



Letter

Activation of the MIF-CD74-AKT pathway by endothelial cells promotes myeloma growth and limits NK cell-mediated killing

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Title: Activation of the MIF–CD74–AKT pathway by endothelial cells promotes myeloma growth and limits NK cell–mediated killing

Multiple myeloma (MM) is a hematologic malignancy characterized by the clonal expansion of plasma cells within the bone marrow (BM). Despite significant therapeutic advances, MM remains incurable for most patients, with frequent refractory disease and relapse¹. A major contributor to treatment resistance and disease persistence is the protective tumor microenvironment (TME) within the BM, which supports myeloma cell survival and proliferation while promoting immune escape². The TME comprises both hematopoietic and non-hematopoietic compartments. The latter includes endothelial cells, osteoblasts, fibroblasts, and adipocytes that actively shape the myeloma niche through dynamic cell-cell interactions and secretion of growth factors, cytokines, immunomodulatory mediators and extracellular matrix components³, fostering a myeloma-protective and immunosuppressive landscape that undermines durable anti-myeloma immunity.

NK cells are key cytotoxic effectors of the innate immune system and can eliminate tumor cells. In MM, NK cells frequently display an exhausted phenotype with reduced activating receptors and impaired cytotoxic capacity⁴. Although NK cell-based therapies, including adoptive NK cell transfer and CAR NK cell approaches, have shown promise and offer advantages such as decreased graft-versus-host disease and reduced likelihood of cytokine release syndrome, their efficacy is limited by poor persistence and TME-mediated resistance⁵. While stromal cell mediated suppression of NK function has been described for mesenchymal stromal cells and osteoblasts^{6,7}, the contribution of endothelial cells, which regulate angiogenesis and immune trafficking, remains less well defined. Given their strategic position in the BM niche, endothelial cells represent an important but understudied regulator of MM progression and therapy resistance. We

investigated mechanisms underlying the tumor protective effects of endothelial cells on MM cells and explored strategies to counter endothelial cell mediated pro-myeloma effects while enhancing NK cell mediated antimyeloma activity.

We first assessed the impact of non-hematopoietic stromal cells on myeloma cell killing by the NK cell line NK92MI as well as primary NK cells using luciferase-based cytotoxicity assays. Luciferase-expressing MM cell lines (MM.1S, NCI-H929, and L363) were cultured with NK92MI cells alone or on a confluent monolayer of stromal cells at defined effector-to-target ratios for up to 48 hours, and target cell survival was measured by luminescence relative to MM cell monocultures. Osteoblasts (hFOB) and BM-derived fibroblasts (HS-5) partially protected MM.1S and NCI-H929 cells, whereas another BM-derived fibroblast cell line (HS-27a) had minimal effect; L363 cells were largely unchanged across conditions (Supplemental Fig. 1). These findings highlight the cell type-specific nature of stromal protection of MM cells within the BM niche. Among the tested stromal populations, human umbilical vein endothelial cells (HUVEC)⁸ exerted the strongest protective effect. HUVECs consistently promoted survival of several MM cell lines in co-culture killing assays with NK92MI or primary NK cells as effector cells, indicating robust vascular niche protection (Fig. 1A, B). Flow cytometry confirmed increased viability and reduced death of MM cells following NK exposure in the presence of HUVECs using Annexin V and DAPI staining (Fig. 1C). A similar pattern was observed in patient-derived primary MM cells, where endothelial cells promoted myeloma viability both in co-cultures with MM cells and in triples cultures with MM and NK cells (Fig. 1D).

HUVEC mediated MM cell protection was not due to suppression of NK cell function, as NK cell activation/effector markers were not reduced in triple cultures (Supplemental Fig.

2A, B), suggesting endothelial-induced tumor-intrinsic reprogramming. RNA-seq of MM.1S cells cultured with HUVECs revealed broad endothelial-induced remodeling, with differential expression centered on growth and survival programs (Fig. 1E-G). The volcano plot highlights *PARP9* upregulation, a PARP family member linked to DNA repair and apoptosis resistance in B-cell malignancies⁹. The heatmap displays the top 100 differentially expressed genes (adjusted $P < 0.05$, $|\log_2 \text{fold change}| > 1$) and shows clear condition-specific clustering, with prominent upregulation of *ICAM1* (adhesion/immune evasion)¹⁰, *FOS/JUN* (survival/proliferation/drug resistance)¹⁴, *JAK3* (B-cell malignancy progression)¹¹, *STAT3* (growth promotion/apoptosis blockade)¹², and *CD320* (proliferation/angiogenesis)¹³. Kaplan-Meier analyses¹⁴ confirmed the prognostic relevance of this signature: high *PARP9* and *JAK3* mRNA expression correlated with inferior overall survival (Fig. S2C). Gene set enrichment analysis (GSEA) (Fig. 1G) revealed significant enrichment of the AKT-driven mTORC1 signaling pathway, a key driver of anabolic growth and proliferation in cancer cells¹⁵. Additionally, the MET pathway was significantly enriched, implicating its role in enhancing cell migration, invasion, and survival (Supplemental Fig. 3)¹⁶.

Endothelial cell mediated protection partly depended on soluble factors. HUVEC supernatant partially protected myeloma cells from NK cell-mediated killing, whereas priming of MM cells by 24-hour preincubation with HUVEC did not reproduce this effect in killing assays in the absence of endothelial cells (Supplemental Fig. 4). Direct MM–HUVEC contact was most protective, indicating contributions from both soluble and contact-dependent signals. (Fig. S4). Cytokine profiling demonstrated that HUVECs secrete several pro-survival factors, including SERPINE1 (PAI-1), CCL2 (MCP-1),

CXCL1, IL-6, CXCL8, and MIF (Fig. 2A, B). Consistent with these findings, proteomic analysis showed increased CXCL8, MIF, SERPINE1, and CCL2 in MM.1S–HUVEC co-cultures, whereas NCI-H929–HUVEC co-cultures exhibited increased CXCL8 and CCL2, MIF, SERPINE1, and MMP1 (Supplemental Fig. 6). SERPINE1, CCL2, MIF, IL-6 and IL-8 also have immunosuppressive functions that may further protect MM cells^{17,18}. MIF secretion was detected in both MM cell and HUVEC monocultures; however, MIF levels in cocultures were lower than the expected additive levels, suggesting that HUVEC-derived MIF may be consumed by MM cells (Fig. 2B). As CD74 is a well-known MM antigen¹⁹, these findings pointed to the MIF–CD74 axis as a likely mediator of endothelial support in MM cells. High CD74 expression was associated with significantly inferior overall survival in both PR (high proliferation) and MY (MAF-like) molecular subgroups, highlighting the clinical relevance of this receptor in MM (Fig. 2C). Furthermore, anti-CD74 treatment markedly reduced the survival advantage conferred by HUVECs, indicating that disruption of CD74 can abrogate endothelial-mediated myeloma survival (Fig. 2D).

We next examined whether endothelial-mediated NK cell resistance could be reversed by enhancing NK cell cytotoxicity. We specifically sought to enhance NK cell mediated anti-myeloma activity by targeting CD6, a co-regulatory receptor expressed on cytotoxic T lymphocytes and NK cells that has emerged as a promising immunotherapy target²⁰. CD6 was detected on NK92MI cells by flow cytometry (Supplemental Fig. 5A). Endothelial-mediated resistance to NK cell killing was overcome by anti-CD6 treatment, which restored cytotoxicity against myeloma cells to near baseline levels (Fig. 2E). To assess broader effects of anti-CD6 on NK cell state, we performed RNA sequencing of NK92MI cells after 24 hours of treatment. Differential gene expression analysis revealed

a distinct set of significantly altered transcripts in treated cells, consistent with a treatment-specific transcriptional program (Supplemental Fig. 5B, C). Gene ontology enrichment analysis further demonstrated that anti-CD6 treatment induced significant enrichment of pathways associated with immune activation and lymphocyte proliferation (Fig. 2F). Flowcytometric analysis showed that while anti-CD6 treatment did not significantly affect CD25, CD44, granzyme B, perforin, or TNF α expression, it consistently increased CD107a levels across conditions, consistent with enhanced NK cell degranulation and cytotoxic potential (Supplemental Fig. 5D). Finally, to determine whether simultaneous targeting of CD74-mediated tumor-intrinsic survival signaling and CD6-mediated NK cell suppression would yield additive anti-myeloma effects, we performed triple-culture assays with anti-CD74, anti-CD6, or both antibodies. In HUVEC-supported triple cultures, anti-CD74 or anti-CD6 alone reduced myeloma survival relative to untreated controls, but combined blockade produced the greatest suppression across time points, lowering MM.1S and NCI-H929 survival to below 50% by 48 h (Fig. 2G). The schematic diagram in Fig. 2H summarizes the endothelial–myeloma–NK cell crosstalk and the proposed anti-myeloma effect of dual CD74/CD6 blockade.

Our findings identify the endothelial niche as an active driver of myeloma growth and protection through MIF–CD74–AKT–mTORC1 signaling. Blocking CD74 disrupts endothelial-mediated tumor cell growth, while CD6 blockade improves NK cell cytotoxicity and enhances their anti-myeloma activity. By simultaneously disrupting tumor immune evasion signals and enhancing NK cell functional engagement, this combinatorial approach is poised to improve antitumor activity while reducing the likelihood of treatment failure driven by the MM associated microenvironment. Together, these findings provide

a mechanistic basis for prioritizing dual CD74/CD6 targeted approaches in translational studies aimed at improving therapeutic responses to NK cell-based immunotherapies in MM.

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Figures and figure legends

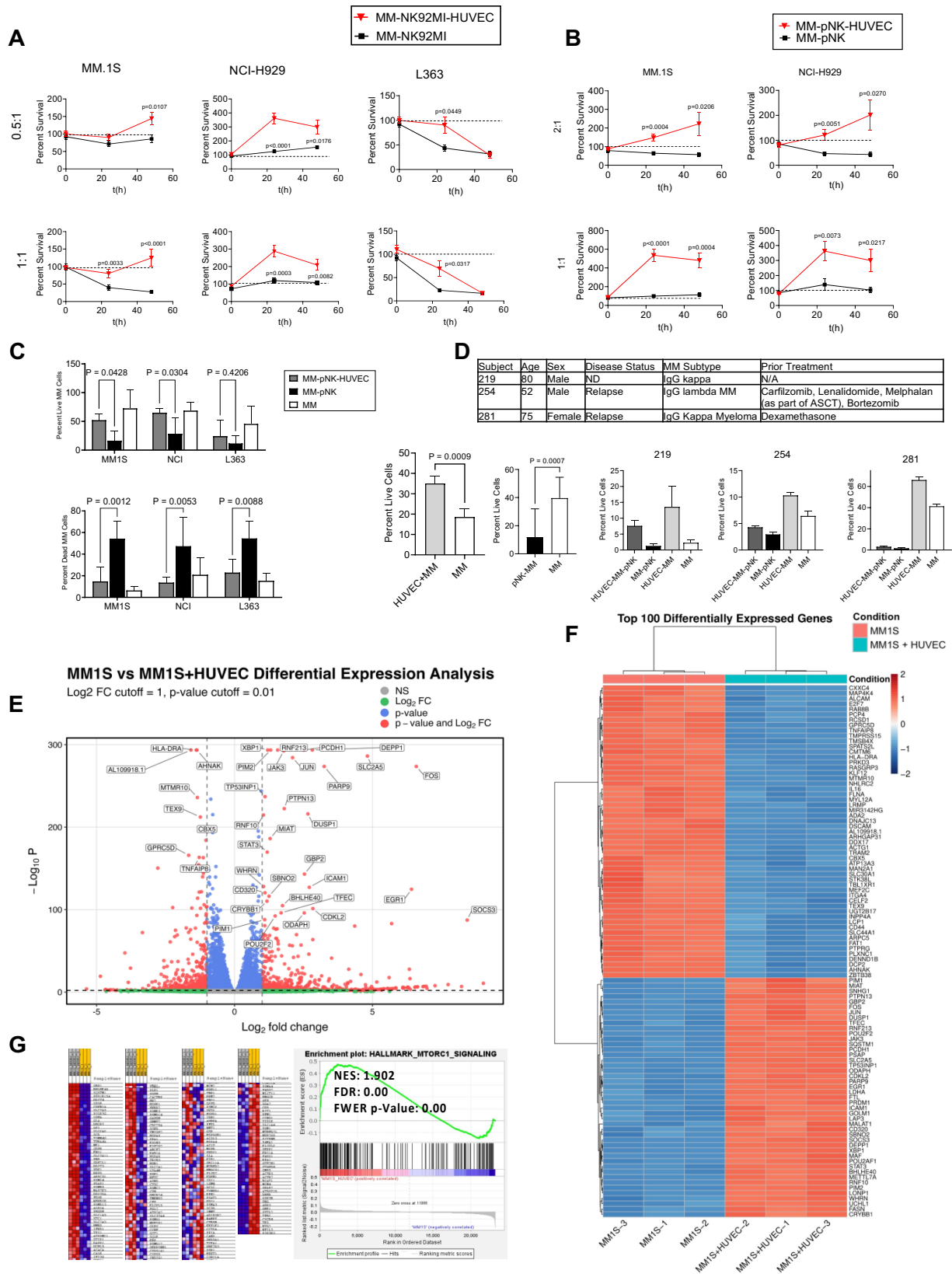


Figure 1. Endothelial cells attenuate NK cell–mediated cytotoxicity against multiple myeloma cells.

(A) Percent survival of MM.1S, NCI-H929, and L-363 cells co-cultured with NK92MI cells at E:T ratios of 1:1 and 0.5:1 for 48 h, in the presence (red) or absence (black) of HUVECs.

(B) Percent survival of MM.1S and NCI-H929 cells co-cultured with primary NK cells (p-NK) at E:T ratios of 2:1 and 1:1 for 48 h, with (red) or without (black) HUVECs. Cell Survival was measured after 24h and 48h by luciferase assay. Data were normalized to respective monoculture controls (dotted line, 100%) and represent mean $\pm$ SEM from at least three independent experiments. Differences between groups were analyzed by Mann-Whitney U test. (A + B)

(C) Annexin V–binding analysis of live (Annexin V⁻/DAPI⁻) and dead (Annexin V⁺/DAPI⁺) MM.1S and NCI-H929 cells cultured with p-NK cells (E:T 1:1) for 24 h, with or without HUVECs. Differences between groups were evaluated by two-way ANOVA. Data are presented as Mean $\pm$ SEM from two independent experiments.

(D) Ex vivo co-culture of primary MM cells from three patients with p-NK cells and HUVECs; the table summarizes clinical characteristics of MM donors, and bar graphs show that HUVEC co-culture increases viability of primary MM cells compared with MM monoculture. Statistical significance was assessed by unpaired two-tailed Mann–Whitney test.

(E-G) RNA sequencing was performed to determine whether HUVEC co-culture alters the transcriptional landscape of MM.1S cells.

(E) Volcano plot showing differential gene expression in MM.1S cells cultured alone versus co-cultured with HUVECs. The x-axis indicates $\log_2$ fold change and the y-axis indicates $-\log_{10}$ P value; genes in red are significantly up- or downregulated in the presence of HUVECs ($P < 0.01$ and $|\log_2 \text{fold change}| > 1$ (total = 13,413 features genes analyzed)).

(F) Heatmap of the top 100 differentially expressed genes in MM.1S cells cultured alone versus co-cultured with HUVECs. Genes were selected based on differential expression criteria (adjusted $P < 0.05$ and $|\log_2 \text{fold change}| > 1$), and variance-stabilizing-transformed counts were row-scaled (z-scores) and hierarchically clustered across three MM.1S and three HUVEC+MM.1S replicates, with column annotation indicating culture condition.

(G) Gene set enrichment analysis (GSEA) demonstrating activation of the AKT1 driven mTORC1 signaling pathway in MM.1S cells following co-culture with HUVECs; NES denotes normalized enrichment score, and FDR denotes false discovery rate.

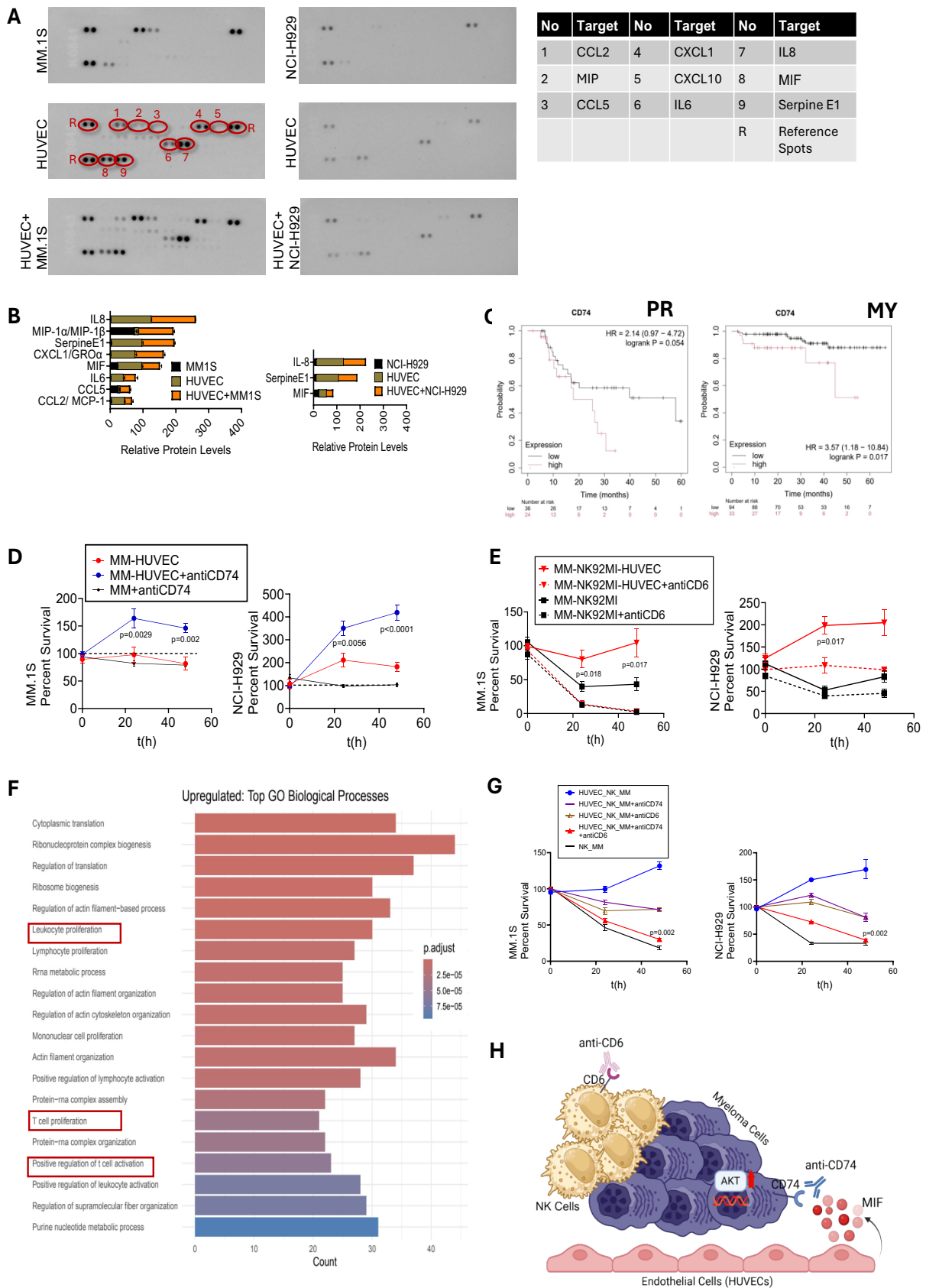


Figure 2. HUVECs secrete pro-survival and immunomodulatory factors in co-culture with myeloma cells.

(A) Cytokine array blots generated using the Proteome Profiler Human Array Kit (ARY005B; R&D Systems; 36 analytes) showing secretion profiles in conditioned media from MM.1S or NCI-H929 monocultures, HUVEC monocultures, and HUVEC–myeloma co-cultures.

(B) Quantification of cytokine array signals after ImageJ-based spot intensity measurement, background subtraction using negative control spots, and normalization to the array's positive control spots.

(C) Kaplan–Meier curves showing overall survival in two molecular subgroups, PR (left) and MY (right), stratified by CD74 expression.

(D) Targeting the MIF–CD74–AKT axis reduces myeloma cell survival. Line graphs show percent survival of MM.1S (left) and NCI-H929 (right) cells cultured alone with HUVECs (blue), with HUVECs plus anti-CD74 (red), or with anti-CD74 alone (black) over time.

(E) Percent survival of MM.1S (left) and NCI-H929 (right) cells cultured with NK92MI cells, with or without HUVECs and anti-CD6 antibody. Data were normalized to respective monoculture controls (dotted line, 100%) and represent mean $\pm$ SEM from at least three independent experiments. (D, E)

(F) Gene ontology (GO) dot plot showing pathways enriched among genes differentially expressed in NK92MI cells after anti-CD6 therapy.

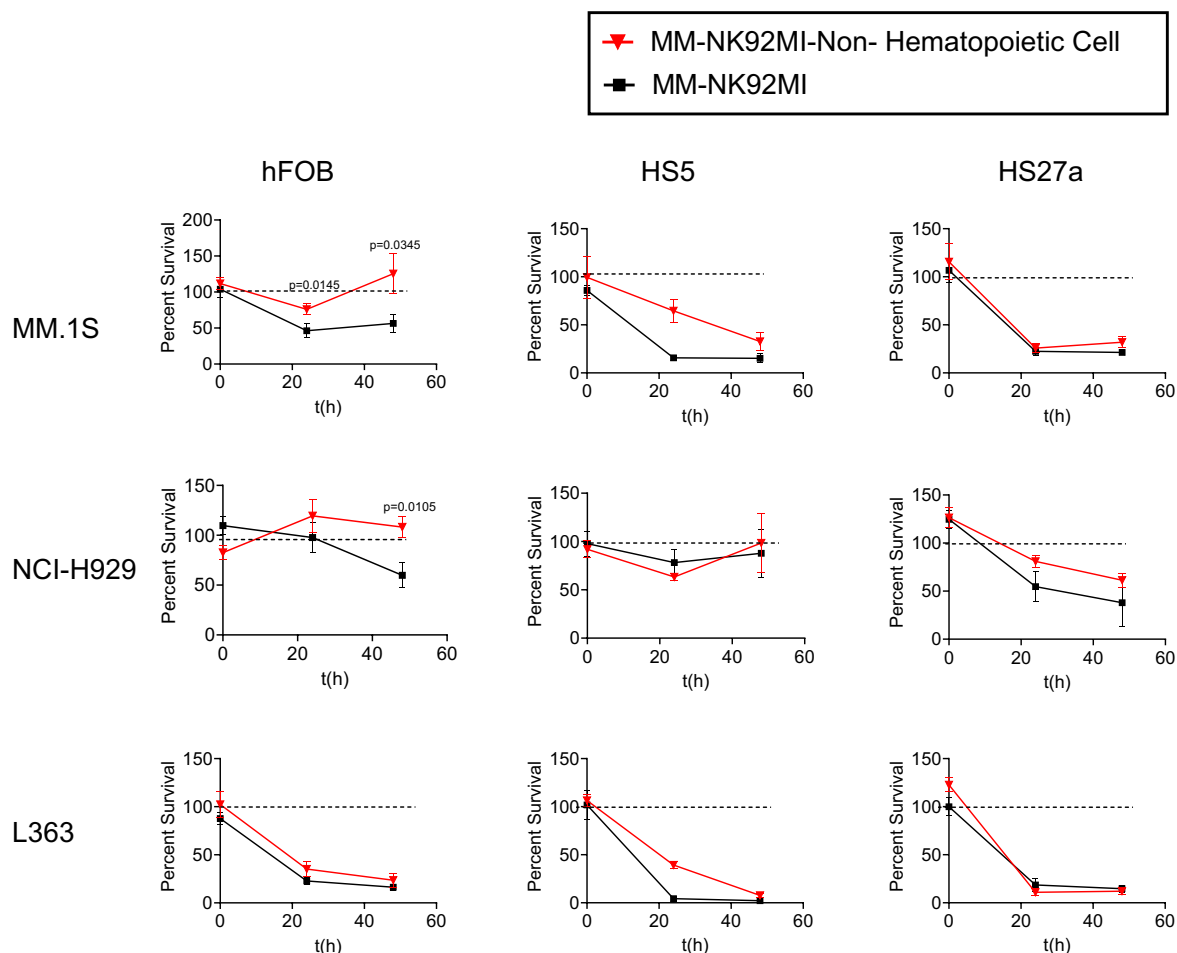
(G) Line graphs showing percent survival of MM.1S (top) and NCI-H929 (bottom) cells over time at an effector-to-target ratio of 2:1 in (HUVEC+MM+pNK) triple cultures without antibody, with anti-CD74 alone, with anti-CD6 alone, or with combined anti-CD74 plus

anti-CD6, alongside NK–myeloma co-cultures without endothelium. Data are presented as mean $\pm$ SEM from at least two independent experiments.

(H) Schematic illustration of the endothelial–myeloma–NK cell axis. Endothelial cells (HUVECs) support myeloma cell growth through MIF–CD74–AKT signaling, making myeloma cells less susceptible to NK cell-mediated killing, whereas blockade of CD74 and CD6 disrupts these pro-survival cues and facilitates NK cell–mediated killing of myeloma cells.

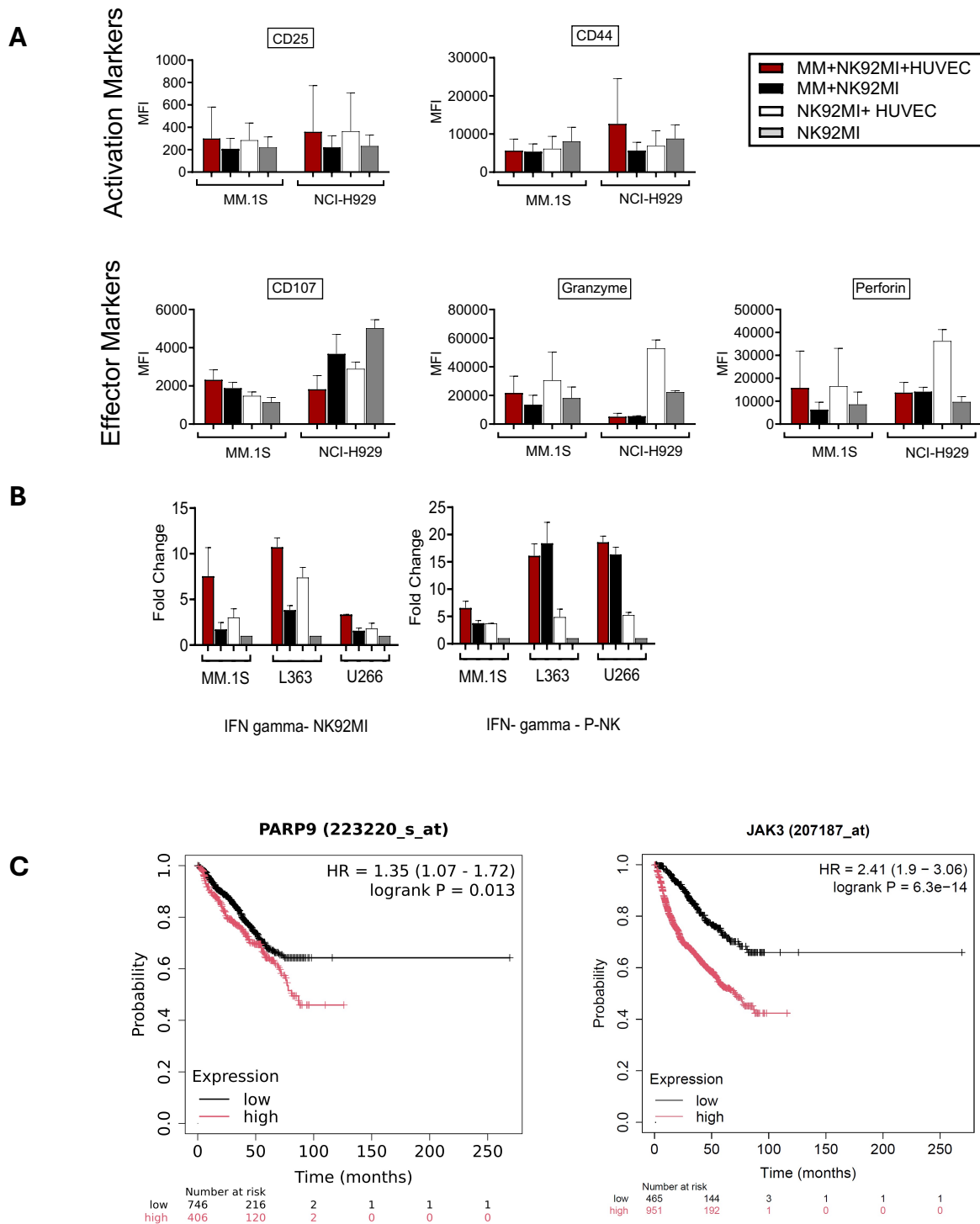
Supplemental Figures

Supplemental Figure 1



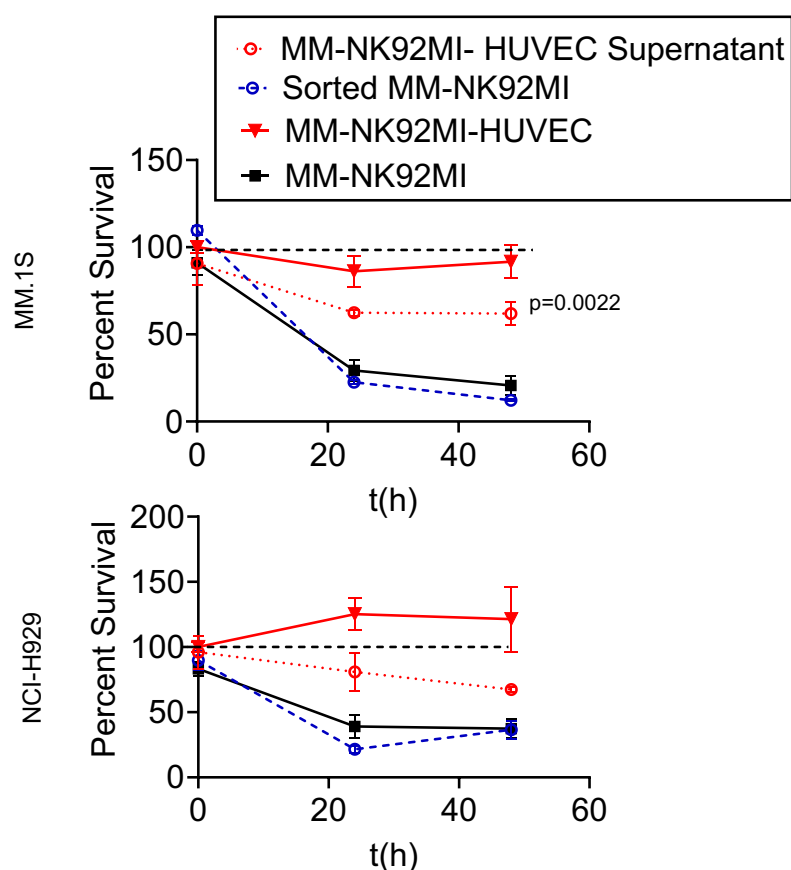
Osteoblasts and fibroblasts differentially protect multiple myeloma cells from NK92MI-mediated cytotoxicity. Survival of MM.1S, NCI-H929, and L363 cells co-cultured with NK92MI cells at an effector-to-target ratio of 1:1 for 48 h in the presence or absence of human osteoblasts (hFOB) or fibroblast cell lines (HS-5 and HS-27a). Percent survival was normalized to respective monoculture controls (dotted line, 100%). Data are presented as mean $\pm$ SEM from at least three independent experiments. Statistical significance was determined using an unpaired two-tailed Mann-Whitney test.

Supplemental Figure 2



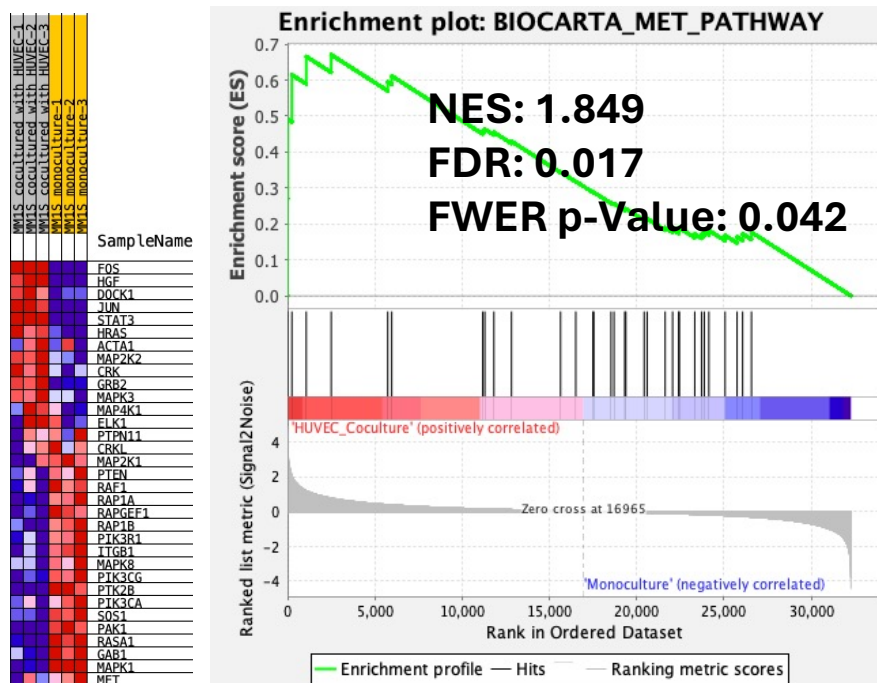
Phenotypic activation and cytokine secretion by NK92MI cells in co-culture with myeloma cells and endothelial cells. (A) Bar graphs show mean fluorescence intensity

of CD25 and CD44 activation markers and CD107a, granzyme B, and perforin effector markers on NK92MI cells after co-culture with MM.1S or NCI-H929 cells under the indicated conditions. No significant reduction in activation or effector marker expression was detected in triple cultures (NK92MI+MM+ HUVEC) compared with NK92MI+ MM co-cultures. (B) IFN-gamma secretion measured in supernatants from NK92MI co-cultures with MM cell lines in the presence or absence of HUVECs. (C) Kaplan-Meier curves showing overall survival in multiple myeloma patients stratified by PARP9 or JAK3 expression.

Supplemental Figure 3

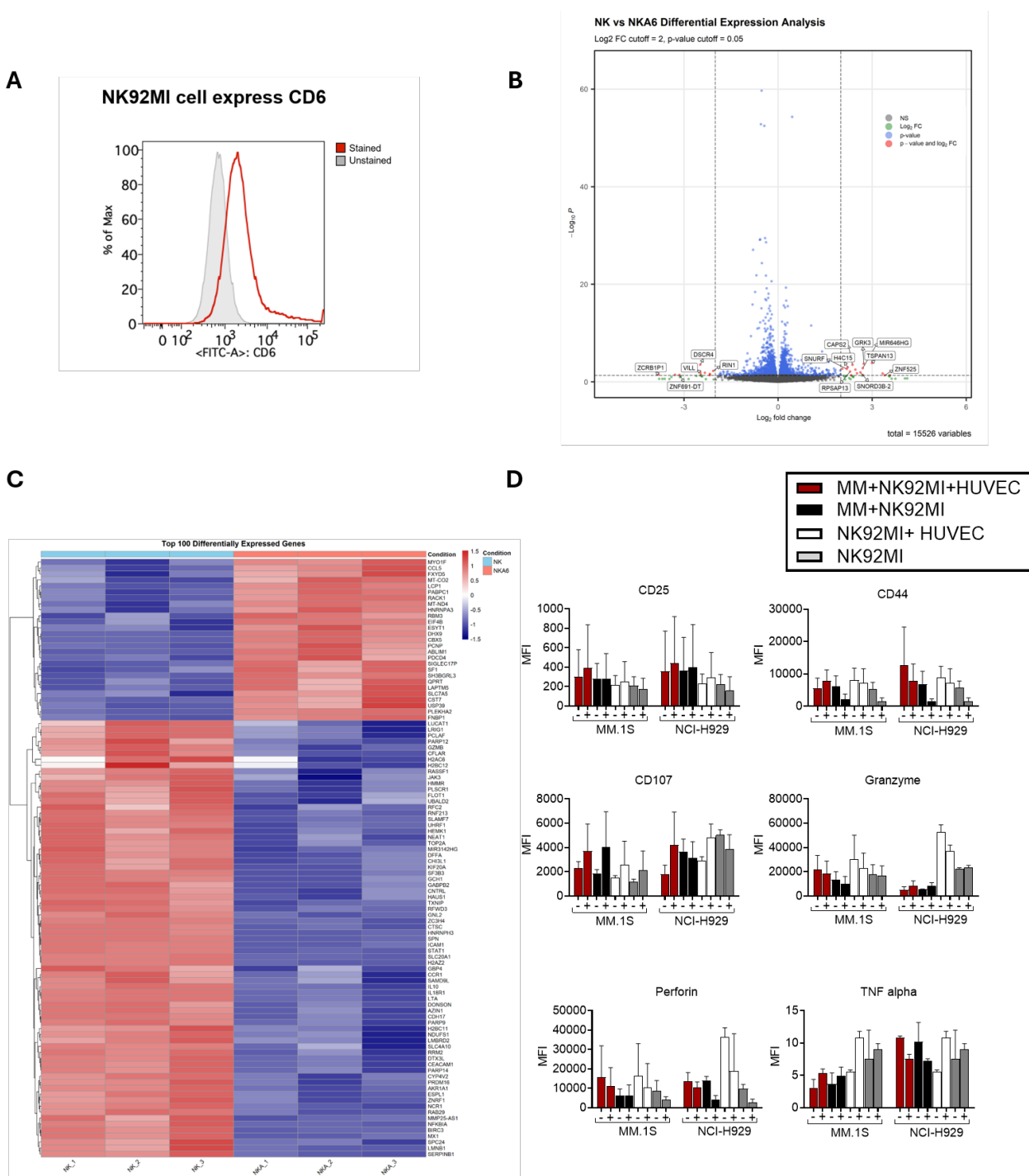
Endothelial cell priming alters myeloma cell susceptibility to NK cell-mediated killing. Percent survival of MM.1S (top) and NCI-H929 (bottom) cells after culture with NK92MI cells under four conditions: direct co-culture with HUVECs, exposure to HUVEC supernatant, sorting after transient contact with HUVECs, and MM-NK92MI co-culture without HUVECs. Data represent mean percent survival $\pm$ SEM over time from 3 independent experiments. Statistical significance was assessed by Mann-Whitney test.

Supplemental Figure 4



Endothelial cells induce activation of the MET signaling pathway in MM cells. GSEA demonstrating activation of the MET signaling pathway in MM.1S cells following co-culture with HUVECs; NES denotes normalized enrichment score, and FDR denotes false discovery rate.

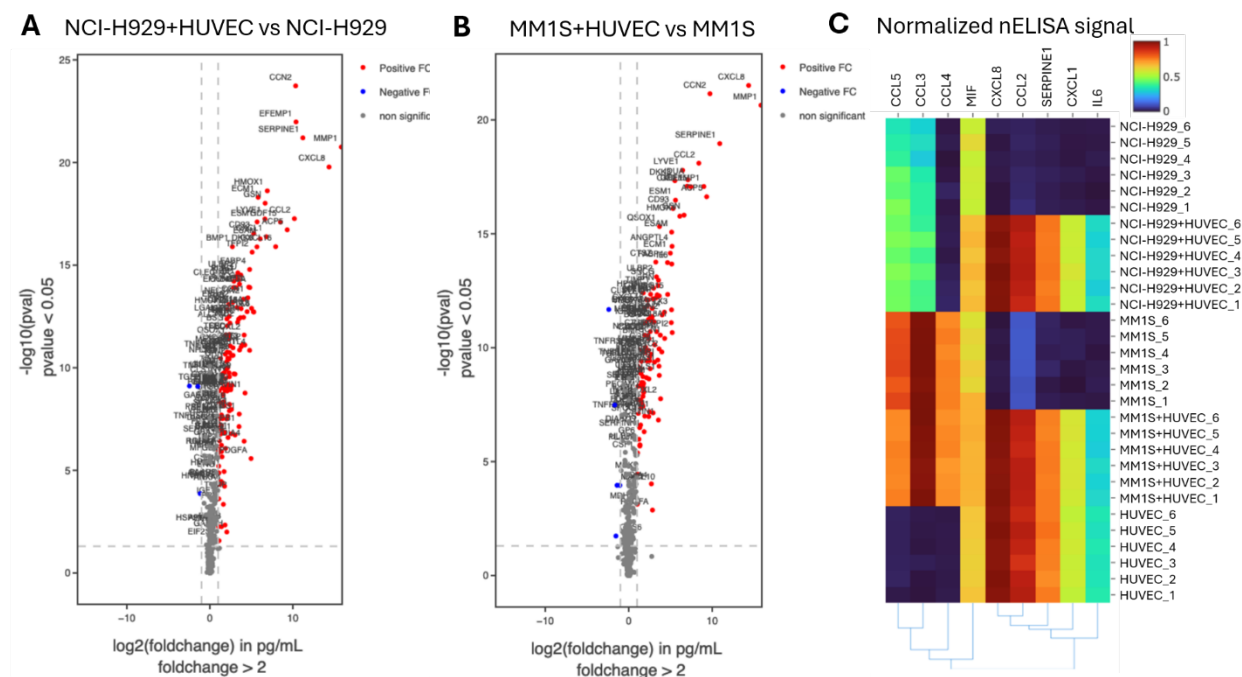
Supplemental Figure 5



Anti-CD6 treatment alters the transcriptional profile of NK cells. (A) Flow cytometric analysis confirming surface expression of CD6 on NK92MI cells, shown as histograms of stained versus unstained controls. (B) Volcano plot displaying differentially expressed genes in NK92MI cells treated with anti-CD6 antibody versus untreated NK92MI controls. Genes meeting significance thresholds ($|\log_2$ fold change > 2 and adjusted $P < 0.05$) are highlighted.

(C) CD6 blockade induces distinct transcriptional changes in NK92MI cells. Heatmap of the top differentially expressed genes in NK92MI cells treated with anti-CD6 antibody (NKA6) compared with untreated control (NK). (D) Bar graphs display mean fluorescence intensity (MFI) of CD25, CD44, CD107a, granzyme B, perforin, and TNF α on NK92MI cells cultured with MM.1S or NCI-H929 myeloma cells in the presence or absence of HUVECs and anti-CD6 antibody. Data are presented as mean $\pm$ SEM from at least 3 independent experiments.

Supplemental Figure 6



HUVEC co-culture alters the secretory profiles of multiple myeloma cells. Volcano plots show changes in soluble-factor levels measured by next-generation multiplexed ELISA in (A) NCI-H929 and (B) MM.1S cells co-cultured with HUVECs compared with the corresponding monocultures. In MM.1S–HUVEC co-culture, prominent upregulated factors included CXCL8, CCN2, MMP1, SERPINE1, and CCL2. In NCI-H929–HUVEC co-culture, the upregulated cytokines included CXCL8 and CCL2, accompanied by increases in secreted factors such as CCN2, EFEMP1, SERPINE1, and MMP1. Red and blue points indicate significantly increased and decreased factors, respectively (fold change >2; $P < 0.05$), while gray points are nonsignificant. (C) Hierarchical clustering heatmap illustrates the relative abundance patterns of select differentially detected factors across the experimental groups.