman CD3 (BW264/56 # 130-113-126) from Miltenyi, alexa fluor 647-conjugated antihuman CD19 (HIB19 #

302220), Fitc-conjugated rat antimouse CD45 (30-F11 # 553080) and PE-conjugated antihuman CD16 (3G8 # 302008) from Biolegend.

scRNAseq

$CD34^+Lin^-$ HSPCs from the same donors used for xeno-transplants were sorted in 384-well plates (Eppendorf) containing 1.2 µL lysis buffer, spun down and frozen (−80 °C).[4] After thawing, lysates were incubated for 3 min at 72 °C and cooled on ice. A total of 2 µL RT mix containing 20 U Maxima RT was added and cDNA was generated and amplified for 21 cycles. cDNA was purified with AMPure beads and libraries were prepared with the Illumina Nextera Kit. Libraries were sequenced on a NextSeq550 with 384 cells per lane.

For single-cell RNA-sequencing (scRNAseq),[4] raw reads were mapped to the reference genome GRCh38 using HISAT2.[5–7] RNA-seq mapping QC was performed using Picard tools (https://broadinstitute.github.io/picard/). Downstream analyses were conducted using R and Bioconductor packages.[8,9] Deep-learning analysis was performed using the Python package keras/TensorFlow[10] implementation in R. Gene set enrichment analysis (GSEA) was performed using the standalone Java implementation of the package.[11]

Statistical analyses

Statistical analyses were performed with GraphPad Prism 9 (version 9.0.2). *P* values < .05 were considered significant.

Results

HSPCs from treated MM patients are impaired in their overall reconstitution potential

To test for function, sorted HSPC (Lin^-CD34^+ cells) from MM patients (Supplementary Table S1) were transplanted into NBSGW recipient mice (Figure 1A). The BM reconstitution ability of HSPCs from treated MM patients, but not from NDMM patients, was impaired when compared to HSPCs from healthy age-matched controls (average chimerism of 6.3% for treated MM vs 16.7% for healthy controls at 10 weeks after transplantation; Figure 1B and C). Determination of the frequency of human $CD19^+$ B-, $CD3^+$ T-, and $CD16^+SSC^{high}$ myeloid cells in BM among engrafted animals further revealed a reduced frequency of B cells in recipients that received HSCPs from treated MM patients when compared to animals transplanted with healthy control HSPCs (47.1% vs 77.0%; Figure 1D and E). Whilst T-cell reconstitution (Figure 1F and G) remained low in all groups, the frequency of myeloid cells (Figure 1H and I) within the NDMM group showed upregulation when compared to animals transplanted with healthy control HSPCs (1.4% vs 0.27%).

HSPCs from MM patients are molecularly distinct from healthy HSPCs

To investigate molecular signatures of HSPCs from MM patients, we performed scRNAseq analyses on BM-derived CD138-depleted[12] $CD34^+$ cells from healthy controls, NDMM or treated MM patients whose function had been already determined in a transplantation assay (Figure 1). We used the SMARTseq2 protocol to ensure complete transcript coverage and a high sequencing depth. Based on published cell markers that included a recent reference dataset,[13] we identified different types of progenitor cells (Figure 2A). HSCs were determined by applying a recently published human HSC signature[14] ($RUNX1^+HOXA9^+MLLT3^+MECOM^+$ $HLF^+SPINK2^+$). Unlike previous reports,[15] we could not detect any difference in the composition of cell subtypes within HSPCs from MM patients compared to HSPCs from healthy controls.

To identify transcriptional signatures that define and separate healthy control cells from NDMM or from treated MM cells at the single-cell level, a Keras/TensorFlow machine learning (ML) model was applied to the complete dataset (split into training and validation sets). In the validation analysis, 55% (control), 94% (NDMM), and 76% (treated MM) of the cells were correctly assigned into their respective groups (Figure 2B).

To identify differentially expressed genes (DEGs) among $CD34^+$ cells from healthy controls and MM patients, expression profiles from single cells were pseudobulked (by aggregating cells by cell types per experimental arm; Figure 2C and Supplementary Figure S2). Overall, and similar to the ML approach, HSPCs from NDMM and treated MM patients showed an expression pattern that is distinct from that of healthy controls.

GMPs, MEPs, and monocytes bear signatures of MM cells

We then identified which cell type among the $CD34^+$ cells represent best the MM signatures by using a random forest ML model. HSCs and pDCs showed the highest misclassification rate in this model, and therefore, the signatures and transcriptional identity of HSCs and Plasmacytoid dendritic cells (pDCs) are quite similar among the three groups. Instead, the misclassification rate of GMPs was the lowest, closely followed by MEPs and Monocytes (Figure 2D and Supplementary Table S2). These cell types, therefore, showed the highest level of correlation to the overall MM signatures (healthy, NDMM, MM), especially in the NDMM samples.

HSPCs acquire a high-risk MM expression profile signature upon induction treatment

Gene sets derived from GSEA of the three types of HSPCs were next compared to the EMC92 expression signature, in which patients defined as high-risk show a reduced overall survival.[16] There was a significant enrichment of the EMC92 signature within the signature that specifically defined $CD34^+$ cells from treated MM samples (Figure 3A). Congruently, the GSEA of MM overlap signatures aggregated from published data sets (MsigDB) also showed a strong positive correlation with the two signatures (Figure 3B). Genes that showed upregulation of expression in both correlation analyses were NCAPG, ZWINT, BIRC5, MCM6 or FANCI, which are all proteins involved in chromosomal biology of dividing cells, which are though also specific to MM, for example, BIRC5.[17] In contrast, there was a negative correlation between the EMC92 (Figure 3C) as well as the EMC92/MM overlap (Figure 3D) signatures.

A GMP focused analyses by GSEA showed significant enrichment of the MM signature genes in both treated (Figure 3D) and NDMM (Figure 3F) samples, while Reactome analyses displayed an almost exclusive upregulation of processes in both NDMM and treated MM.

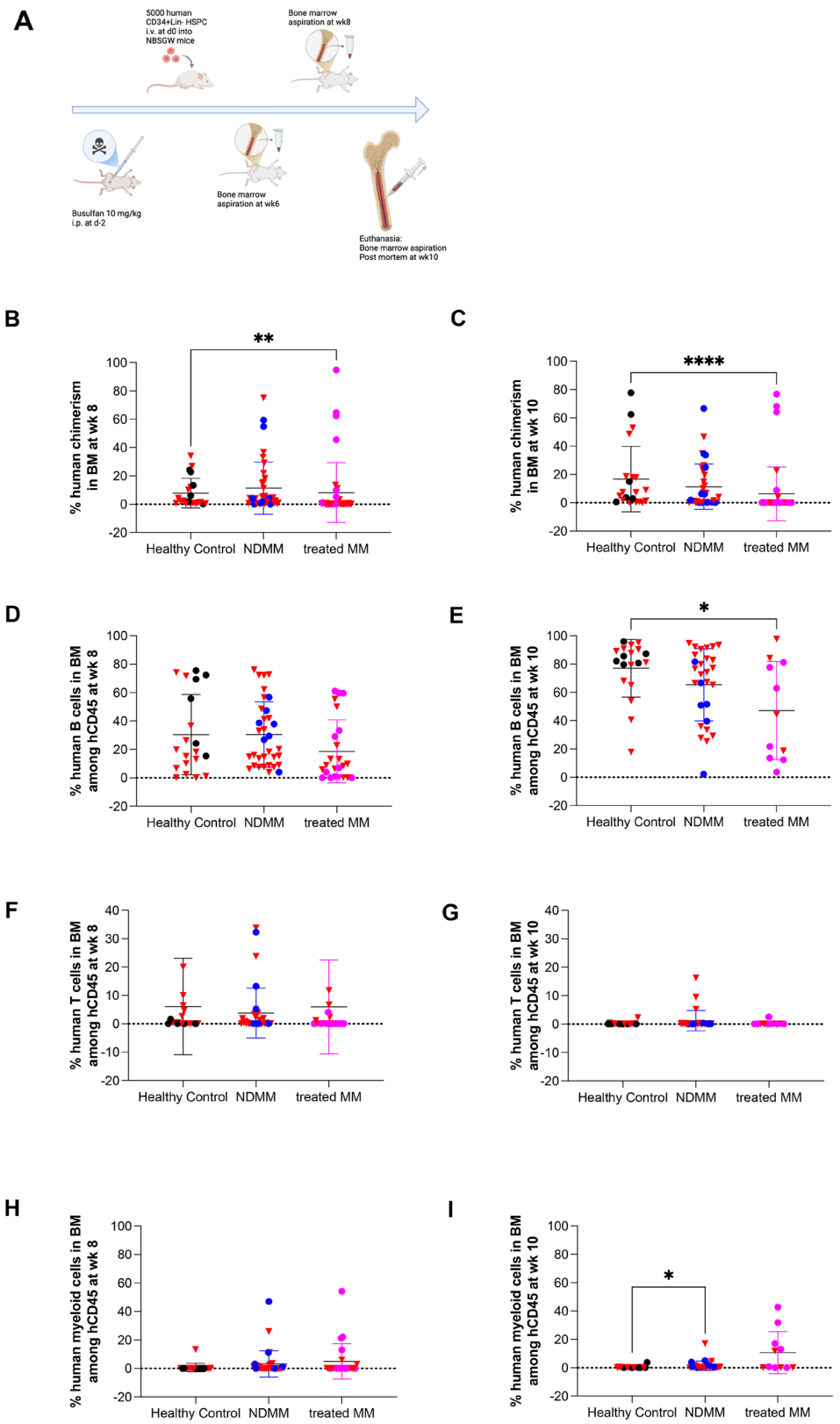


Figure 1. HSPCs from treated MM patients are impaired in their function. (A) Schematic for the *in vivo* workflow. NBSGW mice ($n = 5$ per donor) were injected with busulfan 10 mg/kg body weight intraperitoneally and transplanted with 5000 $CD34^{+}Lin^{-}$ HSPC from either healthy donor ($n = 4$), NDMM ($n = 8$), or treated ($n = 8$) MM patients via tail vein two days after. BM was aspirated at week 6, 8, and 10 post-xenotransplantation (Created in BioRender. https://BioRender.com/z94s637). Frequency of human cells in the BM of transplanted NBSGW mice at (B) 8 weeks and (C) 10-weeks post-transplant from healthy control, NDMM, and treated MM-donors, red triangles indicate recipients transplanted with cells from donors also used for scRNAseq analyses. Emergence of human B-cells at (D) 8 weeks and (E) 10 weeks post-transplant, human T-cells at (F) 8 weeks and (G) 10 weeks post-transplant and human myeloid cells at (H) 8 weeks and (I) 10 weeks post-transplant. Line = mean ± SD. $^{*}P < .05$, $^{***}P < .0005$, $^{****}P < .0001$, Mann–Whitney test.

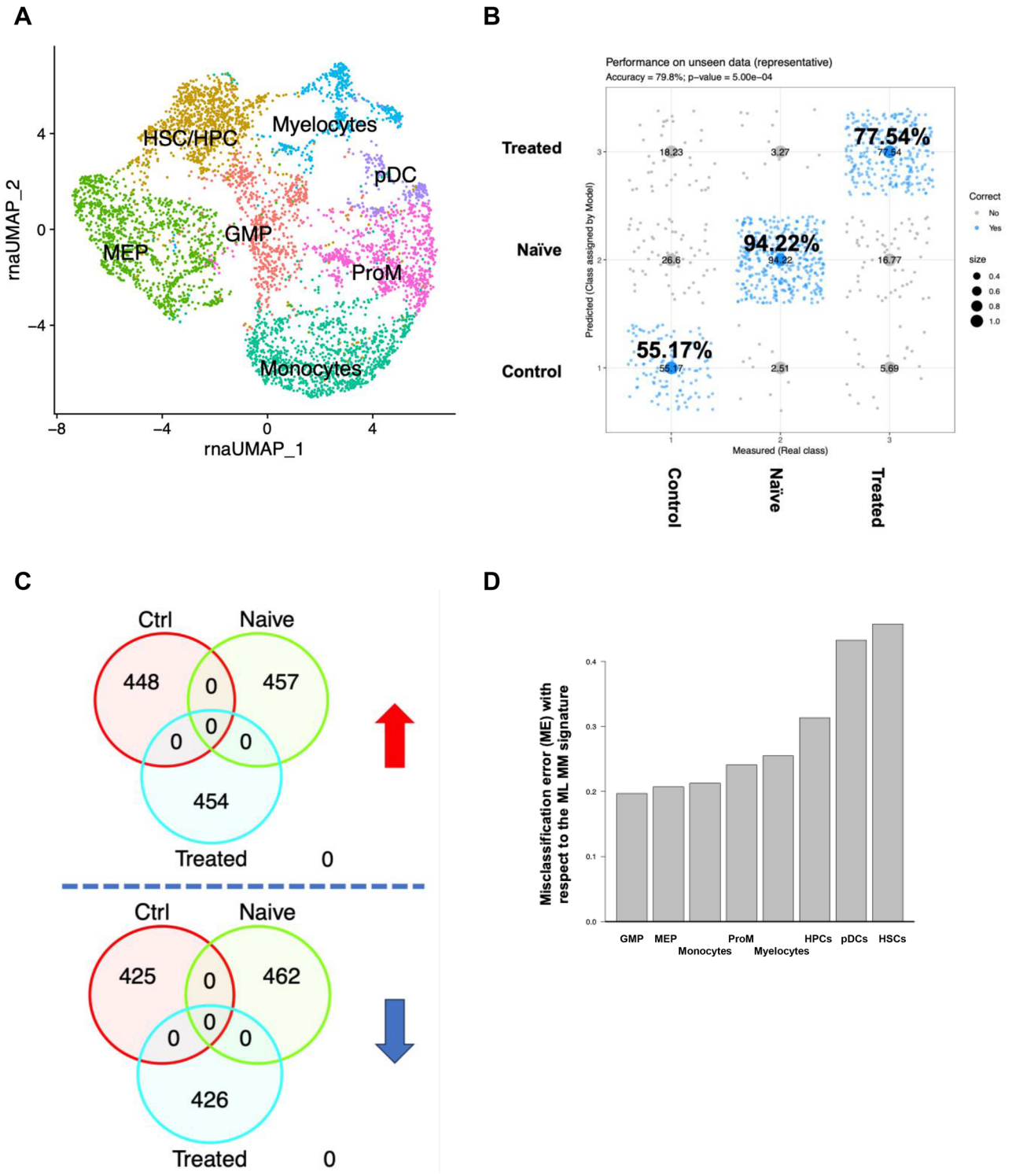


Figure 2. Single cell transcriptomic profile of CD34⁺ cells reveal a distinct disease-linked expression profile of GMPs from MM patients (A) Uniform manifold approximation and projection (UMAP) representation of 2688 single-cell transcriptomes from CD34⁺ HSPCs from BM of healthy control, NDMM, and treated MM patients. Cells shown are GMP ($n = 325$), HSC/HPC ($n = 498$), MEP ($n = 618$), Monocytes ($n = 567$), Myelocytes ($n = 212$), pDC ($n = 111$), and ProM ($n = 357$) (B) Machine learning analysis using a keras/TensorFlow machine learning model to correctly predict gene signatures of HSPCs according to healthy control, NDMM or treated MM. (C) Signature analyses comparing differentially expressed genes in healthy control, NDMM, and treated MM samples ($P < .01$, FDR < 0.25). (D) Error of misclassification with respect to the MM gene signature among distinct types of progenitor cells.

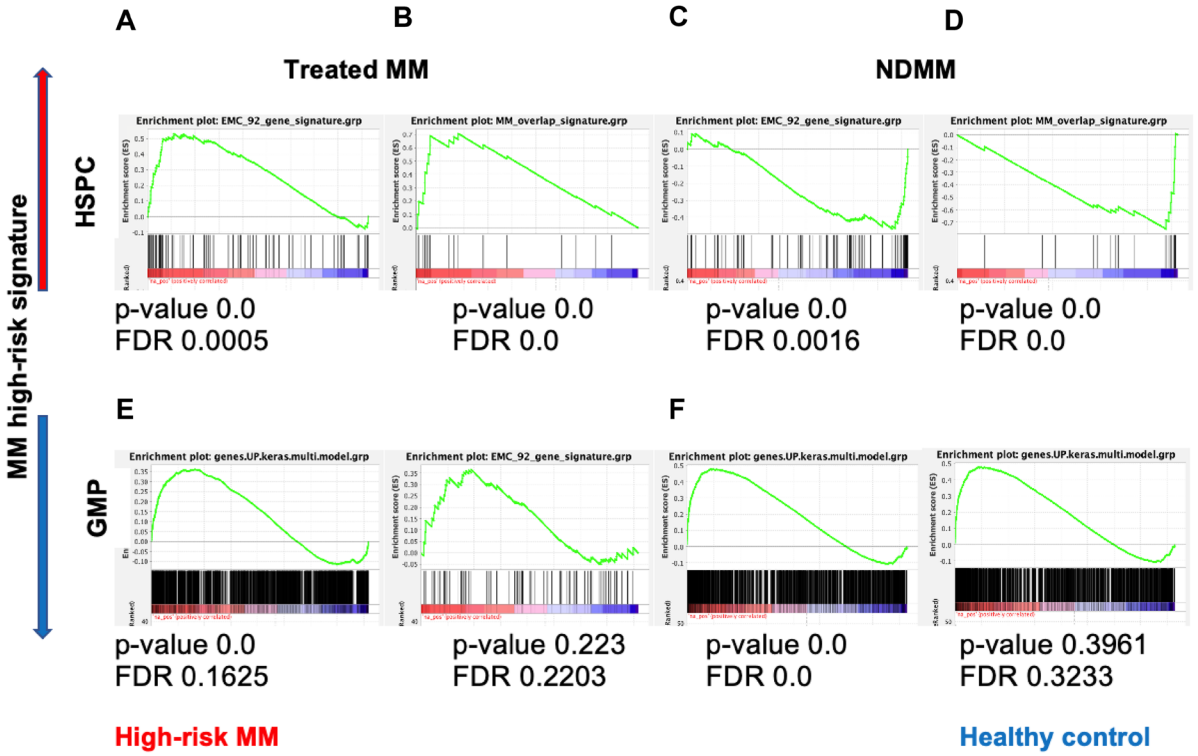


Figure 3. CD34⁺ HSPCs acquire a high-risk MM expression profile signature upon induction treatment. Gene set enrichment analyses (GSEA) from EMC92 signatures and MM overlap signatures, aggregated from published data sets, from (A, B) treated MM and (C, D) NDMM samples. GMP focused analysis from (E) treated and (F) NDMM samples.

Discussion

In our xenotransplant model, HSPCs from treated MM patients demonstrated an impaired overall reconstitution potential and provided a reduced level of B-cells in comparison to HSPCs from healthy controls. Meanwhile, T-cell and myeloid output remained low, in line with reported xenotransplant models.[18] To further understand underlying mechanisms, we combined the *in vivo* experiments with in-depth single cells sequencing analysis. These data imply that (i) HSPCs from MM patients are molecularly distinct from healthy HSPCs, (ii) there is a molecular progression from the NDMM to the treated MM signature, as within treated MM cells, there is still a significant number of NDMM cells but not vice-versa, (iii) while the majority of healthy HSPCs show an expression signature distinct from that of MM HSPCs, the expression profile within healthy HSPCs was quite heterogenous, with over 25% being closer to the NDMM signature and 18% closer to the treated MM signature. Overall, the small number of patient samples per group will limit the generalizability of our data.

While HSCs showed no association to the signatures and the transcriptional identity of HSCs remained unchanged among the groups, the transcriptional make-up of GMPs, MEPs and Monocytes, especially in the treated MM sample, was severely affected. This finding is surprising, as MM is a disease of primarily malignant plasma cells. One explanation might be that GMPs, MEPs and Monocytes are geneologcially closer to the general profile of B-cells, which though is not likely as the alignment is specific for the MM signature. Another possible explanation is, that in MM, malignant B-cells transfer part of their identity on myeloid precursor cells. This hypothesis is further supported by our finding of elevated myeloid output in recipients transplanted with HSPCs from NDMM patients relative to the output in recipients transplanted with HSPCs from healthy controls. Indeed, it has been shown that MM B-cells express high levels of C/EBPbeta, inducing the transdifferentiation of B-cells directly into GMPs, macrophages and granulocytes.[19] It, therefore, remains a possibility that in MM preferentially malignant B-cells undergo, in the presence of high levels of C/EBPbeta signaling, a switch of cell identity. This interpretation though remains speculative and requires additional experimental verification.

Pathways that were distinctly represented (based on the DEGs) in NDMM vs healthy controls (Supplementary Table S3A) included ATF6, EGFR signaling, and NF-kB deregulation. All three of these pathways are mechanistically highly intertwined with C/EBP signaling and serve as C/EBP signaling enhancers.[20–22] Additional experiments will be necessary to determine whether there is a role of C/EBP signaling for the identity of myeloid progenitors in MM. Differentially expressed pathways in treated MM vs healthy controls (Supplementary Table S3B) included mitochondrial biology, apoptosis, or activation of the ATR pathway. Latter regulates repair of DNA damage or apoptosis upon genotoxic stress.[23] Changes, therefore, might be linked to a stem cell intrinsic mechanism of reduced function of HSPCs from treated MM patients in the xeno-transplant setting.

The findings suggest that the elimination of myeloid progenitors (by purification for HSCs) in HSPCs harvests from treated MM patients for subsequent ASCT might improve outcomes by avoiding re-infusion of cells with a dysregulated and disease-linked transcriptional program. It is further possible that the presence of these dysregulated cells in treated MM samples might negatively affect HSCs upon transplantation, as the transcriptional profile of treated MM HSCs was not altered in comparison to healthy HSCs, while their function was severely affected in the xenotransplant assay.

By GSEA, we demonstrate that HSPCs acquire a high-risk MM expression profile signature, associated with a reduced overall survival,[16] upon induction treatment. This implies that treatment promotes a gene expression signature among CD34+ cells that is similar to high-risk MM plasma cells. Still, due to the limited number of patients in this analysis future studies with correlation with clinical outcome data are needed. But indeed, it was recently shown that cytotoxic chemotherapy produces a distinctive mutation signature in HSPCs from treated MM patients.[24,25] Furthermore, GMP-focused GSEA again supports a view that GMPs are linked to MM, with GMP from treated patients further showing connections to a high-risk disease profile of MM.

Conclusion

In summary, our data support a reduced function of HSPCs from treated MM patients. Second, while GMPs or MEPs have not yet been considered to contribute to the BM phenotype in MM,[15] our data show that GMPs, MEPs, Monocytes bear signatures of MM cells, which suggests that these progenitors contribute to phenotypes associated with the disease. A GMP/MEP/Monocyte depleted transplant product might, thus, be beneficial in ASCT. Further studies to investigate the role of GMPs and MEPs in MM, in combination with studies on the role of niche factors such as MSCs from treated MM patient that maintain HSPCs[15] might further add to a better understanding of the disease etiology to inform on new transplant concepts and improve outcomes.

Supplementary material

Supplementary material is available at *Stem Cells Translational Medicine* online.

Funding

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Conflicts of interest

Kull: GSK: Membership on an entity's Board of Directors or advisory committees; Pfizer: Membership on an entity's Board of Directors or advisory committees. Kronke: Janssen: Consultancy, Honoraria, Speakers Bureau; Pfizer: Honoraria; Abbvie: Consultancy, Honoraria, Speakers Bureau; Sanofi: Consultancy, Honoraria, Speakers Bureau; Takeda: Consultancy, Honoraria; Astra Zeneca: Consultancy, Honoraria. Geiger: Mogling Bio: Consultancy, Other: Co-founder.

Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

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