Transcriptional remodeling of the Stromal and Endothelial Microenvironment in MGUS to Multiple Myeloma Progression

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RUNNING TITLE: scRNA-seq of BM microenvironment in MM progression

KEYWORDS
Multiple myeloma, MGUS, single-cell RNA sequencing, Endothelial cells, Stromal cells, tumor microenvironment.

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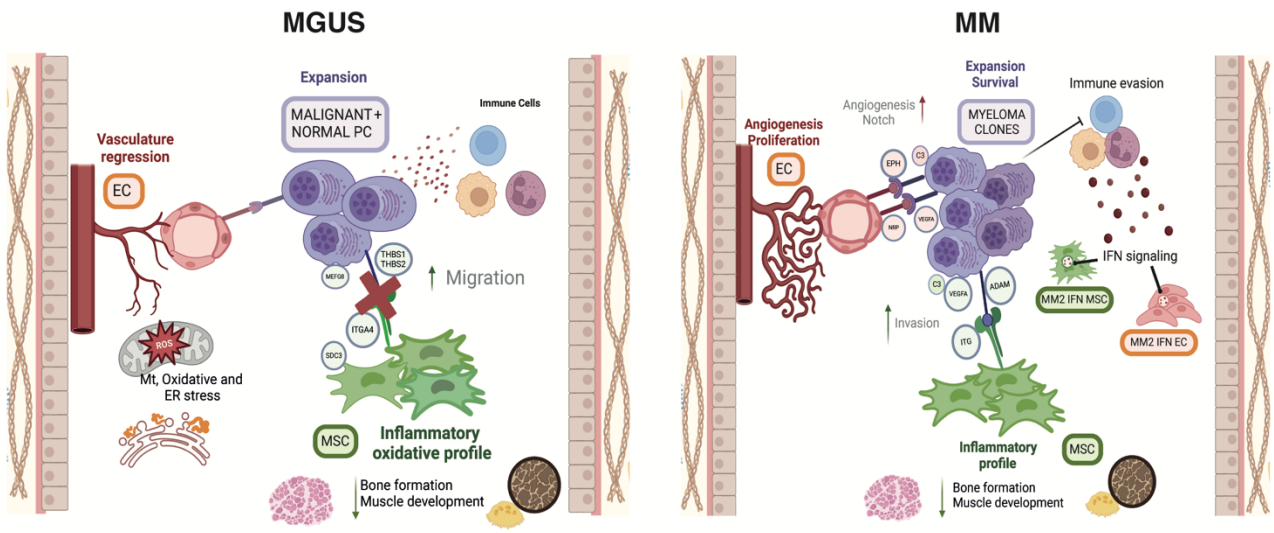
ABSTRACT

The role of the bone marrow (BM) microenvironment in the transition from monoclonal gammopathy of undetermined significance (MGUS) into clinically active multiple myeloma (MM) is not completely determined. To address this issue, we performed single-cell RNA sequencing (scRNA-seq) of BM non-hematopoietic microenvironment cells as well as plasma cells (PC) from two genetically engineered mouse models of MM termed $BI_{c\gamma}$ and $MI_{c\gamma}$ that recapitulate the progression of MGUS into MM. Our results identify distinct transcriptional dynamics between mesenchymal stem cells (MSC) and endothelial cells (EC). While EC acquire a stress state during MGUS, a proliferating and angiogenic profile characterizes MM. On the other hand, MSC compromised their differentiation potential, exhibiting a more inflammatory profile that initiates from the MGUS stage. Interestingly, we identified an interferon (IFN)-related myeloma signature in malignant EC of the BIcγ model, which is also expressed in MSC but not observed in the more aggressive MIcγ model and can be identified in MSC from a subgroup of MM patients. The analysis of the MSC and EC interactions with malignant plasma cells (PC) revealed stage-specific interactions that contribute to angiogenesis, immunomodulation, and MM extravasation. Finally, the translational relevance of our results in humans was confirmed on MSC from newly diagnosed patients with PC neoplasias at different stages of the disease. In summary, these results show a remodeling of the non-hematopoietic BM microenvironment in MM progression, providing potential targets at the tumor-niche interface that may hold clinical significance and complement existing immunotherapies.



KEY POINTS

- EC stress pre-vascular state in MGUS, shifts to angiogenic in MM, while MSC early transcriptional changes in MGUS persist in overt MM.

- Identification of a myeloma-specific IFN signature in the non-hematopoietic BM microenvironment that could define a subgroup of MM patients.

INTRODUCTION

Multiple myeloma (MM) is a B-cell malignancy characterized by the clonal expansion and dissemination of plasma cells (PC) in the bone marrow (BM) that is preceded by asymptomatic precursor conditions, namely monoclonal gammopathy of undetermined significance (MGUS) and smoldering multiple myeloma (SMM)[1]. Despite significant improvement in the response rate and overall survival of MM due to the introduction of novel therapies based on immunomodulatory drugs, proteasome inhibitors, anti-CD38 monoclonal antibodies, and, more recently, T-cell redirecting therapies, patients eventually relapse, and therefore, it is still considered an incurable disease[2]. Treatment of the disease at the stage of SMM in patients with a high risk of progression has been associated with an increase in progression-free survival (PFS) and may result in an increase in long-term survival and even cure of a significant proportion of patients. Understanding the molecular mechanism involved in the progression from precursor stages to symptomatic MM is thus instrumental in the design of therapies directed to prevent the development of overt disease.

The BM microenvironment comprises a complex network of hematopoietic and non-hematopoietic cells, extracellular matrix components, and soluble factors. This microenvironment creates an immunosuppressive and proinflammatory milieu that supports malignant PC growth and survival[3]. Therefore, understanding the intricate interactions between myeloma cells and their surrounding microenvironment is crucial for developing effective therapies that can disrupt supportive signals and overcome drug resistance mechanisms. While most of the studies have focused on the immune component [4–7], mounting evidence suggests that endothelial cells (EC) and mesenchymal stem cells (MSC) also contribute to the survival and growth of myeloma PC through the production of cytokines and the release of extracellular vesicles[8,9]. Moreover, an enhanced inflammatory signature has been identified in MSC of patients with MM[10–12]. However, the role of the non-hematopoietic BM microenvironment in the transition from MGUS to MM remains less understood.

In the current study, we took advantage of our recently described mouse models of MM that recapitulate the principal clinical, genetic, molecular, and immunological characteristics of MM patients[13]. These models are characterized by the presence of a precursor stage equivalent to MGUS that evolves into asymptomatic MM. We performed single-cell RNA sequencing (scRNA-seq) on Fluorescent-Activated Cell Sorted (FACS) BM non-hematopoietic microenvironment cells, specifically EC and MSC, as well as PC from the same animals.

Our results identified distinct transcriptional changes between EC and MSC during the different stages of the disease. While EC are characterized by significant differences between MGUS and MM stage, changes in MSC are already identified at the MGUS stage with an inflammatory profile along with the reduction of structural functions, which is preserved in MM. Interestingly, we identified an interferon (IFN)-related myeloma signature in malignant EC, which is also expressed in MSC. Our work further characterizes interactions between microenvironment cells and malignant PC potentially involved in disease development. While interactions between EC and PC are significantly reduced during MGUS, MSC displayed an increased communication with PC compared to the EC. Finally, using MSC from patients with MGUS, SMM, and MM, we unveil similar transcriptional alterations in human MSC with an inflammatory profile underlying progression into MM.

METHODS

Mice

We used $BI_{c\gamma}$ mice (B6(Cg)-Gt (ROSA)26Sor$^{tm4(Ikbkb)Rsky}$/J mice and B6. Cg-Tg(BCL2)22Wehi/J mice crossed with cγ1-cre mice (B6.129 × 1-Gt (ROSA)26Sor$^{tm1(EYFP)Cos}$/J mice; all from The Jackson Laboratory) that recapitulate the most common changes observed in human MM preceded by an MGUS-like stage[13]. In addition, we used $MI_{c\gamma}$ mice (B6(Cg)-*Gt (ROSA)26Sor$^{tm4(Ikbkb)Rsky}$*/J and C57BL/6N-Gt(ROSA)26Sor$^{tm13(CAG-MYC,-CD2*)Rsky}$/J mice crossed with cγ1-cre mice), a fast progression model with a less marked MGUS stage. As controls, mice carrying a YFP reporter (B6.129 × 1-Gt (ROSA)26Sor$^{tm1(EYFP)Cos}$/J mice; The Jackson Laboratory) were crossed with cγ1-cre mice. To induce PC generation in mice kept in specific pathogen-free (SPF) animal facilities, eight to ten-week-old mice were immunized with sheep red blood cells (SRBCs) every 21 days for 4 months.

For each scRNA-seq experiment, a pool of five or six adult mice per group compromising control, MGUS, and MM stages was used. Mice of both sexes were used in the study. Mice were kept under SPF conditions in

the Center for Applied Medical Research animal facilities at the University of Navarra. Animal experimentation was approved by the Ethical Committee of Animal Experimentation of the University of Navarra and by the Health Department of the Navarra Government.

Murine BM non-hematopoietic niche cells were isolated as previously described[14], sorted with GFP positive (GFP^+) PC using BD FACSAria II, and collected in PBS 1X supplemented with 0.05% UltraPure BSA for subsequent scRNA-seq protocol. Cell viability of sorted cells was assessed using a Nexcelom Cellometer.

Human

For human studies, clinical BM aspirate samples from individuals of both sexes with newly diagnosed MGUS (n = 5), SMM (n =2), or MM (n =10), and bone chip BM samples from healthy donors (HD) (50-80 years of age) undergoing orthopedic surgery (hip or knee replacement) were collected. Written informed consent was obtained according to the Declaration of Helsinki and the Institutional Review Board of the University of Navarra. The clinical characteristics of all individuals are described in **supplemental Table 1**.

After sample collection, red blood cells were lysed, and portions of bones present in bone chip BM samples were crushed and collagenized. After digestion, the collagenized fraction was filtered and pooled with the BM fraction. After cell counting, BM cells were subsequently cryopreserved with FBS and 10% dimethyl sulfoxide (Sigma) in liquid nitrogen until use. Frozen BM samples were thawed, and MSC were prospectively isolated in 100 µl of Lysis/Binding Buffer (Ambion) and stored at -80ºC until further Bulk RNA sequencing (bulk RNA-seq) processing.

Single-cell RNA and Bulk RNA sequencing

The specifics related to the preparation, sequencing, and analysis of the scRNA-seq and bulk RNA-seq libraries are described in the supplemental Methods. Briefly, scRNA-seq genomic sequence reads were analyzed using the CellRanger suite against the mouse reference genome mm10 with default parameters. Next, the analysis of scRNA-seq count tables was performed using R version (v) 4.1.3 and Seurat v 4.3.0[15]. Additional information, including quality control steps, differential expression, gene set enrichment analysis (GSEA), cell-cell communication, and gene regulatory network (GRN) analysis, are fully described in the supplemental Methods.

RESULTS

Single-cell transcriptomic profiling of BM murine cells in control, MGUS, and MM

The BM microenvironment is essential for fostering the growth and survival of MM cells. Using the BIcγ1 myeloma murine model[13], we characterized the influence of the stromal and endothelial BM microenvironment on the progression from MGUS to MM. This model carrying BCL2 and $IKK2^{NF\text{-}\kappa B}$ genetic lesions develops MGUS starting around 6 months of age and progressing to MM in 4 to 6 months. Considering that the current BIcγ1 mice received T-cell-driven immunization with SRBCs, we also included YFPcγ1 mice similarly immunized as controls (**Figure 1A**). Then, we performed scRNA-seq profiling on FACS-sorted non-hematopoietic BM microenvironment cells (Lin^-, $TER119^-$, $CD45^-$, VDO^+, and $7AAD^-$) and myeloma PC (VDO^+, $7AAD^-$ and GFP^+) (**supplemental Figure 1**). After quality control and preprocessing, we obtained a total of 17451 cells across all groups (2654 for control, 6122 for MGUS, and 8675 for MM). We identified 19 distinct clusters comprising MSC, EC as well as a limited number of cells from other populations of immune and non-immune BM cells (**Figure 1B**; **supplemental Figure 2A-B**). Eight of these clusters, based on the expression of *Sdc1*, *Xbp1, Tnfrsf17,* and *Slamf7,* were labeled as PC (clusters 1, 2, 5, 9, 10, 11, 12, and 13, n=7924). Two clusters defined by the expression of *Hba−a1*, *Hba−a2, Hmgb2,* and *Car2* were labeled as erythroblasts (clusters 16, 17, n=257). One cluster, defined by the expression of *S100a8*, *S100a9, Fcer1g,* and *Lyz2* was labeled as neutrophils (cluster 18, n=176) (**Figure 1C; supplemental Figure 2C-D**). EC (clusters 3, 7, and 8, n= 3288) were identified by the expression of canonical genes such as *Cdh5*, *Cav1*, *Ly6c1*, and *Kdr* (**Figure 1C; supplemental Figure 2C-E**). The MSC (clusters 14 and 15, n= 592) specifically expressed mesenchymal and osteolineage (OLN)-primed markers *Cxcl12*, *Pdgfra*, *Lepr*, and *Col1a1* (**Figure 1C; supplemental Figure 2C-F**).

To better understand the roles of BM MSC and EC in myeloma progression, we first conducted a series of confirmatory analyses on both cell populations. The distribution of EC (315 control cells, 262 MGUS, and

2060 MM) was confirmed based on the expression of canonical endothelial markers such as *Cdh5* and *Pecam1* (**Figure 1D; supplemental Figure 2G-H**). Similarly, the distribution of MSC was confirmed by the expression of *Cxcl12* and *LepR* (132 control cells, 143 MGUS, and 229 MM) (**Figure 1E; supplemental Figure 2I-J**). An increase in the number of EC in the BM during MM development was verified by flow cytometry (**supplemental Figure 3**). Overall, these results validate our cell enrichment strategy for the isolation of EC and MSC within the non-hematopoietic BM compartment.

Identification of a myeloma specific IFN signature in the BM microenvironment

The observed increase in EC numbers in the context of MM emphasizes a potential connection between EC and MM progression. Delving into the composition of EC, UMAP analysis revealed a cluster of cells shared across all stages alongside a second cluster of cells ("MM2") that was nearly unique to the MM stage (**Figure 2A**). The predominance of EC from the MM stage within the "MM2" cluster suggested a central role in myeloma. The "MM2" cluster gene signature was characterized by the expression of IFN-inducible genes such as *Ifit2*, *Rsad2*, *Ifit3*, *Isg15*, *Ifi44, Ifit,* and *Oasl2,* as well as, key transcriptional regulators of IFN-dependent immune responses, such as guanylate-binding proteins (GBPs), indicating an upregulation of an IFN response pathway (**Figure 2B**). Over-representation analyses (ORA) confirmed the involvement of IFN-response pathways in "MM2" EC (**Figure 2C**).

To uncover the regulatory mechanisms governing the IFN-like signature, we carried out a cis-regulatory analysis to identify gene-expression-derived regulons using SCENIC[16]. Regulon analysis implicated *Irf7*, *Stat1*, and *Stat2* as master regulators significantly activated in the "MM2" EC subset (**Figure 2D; supplemental Figure 4A-B**). These transcription factors (TFs) are widely recognized regulators of the type 1 IFN induction pathways, which have been linked to regulatory T cell immunosuppressive function observed in myeloma patients[17]. In addition, regulon-specific targets related to T-cell regulation, such as *Trim30d*, *Trim56*, *H2-T22*, *H2-T23*, *H2-Q6*, and *H2-Q7,* showed high expression levels in the "MM2" EC subset (**supplemental Figure 4C**).

As shown in **Figure 2E**, this signature robustly characterizes "MM2" EC in our data and highlights genes that are overexpressed in the abnormal cell subset in MM. Hence, to determine whether this IFN-like signature was unique for EC, we evaluated the score of the gene set related to "MM2" EC across other BM populations. This signature was also significantly upregulated in MSC from BIcγ1 mice with MM, suggesting a shared immunomodulatory response of the BM microenvironment to the disease (**Figure 2F**). In other cell types, including PC, neutrophils, and immature erythrocytes, among others, an IFN signature was observed at the MM stage (**supplemental Figure 4D**). As the signature is not present in cells from control or precursor stages, we concluded that this upregulation is specific to the malignant stage. Consequently, we termed this MM-IFN signature to highlight their association with IFN pathways and MM phenotype.

Interestingly, when we analyzed the MM-IFN signature in the MIcγ1 model[13], an additional model characterized by rapid MGUS-to-MM transition, we did not observe a statistically significant enrichment of the IFN signature in any of the stages in either population (**Figure 2G supplemental Figure 4E**). This finding prompted us to further investigate this MM-IFN signature in humans. Using a single-cell publicly available dataset containing stromal cells from both MM patients and HD[11], we uncovered an enrichment of the murine MM-IFN fingerprint in a subset of MM patients, independent of the genomic alterations (**supplemental Figure 4F-G**). Likewise, previous studies have described a similar IFN-inducible signature with highly overlapping marker genes in PC and immune microenvironment cells from certain MM patients[4,5]. Taken together, our findings suggest that IFN-signaling is a coordinated response within the BM microenvironment populations and could define a subgroup of patients with MM.

EC are characterized by a stress pre-vascular state during MGUS stage

We next analyze those EC that did not show a specific IFN signature. To investigate deeper into the molecular mechanisms of myeloma progression, we performed a differential expression analysis among non-IFN EC at the different stages of the disease. A first global analysis uncovered a distinct transcriptome profile between EC from MGUS in comparison with control and MM cells (**Figure 3A**). Interestingly, EC from MGUS were characterized by overexpression of *Slpi*, *Mt1*, *Mt2*, and *Hspa5*, genes related to protein folding, endoplasmic reticulum stress and metal ion homeostasis, suggesting a stress state of EC at the onset of the disease

(**supplemental Table2; Figure 3B; supplemental Figure 5A-B**). These results suggest that the upregulation of *Mt1* and *Mt2* in EC from MGUS may be involved in neovessel formation and may represent a "pre-vascular phase" in the progression to MM. Similarly, the *Hspa5* gene (also called *Grp78*) favors pro-survival signaling and contributes to tumor angiogenesis[18]. Notably, during the MGUS stage, there was a significant upregulation of *Cd74*, *Tmem176a,* and *Nme2* (**supplemental Figure 5C**). While these genes have documented roles in various pathologic conditions, their implication in MGUS pathogenesis within the endothelium has not been previously reported[19–21].

Then, to comprehend how EC might contribute to disease progression, we performed a differential expression analysis comparing EC from MM and MGUS. Overall, 180 genes were differentially expressed in EC between both stages, including 175 upregulated and 5 downregulated genes (**supplemental Table 3; Figure 3C**). ORA revealed enrichment for biological processes involving angiogenesis, EC migration, differentiation, and epithelial cell proliferation in EC from MM, reflecting the vascular phenotype (**Figure 3D**). We also found overexpressed genes strongly related to lipid metabolism, reflecting the potential role of lipoproteins and lipids in MM[22]. Intriguingly, among the most upregulated genes in MM EC, *Sox18* was particularly interesting as this gene is critical for vascular development and is involved in the proliferation and migration processes of various malignant tumors[23](**supplemental Figure 5D**). In addition, genes such as *Fabp4* and *Cav1* were also upregulated in EC from MM, suggesting their role in malignant transformation.

We expanded our analysis to the MIcγ1 model. Global transcriptional changes showed a similar stress transition state akin to that seen in the BIcγ1 model, marked by the upregulation of protein folding functions (**supplemental Figure 6A**). Interestingly, genes upregulated at the MGUS stage of the MIcγ1 model mirrored those elevated at the MM stage in the BIcγ1 model, supporting the rapid disease progression (**supplemental Figure 6B**). Likewise, we observed an increased vascular phenotype during the MGUS stage, along with the upregulation of *Anxa2*, *Anxa5*, *S100a6*, and *S100a10* in MM genes associated with a more aggressive disease[24,25] (**Figures S6C-F**).

Next, we directed our attention to the GRNs deregulated in non-IFN EC in MGUS and MM. We focus on the BIcγ1 model, as this model better mimics the slower progression observed in humans. The regulon activity analysis revealed a global decline in the activity of regulons during disease progression (**Figure 3E; supplemental Figure 7A-B**). Xbp1 regulon displayed the highest activity in MGUS EC (**Figure 3F; supplemental Figure 7B-C**). *Xbp1* has been reported to promote endothelial proliferation and angiogenesis through VEGF signaling in EC[26]. Furthermore, the overrepresentation of Fos regulon in EC from MGUS and MM suggested the importance of *Fos* not only in PC but also in BM EC[27] (**supplemental Figure 7C**). Interestingly, regulons from the ETS TFs family and Sox18 regulon recovered their activity in MM, which may imply the involvement of these regulons in the pathogenesis of the disease (**Figure 3F; supplemental Figure 7C**). Thus, our regulon network offers a comprehensive view of the interplay between crucial TFs and their targets that could drive the aberrant gene regulatory program of the endothelium during progression. Globally, our results support a transition state during progression from MGUS to MM driven by activation of molecular programs associated with angiogenesis and vascularization.

MSC undergo early transcriptional changes during pre-malignant MGUS that persist in overt MM

Recognizing MSC as crucial contributors to the MM microenvironment, we sought to unravel the distinct transcriptional alterations that arise during myeloma progression in non-IFN MSC. Global gene expression analysis revealed a similar transcriptional signature between MGUS and MM MSC, suggesting that the deregulation associated with the disease begins as early as the premalignant stage in the mesenchyme (**Figure 4A**). GSEA revealed an impaired osteogenic and adipogenic differentiation capacity of MSC during MM development, which is in line with previous reports in MM[9,10,28] (**Figure 4B; supplemental Table 4**). We also found a downregulation of genes involved in muscle development and regulation of smooth muscle cell proliferation, such as *Pi16*, *Myl6*, *Enpp2, Nupr1*, and *Shox2*, which have not been previously implicated in MM.

As changes were already identified during the MGUS stage, we compared MSC from MGUS and control (**supplemental Table 5**). MGUS MSC exhibited a significant overexpression of oxidative phosphorylation and ATP metabolism (*Uqcrq*, *Atp5j2*, *Atp5b*, *Cox6a1*, and *Cox5a*), protein folding (*Cd74*, *Hsp90b1*, *Prdx4*,

Pdia4, *Sh3glb1*, and *Hspe1*) and RNA processing (*Npm1*, *Prpf4b*, *Snrpd2*, *Srrm2*, and *Hnrnpa2b1*) (**Figure 4C-D**). Overexpression of *Cd74*, both in MSC and EC in MGUS stage, suggests its potential role in myeloma progression. Despite most of the transcriptional changes developed at the MGUS stage, we investigated the limited changes between MGUS and MM. Only 35 genes, of which 21 were upregulated and 14 were downregulated, showed differential expression between MM and MGUS MSC (**Figure 4E; supplemental Table 6**). ORA and GSEA revealed that functions related to the immune response were the most significantly affected, resembling the inflammatory profile described in MSC from MM patients[11] (**Figure 4F-G**). Likewise, by analyzing the transcriptional differences in MSC from MIcγ1 mice, we found highly overlapping genes with the previous large-scale analysis of the BIcγ1 model (**supplemental Figure 8**), highlighting the compromised osteogenic, adipogenic, and myogenic differentiation during myeloma development.

Finally, a regulon-identification analysis was conducted to gain a better understanding of the master regulators underlying molecular regulation of MSC during each stage of the disease in BIcγ1 model (**Figure 4H; supplemental Figure 9A**). Control MSC were highly enriched in well-known TFs mediating osteochondrogenic and adipogenic differentiation, including *Sp7*, *Dlx5*, *Sox8*, *Sox9,* and *Cebpa*, as well as an enhanced activity of Klf4 regulon, consistent with the impaired myogenic phenotype disclosed in MSC from MGUS and MM. Our analysis revealed that MGUS MSC exhibited high activity of AP-1 TFs family members that have been implicated in the development of MM[29]. Furthermore, in line with previous studies[30], Egr1 regulon showed lower activity in MSC from MM compared to MSC from MGUS. Interestingly, both MGUS and MM MSC showed high levels of Yy1 and Xbp1 regulons (**supplemental Figure 9B**). *Yy1* is known to contribute to tumorigenesis, while *Xbp1* supports MM cell growth and osteoclast formation[31,32]. On the other hand, Elk3 regulon was found to be active only in MSC from MM, suggesting a more invasive role as described in other cancers[33] (**supplemental Figure 9C**). Overall, our results suggest that MSC undergo a transcriptional shift at the stage of MGUS that persists throughout the course of the disease.

Altered crosstalk between EC and MSC with malignant PC is potentially associated to myeloma development

The crosstalk between tumor cells and the BM microenvironment plays a vital role in the pathogenesis of malignancy. Hence, to further understand the molecular mechanism underlying niche-PC communication pathways and how they change upon the development of the disease, we analyzed cellular communication networks through ligand-receptor (L-R) analysis using LIANA[34] (**Figure 5A**). Focusing our analysis on the interactions established between EC and PC, we identified interactions present in controls as well as the different stages of the disease (**supplemental Figure 10A; supplemental Table 7**). We next focused on specific interactions associated with malignant PC. We observed a decrease in the number of interactions during the MGUS stage, followed by an increase at the myeloma stage (**Figure 5A**). Interactions, including *Fn1* as ligand and *Insr* as receptor in EC and the *Cd93* as receptor in PC, were identified only in controls, while *Apoe* as ligand in EC and *Adam9*, *C3,* and *Vegfa* as ligand in PC were only found in MM (**Figure 5B**). These results support the role of angiogenesis in myeloma progression through *Vegfa* secretion from PC, which interacts predominantly with *Kdr*, *Nrp1*, and *Nrp2* receptors in EC. Interestingly, although the later interactions are poorly characterized in MM, several reports have correlated the high expression of Nrps with poor outcomes in multiple cancer subtypes[35]. Another disease-specific interaction was the *Efna1-Epha8*, which was predicted exclusively in MM, further implicating the ephrin pathway network in disease progression (**supplemental Figure 10B**).

We next examined in detail the potential interactions between MSC and PC. Interestingly, the L-R interactome analysis of communication between the PC and the BM microenvironment revealed that most interactions are borne from MSC, and these interactions decreased along disease progression (**Figure 5A; supplemental Figure 10C; supplemental Table 8**). Focusing on specific interactions observed during the different disease stages, we observed a decrease in *Itga4*-mediated communication during disease progression. Specifically, the loss of *Thbs1-Itga4* and *Thbs2-Itga4* interactions may be related to malignant PC extravasation (**Figure 5C**). MGUS and MM PC participate in different outgoing and incoming interactions that were not predicted in healthy controls and involved *Mfge8* and *Sdc3,* respectively (**Figure 5C, supplemental Figure 10D**). *MFGE8*, in addition to controlling apoptosis, cell remodeling, and immunomodulation, has been reported to promote disease progression through the interaction with integrin αvβ3 in melanoma microenvironment[36]. Further analysis of cellular communication during the MM stage showed specific interactions related to cell motility and adhesion processes (**supplemental Figure 10D**). MM PC overexpressed *Adam9* and *Adam17*, which are

predicted to bind to *Itgav* as well as *Itgb1* on MSC (**Figure 5C; supplemental Table 8**). Several studies have highlighted the crucial role of ADAMs in the reorganization of the extracellular matrix, which promotes cancer egress and invasion in various types of tumors[37].

Of note, we uncovered certain L-R pairs that were common between EC and MSC with PC along the progression of the disease (**Figure 5D**). Multiple predicted interactions between MSC and EC with PC via the *Itgb5* receptor initiated during the MGUS stage. Moreover, MGUS and MM PC interacted with both MSC and EC populations by secreting *Vegfa*, indicating the importance of angiogenesis in MM. Additionally, the interactions of *C3*-mediated responses between PC and the non-hematopoietic BM microenvironment observed in MM suggest that *C3* released by PC may maintain the immunosuppressive environment of MM, as described in other types of cancer. Overall, the description of specific interactions between EC, MSC and PC during the different stages of the disease should contribute to generating new hypotheses to improve the understanding of the pathogenesis during MM progression.

Transcriptional dynamics of human MSC during myeloma progression

To validate some of the results generated with the mouse models, we performed bulk RNA-seq with MSC obtained from BM samples of aged-matched HD, newly diagnosed MGUS (n = 5), SMM (n =2), or MM (n =10) patients isolated by FACS (**Figure 6A, supplemental Figure 11**). PCA revealed a clear distinction between human MM-MSC and HD-MSC, with MGUS-MSC and SMM-MSC mapped in between them, showing an evolving transcriptional trajectory (**Figure 6B**).

The comparison between MGUS-MSC and HD-MSC revealed 180 differentially expressed genes (DEGs) (**supplemental Figure 12A; supplemental Table 9**). We found a downregulation of genes related to osteoblast differentiation and osteogenesis, such as *TNN, SPP1,* and *A2M*, that is maintained in MSC from MM patients. Besides, MSC from MGUS patients showed a significant upregulation of genes related to cell cycle, protein folding, and ubiquitination, as well as the suppression of MSC senescence (**supplemental Figure 12B**). Remarkably, MSC from MGUS patients revealed enrichment in terms related to mitochondrial activity and ATP metabolism (*MCUR1, MCUB, ATP6V0D1*) and oxidative stress (*ZNF622, HIF1A*), as previously stated in our mouse data. The deregulation of these signaling pathways in MSC from MGUS patients suggests that alterations in the tumor microenvironment play a crucial role in MM pathogenesis.

The comparison between MSC from MGUS and MM patients yielded the fewest number of DEGs, consistent with the results obtained in the mouse model where MGUS and MM MSCs showed high transcriptional similarity. Specifically, we identified 74 DEGs (41 upregulated and 33 downregulated) defining stromal changes in active MM (**supplemental Figure 12C; supplemental Table 10**). Functional enrichment analysis revealed a significant increase in inflammatory function in MM-MSC, suggesting that a pro-inflammatory microenvironment is required for transition from premalignant stages to active myeloma (**supplemental Figure 12D**). In addition, MSC from MM patients demonstrated an upregulation of certain genes associated with Epithelial-Mesenchymal Transition (EMT), such as *PHLDA1*, *FOXC2*, *PPA1*, and *CTNNB1*. This finding aligns with the potential of MSC to induce EMT through communication via extracellular vesicles, a process that has been linked with more aggressive MM and the acquisitions of drug resistance[38].

The pairwise comparison of MSC from HD *versus* MM patients confirmed the defective osteogenic differentiation and a dysfunctional immunomodulatory ability in MM-MSC (**Figure 6C, supplemental Figure 12E-F; supplemental Table 11**). Interestingly, consistent with our results in the mouse model (**Figure 4B**), human MM-MSC showed impaired myogenic differentiation, with downregulating of genes related to skeletal muscle differentiation, compared with HD-MSC (**supplemental Figure 12F**). GSEA revealed enrichment of IFN response and TNF-α signaling via NF-KB consistent with the results from our mouse model. In addition, the IFN signature we previously identified in the murine MM BM microenvironment was associated with a specific subset of MM patients when we projected it onto our human bulk RNA-seq data (**Figure 6E, supplemental Figure 12G**). Notably, this IFN enrichment was not associated with any consistent genetic alteration across patients.

Finally, we sought to determine the changes in TFs activity during myeloma progression. Several TFs activity scores in MSC from MGUS and MM patients showed an upregulation during disease progression (**Figure 6F**).

Specifically, enhanced activity of TFs related to the NF-KB pathway, such as *NF-KB, NKFB1,* and *RELA*, were observed in MSC from MGUS and MM patients. Furthermore, MM-MSC exhibited increased activity of the IFN regulatory factors, *IRF1* and *IRF3*, which again emphasized the importance of the IFN signaling for the disease. In conclusion, the transcriptional reprogramming of MSC during disease progression supports a consistent pattern shared by both human patients and mouse models.

DISCUSSION

Over the last decades, our understanding of MM biology has advanced significantly, with scRNA-seq analysis driving profound insights into MM heterogeneity, tumorigenesis, and extramedullary metastasis[39–43]. An important part of this novel understanding is the evidence that the BM microenvironment contributes to the outgrowth and relapse of this neoplasia and exerts a strong supportive function for the expansion of malignant PC[4,11,44,45]. Although significant progress has been made, research has mainly concentrated on PC and their immune microenvironment, highlighting the need for additional exploration of other components of the BM microenvironment. Here, using our BIcγ and MIcγ murine models[13], which mirrors MM pathogenesis, we provide a comprehensive overview of the transcriptional deregulation within the BM EC and MSC during the transition from healthy BM to MGUS and eventually MM. Our single-cell transcriptomic analysis has revealed the temporal remodeling of the BM microenvironment during MM development, detailing the longitudinal transcriptional alterations and the dynamic interplay between PC, EC, and MSC. Moreover, using bulk RNA-seq of human MSC from MGUS, SMM, and MM patients, we observe consistency of transcriptional reprogramming of these cells across human patients and mouse models.

A fundamental finding of our study is the identification of a myeloma-specific IFN signature that affects several cellular components of the BM microenvironment. Notably, we observed that this MM-IFN signature is not exclusive to EC, but also extends to MSC, suggesting a shared response of the BM microenvironment during myeloma progression. While the IFN-related signature has previously been reported in PC and the immune environment[4,5,46], our data reveal that, in fact, non-hematopoietic cells also exhibit the IFN fingerprint. This IFN-related signature is marked by the upregulation of IFN-inducible genes and involved master regulators such as *Irf7*, *Stat1*, and *Stat2*. Previous studies highlighted *STAT3* as an oncogenic driver in MM and reported the role of *STAT1* in promoting MM cell migration and BM homing[47]. The heightened correlation between Stat signaling and the IFN in the EC and MSC from MM expands the emerging tumor-promoting roles described for Stat1 and Stat2 pathways.

This IFN-inducible signature not only suggests an active response pathway but also unveils a new potential role in immune modulation within the non-hematopoietic BM microenvironment. We speculate that the reprogramming of EC and MSC into IFN-inducible signaling may contribute to the remodeling of immune functions, potentially promoting T-cell exhaustion under inflammatory conditions[17]. This association hints at a complex interplay between the immune system and the BM microenvironment in myeloma, suggesting the immunomodulatory potential of EC and MSC[48]. Extending our observations to human MM data, we reinforce the relevance and applicability of the identified MM-IFN signature to the clinical setting. The enrichment of the murine IFN fingerprint in MSC from a subset of MM patients, along with its correlation with previous studies[4,5], may help to define a narrower patient group within the MM heterogeneity. This role of IFN signaling shaping the BM environment during myeloma progression, could be particularly relevant in patients with a more inflammatory component.

Examining the molecular mechanisms governing myeloma progression within non-IFN EC identified transcriptional and regulatory changes during MGUS with the upregulation of genes related to protein folding and metal ion homeostasis, such as heat shock proteins and metallothioneins. The upregulation of genes such as *Slpi*, *Mt1*, *Mt2*, and *Hspa5*, known for their roles in tumor angiogenesis and pro-survival signaling[49,50] may serve as promising early indicators of myeloma development in EC. Remarkably, our analysis unveils unexpected upregulation of genes like *Cd74*, *Tmem176a*, and *Nme2* in MGUS EC, indicating their potential involvement in MM pathogenesis, although their precise roles require further elucidation. Comparing the transcriptional profiles of MGUS and MM EC reveals dynamic changes associated with disease progression, including alterations in angiogenesis, endothelial migration, and lipid metabolism, reflecting the vascular phenotype and metabolic alterations characteristic of MM EC. The upregulation of *Sox18*, a key regulator of vasculature development and cancer progression[23], in MM EC highlights its targeting potential.

Unlike EC, transcriptional changes in MSC were already present during the MGUS stage. These changes mirror those seen in advanced MM, including impairment in osteogenic and adipogenic differentiation, as previously reported in myeloma[9,10]. Thereby, MGUS MSC may exhibit a reshaping of their differentiation capacity and metabolism to support the growth of MM cells from the early stages of the disease[51]. Despite these similarities between MGUS and MM-MSC, comparative gene expression analysis showed additional changes in MSC from MM. In a recent study, De Jong et al. identified a sub-population of MSC within the microenvironment of MM patients, exhibiting an inflammatory transcriptional signature[11]. Building upon these findings, we observed the enrichment of cytokines, TNFα, and NF-κB signaling pathways, supporting the contribution of MM MSC to the pro-inflammatory tumor microenvironment. Additionally, the upregulation of type 1 IFN signaling pathways in MM MSC reinforces the essential role of IFN in the progression of the disease. The selective regulation of MGUS MSC by Egr1 and AP-1 TF[29,30], as well as the involvement of the Elk3 regulon in maintaining an invasive phenotype in MM MSCs, highlight the complex transcriptional networks underlying disease pathogenesis, making them potential biomarkers and therapeutic targets for MM treatment.

Understanding the intricate molecular crosstalk between the BM microenvironment cells and malignant PC is crucial for unraveling the mechanisms underlying tumor progression. Our cell communication analysis identified a tapestry of interactions targeting both myeloma cells and their surrounding microenvironment that shapes the landscape from MGUS to MM. This L-R analysis revealed altered crosstalk between EC, MSC cells, and PC, primarily orchestrated by MSC. To our knowledge, this study represents the first investigation into the predicted interactions between endothelial and mesenchymal resident cells with malignant PC along the continuum of MM disease progression. Analysis of interactions between EC and PC uncovered a communication decline in the MGUS stage that was restored in MM, highlighting the evolving relationship between them during myeloma progression. These interactions, especially those involving *Vegfa* secretion from PC and its interactions with *Kdr*, *Nrp1*, and *Nrp2* endothelial receptors, emphasize the importance of angiogenesis as a hallmark of MM development. Additionally, identifying specific interactions exclusively in MM, such as *Efna1-Epha8*, implicates the ephrin pathway network in disease development. The involvement of this axis in the pathogenesis of other cancers, such as glioblastoma, melanoma, or breast cancer, has been described[52]. Remarkably, predicted interactions between MSC and PC were associated with adhesion, extracellular matrix organization, and migration pathway networks, suggesting their crucial role in promoting MM cell extravasation, emphasizing the substantial role of MSC-PC crosstalk as a potential contributor to the dissemination of the disease.

Challenges associated with the scarcity of BM stromal and EC hinder scRNA-seq studies in humans. Our bulk RNA-seq results showed that MSC from human patients exhibit compromised differentiation capacities, which is also apparent in the early stages of disease progression, in line with our findings in mice. Similarly, human MSC also experiences a metabolic swift in MGUS stage associated with increased protein folding ability. Human MM-MSC exhibit higher expression of angiogenesis and inflammation-related genes This inflammatory microenvironment could aid in the immune evasion of MM by suppressing T cells, which could be enhanced by the MM-MSC overexpressing genes such as *LGALS9* or *FADD*[53,54]. On the contrary, some genes related to extracellular matrix organization such as *SDC2*, *SDC4*, *MMP14* or *LAMC1* that appear to be upregulated in human MM-MSC, are downregulated in murine MM MSC. This discrepancy between both species could be explained by the heterogeneity of MM patients and their different clinical characteristics. Notably, all the mentioned genes are linked to patients with poor outcomes, which might not align with the characteristics of our mouse BIcγ1 model.

In summary, the utilization of the BIcγ1 myeloma murine model, which recapitulates the progression from MGUS to full-blown MM, coupled with the utilization of scRNA-seq and the bioinformatic toolkit associated (e.g., GRN and cellular interactome among others), enables a comprehensive dissection of the non-hematopoietic BM microenvironment. The myeloma-specific IFN signature, the distinct transition stress state in MGUS EC, the early transcriptional shifts in MSC, and the evolving cellular communication networks collectively reinforce the role of the stromal and endothelial BM microenvironment in MM development from premalignant stages. Additionally, validating our murine model findings in human patients strengthens the translational relevance of our study and emphasizes the importance of further investigation into the molecular

mechanisms underlying MM progression. Overall, these results suggest that changes in the non-hematopoietic fraction of the BM microenvironment could create a more permissive microenvironment that favors the development of MM from premalignant stages.

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AUTHORSHIP CONTRIBUTIONS
I.C. and M.C. processed murine and human BM samples. I.C. conceptualized the study, analyzed and interpreted the scRNA-seq experiments data, and wrote the paper; M.C. analyzed and interpreted the bulk RNA-seq experiments data; A.B. performed the cell-cell computational analysis; M.L. and J.A.M.C. designed and provided the mice models; A.V., P.S.M., and P.A.R. performed the scRNA-seq and bulk RNA-seq experiments. D.A. and A.L. performed the FACS sorting. M.M.B., D.Q.A., I.S.G., and J.R. provided the BM samples from HD; P.R.O. and L.E.T.A provided the BM samples from MGUS, SMM, and MM patients; D.G.C., with help from M.H., I.M.S, supervised the computational work; I.A.C. and B.S. designed the research experiments; F.P., D.G.C. and I.A.C conceived and directed the research project.
All authors actively participated in the discussions underlying this manuscript. I.C., M.C., I.A.C., D.G.C., and F.P. discussed the results and wrote the final manuscript. All authors contributed to, read, and approved the final manuscript.

DISCLOSURE OF CONFLICTS OF INTEREST
A patent on the knowhow and experimental use of the BIcγ1 and MIcγ1 mouse models of MM has been licensed to MIMO Biosciences. The authors declare no competing interests.

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FIGURE LEGENDS

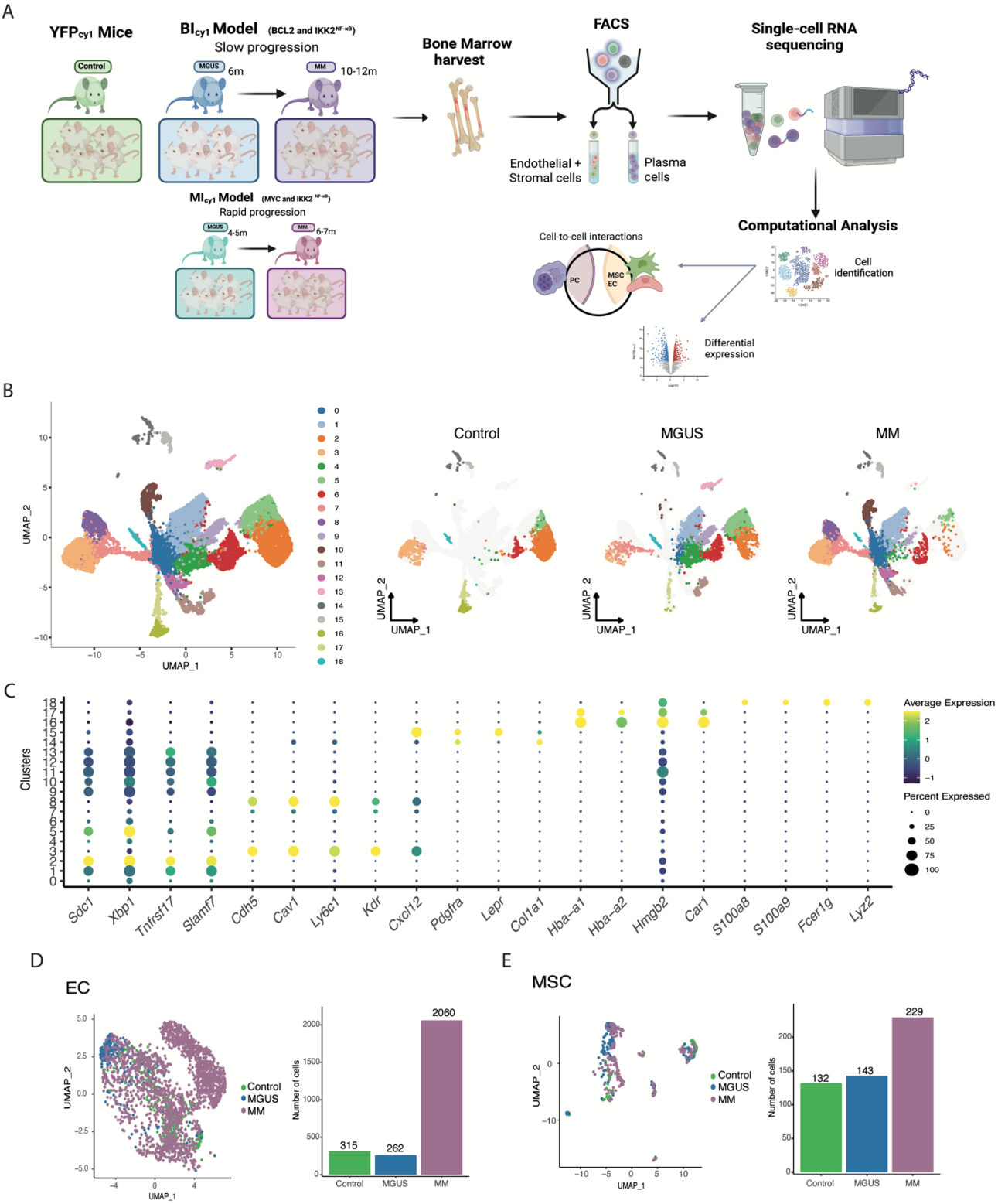

Figure 1. Single-cell transcriptomic profiling of BM murine cells in control, MGUS, and MM. (A) Experiment overview. Schematic view of control and BIcγ1 mice and experimental workflow for the isolation and scRNA-seq analysis of PC and BM MSC and EC cells. (B) UMAP representation of murine BM microenvironment control, MGUS, and MM cells colored by cell clustering after merging samples. Right panel: Original UMAP split into control, MGUS, and MM datasets. (C) Dot plot of canonical markers used to define PC, EC, MSC, erythrocyte precursors, and neutrophils. The dot size reflects the percentage of cells within the cluster expressing each gene, and the color represents the average expression level. (D-E) Left panel:

UMAP projection showing the distribution of control, MGUS, and MM cells for EC (D) and MSC (E) Right panel: Bar plot depicting the total number of cells per stage for EC (D) and MSC (E).

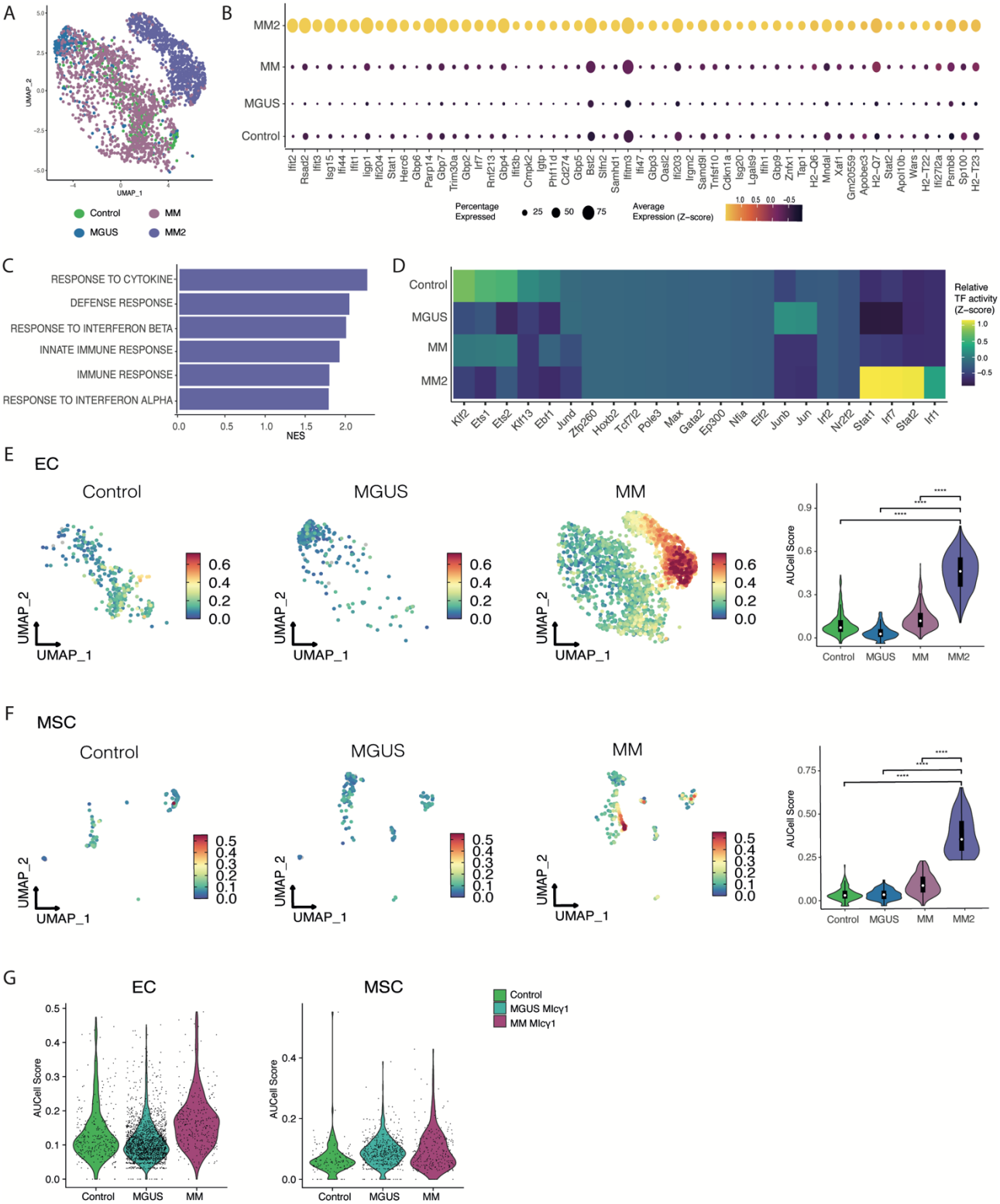

Figure 2. Identification of a myeloma specific IFN signature in the BM microenvironment. (A) UMAP dimension reduction of EC revealing a transcriptionally distinct population in MM. (B) Dot plot visualization of the markers defining EC MM2 cluster. Average gene expression is shown by color intensity, and the percentage of cells expressing each gene is shown by size. (C) Bar charts of enriched Gene Ontology (GO) terms from GSEA (FDR < 0.05) computed using MM2 cluster markers. The horizontal axis represents the

normalized enrichment scores (NES). (D) Heatmap representing z-scaled mean regulon activity score of inferred top specific regulons for each stage. (E) Left panel: UMAP plot illustrating the distribution of the IFN-related signature in EC across control, MGUS, and MM. Right panel: Violin plots showing AUC-score for IFN-related signature within EC in control, MGUS, MM and MM2 cells. (F) Left panel: UMAP plot illustrating the distribution of the IFN-related signature in MSC across control, MGUS, and MM. Right panel: Violin plots showing AUC-score for IFN-related signature within MSC in control, MGUS, MM and MM2 cells. (G) Violin plots of the AUC-score for IFN-related signature within EC (left panel) and MSC (right panel) in control, MGUS, and MM cells from the MIcγ1model.

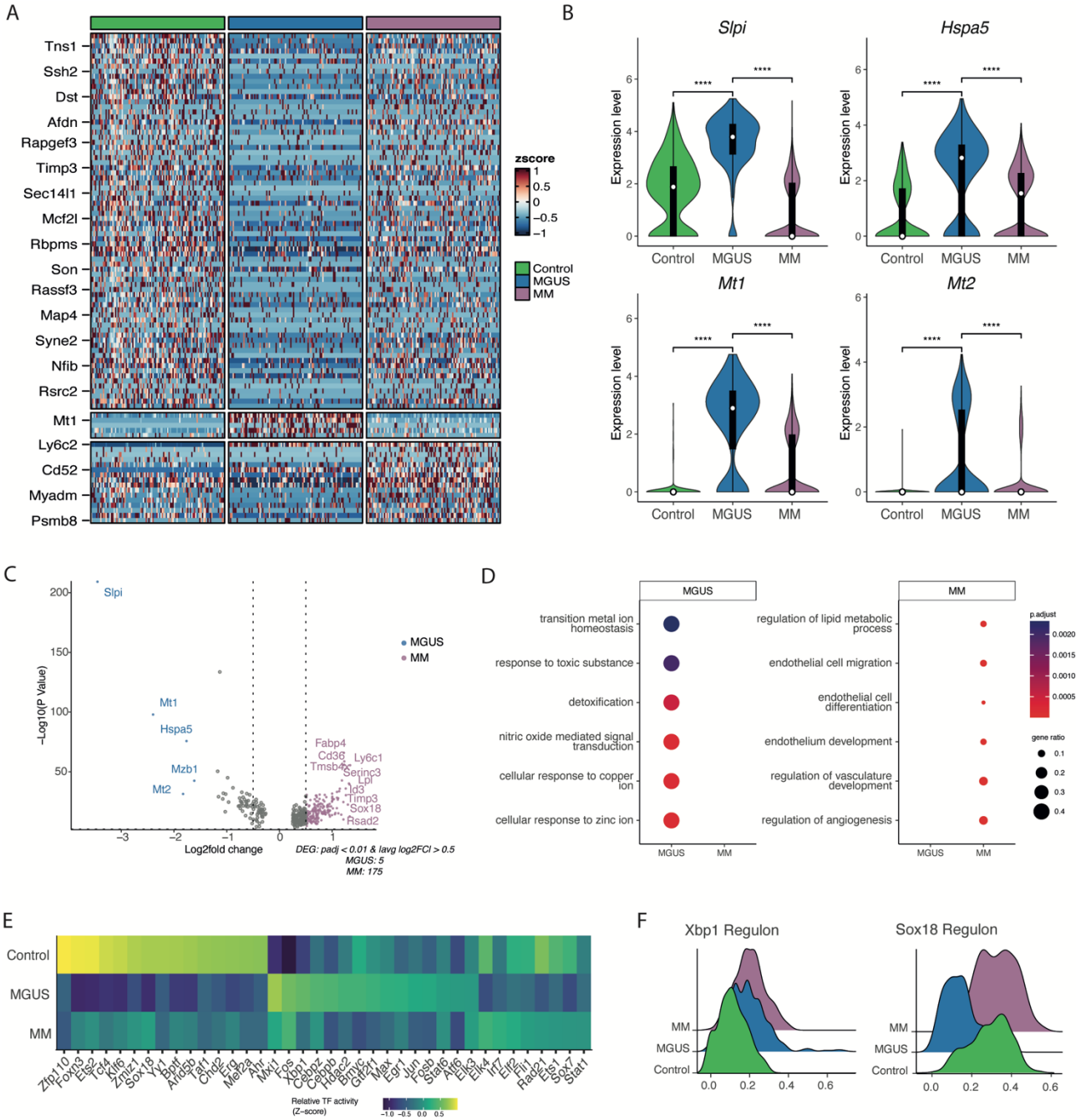

Figure 3. EC are characterized by a stress pre-vascular state during MGUS stage. (A) Heatmap of the DEG between control, MGUS, and MM cells. Color represents the z-score scaled expression values. (B) Violin plots showing the distribution of expression of genes upregulated in MGUS EC. (C) Volcano plot of the DEGs in MGUS vs. MM EC. The y-axis represents the -log10(p-value) and the x-axis represents the log2(fold change). The dot's color denotes the disease stage for which that DEG was detected, with grey dots representing

non-significant genes. (D) Significant terms derived from ORA between EC from MGUS and MM. (E) Heatmap representing z-scaled mean regulon activity score of inferred top specific regulons for EC in each stage. (F) Density plots showing the activity score of Xbp1 and Sox18 regulons in EC.

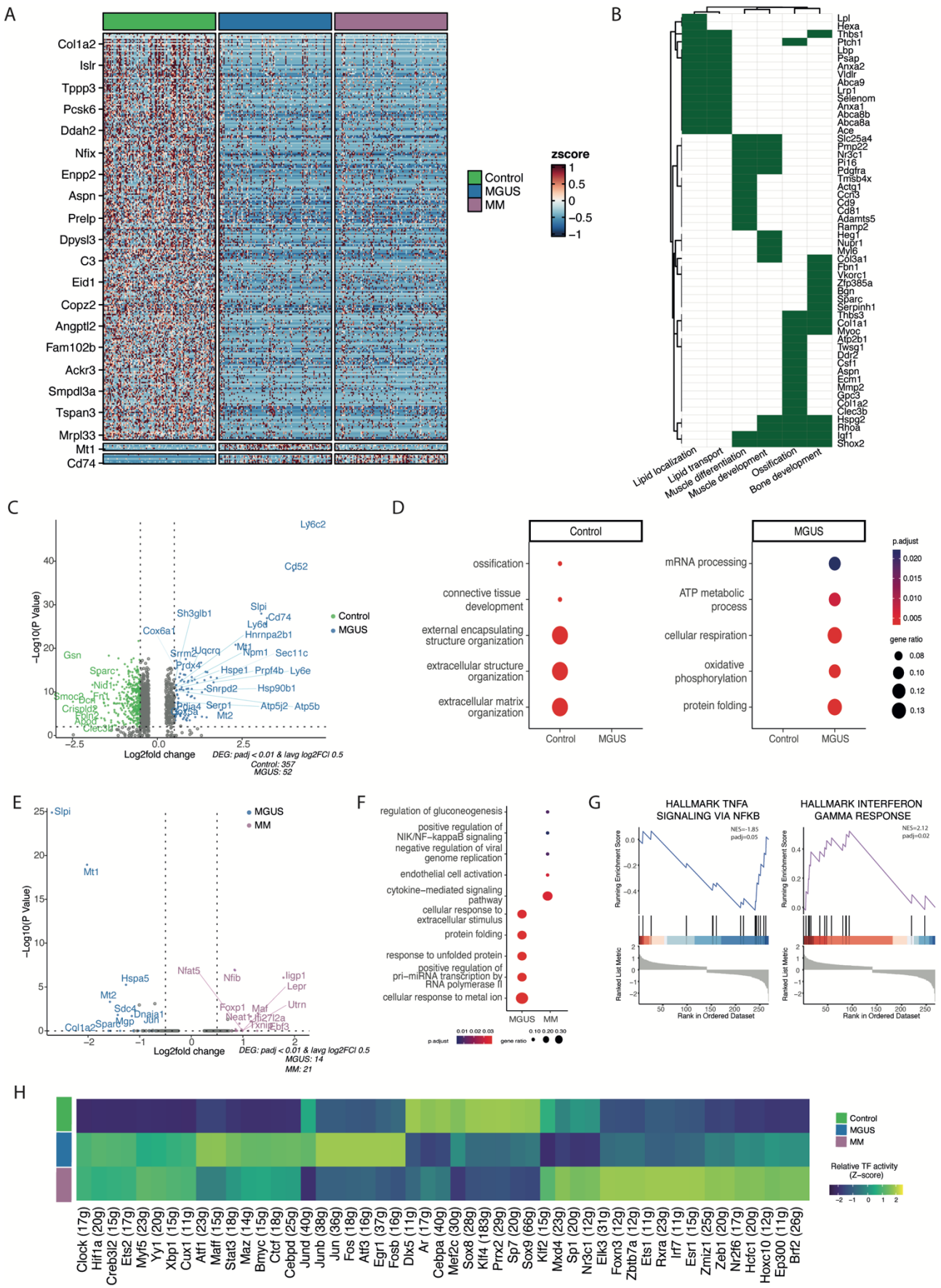

Figure 4. MSC undergo early transcriptional changes during pre-malignant MGUS that persist in overt MM. (A) Heatmap depicting DEG between MSC in control, MGUS, and MM. Color represents the z-score scaled expression values. (B) Heatmap of significantly enriched GO terms in control MSC with the associated

genes. The green color represents the presence of the gene in each GO term. (C) Volcano plot of the DEGs from the comparison between MSC from control and MGUS. The y-axis represents the -log10(p-value), and the x-axis represents the log2(fold change) of the gene. The dot's color denotes the disease stage for which that DEG was detected, with grey dots representing non-significant genes. (D) Significant terms derived from ORA of MSC from control vs MGUS. (E) Volcano plot of the DEGs in MSC from MGUS vs. MM. (F) ORA between MSC from MGUS and MM. (G) GSEA plots for the gene sets 'TNFα signaling via NFκB' (left panel) and 'Interferon gamma response' (right panel) in MGUS vs MM MSC. (H) Heatmap of mean regulon activity score of significantly enriched regulons in each stage. The color scale represents the z-score of the activity scaled by row.

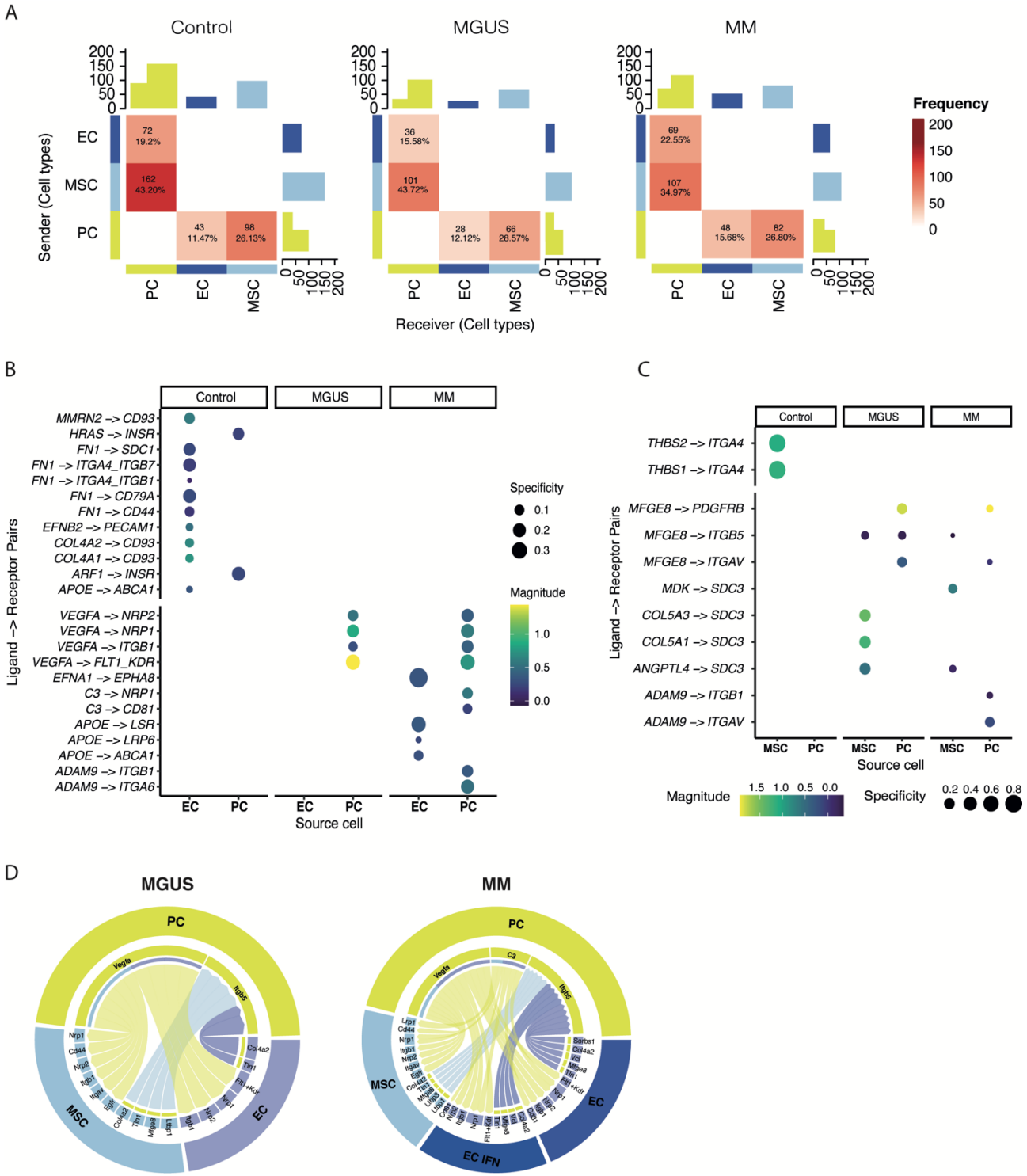

Figure 5. Altered crosstalk between EC and MSC with PC are potentially associated to myeloma

development. (A) Heatmap showing the number of predicted cellular interactions between EC, MSC and PC. Block sizes and colors are proportional to the number of ligand-receptor pairs. (B-C) Dot plots of representative ligand-receptor interactions between PC and EC (B) or MSC (C) during disease progression. Dot size and color indicate the specificity and the strength of the interaction, respectively. (D) Chord diagram showing shared relevant signaling network between EC, MSC and PC in the MGUS and MM stages. Colors and width represent signal senders and strength, respectively.

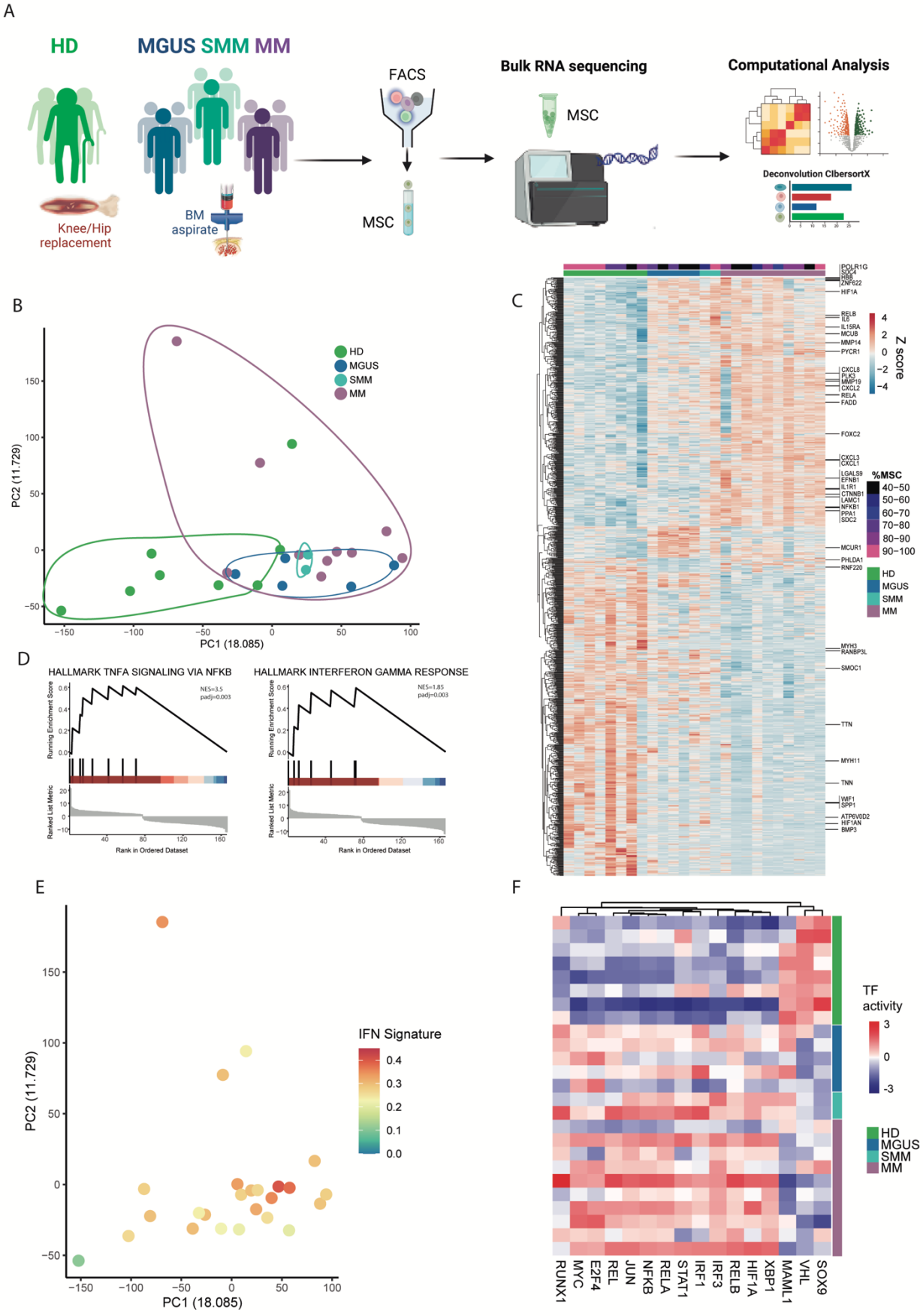

Figure 6. Transcriptional dynamics of human MSC during myeloma progression. (A) Experimental

workflow used for the isolation and bulk RNA-seq analysis of human BM MSC. (B) PCA of MSC expression profiles shows clustering by disease stage. The color of the dots represents disease stages. (C) Summary heatmap showing the expression profiles across samples of DEGs identified in each pairwise comparison along disease progression. (D) GSEA plots for the gene sets 'TNFα signaling via NFκB' (left panel) and 'Interferon gamma response' (right panel) in the comparison between HD and MM patients. (E) PCA plot representing the IFN signature score per patient. (F) Heatmap representing the variation of TF activity score across samples.

SUPPLEMENTARY MATERIAL

Supplemental Methods

Supplemental Figures

- Supplemental Figure 1: Cell sorting strategy for murine scRNA-seq of BM niche cells.
- Supplemental Figure 2: scRNA-seq Analysis of murine BM niche cells.
- Supplemental Figure 3: Flow cytometric quantification of the percentage of EC, MSC, and OB in control, MGUS, and MM BIcγ1 mice.
- Supplemental Figure 4: Additional information on the MM IFN-related signature and validation in the MIcγ1 model and scRNA-seq human data.
- Supplemental Figure 5: Additional visualizations of transcriptional differences detected between control, MGUS, and MM EC excluding MM IFN cells.
- Supplemental Figure 6: Transcriptional remodeling of EC during myeloma progression in the MIcγ1 model.
- Supplemental Figure 7: Gene regulatory network of EC during MM progression excluding MM IFN signal.
- Supplemental Figure 8: Transcriptional remodeling of MSC during myeloma development in the MIcγ1 model.
- Supplemental Figure 9: Gene regulatory network of MSC during MM progression excluding MM IFN signal.
- Supplemental Figure 10: Cell-cell communication analysis between niche and PC.
- Supplemental Figure 11: Cell sorting strategy for isolation of human BM MSC.
- Supplemental Figure 12: Additional information on the transcriptional alterations of human MSC during myeloma progression.
- Supplemental Figure 13: Evaluation of MSC proportion in bulk RNA-seq human samples.

Supplemental Tables

- Supplemental Table 1: Clinical characteristics of all individuals of bulk RNA-seq.
- Supplemental Table 2: DEGs between MGUS and control EC.
- Supplemental Table 3: DEGs between MM and MGUS EC.
- Supplemental Table 4: ORA between all stages MSC.
- Supplemental Table 5: DEGs between MGUS and control MSC.
- Supplemental Table 6: DEGs between MM and MGUS MSC.
- Supplemental Table 7: Full list of significant L-R pairs involved in EC and PC communication.
- Supplemental Table 8: Full list of significant L-R pairs involved in MSC and PC communication.
- Supplemental Table 9: Comparison between human MGUS-MSC and HD-MSC.
- Supplemental Table 10: Comparison between human MM-MSC and MGUS-MSC.
- Supplemental Table 11: Comparison between human MM -MSC and HD-MSC.
- Supplemental Table 12: Percentage of BM PC infiltration per mouse.