

Immunogenomic identification and characterization of granulocytic myeloid derived suppressor cells in multiple myeloma

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Abstract:

Granulocytic myeloid-derived suppressor cells (G-MDSCs) promote tumor growth and immunosuppression in multiple myeloma (MM). However, their phenotype is not well-established for accurate monitoring and clinical translation. Here, we aimed at providing the phenotypic profile of G-MDSCs based on their prognostic significance in MM, immunosuppressive potential, and molecular program. The pre-established phenotype of G-MDSCs was evaluated in bone marrow samples from controls and MM patients using multidimensional flow cytometry and, surprisingly, we found that CD11b⁺CD14⁺CD15⁺CD33⁺HLADR⁺ cells overlapped with common eosinophils and neutrophils, which were not expanded in MM patients. Thus, we relied on automated clustering to unbiasedly identify all granulocytic subsets in the tumor microenvironment: basophils, eosinophils, immature, intermediate and mature neutrophils. In a series of 267 newly-diagnosed MM patients (GEM2012MENOS65 trial), only the frequency of mature neutrophils at diagnosis was significantly associated with patients' outcome, and a high mature-neutrophils/T-cell ratio resulted in inferior progression-free survival ($P < .001$). Upon FACS sorting of each neutrophil subset, T cell proliferation decreased in presence of mature neutrophils (0.5-fold; $P = .016$) and the cytotoxic potential of T cells engaged by a BCMAxCD3 bispecific antibody increased notably with the depletion of mature neutrophils (4-fold; $P = .0007$). Most interestingly, RNAseq of the three subsets revealed that G-MDSCs-related genes were specifically upregulated in mature neutrophils from MM patients vs controls due to differential chromatin accessibility. Taken together, we established a correlation between the clinical significance, immunosuppressive potential and transcriptional network of well-defined neutrophil subsets, providing for the first time, a set of optimal markers (CD11b/CD13/CD16) for accurate monitoring of G-MDSCs in MM.

Conflict of interest: COI declared - see note

COI notes: A.O. participated in advisory boards for Amgen, Celgene and Janssen. F.d.A. reports honoraria for lectures from Celgene, Janssen, Amgen and Takeda, and consultancy with Amgen, Celgene and Janssen. M.-V.M. has received honoraria from lectures or participation in advisory boards from Janssen, Celgene, Amgen, Takeda, Abbvie, Adaptive, GSK, Pharmamar, EDO, Oncopeptides. L.R. reports honoraria from Janssen, Celgene, Amgen, and Takeda. J.B. reports honoraria for lectures from Janssen, Amgen, Celgene, Takeda and Oncopeptides. J.-J.L. reports honoraria from and membership on board of directors or advisory committees with Takeda, Amgen, Celgene, and Janssen. J.F.S.M. reports consultancy for Bristol-Myers Squibb, Celgene, Novartis, Takeda, Amgen, MSD, Janssen, and Sanofi and membership on board of directors or advisory committees with Takeda. B.P. reports honoraria for lectures from and membership on advisory boards with Amgen, Bristol-Myers Squibb, Celgene, Janssen, Merck, Novartis, Roche, and Sanofi; unrestricted grants from Celgene, EngMab, Sanofi, and Takeda; and consultancy for Celgene, Janssen, Sanofi and Takeda. The remaining authors declare no competing financial interests.

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KEY POINTS

- There is a progressive gradient of immunosuppression from immature into mature neutrophils present in the myeloma microenvironment
- CD11b⁺CD13⁺CD16⁺ mature neutrophils are epigenetically deregulated and their abundance in the myeloma microenvironment is prognostic

ABSTRACT

Granulocytic myeloid-derived suppressor cells (G-MDSCs) promote tumor growth and immunosuppression in multiple myeloma (MM). However, their phenotype is not well-established for accurate monitoring and clinical translation. Here, we aimed at providing the phenotypic profile of G-MDSCs based on their prognostic significance in MM, immunosuppressive potential, and molecular program. The pre-established phenotype of G-MDSCs was evaluated in bone marrow samples from controls and MM patients using multidimensional flow cytometry and, surprisingly, we found that CD11b⁺CD14⁺CD15⁺CD33⁺HLADR⁺ cells overlapped with common eosinophils and neutrophils, which were not expanded in MM patients. Thus, we relied on automated clustering to unbiasedly identify all granulocytic subsets in the tumor microenvironment: basophils, eosinophils, immature, intermediate and mature neutrophils. In a series of 267 newly-diagnosed MM patients (GEM2012MENOS65 trial), only the frequency of mature neutrophils at diagnosis was significantly associated with patients' outcome, and a high mature-neutrophils/T-cell ratio resulted in inferior progression-free survival ($P < .001$). Upon FACS sorting of each neutrophil subset, T cell proliferation decreased in presence of mature neutrophils (0.5-fold; $P = .016$) and the cytotoxic potential of T cells engaged by a BCMAxCD3 bispecific antibody increased notably with the depletion of mature neutrophils (4-fold; $P = .0007$). Most interestingly, RNAseq of the three subsets revealed that G-MDSCs-related genes were specifically upregulated in mature neutrophils from MM patients vs controls due to differential chromatin accessibility. Taken together, we established a correlation between the clinical significance, immunosuppressive potential and transcriptional network of well-defined neutrophil subsets, providing for the first time, a set of optimal markers (CD11b/CD13/CD16) for accurate monitoring of G-MDSCs in MM.

INTRODUCTION

Myeloid derived suppressor cells (MDSCs) are described as a mixture of immature cells that have common biological activity. They are able to influence innate and adaptive immune responses through depletion of L-arginine, generation of oxidative stress, induction of cytotoxic T-cell apoptosis and activation of T regulatory cells.¹ All these mechanisms lead to immune suppression.^{2,3}

MDSCs have been extensively studied in mice and less frequently in humans. Whilst in mice they are identified based on expression of Gr-1 and CD11b, the immunophenotype of their human counterpart remains unclear.⁴ Commonly, they are defined as a CD11b⁺CD33⁺HLADR^{-/lo} subset amongst mononucleated cells isolated after density gradient.^{3,5} Cells with such phenotype were found to be rare in healthy adults (HA) but expanded in patients with cancer. Two subsets of MDSCs have been identified based on the additional expression of CD14 – monocytic MDSCs (M-MDSCs) – or CD15 – granulocytic MDSCs (G-MDSCs).^{2,4-6}

In multiple myeloma (MM), malignant plasma cells colonize and modify the bone marrow (BM) microenvironment through cytokine production and bidirectional interactions with other cell types. Namely, it has been suggested that MM cells induce MDSC development and survival whereas MDSCs promote tumor growth and induce immune suppression.⁷⁻¹⁰ In addition, anti-myeloma therapies such as dexamethasone, melphalan or cyclophosphamide and even immunomodulatory drugs could expand and potentiate MDSC immunosuppressive effects, most likely as a counter-regulatory mechanism.⁸ By contrast, recent data suggest that daratumumab acts, amongst other modes of action, by depleting MDSCs.^{6,7,11} Thus, MDSC suppression could become an important strategy to increase and prolong the efficacy of novel immunotherapies (e.g. CAR T cells or T cell engager bispecific antibodies) but, in such case, precise knowledge on the phenotype of MDSCs would be required for its clinical monitoring in the MM tumor microenvironment.

Enumeration of putative G-MDSCs was shown to correlate with MM burden but never with patients' survival.^{12,13} However, it is challenging to dissect casual effects and mechanistic functions based solely on tumor burden. This study overcomes these limitations by integrating clinical, functional and molecular data on granulocytic cells from the tumor microenvironment, and provides a set of markers for optimal monitoring of G-MDSCs in MM.

PATIENTS AND METHODS

Subjects and treatment. A total of 388 BM samples from 22 HA and 366 patients with MM were analyzed in this study (median age of 54 and 64 years, respectively). Only samples with >90% viability (according to the percentage of debris identified by flow cytometry) were used for downstream analysis. Of the 366 MM patients, 267 were enrolled in the PETHEMA/GEM2012MENOS65 clinical trial (NCT01916252) and this cohort was selected to determine the prognostic value of the distribution of various granulocytic subsets in the tumor microenvironment. Briefly, patients received six induction cycles of bortezomib, lenalidomide and dexamethasone (VRD), autologous stem cell transplant conditioned with Bu-Mel or Mel-200 high-dose therapy, and two consolidation cycles of VRD.¹⁴ Afterwards, patients were enrolled in the PETHEMA/GEM2014MAIN clinical trial (NCT02406144) that randomized maintenance with RD or RD plus ixazomib for two years, after which patients continued with RD for three additional years if minimal residual disease (MRD) positive, or stopped therapy if MRD negative.¹⁵ Median follow-up is 39 months (range, 7 – 57 months). Each study site's Independent Ethics Committee approved the protocol and informed consent forms, required prior to patient enrollment. The study was conducted per the ethical principles of the Declaration of Helsinki.

Multidimensional flow cytometry (MFC). MFC was used to evaluate the pre-established phenotype of G-MDSCs^{2,3,6-9,12,16} in BM samples from HA (N = 7) and MM patients (N = 10), and to compare their phenotype in paired BM and peripheral blood (PB) samples from MM patients (N = 5). Briefly, the EuroFlow lyse-wash-and-stain standard sample preparation protocol (SOP) adjusted to 10⁶ nucleated cells, together with the 8-color combination of the monoclonal antibodies (mAb) HLADR-BV421, CD45-OC515, CD15-FITC, CD13-PE, CD33-PerCPCy5.5, CD16-PECy7, CD11b-APC and CD14-APCH7, were selected to identify CD11b⁺CD14⁻CD15⁺CD33⁺HLADR⁻ cells and compare their frequency in BM samples from HA vs MM patients using the Infinicyt software (Cytognos SL, Salamanca, Spain). Screening of different granulocytic subsets in newly-diagnosed patients enrolled in the PETHEMA/GEM2012MENOS65 study was performed with the following combination of mAb: HLADR-PacB, CD45-OC515, CD36-FITC, CD13-PE, CD34-PerCPCy5.5, CD117-PECy7, CD11b-APC and CD71-APCH7. EDTA-anticoagulated BM samples were processed within 24h after collection following the EuroFlow lyse-wash-and-stain SOP, and data acquisition was performed in a FACSCanto II flow cytometer (Beckton Dickinson –BD- Biosciences, San Jose, CA) using the FACSDiva 6.1 software (BD). FCS files obtained from 55 patients studied in one of the three central laboratories of the Spanish Myeloma Group (GEM/PETHEMA)

were used as a discovery dataset and analyzed with a semi-automated pipeline that performs batch-analyses of flow cytometry data to avoid variability intrinsic to manual analysis, and unveils full cellular diversity based on unbiased clustering (described in detail in Supplemental Methods). Briefly, this strategy allowed the systematic identification and quantification of a variable number of cell clusters, which grouped according to the similarity of antigen expression profiles by using the bioinformatic algorithm FlowSOM (Supplemental Figure 1).¹⁷ After unbiased identification of cell clusters in the discovery dataset, we performed manual analysis to quantify them in the remaining 212 patients enrolled in the PETHEMA/GEM2012MENOS65 clinical trial.

3D cultures. An organoid 3D model was developed to test the effect of daratumumab (10 µg/mL) on granulocytes from BM samples of MM patients (N = 3). Cells were lysed with 1X BulkLysis buffer (Cytognos) and 1x10⁶ cells were embedded in 30µL of Matrigel Matrix (Corning) and fibronectin (ratio matrigel:fibronectin 2:1). This mix was seeded per well in a 48-well plate (Cellstar®) and left 40' in the incubator so that the matrigel solidifies. Afterwards, we added 300µL of RPMI1640 medium (10% FBS, 1% L-Glu, 1% Penicillin-Streptomycin) supplemented with 10% of plasma from the same BM sample, IL-6 100 nM and IGF1 100 nM per well. Organoids were maintained in culture for 10 days at 37°C and daratumumab (10 µg/mL) was added to the medium on days 1 and 5 of culture. Finally, organoids were desegregated with Cell Recovery Solution (Corning) and labelled with HLA-DR-BV421, CD45-OC515, CD16-FITC, CD13-PE, CD34-PerCP-Cy5.5, CD117-PE-Cy7, CD11b-APC and CD10-APCH7. Data acquisition was performed in a FACSCantoll flow cytometer using the FACSDiva software and data analysis was performed using the Infinicyt software. In addition, we analyzed the percentage of various granulocytic subsets present in BM samples from 36 MM patients collected before and after treatment with daratumumab, which were stained with the next-generation flow (NGF) mAb panel designed to monitor plasma cell clonality (tube 1: CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, CD117-APC, CD81-APCH7 and; tube 2: CD138-BV421, CD27-BV510, CD38-FITC, CD56-PE, CD45-PerCPCy5.5, CD19-PECy7, cyKAPPA-APC, cyLAMBDA-APCH7).¹⁸ The first mAb combination of the NGF panel was used to enumerate immature neutrophils (CD38⁺, CD45^{dim}, CD117⁺, SSC^{hi}), intermediate and mature neutrophils (CD38^{-/lo}, CD45^{dim}, CD81⁻, CD117⁻, SSC^{hi}), basophils (CD38^{hi}, CD45^{dim}, CD81⁻, CD117⁻, SSC^{lo}), and eosinophils (CD38^{-/lo}, CD45^{bright}, CD81^{bright}, CD117⁻, SSC^{hi}).

Fluorescence-activated cell sorting (FACS). Cells with the pre-established G-MDSCs phenotype (CD11b⁺CD14⁺CD15⁺CD33⁺HLADR⁻) and three maturation stages of the neutrophil lineage were sorted from a total of HA (N = 15) and MM patients (N = 45) using a FACS Aria II flow cytometer (BD). Cells were stained with 7-AAD or Sytox Blue Dead to exclude dying events. Cells were stored in Lysis/Binding Buffer (Invitrogen™, CA, USA) for RNA sequencing (RNAseq) or in PBS+0.005% BSA until processing for the assay for transposase accessible chromatin with high-throughput sequencing (ATACseq), or used immediately for morphological assessment or functional assays. These are described in Supplemental Methods.

RNAseq data from mesenchymal stem cells (MSCs) isolated by FACS from BM aspirates of age-matched HA (N = 8) and MM patients (N = 56),¹⁹ was used to compare the expression levels of genes coding for TGF- β and other soluble mediators potentially involved in the modulation of the BM tumor microenvironment.

Statistical analysis. The Kruskal-Wallis test was used to estimate the statistical significance observed between groups in T cell immunosuppression assays. The t-Student test was used to evaluate differences between groups in experiments measuring T cell proliferation, as well as to evaluate significant associations between patients' clinical data and the distribution of various granulocytic subsets. Progression-free survival (PFS) was defined from the time of MFC assessment at diagnosis until disease progression or death from any cause, estimated using the Kaplan-Meier method, and compared with the use of a two-sided stratified log-rank test. Patients were stratified into groups according to the median value of each cell type (or cell ratio) in the whole population. Statistical analyses were performed using the GraphPad Prism software (version 7, San Diego, CA) and SPSS (version 25.0.0, IBM, Chicago, IL). *P* values <0.05 were considered as statistically significant.

RESULTS

Characterization of G-MDSCs based on conventional criteria. In humans, G-MDSCs have been previously defined as a unique (rare) population displaying a CD11b⁺CD14⁺CD15⁺CD33⁺HLADR⁺ phenotype, comprising approximately 1% of BM nucleated cells in HA and up to 25% in MM patients.²⁰ However, we found that the frequency of CD11b⁺CD14⁺CD15⁺CD33⁺HLADR⁺ cells (gating strategy in Supplemental Figure 2) among total BM nucleated cells was similar between HA (N = 7) and MM (N = 10) patients (medians of 28% vs 24%; *P* = .49) (Figure 1A). Moreover, rather than defining a unique population, CD11b⁺CD14⁺CD15⁺CD33⁺HLADR⁺ cells included a mixture of neutrophil subsets (i.e. metamyelocytes, band/mature neutrophils) plus eosinophils (Supplemental Figure 2).

Since various granulocytic subsets were identified within putative G-MDSCs according to conventional phenotypic criteria, we decided to perform an unbiased analysis based on automated clustering using the antigens described above and others reported as potentially relevant⁴ for MDSC isolation, to unveil how many granulocytic clusters were present in BM samples from HA and MM patients. This strategy led to the identification of eosinophils, basophils and three well-defined neutrophil maturation stages according to differential expression of CD11b, CD13 and CD16 in HA and MM patients: immature (CD11b⁺CD13^{low}CD16⁺), intermediate (CD11b⁺CD13^{low}CD16⁺) and mature (CD11b⁺CD13⁺CD16⁺) neutrophils as confirmed by the expected shape of their nucleus (Figure 1B-C). Of note, the mean frequency of each of the five granulocytic subsets was similar between HA and MM patients (Figure 1D), and so was the percentage of each neutrophil subset within total neutrophils (data not shown). There were no differences in the phenotypic profile of mature neutrophils present in matched BM and PB samples from MM patients (N = 5) though, as expected, immature and intermediate neutrophils were absent in PB (Supplemental Figure 3A-B). On transcriptional grounds, mature neutrophils from BM and PB of MM patients clustered together and apart from those of HA (Supplemental Figure 3C).

Daratumumab has no long-term *in vitro* effect on BM granulocytes from MM patients. Based on previous data indicating that daratumumab depletes G-MDSCs¹¹, we treated primary BM aspirates from MM patients (N = 3) with daratumumab to compare the number and phenotype of granulocytic cells before vs after treatment, and thereby infer the antigen expression of putative G-MDSCs depleted by the drug. Samples were cultured in an organoid 3D model to enable long-term treatment (Supplemental Figure 4A). As expected, daratumumab induced a significant depletion of tumor plasma cells (Supplemental Figure 4B), but no differences were found

regarding the percentage of CD11b⁺CD14⁺CD15⁺CD33⁺HLADR⁺ cells (Supplemental Figure 4C) or various granulocytic subsets (Supplemental Figure 4D) after 10-day treatment with daratumumab. These results were confirmed *ex vivo*, where the percentage of various granulocytic subsets was similar in paired BM samples from MM patients (N = 36) analyzed before and after treatment with daratumumab (Supplemental Figure 4E).

Clinical significance of granulocytes in the tumor microenvironment. Since the pre-established phenotype of human G-MDSCs do not allowed the identification of a unique population of myeloid cells in the BM of HA and MM patients (nor in different percentage), and that no myeloid cells were depleted by daratumumab *in vitro* to allows us identifying the phenotype of G-MDSCs, we sought to define their phenotypic profile based on the identification of granulocyte subsets with clinical significance in patients with newly-diagnosed MM (N = 267). Overall, the frequency of basophils, eosinophils and immature (CD11b⁺CD13^{low}CD16⁺), intermediate (CD11b⁺CD13^{low}CD16⁺) and mature (CD11b⁺CD13⁺CD16⁺) neutrophils had no significant association with clinical parameters including cytogenetic alterations (Supplemental Figure 5A). However, we noted the presence of unique patient subgroups based on differential predominance of neutrophils, nucleated red blood cells, tumor cells and T cells in the tumor microenvironment (Figure 2A-B). On prognostic grounds, we found that only the percentage of mature neutrophils and not any other granulocytic subset had a significant impact in PFS (Supplemental Figure 5B); patients with >32% CD11b⁺CD13⁺CD16⁺ BM cells had 3-year PFS rates of 66% vs 79% in cases with ≤32% mature neutrophils (Figure 2C). Due to the relationship between MDSCs and T cell immunosuppression, we then explored if a mature-neutrophil to T-lymphocyte ratio was prognostically relevant. Accordingly, patients with higher ratios (>3.4) had significantly inferior PFS when compared to cases with lower ratios (3-year PFS rates of 85% vs 60%, respectively; *P* < .001) (Figure 2D).

Progressive immunosuppression from immature to mature neutrophils. As a result of the prognostic value found in regard to the frequency of mature neutrophils in the tumor microenvironment of MM patients, we then investigated the immunosuppressive potential of these cells with two functional assays: i) the proliferation rate of autologous T cells in presence of CD3/CD28 stimulatory beads (N = 14, Figure 3A) and, ii) the cytotoxic potential of autologous T-cells against MM cells using a BCMAxCD3 bispecific antibody (N = 10, Figure 3B). Interestingly, we noted a significant decrease in T cell proliferation when these were stimulated in the presence

of mature neutrophils from MM patients (0.5-fold, $P = .016$) but not from HA (Figure 3C). By contrast, immature- and intermediate neutrophil subsets from MM patients or HA had no impact in T cell proliferation. In addition, we noted that the cytotoxic potential of T cells engaged by a BCMAxCD3 bispecific antibody progressively increased with the depletion of immature, intermediate and mature neutrophils (2-fold, 3-fold and 4-fold, respectively; $P \leq .03$) (Figure 3D).

Molecular characterization of neutrophil differentiation in normal and tumor BM.

In light of the progressively increasing gradient of immunosuppression from immature to mature neutrophils, we decided to investigate if differences in the functional behavior of each neutrophil subset were related to transcriptional modulation following their differentiation. Thus, unsupervised clustering after RNAseq of immature, intermediate and mature neutrophils from HA and MM patients (each, $N = 8$) showed accurate segregation per cell-type and not subjects (Figure 4A), thereby validating CD11b, CD13 and CD16 as robust markers to identify and isolate neutrophil stages with different transcriptional profiles. Specific analysis of genes coding for cytokine/chemokine soluble mediators based on the KEGG cytokine-cytokine receptor interaction pathway list (Supplemental Table 1), unveiled significantly different levels in 21 genes and various patterns of differential expression in immature, intermediate and mature neutrophils (Supplemental Figure 6A-B). Notably, most of these patterns were identical in HA and MM patients, except for the *CXCL1*, *PTGS2*, *TGFB1*, *TNFSF13B*, *VEGFA*, *CCL4* and *IL1B* genes since their expression levels were significantly altered in mature neutrophils from MM patients (Supplemental Figure 6C). Accordingly, unsupervised clustering at the subset level showed incomplete segregation between HA and MM regarding the transcriptional profile of immature and intermediate (Supplemental Figure 7) but not mature neutrophils (Figure 4B), which segregated all HA and MM patients based on differentially expressed genes. Interestingly, gene set enrichment analysis unveiled that mature neutrophils from MM patients displayed reduced anti-viral and anti-cancer type I and II IFN transcriptional response, as well as increased activation of transcriptional pathways related with inflammation such as TNF- α , IL2-STAT5 and TGF- β signaling (Figure 4C).

TGF- β transcriptional rewires mature neutrophils. Based on our transcriptomic findings and on the prominent role of TGF- β in the MM tumor microenvironment^{21,22}, we exposed mature neutrophils from HA ($N = 3$) to TGF- β and investigated if this cytokine could contribute to a shift in their transcriptional profile into a similar program to that found in mature neutrophils from MM patients. Thus, we focused on the top-ten

differentially expressed genes between mature neutrophils from HA and MM patients identified above, and compared their expression levels in MM patients vs mature neutrophils from HA after treatment with TGF- β (Figure 4D). Accordingly, we found no significant differences ($P > .05$) in the expression of these genes, suggesting that TGF- β significantly contributes to the molecular reprogramming of mature neutrophils. Interestingly, MSCs from MM patients (N = 56) had similar expression levels of TGF- β as compared to those from age-matched HA (N = 8), but genes coding for pro-inflammatory molecules (*CXCL2*, *CXCL3* and *PTGS2*), growth-factors (*IL-6*, *BAFF*) and angiogenetic mediators (*IL-8*) that eventually, may also shift the transcriptome of mature neutrophils, were found to be upregulated in MSCs from MM patients (Supplemental Figure 8).

The transcriptional network of mature neutrophils is epigenetically deregulated in MM. Under the hypothesis that the transcriptional changes found in mature neutrophils from MM patients resulted from epigenetic modulation as consequence of the altered cellular and cytokine content in the tumor microenvironment, we integrated RNAseq data with chromatin accessibility profiling through ATACseq in mature neutrophils from BM aspirates of HA (N = 3) and MM patients (N = 3). A mean of 23,214 open chromatin sites (peaks) in nucleosome free regions in the six different samples was reported and, using a generalized linear model (DESeq2, $Padj < .1$), we identified 1,445 differentially accessible peaks between mature neutrophils from HA vs MM patients. Among these peaks, 678 showed an increase and 767 a decrease in chromatin accessibility in MM. To assess their biological relevance, differential peaks were annotated to the nearest gene based on their distance to transcription start sites (TSSs). Of note, 50% of these peaks were in potential promoter regions within 3 kb of a TSS, thus suggesting that these gains/losses in accessibility could exert regulatory activity (Supplemental Figure 9A). Accordingly, we performed a gene ontology enrichment analysis on genes that are “closing” or “opening” in MM-derived mature neutrophils. Interestingly, we found several functions (the top ten are described in Table 1) associated with closed regions that predicted for altered neutrophil immunity in MM, while no significant functions were found associated with open regions.

Based on paired ATACseq and RNAseq data, we found a significant correlation between gains or losses of chromatin accessibility near TSS and gene expression for each normal and tumor derived neutrophil samples (Supplemental Figure 9B). To confirm these results, we selected *CD83* that showed significantly higher mRNA expression in MM vs HA as well as concordant transcriptional and chromatin accessibility data, and confirmed its increased protein expression in MM by flow

cytometry (Supplemental Figure 9C). Most importantly, we observed a significant positive correlation between MM-specific changes in gene expression levels and chromatin accessibility at gene promoters in mature neutrophils ($P = 8.17 \times 10^{-6}$) (Figure 5A). Furthermore, gene ontology enrichment analysis of differentially expressed genes in mature neutrophils from MM patients unveiled a significant downregulation in functions related with neutrophil immune activation, in accordance with chromatin accessibility (Figure 5B).

These results led us to investigate if DNA demethylation induced by CM-272, a selective and reversible inhibitor of histone methyltransferase G9a and DNA-methyltransferase²⁴, could open chromatin regions that were closed in mature neutrophils from MM patients, and re-induce expression of genes related with neutrophil immune activation. Accordingly, we found dose-dependent transcriptional changes in mature neutrophils from MM patients after treatment with CM-272 (Figure 5C), and validated its mode of action by confirming increased expression of several type I IFN related genes (Supplemental Figure 10).²⁴ Most importantly, we observed a significant enrichment of upregulated genes related with neutrophil activation (Figure 5D), which suggests that hypomethylating agents could potentially be used to revert the immunosuppressive signature of mature neutrophil present in the tumor microenvironment. Accordingly, we noted that the cytotoxic potential of T cells engaged by a BCMAxCD3 bispecific antibody was restored and even enhanced when mature neutrophils were pretreated with CM-272 (Supplemental Figure 11).

DISCUSSION

Emerging immunotherapies have shown efficacy in the treatment of early and late stage MM.²⁵⁻²⁷ Thus, better understanding of the complexity and diversity of the tumor immune milieu is warranted to improve the ability to predict, monitor and guide immunotherapeutic responsiveness. Here, by integrating the clinical significance with the immunosuppressive potential and molecular network of various granulocytic subsets in the BM, we provided for the first time a set of markers for optimal monitoring of G-MDSCs in MM.

There is growing interest in targeting immunosuppressive cells to optimize T cell activity and immunotherapy efficacy in MM. However, the ability to specifically target immunosuppressive cells while preserving the function of antitumor immune cells remains a challenge in the absence of specific cell markers, and MDSCs are a good example of this conundrum. MDSCs were first described in 2007²⁸ but since then, the few studies performed in humans have commonly required isolation by density centrifugation of PB samples due to the lack of markers to isolate G-MDSCs from other cells in the tumor microenvironment.⁴ Interestingly, these low-density granulocytes were found to be a heterogeneous mix of both banded and segmented neutrophils^{4,29}, but not of more immature stages. These findings are consistent with the observation made in this study that there is a gradient of progressive immunosuppression from immature into mature neutrophils, reaching its maximum at the banded/segmented stage.

The G-MDSC specific GR-1 surface antigen is only present in mice and, therefore, human G-MDSCs have been attributed a broader CD11b⁺CD14⁻CD15⁺CD33⁺HLADR⁻ phenotype. However, we showed that this combination of markers could not distinguish G-MDSCs from common neutrophils; in fact, most maturing granulocytes are CD11b positive, CD15 is also expressed in eosinophils, and CD33 is present in all myeloid cells. CD16 has been proposed as an additional marker³⁰ to identify G-MDSCs, but alone is not sufficient because it is also expressed in non-classic monocytes that, coincidentally, downregulate CD14.³¹ Thus, in the absence of established markers, human G-MDSCs can only be defined by their functional hallmarks (T cell suppression and arginase 1 [ARG1] expression). Here, we showed that mature neutrophils (and no other granulocytic subset) present in the MM tumor microenvironment correlates with patients' outcome. Furthermore, these cells exerted the strongest T cell immunosuppression and expressed higher levels of inflammatory cytokines such as *TGFB1*, *TNF* and *VEGFA*, together with the increase of the NFκB and of other G-MDSC associated markers (e.g. *PTGS2*, *CSF1*, *IL-8*, *IRF1*, *IL4R*,

STAT1, *STAT3*, *STAT6*) when compared to intermediate and immature neutrophils.³²⁻

³⁴ Thus, we propose that in MM, G-MDSCs are CD11b⁺CD13⁺CD16⁺ neutrophils.

Structured models of transcriptional, phenotypic, and functional diversity are instrumental for better understanding of immune cell biology. However, unlike other myeloid cells in which diverse functional properties have been linked to specific molecular programs, the transcriptional heterogeneity behind functional diversity of neutrophils remains largely unknown.³⁵ Here, after identifying surface markers enabling the tracking of immunosuppressive neutrophils (ie. G-MDSCs) within the MM tumor microenvironment, we showed that maturing neutrophil subsets have unique gene expression profiles that are rewired into an immunosuppressive state through epigenetic modulation in the BM of patients with cancer. It has been suggested that TGF- β , an immunosuppressive cytokine produced by tumor cells from various cancer types, polarizes neutrophils to a pro-tumorigenic phenotype.^{36,37} Here, we have shown that exposure to TGF- β was able to shift the transcriptional profile of mature neutrophils from HA into a similar program to that found in MM, and future studies are warranted to investigate a possible correlation between TGF- β levels and the immunosuppressive potential of G-MDSCs in BM aspirates. Additional research should also be performed to identify which other players in the BM milieu may contribute to this phenomenon. Finally, we showed that the molecular network of mature neutrophils from MM patients could be modified by epigenetic drugs and thereby, prevent their immunosuppressive effect in T cells engaged by a BCMAxCD3 bispecific antibody. Thus, this study proposes further investigation of their biology to identify targeted therapies for rewiring of G-MDSCs and increase the successful application of immunotherapy in MM and other tumors.

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AUTHORSHIP CONTRIBUTIONS

C.P., C.B. and B.P. conceived the idea and designed the study protocol. C.P. and C.B. analyzed flow cytometry data. D.A. performed cell sorting. C.P. performed *in vitro* experiments. C.P., S.S. and A.V.Z. performed next-generation sequencing; C.B. and I.G. analyzed sequencing data. M.J.C., J.M.L., F.J.G-A., G.A., R.G.S., M.G., C.B., F.P., S.M., A.Orfao., A.Oriol, Al.T., MA.E., A.S., R.d.P, F.dA., M.T.H., L.P., R.M., L.R., MV.M, JJ.L., J.B., and J.F.S.M. provided study material and/or patients. C.P. and C.B. performed statistical analysis. C.P., C.B. and B.P. wrote the manuscript. All authors reviewed and approved the manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST

A.O. participated in advisory boards for Amgen, Celgene and Janssen. F.d.A. reports honoraria for lectures from Celgene, Janssen, Amgen and Takeda, and consultancy with Amgen, Celgene and Janssen. M.-V.M. has received honoraria from lectures or participation in advisory boards from Janssen, Celgene, Amgen, Takeda, Abbvie, Adaptive, GSK, Pharmamar, EDO, Oncopeptides. L.R. reports honoraria from Janssen, Celgene, Amgen, and Takeda. J.B. reports honoraria for lectures from Janssen, Amgen, Celgene, Takeda and Oncopeptides. J.-J.L. reports honoraria from and membership on board of directors or advisory committees with Takeda, Amgen, Celgene, and Janssen. J.F.S.M. reports consultancy for Bristol-Myers Squibb, Celgene, Novartis, Takeda, Amgen, MSD, Janssen, and Sanofi and membership on board of directors or advisory committees with Takeda. B.P. reports honoraria for lectures from and membership on advisory boards with Amgen, Bristol-Myers Squibb,

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DATA SHARING

For original data, please email the corresponding author.

Table 1. Gene ontology enrichment analysis on genes that are closing or opening in mature neutrophils of MM patients as compared to healthy adults. Several functions associated with closed regions predicted for altered neutrophil immunity in MM, while no functions were significantly associated with open regions.

ID	Description	p value	p.adjust
GO:0042119	neutrophil activation	1.72e-11	3.56e-8
GO:0043312	neutrophil degranulation	2.27e-11	3.56e-8
GO:0002283	neutrophil activation involved in immune response	2.82e-11	3.56e-8
GO:0036230	granulocyte activation	2.83e-11	3.56e-8
GO:0002446	neutrophil mediated immunity	6.15e-11	6.18e-8
GO:0031325	positive regulation of cellular metabolic process	1.08e-10	8.60e-8
GO:0051173	positive regulation of nitrogen compound metabolic process	1.20e-10	8.60e-8
GO:0009893	positive regulation of metabolic process	3.33e-10	2.09e-7
GO:0043299	leukocyte degranulation	5.13e-10	2.87e-7
GO:0002275	myeloid cell activation involved in immune response	9.00e-10	4.29e-7

FIGURE LEGENDS

Figure 1. Characterization of G-MDSCs based on conventional criteria. (A) Bone marrow (BM) samples from multiple myeloma (MM) patients (N = 10) and healthy adults (HA) (N = 7) were stained with HLADR-BV421, CD45-OC515, CD15-FITC, CD13-PE, CD33-PerCPCy5.5, CD16-PECy7, CD11b-APC and CD14-APCH7 monoclonal antibodies. Cells with a CD11b⁺CD14⁻CD15⁺CD33⁺HLADR⁻ phenotype represent approximately 25% of total BM nucleated cells both in HA and MM patients. **(B)** Unbiased analysis based on uniform manifold approximation and projection (UMAP) according to the expression levels of HLADR, CD45, CD15, CD13, CD33, CD16, CD11b and CD14, unveiled various granulocytic subsets (neutrophils, eosinophils and basophils) in BM samples from HA and MM patients. **(C)** UMAP of the neutrophil population led to the identification of three neutrophil maturation stages according to differential expression of CD11b, CD13 and CD16: immature (CD11b⁻CD13^{-/lo}CD16⁻), intermediate (CD11b⁺CD13^{-/lo}CD16⁻) and mature (CD11b⁺CD13⁺CD16⁺) neutrophils. Cellular maturation was confirmed on cytopinned cells from the three different populations by evidencing the classic changes in nuclear shape **(D)** The frequency of each granulocytic subset was similar between HA and MM patients. Bar graphs represent the mean and lines the standard deviation.

Figure 2. Clinical significance of granulocytes in the tumor microenvironment.

(A) Unbiased immune monitoring of the tumor microenvironment based on UMAP of bone marrow (BM) samples of newly-diagnosed multiple myeloma (MM) patients (N = 55). **(B)** Unsupervised clustering of MM patients based on cellular composition of the tumor microenvironment. **(C)** Progression-free survival (PFS) according to high (>32%) vs low (≤32%) abundance of mature (CD11b⁺CD13⁺CD16⁺) neutrophils: 3-year PFS rates of 66% vs 79%, respectively; *P* = .0391. **(D)** PFS according to high (>3.4) vs low (≤3.4) mature-neutrophil to T-lymphocyte ratio: 3-year PFS rates of 60% vs 85%, respectively; *P* < .0001. NRBCs: nucleated red blood cells.

Figure 3. Progressive immunosuppression from immature to mature neutrophils.

(A) FACSsorting of the three neutrophil subsets from bone marrow (BM) samples of multiple myeloma (MM) patients (N = 10) and HA (N = 4) was performed to culture each subset with autologous T cells previously stimulated with CD3/CD28 antibodies and labelled with violet proliferation dye (VPD). After a 4-day incubation, VPD intensity was measured on total T cells. **(B)** Total BM samples vs BM samples depleted of each neutrophil subset (i.e. BM w/o CD11b⁻CD13^{-/lo}CD16⁻, BM w/o CD11b⁺CD13^{-/lo}CD16⁻ and BM w/o CD11b⁺CD13⁺CD16⁺) from MM patients (N = 10), were treated with 30 nM

of a BCMAxCD3 bispecific antibody and left in culture for 24h. **(C)** Significant decrease in T cell proliferation when these were stimulated in the presence of mature neutrophils from MM patients (0.5-fold, $P = .016$), but not the immature- and intermediate subsets from MM patients or HA. **(D)** Cytotoxic potential of T cells engaged by a BCMAxCD3 bispecific antibody progressively increased with the depletion of immature, intermediate and mature neutrophils (2-fold, 3-fold and 4-fold, respectively; $P \leq .03$). Bar graphs represent the mean and lines the standard error of the mean. The statistical significance was evaluated using the t-Student test for proliferation analysis and the Kruskal-Wallis test for the immunosuppression assay.

Figure 4. Molecular characterization of neutrophil differentiation in normal and tumor BM. **(A)** Unsupervised clustering after RNAseq of immature, intermediate and mature neutrophils from HA and MM patients (each, $N = 8$) showed accurate segregation per cell-type and not subjects. **(B)** Whole transcriptome profiling through RNAseq segregates mature neutrophils from HA and MM according to 108 genes differentially expressed ($P < .05$). **(C)** Gene set enrichment analysis showed that mature granulocytes from MM have increased activation of pathways related to inflammation and reduced anti-viral and anti-cancer type I and II IFN transcriptional response. **(D)** Mature neutrophils from HA ($N = 3$) were treated with TGF- β and expression levels of the top 10 differentially expressed genes between MM and HA neutrophils (i.e. figure 4B) were analyzed. There were no significant differences when we compared mature neutrophils from MM patients vs HA treated with TGF- β ($P > .05$).

Figure 5. The transcriptional network of mature neutrophils is epigenetically deregulated in MM. **(A)** Correlation between gains and losses of chromatin accessibility near TSS and gene expression for each sample. Significant positive correlation between MM-induced changes in gene expression levels and chromatin accessibility at gene promoters in mature granulocytes ($P = 8.17e^{-6}$). **(B)** GO enrichment analysis on differentially expressed genes underscored functions related to neutrophil activation in MM. **(C)** Transcriptional analysis of mature neutrophils from MM patients ($N = 3$) treated with CM-272 segregated samples according to exposure and concentration of the drug. **(D)** Gene ontology enrichment analysis based on upregulated genes in mature neutrophils from MM patients after treatment with CM-272.

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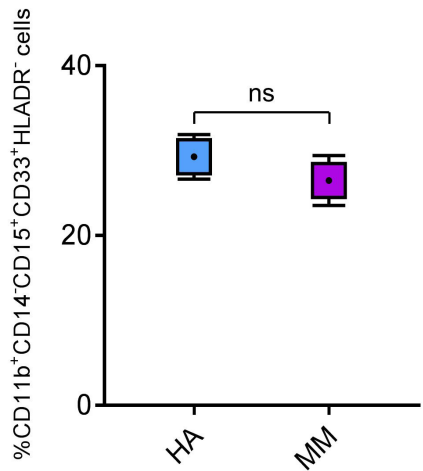
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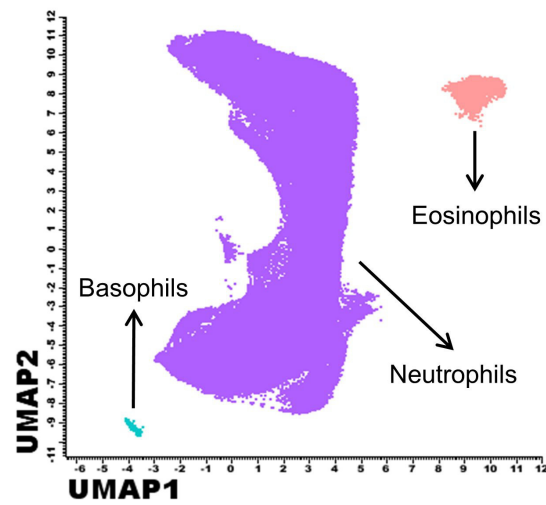
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Figure 1

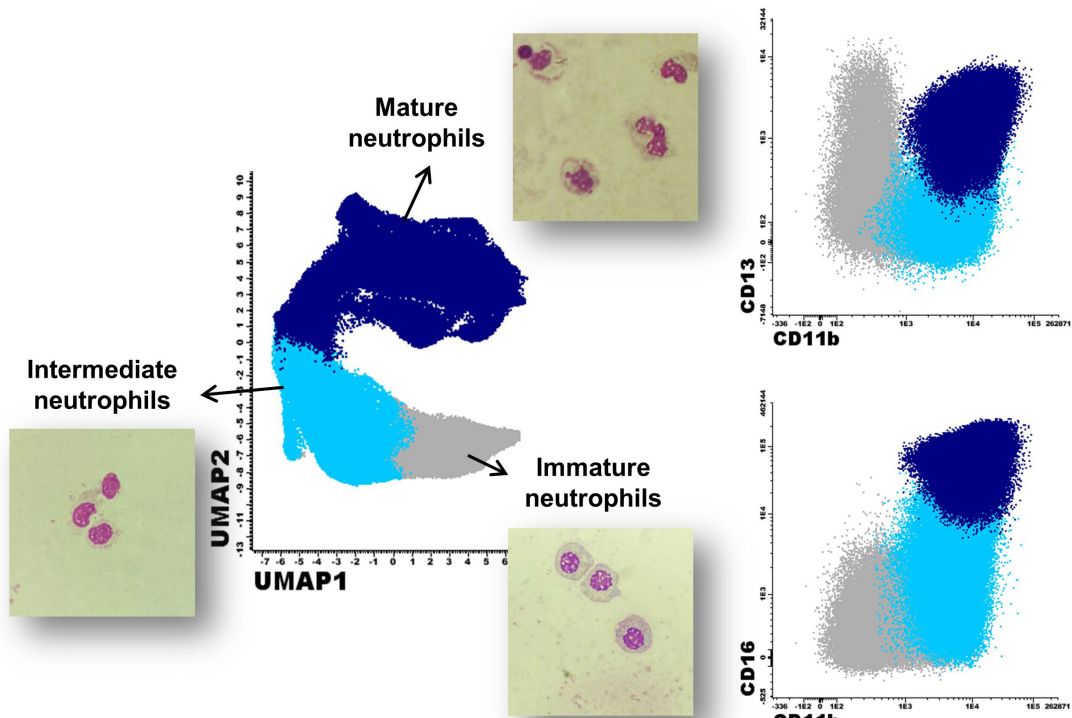
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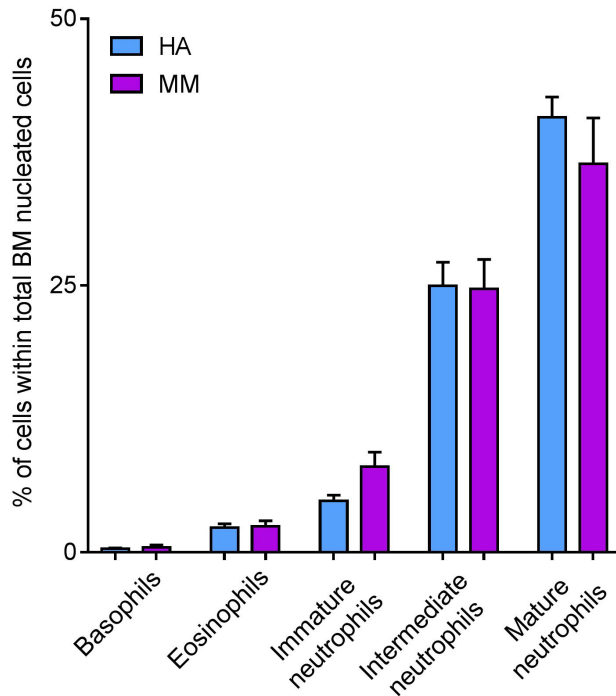
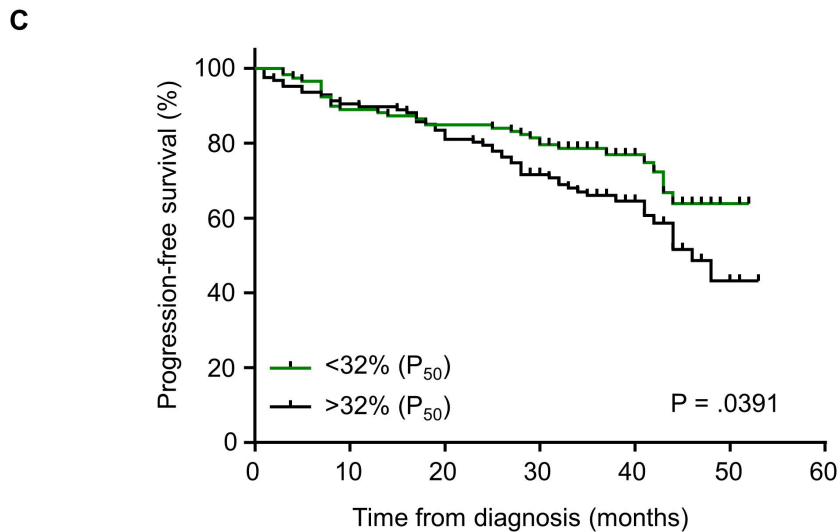
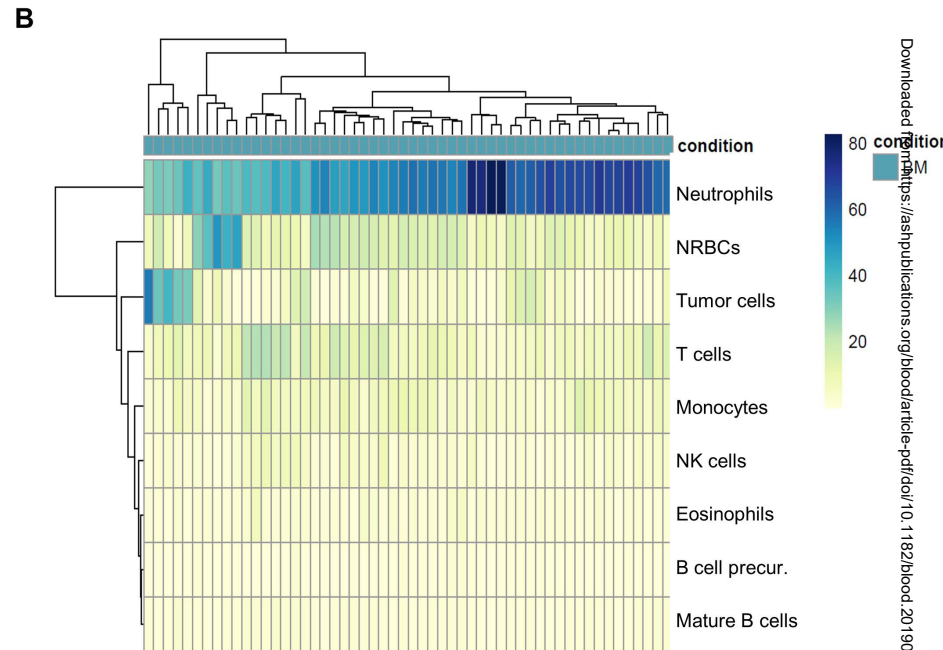
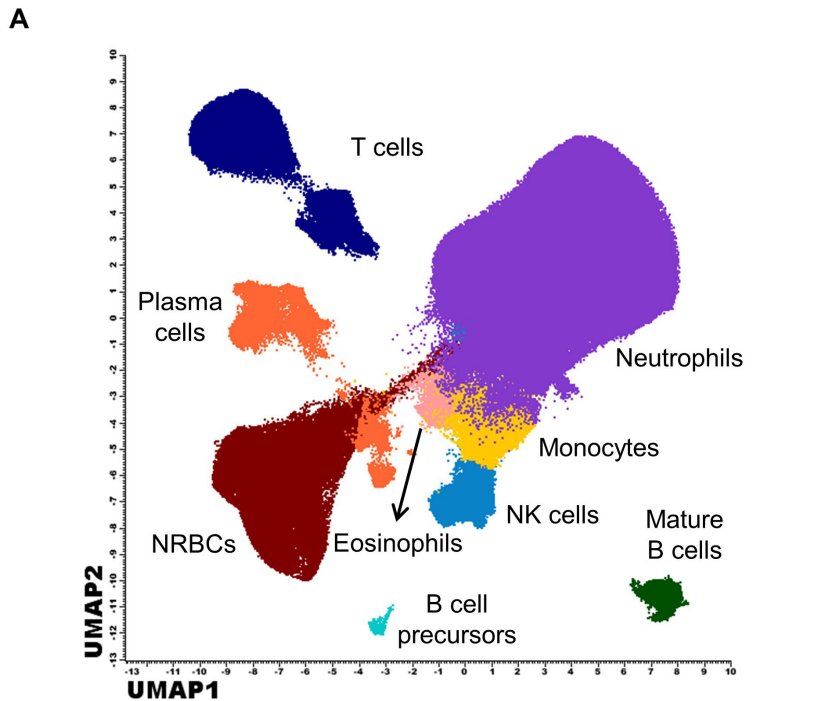
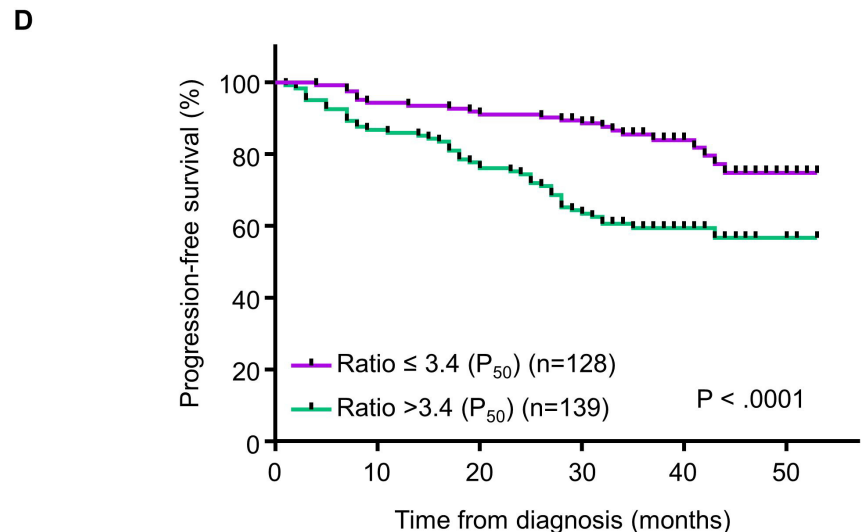


Figure 2



Number of subjects at risk:

Group $<32\%$	129	116	112	99	41	9	0
Group $>32\%$	138	126	116	94	40	4	0



Number of subjects at risk:

Group ≤ 3.4	134	127	122	117	47	7
Group >3.4	133	115	102	76	33	5

Figure 3

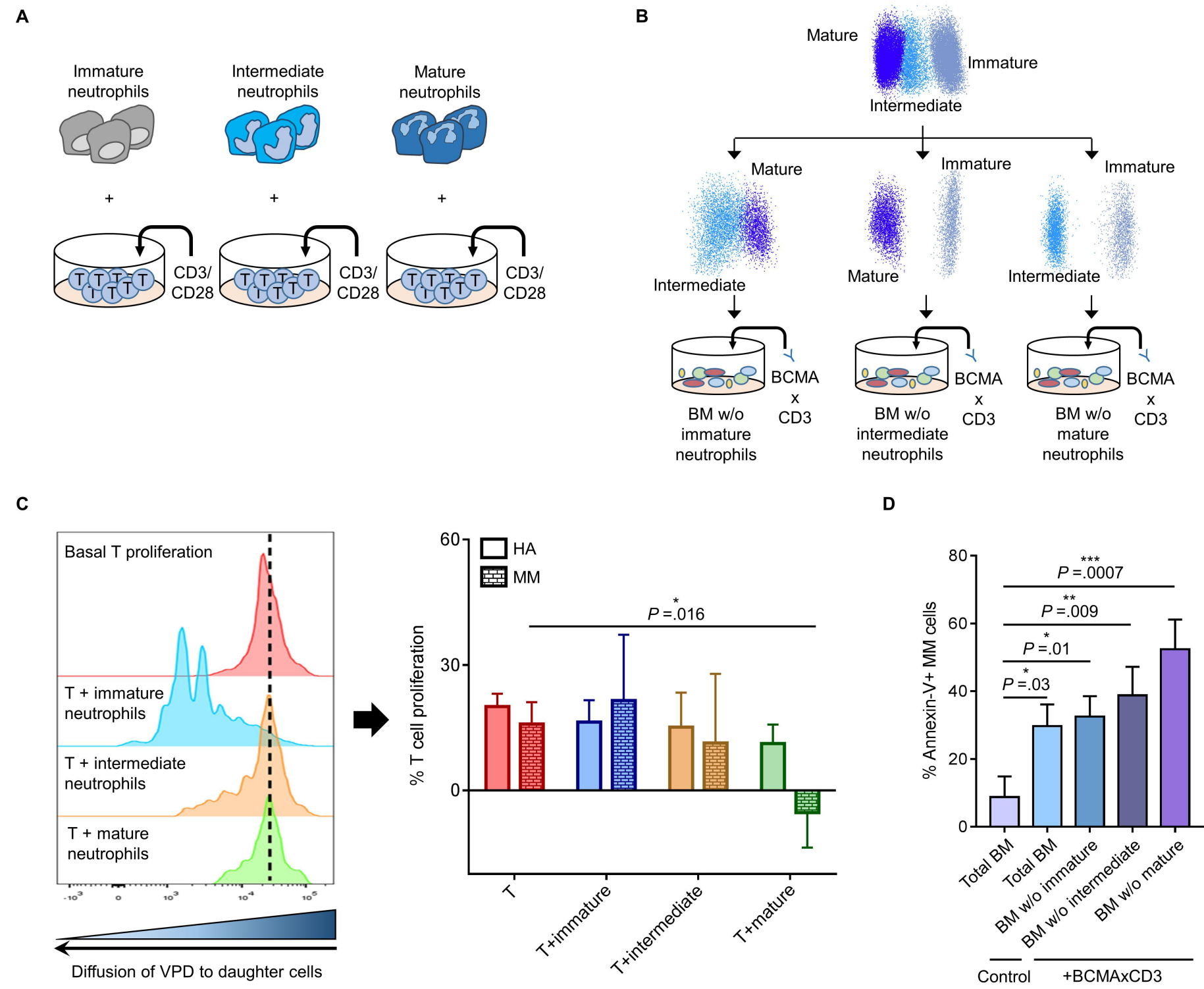


Figure 4

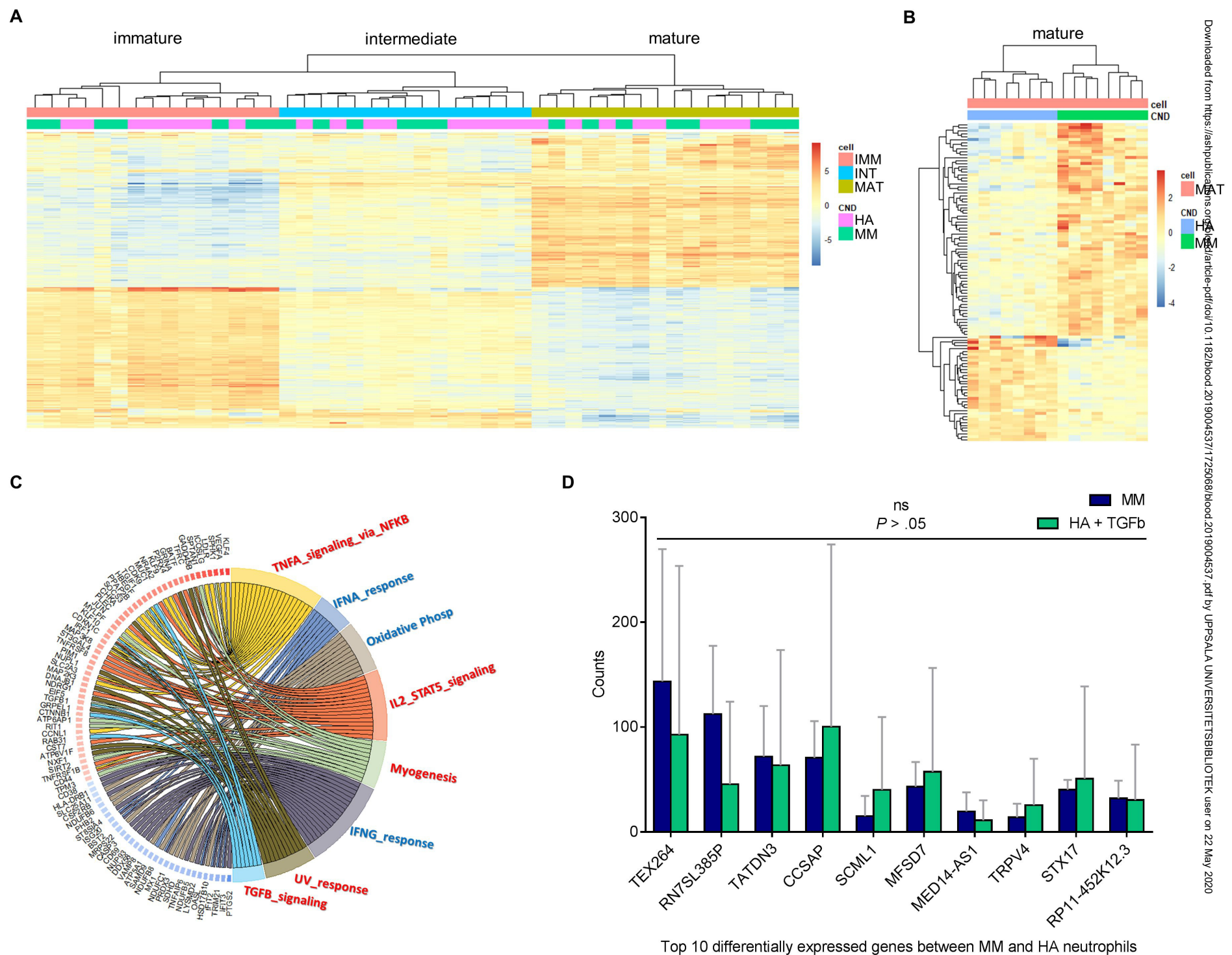


Figure 5

