Altered mesenchymal and endothelial subsets in interstitial bone marrow and focal lesions in myeloma patients and SCID-hu mice

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Abstract

In myeloma, the bone marrow (BM) stroma mediates tumor growth directly and indirectly through the alteration of BM niches. The mesenchymal and endothelial cell subsets altered in the interstitial BM and focal lesions (FL) of patients newly diagnosed with myeloma, as well as in the myeloma-supportive human bone of the SCID-hu mouse model, were identified using single-cell atlases and gene expression profiling. The mesenchymal compartment showed enriched cells reflecting matrix cancer-associated fibroblasts (CAF) and vascular CAF/pericytes in FL compared to interstitial BM and in myeloma interstitial BM compared to healthy donors. Patients with myeloma possessed inflammatory mesenchymal stem cell (MSC) subsets that expressed genes resembling various CAF, including antigen-presenting CAF and genes composing the diagnostic three-gene MSC score for myeloma. The vascular compartment in FL showed reduced expression of genes representing specialized bone-remodeling endothelial cells and upregulation of genes reflecting angiogenic endothelial cells. We identified stroma factor-expressing *CYR61*/CCN1^{+} myeloid cells that were detected in myeloma but not in donors' bones. *CYR61*/CCN1^{+} myeloid cells co-expressed CD14, and their numbers were lower in the interstitial BM of patients with high-risk *versus* low-risk disease, and rare in FL. These cells were enriched in the BM aspirate lipid layer. The SCID-hu model showed changes in mesenchymal and endothelial cell subsets resembling clinical FL, except for inflammatory mesenchymal cells, which were present in the model but suppressed in FL. Overall, this study provides a comprehensive assessment of the altered stroma in myeloma and identifies previously unappreciated microenvironmental cell subsets. Specimens and data were obtained from patients enrolled in our TT2-TT5 Total Therapy clinical trials (*clincaltrials gov. Identifier*: *NCT00083551, NCT00081939, NCT00572169, NCT00734877, NCT00869232*) before initiation of treatment.

Introduction

The malignant plasma cells in multiple myeloma (MM) typically reside within the hematopoietic bone marrow (BM), often in a form of focal lesions (FL), resulting in alterations in the BM microenviroment.[1,2] Under normal physiological conditions and in MM, the main non-hematopoietic compartment in BM primarily includes cells of mesenchymal and endothelial origin.[3-5] Since the landmark publications by Caligaris-Cappio *et al*., 1991 on the role of stromal cells in the growth of MM,[6] and the clinical study by Barlogie *et al*., 2006 integrating thalidomide as a drug possessing antiangiogenic activity,[7] many studies sought to understand the stroma components in MM, mainly mesenchymal stem cells (MSC),[8,9] osteoblasts,[10] adipocytes,[11] osteocytes,[12] and angiogenic endothelial cells (EC).[13] FL are exceptional sites presenting an altered ecosystem with regard to suppressed hematopoietic cells and enrichment of matrix genes-expressing MSC and neoangiogenic EC,[5,14] suggesting a microenvironment that is optimal for the expansion of MM cells.

Global gene expression profiling (GEP) of whole bone biopsies were used to characterize changes in main hematopoietic and non-hematopoietic BM cells in MM.[15] These GEP data also helped identify *CYR61* and its encoding protein, CCN1, as a stroma gene upregulated in

MM bone and associated with a favorable prognosis.[16] We also created a diagnostic three-gene MSC score for MM[17] and identified specific cell population alterations in MM, such as suppression of small adipocytes adjacent to the hematopoietic cells in red marrow[11] and detached pericytes in high infiltrating areas.[18]

Recently, various cellular atlases were established based on single-cell RNA sequencing (scRNA-seq) of bone and BM stromal cells[8,9,19,20] and of cancer-associated fibroblasts (CAF).[21] These cellular atlases and ample GEP data of bone biopsy samples from healthy individuals and MM patients and of magnetic-resonance imaging (MRI)-guided focal FL from MM patients provide an opportunity to link single-cell-defined cellular subsets in clinical biopsies. Moreover, we previously demonstrated that the experimental SCID-hu model reproducibly supports primary MM.[22-24] Underscoring the cellular stroma that supports primary MM in this model is translationally important for understanding clinical disease. Overall, our goals in this study are to identify the main mesenchymal and endothelial subsets altered in MM and shed light on those stroma subsets that could serve as diagnostic biomarkers and targeted to control disease progression.

Methods

Patient samples

Specimens were obtained after institutional review board approved by the University of Arkansas for Medical Sciences in accordance with the Declaration of Helsinki. All MM samples were collected from MM patients that had been enrolled into our TT2-TT5 protocols, a detailed description of which has been published previously.[15,17] All TT protocols were approved by the Institutional Review Board of the University of Arkansas for Medical Sciences. All patients signed informed consent prior to enrollment in keeping with institutional, federal, and international guidelines.

Cellular clustering

The main CAF, mesenchymal and endothelial cell types, were selected based on several publicly available cellular atlases that applied single-cell RNA sequencing (scRNA-seq) technology.[19-21] The clustering of MSC and endothelial cells using scRNA-seq from BM aspirates of control healthy donors and patients newly diagnosed with MM (NDMM) was described by de Jong *et al.*, 2021[8] and 2024.[9] We selected representative top genes of the main cell types, focusing on genes unique to each cell type. The publicly available whole bone biopsy GEP data from age-matched healthy donors and newly diagnosed MM patients are described in detail elsewhere.[15,17] Briefly, GEP data were available for baseline BM biopsy samples from healthy donors (N=34) and patients with NDMM (N=354).[15,17] Also, paired biopsy samples (i.e., from the same MM patient) of interstitial random BM of the iliac crest or from computed tomography (CT)-guided fine-needle biopsies of MRI-defined FL (N=49) were available. GEP procedure and data of the nonmyelomatious human bone from SCID-hu mice (N=6) are described elsewhere.[25]

Immunohistochemistry

Immunohistochemistry (IHC) was performed on histological, clinical bone biopsies taken from the iliac crest of patients with monoclonal gammopathy of undetermined significance (MGUS)/smoldering MM (SMM) (N=10) and ND-MM patients whose MM cells were molecularly classified based on GEP gene score[26] as low-risk (N=7) and high-risk (N=6). Control samples were obtained from femur heads of age-matched individuals who underwent orthopedic surgery at the University of Arkansas for Medical Sciences (N=5).[11] The preparation of BM cells from ficolled BM aspirates and lipid layers on cytospins is described in detail elsewhere.[11] The CCN1 primary antibody was purchased from Atlas Antibodies (Stockholm, Sweden). An Olympus BH2 microscope (Olympus, Melville, NY, USA) was used to obtain images with a SPOT 2 digital camera (Diagnostic Instruments Inc., Sterling Heights, MI, USA). Adobe Photoshop version 10 (Adobe Systems, San Jose, CA, USA) was used to process the images.[11]

CCN1^{+}CD14^{+} hematopoietic cells

BM aspirate and BM lipid layer cells were subjected to multicolor flow cytometry using CD45 and CD14 (BD Biosciences), and CCN1 (Atlas Antibodies) conjugated antibodies. Briefly, the viable cells were initially stained for cell surface CD45 and CD14, then fixed with Histochoice (Sigma-Aldrich, St. Louis, MO) and stained for cellular CCN1. When indicated, CD14^{+} cells were isolated from the BM lipid layer fraction by bead separation using CD14 immunobeads from Miltenyi Biotec (Auburn, CA) and then subjected to immunohistochemistry for detection of CCN1 expression.

Statistical analysis

The GEP values, expressed as a difference in log2 values between the analyzed groups, were compared using Student's *t* test. Numbers of CCN1^{+} myeloid cells in bone sections were quantified in four overlapping millimeter-square areas in x20 original magnification images, and the numbers of cells between groups were compared using Student's *t* test.

Results

Altered subsets of mesenchymal cells and endothelial cells

We used several scRNA-seq-based atlases that char-

acterize the main CAF subsets in solid tumors[21,27] and the main EC subsets in BM[19,20] and used GEP data from whole bone biopsies[15,17] to shed light on changes in the expression of genes that characterize these subsets in MM. We utilized three sets of GEP data: MRI-guided FL samples and interstitial BM samples from NDMM patients (MMBX) or from healthy donors (normal biopsies, NBX). Based on Cords *et al.*, 2023[21,27] there are seven main types of CAF, including matrix (mCAF), inflammatory (iCAF), vessel-associated (vCAF), interferon-response (ifnCAF), antigen-presenting (apCAF), reticular-like (rCAF), and dividing (dCAF), while pericytes are included as a separate cell type. We focused on mCAF and iCAF, which are the two most abundant CAF types. Due to their overlapping nature, we combined the vCAF and pericytes into one group and the ifnCAF and rCAF into another group. We excluded apCAF and dCAF because the top genes that define them are not cell-type specific. After selecting the top genes for each group, we examined their expression in the GEP data. We found that the expression of genes reflecting mCAF (e.g., *MMP11*), pericytes/vCAF (e.g., *COL4A1, RGS5*), and infCAF/rCAF (e.g., *CXCL9*) were higher in FL compared to MMBX, while expression of genes reflecting iCAF (e.g., *APOD, CXCL12*) was underexpressed in FL compared to MMBX (Figure 1A). When comparing MMBX and NBX there was a similar pattern of changes with regard to mCAF, pericytes/vCAF, and infCAF/rCAF as seen in FL, though the changes in iCAF genes were not as consistent in FL compared to MMBX (Figure 1B). In particular, *CXCL14* was underexpressed in FL yet expressed at higher levels in MMBX compared to NBX. Overall, these findings indicate increased expression of genes associated with matrix, vascular, and interferon-induced mesenchymal cells in the interstitial BM of MM patients, with the highest expression in FL. In contrast, the expression of genes reflecting inflammatory mesenchymal cells (iCAF) was significantly reduced in FL and, to a lesser extent, in MMBX compared to NBX.

With regard to the BM vasculature, we incorporated the recent classification of BM EC by Mohanakrishnan *et al.*, 2024[20] who segregated the EC into five subsets, including metaphyseal (mpEC), bone marrow (bmEC), arterial (aEC), remodeling (rEC), and proliferating (pEC). According to the authors, mpEC reflect previously recognized type H EC and bmEC reflect type L EC, while rEC are previously unrecognized specialized post-arterial capillaries that facilitate bone remodeling. After selecting the top genes for each group, we examined their expression in the GEP data, similar to that performed based on CAF classification. The FL were characterized by underexpression of genes defining rEC (e.g., *CTNNBIP1*) and bmEC (e.g., *SELP*) and upregulation of genes associated with mpEC (*VWA1*) compared to MMBX. The genes reflecting the aEC subset were not significantly different between FLs and MMBX (Figure 1C). MMBX and NBX had similar patterns of change with regard to rEC and mpEC; however, there were no clear differences with regard to bmEC, whereas genes associated with aEC had higher expression in MMBX compared to NBX (Figure 1D).

To assess the impact of specific cellular subsets on the response to therapies, we compared the overall survival of Total Therapy patients treated without novel drugs (TT2-), with the addition of thalidomide (TT2+), or with the addition of thalidomide and bortezomib (TT3a).[28] We focused on genes representing the subsets mCAF, iCAF, rEC, and mpEC. Higher expression of a gene related to iCAF and lower expression of genes related to mCAF and mpEC were associated with favorable outcomes in TT2+ and TT3a. In contrast, higher expression of the gene related to rEC was associated with a favorable outcome in TT3a only (*Online Supplementary Figure S1*). Overall, rEC, which are essential for bone remodeling,[20] were downregulated in MM, while mpEC, which are also associated with angiogenesis in bones,[29] were upregulated in MM, particularly in FL.

Inflammatory mesenchymal stem cells in multiple myeloma express genes associated with distinct cancer-assciated fibroblasts and with the prognostic mesenchymal stem cell gene score

We looked for the expression of genes that are associated with the main CAF types in individual MSC from BM samples of control healthy donors and newly diagnosed MM patients described by de Jong *et al.*, 2021.[8] Their analyses identified five subsets of MSC, where MSC1 and MSC2 subsets were defined as inflammatory MSC (iMSC) and the MSC5 subset was defined as early or transitory cells prior to becoming iMSC. In addition, two subsets of EC were identified (EC and SEC) as well as a small subset of osteogenic like cells (OLC) (Figure 2A). The iCAF genes *APOD* and *IGF2* were expressed by all MSC subsets, whereas *CXCL14* was more specific to the inflammatory MSC1, MSC2, and MSC5 subsets. The mCAF genes *MMP11*, *COL3A1*, and *FN1* were expressed by all subsets, though MSC5 cells had the highest expression. Various MSC subsets expressed vCAF genes, whereas only a few MSC expressed infCAF genes. Notably, apCAF genes such as *CD74*, *HLA-DRA*, and *HLA-DPA1* were expressed by various MSC subsets, with higher expression in the inflammatory MSC1, MSC2a, and MSC5 subsets than in the MSC3 and MSC4 subsets (Figure 2A). Further, we examined apCAF gene expression in MSC. *CD74, HLA-DRA*, and *HLA-DRB1* were expressed in higher proportions in MSC1 and MSC5 subsets and in MM MSC compared to control MSCs (*Online Supplementary Figure S2*). These data indicate that the inflammatory MSC in MM are heterogenous and expressed various markers that reflect various CAF types.

We previously established a prognostic three-gene MSC score based on the expression of the MSC-associated genes *COL4A1*, *ITGBL1*, and *NPR3*.[17] Higher expression of

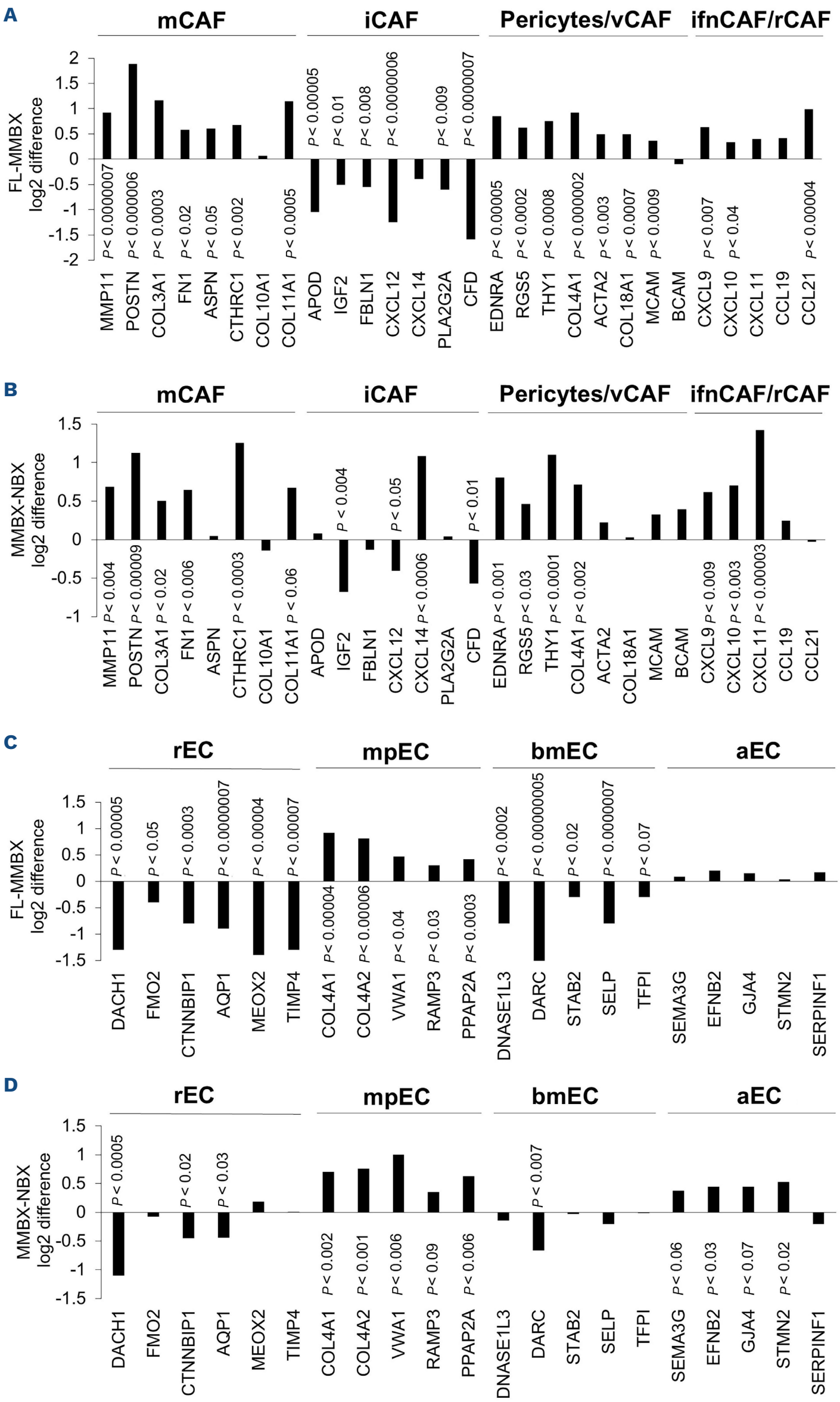

Figure 1. Cancer-associated fibroblasts and endothelial cells subsets in multiple myeloma interstitial bone marrow and focal lesions. Altered expression of genes defining cancer-associated fibroblasts (CAF) and bone marrow endothelial cell (BM EC) subsets in whole bone biopsies taken from interstitial BM of healthy donors (NBX) and from interstitial BM (MMBX) and focal lesions (FL) of newly diagnosed patients with multiple myeloma (MM). (A, B) Differences in gene expression based on CAF classification between paired FL and interstitial BM of patients with MM (A) and between MM interstitial BM and healthy donors interstitial BM (B). (C, D) Differences in gene expression based on EC classifications between paired FL and interstitial BM of patients with MM (C) and between MM interstitial BM and healthy donors' interstitial BM (D). mCAF: matrix CAF; iCAF: inflammatory CAF; pericytes/vCAF: pericytes/vascular CAF; ifnCAF/rCAF: interferon-response CAF/reticular-like CAF; rEC: remodeling EC; mpEC: metaphyseal EC; bmEC: bone marrow EC; aEC: arterial EC.

COL4A1 and lower expression of *ITGBL1* and *NPR3* were associated with shorter PFS and overall survival in our TT3 clinical trials (Figure 3A); *COL4A1* has higher expression, whereas *ITGBL1* and *NPR3* have lower expression in cases with a high-risk MSC gene score compared to those with a low-risk score (Figure 3B). To indicate which cell types express these genes in whole bone, we examined the expression of these genes in publicly available scRNA-seq atlases. Based on the atlas by Bixel *et al.*, 2024,[19] which characterizes the main mesenchymal and EC in injured calvarias, *Col4a1* was mainly expressed by EC and to a lesser extent by bone marrow stromal cells (BMSC). *Itgbl1* was mainly expressed by FB-1 and FB-2 (fibroblasts 1 and 2 subsets, respectively), whereas *Npr3* was expressed by FB-2 and few EC (Figure 3C). Based on Dolgalev *et al.*, 2021,[30] *Col4a1* is expressed by various mesenchymal cells and EC, *Itgbl1* is expressed mainly by osteoblasts and fibroblasts, and *Npr3* is expressed by small subsets of Adipoq-lineage mesenchymal stem and progenitor cells (MSPC-Adipo), EC, and Schwann cells (Figure 3D). The MSC classification for clinical MM by de Jong *et al.*, 2021[8] was performed on MSC sorted from BM aspirates and not whole bone biopsies. Nevertheless, in this MSC classification, *ITGBL1* is expressed mainly by OLC and the MSC1 and MSC5 subsets, whereas *NPR3* is expressed by a few cells that mainly belong to the MSC1 and MSC2 subsets. *COL4A1* is expressed mainly by EC and the MSC5 subset, and iMSC belong to the MSC1 subset (Figure 3E). Overall, these data indicate that the expression patterns of these three genes are distinctive for specific cell subsets in normal bone. Additionally, in MM they are also expressed by iMSC.

Studies by de Jong *et al.*, 2021, 2024[8,9] suggested that a reciprocal interaction between MSC and neutrophils leads to an inflammatory phenotype in both cell types. We found that higher expression of genes associated with iMSC and neutrophils (e.g., *CXCL2, IL8, IL1B, BAFF, APRIL*) correlated with favorable overall survival in in the TT3A trial (*Online Supplementary Figure S3*).

CYR61/CCN1 is traced to a unique subset of myeloid cells that are associated with a favorable disease stage

We previously discovered that *CYR61* is upregulated in MM bone biopsies and that the level of its encoding protein, CCN1, increased in patients with MGUS, SMM, or MM.[16] Higher *CYR61* expression and CCN1 levels were associated with a favorable outcome at different stages of the disease.[16] *CYR61*/CCN1 was initially recognized as a stroma marker in bone. Surprisingly, our initial attempt to characterize the spatial expression of CCN1 by IHC in patients' biopsies revealed that CCN1 was also expressed by hematopoietic cells, particularly cells of myeloid/monocytic lineage. Therefore, we sought to shed light on the cellular origin of *CYR61*/CCN1 in MM. We used two main

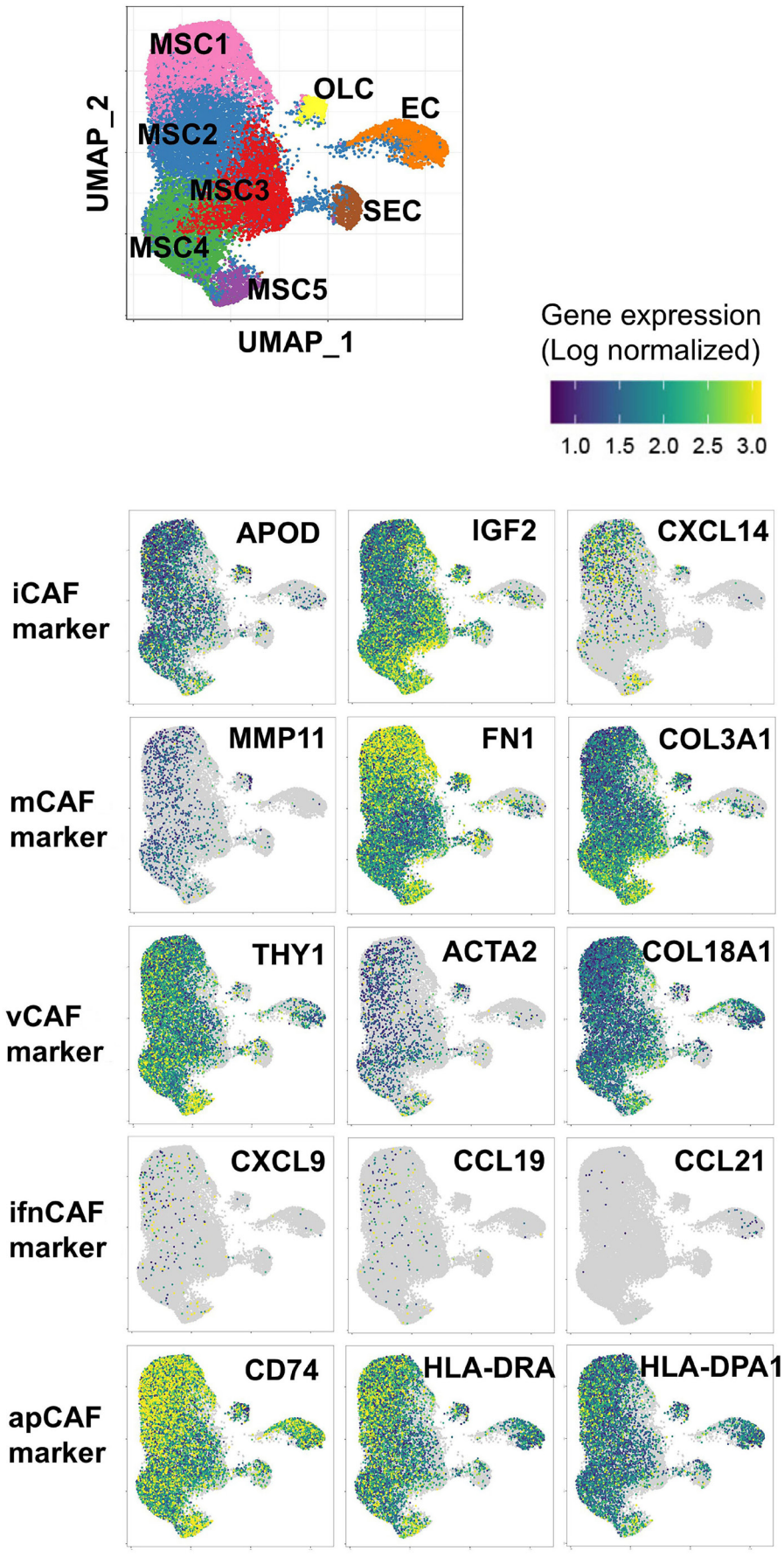

Figure 2. Expression of genes related to various cancer-associated fibroblasts in bone marrow mesenchymal stem cells. The publicly available single-cell RNA sequencing (scRNA-seq)-based atlas by de Jong *et al.*, 2021[8] was used to demonstrate gene expression in the non-hematopoietic cells from bone marrow (BM) of healthy donors and patients with multiple myeloma (MM). (A) Stromal cell classifications showing the 5 mesenchymal stem cells (MSC) subsets (MSC1-5), osteogenic-like cells (OLC), endothelial cells (EC), and the undefined SELP$^+$ EC (SEC) subset. MSC1 and MSC2 are classified as inflammatory MSC that are mostly detected in MM samples. (B) Expression of indicated genes that define inflammatory CAF (iCAF), matrix CAF (mCAF), vascular CAF (vCAF), antigen-presenting CAF (apCAF), and interferon-response CAF (ifnCAF) (3 representative genes for each subset).

approaches: the use of atlases classifying stroma cells and the quantification of CCN1^{+} myeloid cells in clinical histological bone sections.

The atlas based on injured calvaria by Bixel *et al.*, 2024[19] revealed that murine *cyr61* was induced in injured calvaria and expressed mainly by osteoblasts and subsets of BMSC and EC (*Online Supplementary Figure S4*). Based on de Jong *et al.*, 2021,[8] *CYR61* is expressed by OLC, EC, and all MM MSC subsets including the inflammatory MSC1 and MSC2 subsets (*Online Supplementary Figure S5*). We also examined *CYR61* expression in various hematopoietic cells in the de Jong *et al.*, 2021 atlas,[8] and found that *CYR61* was detected within the BM CD38^{-} hematopoietic cells, mainly in few monocytes, GZMK^{+} effector T cells, and mature B cells from MM patients but not control healthy individuals (Figure 4). Some BM CD38- hematopoietic cells also expressed additional mesenchymal markers such as *COL1A1, FN1, THY1*, and *CTGF* (*Online Supplementary Figure S6*). These findings indicate that in MM, *CYR61* is expressed by cells of non-hematopoietic and hematopoietic origin, whereas in healthy bone samples it is mainly expressed by non-hematopoietic cells.

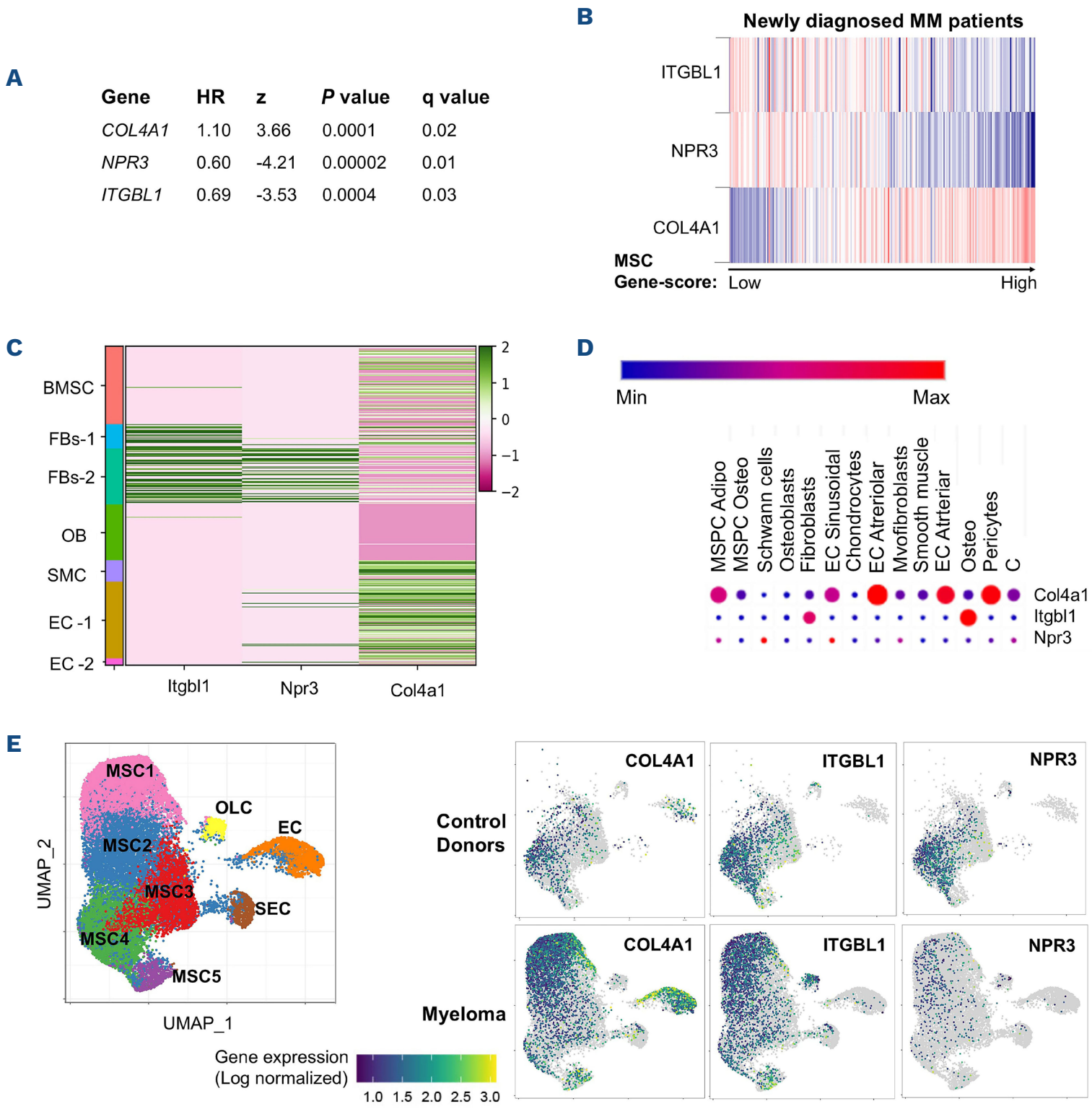

Figure 3. Expression of the three genes composing the prognostic mesenchymal stem cell gene score in various cellular atlases. Expression of the 3 genes, *COL4A1, ITGBL1*, and *NPR3*, described by Schinke *et al.*, 2018,[17] were analyzed in multiple myeloma (MM) bone biopsies and by mesenchymal stem cells (MSC) and bone marrow (BM) atlases. (A) Association between expression of the 3 genes and survival of TT3 patients.[17] (B) Expression of the 3 genes in bone biopsies of newly diagnosed patients.[17] (C) Expression of the 3 genes based on an atlas by Bixel et al., 2024.[19] (D) Expression of the 3 genes based on an atlas by Dolgalev *et al.*, 2021.[30] (E) Expression of the 3 genes in non-hematopoietic cells based on an atlas by de Jong *et al.*, 2021.[8] BMSC: bone marrow stromal cells; FB: fibroblasts; OB: osteoblasts; SMC: smooth muscle cells; EC: endothelial cells.

In the second approach, we quantified the numbers of CCN1⁺ myeloid cells in bone sections from healthy individuals and patients with MGUS/SMM and NDMM. The NDMM samples were from low-risk and high-risk MM cases.[26] CCN1⁺ myeloid cells were rarely detected in bone of age-matched healthy individuals but were apparent in higher frequency in cases with MGUS/SMM or MM. CCN1⁺ myeloid cells were higher in low-risk than high-risk MM cases and rarely detected within FL (Figure 5A-D). CCN1⁺ was also expressed by adipocytes in the red marrow, and CCN1⁺ myeloid cells frequently resided proximally to adipocytes (Figure 5A). CCN1 was not expressed by neoangiogenic EC frequently established in highly infiltrated areas but was observed in mesenchymal cells surrounding established vessels (Figure 5B). These findings indicate a higher prevalence of CCN1⁺ myeloid cells in patients at premalignant stages and patients with low-risk MM. We could not detect CCN1 in the buffy coat of the fi-colled BM aspirates, whereas a previous study detected CCN1⁺ cells in the lipid layer of BM aspirates of patients with MM.[31] Therefore, we performed CCN1 immunohistochemistry (IHC) on cytospin cells from ficolled BM and the lipid layer fraction and detected a high frequency of CCN1⁺ myeloid cells in the lipid layer only (Figure 5E, F). We isolated CD14⁺ cells from the lipid layer and detected CCN1 expression in a subset of these CD14⁺ cells using IHC (Figure 5G, H). Using flow cytometry we quantified the proportions of CCN1⁺ CD45⁺CD14⁺ cells in ten paired samples, and found enrichment of these cells in the BM aspirate lipid layer compared to standard ficolled BM aspirate (Figure 5I, K). Overall, our work discovered unique CCN1-producing hematopoietic cells that are induced in response to MM. These cells are close to adipocytes within the BM niche, and their frequency is higher in MGUS/SMM or low-risk MM cases than in high-risk MM cases.

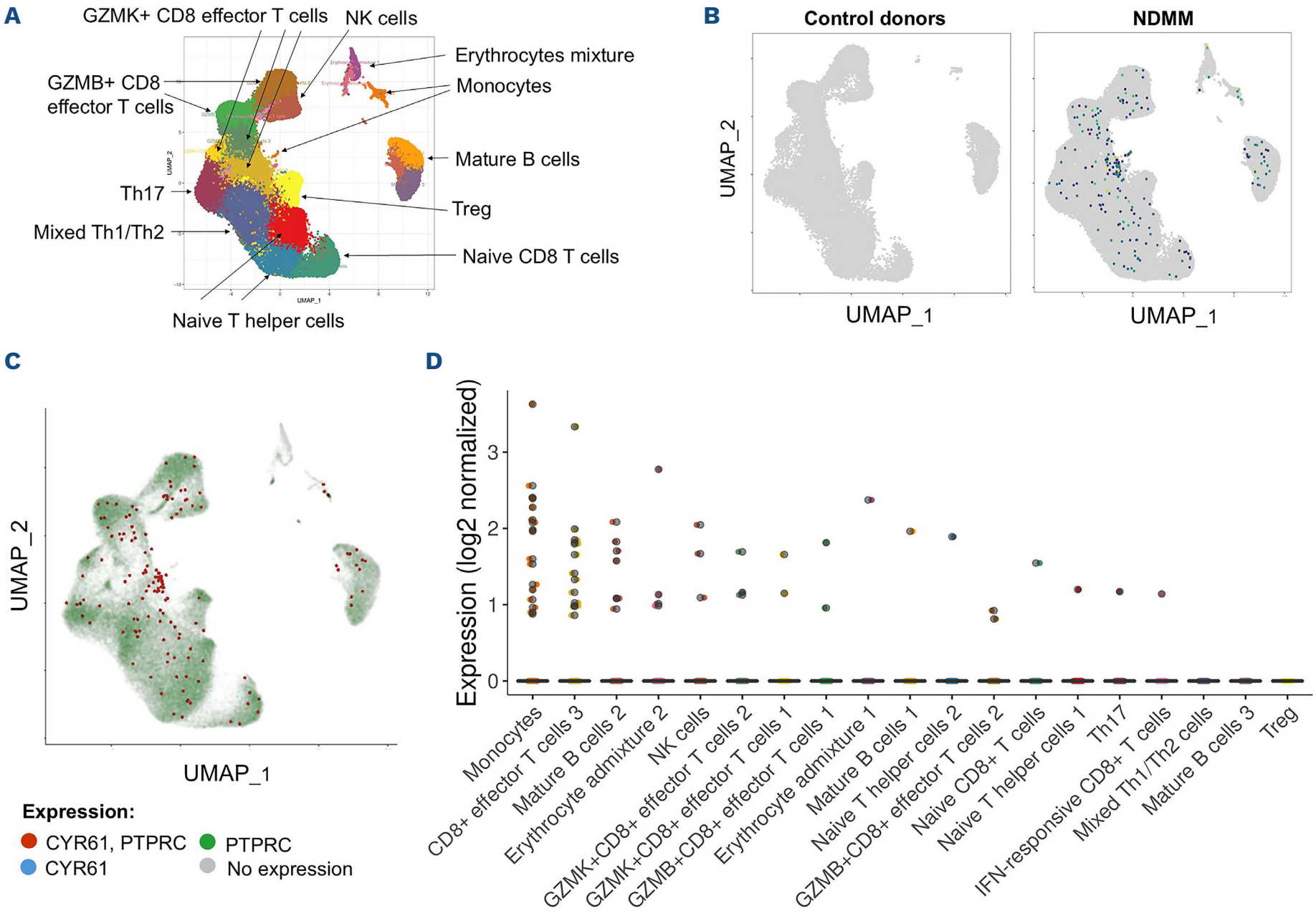

Figure 4. Expression of *CYR61* in bone marrow CD38⁻ hematopoietic cells. The publicly available single-cell RNA sequencing (scRNA-seq)-based atlas by de Jong *et al*. 2021[8] was used to demonstrate gene expression of *CYR61* in hematopoietic cells from bone marrow (BM) of healthy donors and patients with multiple myeloma (MM). (A) BM hematopoietic CD38⁻ cells' classification based on the atlas. (B) CYR61 expression in BM hematopoietic CD38⁻ cells from control donors and newly diagnosed MM patients (NDMM). (C) Co-expression of *CYR61* and *PTPRC* (CD45) in CD38⁻ hematopoietic cells from patients with MM. (D) *CYR61* expression in specific hematopoietic cells' subsets. NK: natural killer; Treg: regulatory T cells.

Altered subsets of mesenchymal and endothelial cells in the primary multiple myeloma-permissible SCID-human mouse model

We have previously demonstrated that the SCID-hu model is permissible to the growth of primary MM cells.[22,23] In this model, a fetal femur or tibia was implanted subcutaneously, and 6 weeks after, MM cells were directly injected into the engrafted fetal bone. We have demonstrated that microenvironmental cell types that are impacted by MM are of human origin, including osteoclasts, osteoblasts, and neoangiogenic EC.[23,32,33] The overall human hematopoiesis within the implanted bones is low. Using the same cell classification methods as for Figure 1, we examined changes in mesenchymal and endothelial cell gene expression in the non-myelomatous SCID-hu model compared to bones from healthy individuals (NBX). Based on CAF classification, genes reflecting mCAF, iCAF, and pericytes/vCAF were expressed at higher levels in the SCID-hu model compared to in NBX. *CXCL12*, an iCAF gene (encoding SDF1), was underexpressed in the SCID-hu model relative to bones from healthy individuals. Similar underexpression of the infCAF/rCAF genes, *CXCL9* and

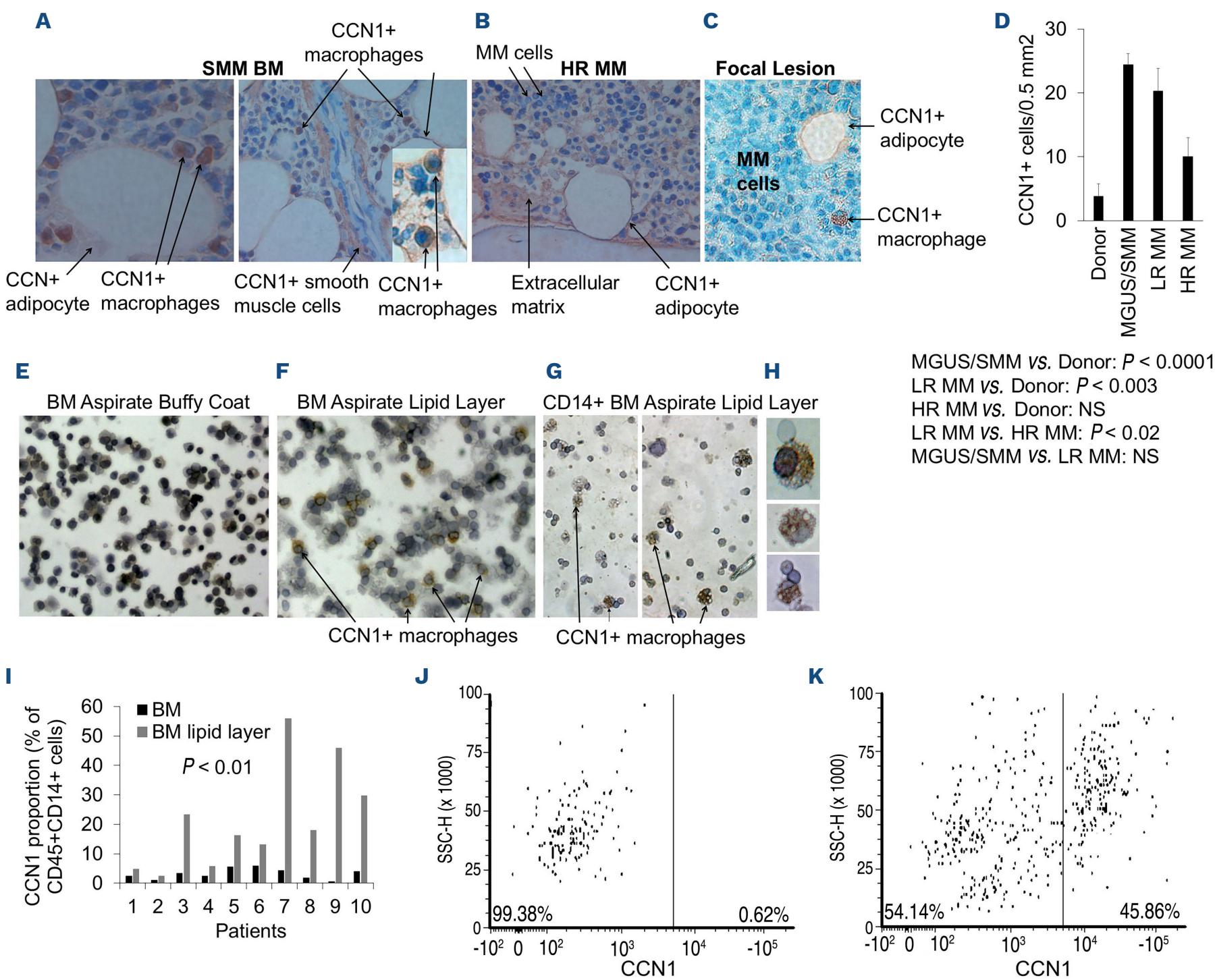

Figure 5. Expression of CCN1 in bone biopsies and bone marrow aspirates. CCN1 expression was determined by immunohistochemistry (IHC). (A-D) CCN1 IHC in bone biopsy sections (x20 magnification) demonstrating detection of CCN1 in indicated cells in smoldering multiple myeloma patients (SMM) (A), high-risk MM (HR MM) (B), and focal lesion (C), and quantification of numbers CCN1⁺ myeloid cells in indicated groups (D). (E-H) CCN1 IHC (x20 magnification) in ficolled BM (E) and lipid layer (F) cells of the same sample. CD14⁺ cells isolated from the lipid layer fraction (G, x20 magnification) and single cells shown in higher magnification (H, x40). (I-K) Flow cytometry analysis of paired BM cells and BM lipid layer cells. Quantification of proportions of CCN1⁺ cells of 10 paired samples (MM: patients 1-9; monoclonal gammopathy of undetermined significance [MGUS]: patient 10). (I) Flow cytometry showing the expression of CCN1 among cells gated on CD45⁺CD14⁺ population in ficolled BM cells (J) and BM lipid layer cells (K) of the same patient.

CXCL11, was also observed (Figure 6A).
Based on EC classifications, genes reflecting mpEC and aEC were expressed at higher levels in the SCID-hu model than in NBX, whereas genes reflecting rEC and bmEC were underexpressed in the SCID-hu model compared to NBX (Figure 6B). The rEC gene *MEOX2* and the bmEC genes *STAB2* and *TFPI* were expressed at higher levels in the SCID-hu model compared to NBX. Overall, the stroma in the SCID-hu model resembles some similarities with the changes observed in clinical FL shown in Figure 1.

Discussion

We present an analysis of the altered stromal cell subsets in MM, identifying previously unrecognized cell subsets in interstitial BM and FL. The altered expression of mesenchymal and endothelial genes resembles CAF subsets, in environments permissible to MM, such as clinical FL and human bone engrafted in SCID-hu mice.
We highlight cells expressing MSC markers that constitute the prognostic three-gene score: *COL4A1, ITGBL1*, and *NPR3*. Elevated *COL4A1* expression indicates increased angiogenic and matrix mesenchymal cell activity, whereas *ITGBL1* and *NPR3* are highly expressed by a distinct subset of mesenchymal cells, likely suppressed as the disease progresses. The mesenchymal cells resembling matrix CAF and vascular CAF are abundant in FL, while antigen-presenting inflammatory mesenchymal cells are suppressed in FL but present in the interstitial BM of patients with MM. The iMSC in MM

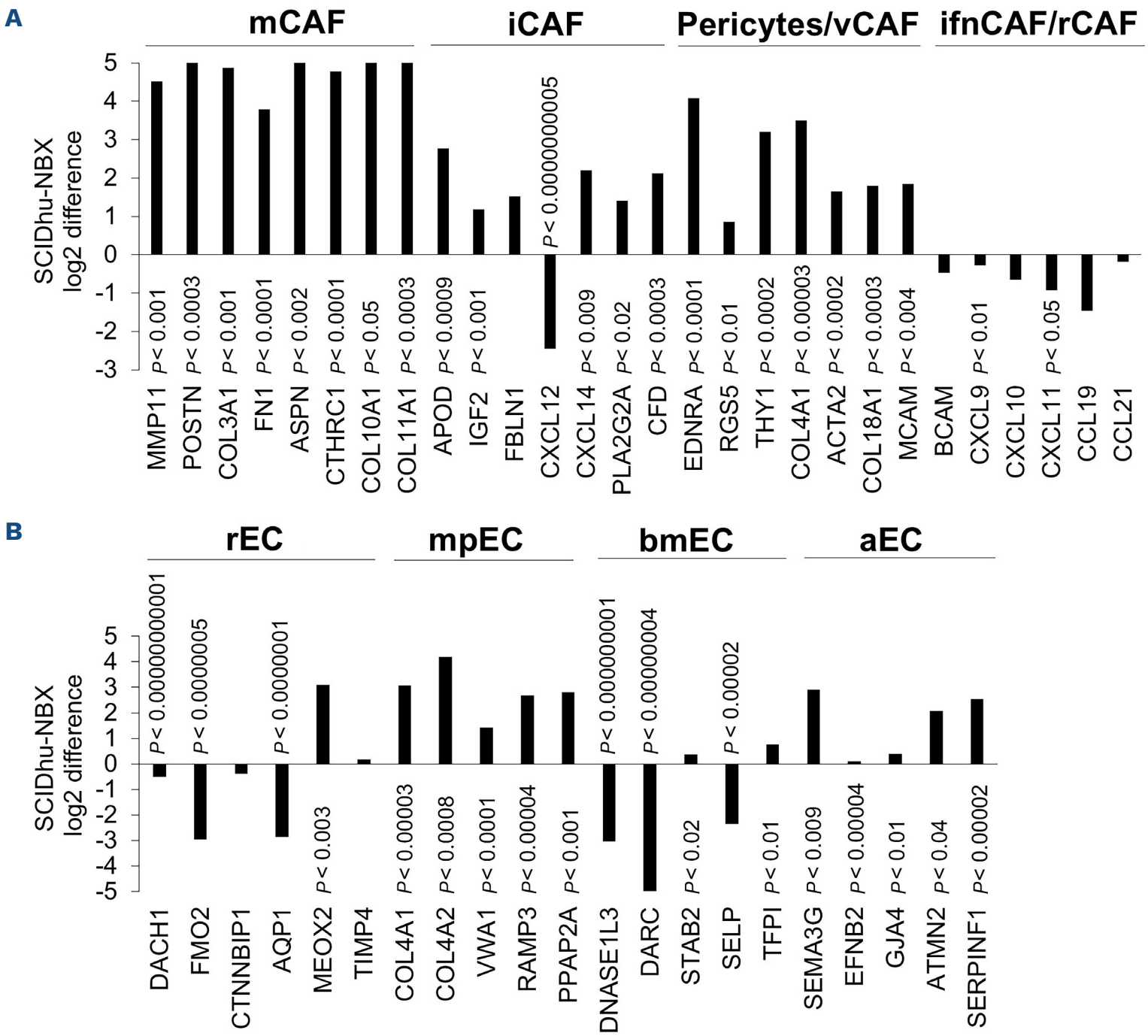

Figure 6. Cancer-associated fibroblasts and endothelial cells subsets in the SCID-human model. Altered expression of genes defining cancer-associated fibroblasts (CAF) and bone marrow endothelial cells (EC) subsets in the whole human bone engrafted in SCID-human (SCID-hu) mice and whole bone biopsies taken from healthy donors (NBX). (A) Differences in expression of indicated genes based on CAF classification between the SCID-hu model and healthy donors. (B) Differences in expression of genes based on EC classifications between the SCID-hu model and healthy donors. mCAF: matrix CAF; iCAF: inflammatory CAF; pericytes/vCAF: pericytes/vascular CAF; ifnCAF/rCAF: interferon-response CAF/reticular-like CAF; rEC: remodeling EC; mpEC: metaphyseal EC; bmEC: bone marrow EC; aEC: arterial EC.

are heterogeneous, expressing markers indicative of various CAF types, particularly antigen-presenting MSC. Regarding BM vasculature in MM, especially in FL, we observed reduced expression of genes associated with specialized bone remodeling rEC and upregulation of genes reflecting mpEC, likely representing a source of neoangiogenic EC. We identify CCN1$^+$ myeloid cells as a unique cell type that may function as stromal-like cells by producing stromal factors such as CCN1 and matrix-associated genes. Additionally, we point to other MSC subsets, such as antigen-presenting MSC, which may play a role in immunity.

The identification of iMSC subsets in MM and CAF subsets in solid tumors opens an avenue to understand the role of these subsets in tumor progression. In MM, iMSC appear to be induced by inflammatory neutrophils.[8,9] However, these iMSC express genes associated with favorable outcomes in MM, such as *ITGBL1, NPR3,* and *CYR61,*[16,17] whereas a recent study showed that inflammatory senescence phenotype in MGUS is associated with delayed progression.[34] iMSC also harbor a subset of antigen-presenting MSCs. Another finding is that inflammatory mesenchymal cells seem to be depleted in FL. Their depletion could be due to multiple mechanisms such as (i) depletion of inflammatory hematopoietic cells[14] shown to promote inflammatory MSC;[9] (ii) the expanding MM cells unselectively deplete hematopoietic cells;[14] (iii) a reduced pool of MSC capable of differentiation to lineages such as osteoblasts and adipocytes;[11] or (iv) due to expansion of other types of MSC that are equivalent to matrix CAF and vascular CAF in solid tumors[27] and MM FL.[5,18]

Further studies are needed to understand how these cellular changes affect T cells and their anti-MM immunity. The MM BM is rich in T-cell chemokines like CCL19, CCL21, and CXCL10. However, suppression of normal vasculature (e.g., bmEC, rEC) may hinder their homing to the BM. Additionally, mCAF producing high levels of extracellular matrix proteins (e.g., FAP) and TGF-β can inhibit CD8$^+$ T cell anti-tumor activity while promoting regulatory T cells.[35] Some iMSC also express markers of immune cells such as *CD14, CD74*, and *HLA-DRA*, suggesting that they are capable of antigen-presenting, previously demonstrated in fibrocytes, a subset of mesenchymal cells of hematopoietic origin.[36,37]

Studies by de Jong *et al*., 2021, 2024[8,9] suggested that iMSC stimulate the production of MM growth factors such as BAFF.[8,9] However, we found that higher expression of inflammatory-associated genes was associated with better outcomes. Additionally, recent reports have shown that the BM cellularity of MM patients achieving MRD negativity[38] and long-term remission,[39] as well as MGUS patients with sustained stable disease[34] is associated with subsets of cells harboring an inflammatory phenotype. Therefore, the role of iMSC in MM progression requires further investigation.

Our work on *CYR61*/CCN1 opened a window to understanding the complexity of the BM microenvironment in MM. We found that *CYR61* is uniquely induced by MM and expressed by both iMSC and small subsets of hematopoietic cells. Secondly, we showed that CCN1$^+$ cells, particularly those that express CD14 and resemble monocytic/macrophage morphology, are evident in MM bone, yet their frequency is lower in high-risk MM, and they are rare in FL. Moreover, CCN1$^+$ myeloid cells were detected in the BM aspirate lipid layer but rarely within the standard ficolled BM aspirate cells. They were detected in histological bone sections close to adipocytes and established vessels in the red marrow. We have previously shown that CCN1 can prevent MM-induced bone disease.[16] Interestingly, *CYR61* is listed as one of the genes that define the specialized rEC, though not one of the top genes.[20] CCN1 is actively involved in efferocytosis, a process by which apoptotic cells are removed by phagocytic cells, thus minimizing damage-associated molecular pattern molecules.[40] CCN1 also restricts fibrosis by inducing senescence on myofibroblasts in wound healing.[41] Taken together, CCN1$^+$ cells described in our study may play a role in maintaining the normal BM niche in MM.

The analysis of EC subsets permitted differentiating subsets enriched with more neoangiogenic EC than normal EC and determined their alteration in interstitial MM BM and FL. The suppressed specialized bone remodeling rEC suggests that the depletion of these cells may contribute to impaired bone formation in osteolytic lesions. Angiogenesis and osteogenesis were thought to be coupled during bone remodeling;[29] however, a recent study suggests that angiogenesis precedes osteogenesis in injured bone.[19] The bmEC are also suppressed in FL, whereas the mpEC seem to be enriched in FL. The mpEC correspond to type H vessels, whereas the bmEC correspond to type L vessels.[29] In physiological conditions, type H vessels/mpEC play an important role in bone formation, and they weaken with age.[29] However, the enrichment of type H vessels/mpEC in MM FL likely results from increased neoangiogenesis.[5] Overall, depletion of rEC and bmEC while enriching mpEC may contribute to the well-known uncoupling processes of bone formation and bone resorption in MM[10,24] and disruption of the endothelial niches.

Finally, our analysis of human bones from SCID-hu mice had two main proposes: (i) providing new insight that shed light on the cellular microenvironment that allows the growth of primary MM even from patients with low-risk disease,[22,23] and (ii) showing a similar pattern of changes in the mesenchymal and endothelial compartments in clinical FL and in the SCID-hu model strengthening our understanding of stroma components in MM progression. In FL and the SCID-hu model, there was higher activity of mCAF, vCA/pericytes, and mpEC and lower expression of genes defining rEC and bmEC. The increased activity of aEC and iCAF in the SCID-hu model mirrors changes

in the clinical interstitial BM, which are crucial for the MM niche. However, the reduced activity of infCAF/rCAF and the absence of T cells in the SCID-hu model highlight its limitation in fully capturing the cellular complexity of the MM niche.

In summary, we described subsets of mesenchymal and endothelial cells altered in clinical FL and in the permissible SCID-hu model for myeloma, showed that the iMSC in MM contain mixed CAF-like cell populations and identified unique CCN1^{+} myeloid cells associated with favorable disease stages. Future studies are necessary to determine the role of these subsets in MM progression and response to novel immunotherapies.

Disclosures

No conflicts of interest to disclose.

Contributions

WL performed the immunohistochemistry, prepared the bone cells for analysis, and helped analyze the data. MZ, FvR, and BB provided patient materials and clinical insights. SY designed and directed the research, conceptualized the work, analyzed and interpreted the data, and wrote the paper.

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Data-sharing statement

The GEP analyses of bone biopsies from MM patients and healthy donors are available as described by Danziger et al. PLoS Med. 2020;17:e1003323. The data on the three-genes MSC score is described by Schinke et al. Cancer Res. 2018;24:2913-2919. The scRNA-Seq data of immune cells in BM of patients with MM and healthy donors are available as described in De Jong et al. Nat Immunol. 2021;22:769-780.

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