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Abstract

Tissue inflammation is shaped by reciprocal interactions between immune cells and stromal niches, yet how leukemic myelopoiesis rewires these circuits remains poorly understood. Here, we identify suppression of interferon regulatory factor 8 (IRF8) in hematopoietic cells as a driver of inflammatory bone marrow niche remodeling in chronic-phase myeloid leukemia (CP-CML). IRF8 expression was reduced in human and murine CML and restored following interferon- α therapy.

In mice, hematopoietic IRF8 deficiency induced aberrant neutrophil expansion, extramedullary hematopoiesis, stromal activation and early marrow fibrosis. Single-cell transcriptomics revealed that IRF8-deficient neutrophils engaged perivascular stromal cells, CXCL12-abundant reticular cells and endothelial cells through S100A8/A9–TLR4 and TGF- β –integrin signaling, associated with hypoxia, vascular remodeling and extracellular matrix programs. Mechanistically, IRF8 directly bound the S100a8 locus and restrained the alarmin program in myeloid cells. Conversely, an IRF8-deficient niche amplified BCR–ABL1-driven leukemia and inflammatory alarmin production. These findings identify IRF8 as a hematopoietic regulator of inflammatory neutrophil–stromal communication and reveal how its suppression promotes pathological remodeling of the leukemic bone marrow niche.

Introduction

Tissue inflammation is shaped by reciprocal interactions between hematopoietic cells and their stromal microenvironment. In the bone marrow, these interactions are essential for preserving hematopoietic homeostasis, yet chronic myeloid inflammation can disrupt this balance and induce profound niche remodeling, including vascular alterations, altered bone turnover, and fibrosis. How aberrant myelopoiesis is translated into structural and functional changes of the bone marrow niche remains incompletely understood.

Chronic myeloid leukemia (CML), a clonal myeloproliferative neoplasm driven by the BCR-ABL1 fusion oncoprotein, provides a clinically relevant model to study this process. Beyond the expansion of the malignant clone, CML is associated with splenomegaly, substantial remodeling of bone marrow microenvironment, including increased angiogenesis, altered bone turnover and fibrosis¹. Bone marrow fibrosis occurs in up to half of newly diagnosed patients and has been linked to inferior clinical outcomes and suboptimal responses to tyrosine kinase inhibitor (TKI) therapy². These findings underscore the clinical relevance of microenvironmental alterations in CML and indicate that disease pathogenesis extends beyond the leukemic cells, involving dynamic changes within the bone marrow niche. Despite increased recognition of stromal involvement in CML², the mechanisms driving microenvironmental remodeling and fibrosis remain incompletely understood, although often attributed to leukemic cytokines.

Defining these early events in patients is challenging. Contemporary clinical monitoring of CML relies primarily on peripheral blood counts and molecular assessment of BCR-ABL1 transcripts, whereas repeated bone marrow biopsies are rarely performed once cytogenetic and molecular responses are achieved. As a result, longitudinal access to bone marrow tissue is limited, and the cellular circuits linking leukemic hematopoiesis to niche inflammation, stromal activation, and fibrotic remodeling remain difficult to capture in human disease.

The transcription factor interferon regulatory factor 8 (IRF8) is a central regulator of myeloid differentiation, inflammatory responses, and tumor suppression. In CML, IRF8 expression has been reported to be suppressed^{3,4}. Loss of IRF8 promotes the expansion of immature myeloid populations and enhances inflammatory signaling. We therefore hypothesized that IRF8-deficient hematopoiesis provides a tractable model to dissect how dysregulated myelopoiesis perturbs hematopoietic-stromal communication during chronic-phase CML.

To test this, we used IRF8-deficient (*Irf8^{Δ/Δ}*) mice and applied single-cell transcriptomics to dissect cellular interactions within the bone marrow niche. These analyses uncovered an inflammatory communication circuit between neutrophil populations and perivascular stromal cells, associated with splenomegaly, hypoxic stress, and activation of fibrotic remodeling programs. IRF8 loss induced marked upregulation of the alarmins S100A8 and S100A9, inflammatory mediators previously implicated in fibrosis progression in Philadelphia-chromosome negative myeloproliferative neoplasms (MPNs) and identified as a therapeutically targetable axis in myelofibrosis⁵. Mechanistically, IRF8 bound the S100A8 locus, indicating that IRF8 directly restrains this alarmin-driven inflammatory program. Together, these findings identify IRF8-dependent neutrophil-stromal signaling axis that links myeloid dysregulation to inflammatory remodeling of the bone marrow niche in CML.

Results

Loss of *Irf8* recapitulates a chronic-phase CML-like phenotype with inflammatory niche remodeling

IRF8 is a key regulator of myeloid differentiation and inflammatory responses, and its suppression has been implicated in CML. We therefore first asked whether reduced IRF8 expression is a disease-associated feature of human CML and whether this distinguishes CML from other myeloproliferative neoplasms. IRF8 expression was selectively reduced in CML compared with controls and polycythemia vera (PV), indicating that IRF8 suppression is not a general consequence of myeloproliferation but is associated with BCR-ABL1-driven disease (Fig. 1A). Notably, IRF8 expression was restored following effective interferon- α therapy⁶ linking IRF8 suppression to active CML and suggesting that IRF8 may mark a reversible immune-regulatory state in CML (Fig. 1A). Consistent with these findings, IRF8 expression was also reduced in BCR-ABL-driven murine models⁷. Together, these results identify IRF8 suppression as a CML-associated hematopoietic alteration and prompted us to investigate whether *Irf8* deficiency could model chronic-phase CML-like immune and niche remodeling.

Irf8-deficient mice displayed pronounced myeloid skewing in the bone marrow with marked expansion of mature Ly6G⁺ granulocytes (Fig. 1B), one of the defining CP-CML features. This was accompanied by broad suppression of non-granulocytic hematopoiesis, including reduced monocyte, dendritic cell (DC), T-cell, B-cell, CD41⁺, and erythroid lineage (Pre MegE, MkPs, Pre CFU-E, CFU-E, Pro-Ery and Ter119⁺) and hematopoietic stem cell and progenitor (LT-HSC, ST-HSC, CMP, GMP, MEP) cells compared with controls (Fig. 1B; Supplementary Fig. 1C,E,G). Despite this pronounced marrow skewing, peripheral blood counts remained largely preserved and systemic inflammation was mild, consistent with an indolent chronic-phase-like state rather than aggressive leukemia (Supplementary Fig. 1A). Circulating leukocyte subsets mirrored the marrow phenotype, with expansion of mature Ly6G⁺ granulocytes and reduced monocytes, dendritic cells, and CD41⁺ cells (Supplementary Fig. 1B).

Irf8-deficient mice also developed prominent splenic remodeling. Spleens were markedly enlarged and showed disrupted white pulp architecture with reduced white pulp areas (Fig. 1D-F; Supplementary Fig. 1I). This architectural remodeling was accompanied by depletion of white-pulp-associated immune populations, including B-cells, T-cells, and DCs, and expansion of red-pulp hematopoiesis, marked by increased hematopoietic stem and progenitor cells, monocytes, erythroid-lineage cells, and megakaryocytic populations (Fig. 1C; Supplementary Fig. 1D,F,H). Thus, *Irf8* loss induces splenomegaly and extramedullary hematopoiesis, two cardinal features of chronic myeloid disease.

In the bone marrow, *Irf8* loss induced striking megakaryocyte remodeling. Despite an overall normocellular marrow, *Irf8*-deficient mice showed increased numbers of enlarged, partially clustering megakaryocytes (Mks) and appearance of hypolobulated micromegakaryocytes. Megakaryocytic expansion and clustering were also evident in the spleen (Fig. 1G-I), and nearest-neighbor analysis confirmed increased megakaryocyte density and altered spatial organization in *Irf8*-deficient tissues (Fig.

1H–K; Supplementary Fig. 1J–L). Immunofluorescence staining further revealed close interactions between Ly6G⁺ neutrophils and CD61⁺ megakaryocytes, consistent with megakaryocyte–neutrophil emperipoiesis (Fig. 1L). These abnormalities mirror characteristic megakaryocytic changes observed in human chronic-phase CML, including megakaryocyte clustering, micromegakaryocytes, nuclear hypolobulation, and emperipoiesis.

Finally, *Irf8* deficiency was associated with early extracellular matrix remodeling. Reticulin staining revealed mild bone marrow fibrosis, confirmed by collagen I immunofluorescence (Supplementary Fig. 1M). Dysplastic white pulp architecture was also observed in the spleen of *Irf8*^{Δ/Δ} mice. Thus, the *Irf8*-deficient phenotype extends beyond altered hematopoietic output and includes structural remodeling of hematopoietic niches.

Collectively, these findings demonstrate that *Irf8* deficiency recapitulates central features of chronic-phase CML, including granulocytic expansion, splenomegaly, extramedullary hematopoiesis, megakaryocytic dysplasia, and early fibrotic remodeling. This establishes *Irf8* deficiency as a chronic-phase CML-like model and links impaired myeloid immune regulation to inflammatory remodeling of the bone marrow niche.

***Irf8* deficiency drives compartment-specific stromal remodeling in bone marrow and spleen**

Having established that *Irf8* deficiency recapitulates key hematopoietic, megakaryocytic, and fibrotic features of chronic-phase CML, we next asked how loss of *Irf8* affects the stromal compartments that organize hematopoietic and immune function. Early niche remodeling in CML remains difficult to study in patients, particularly at stages preceding overt fibrosis. The *Irf8*-deficient model therefore provided an opportunity to resolve how altered myeloid immune regulation is coupled to stromal reorganization in bone marrow and spleen.

We first performed flow cytometric profiling of mesenchymal and endothelial populations in the bone marrow. *Irf8*-deficient mice showed increased frequencies of vascular-associated stromal populations within viable lineage-negative cells, including arteriolar, peri-sinusoidal, and lymphatic endothelial cells (Fig. 2A; Supplementary Fig. 2A). Sinusoidal endothelial cells and pericytes showed a similar trend (Fig. 2A; Supplementary Fig. 2A). In parallel, mesenchymal stromal composition was altered: Lepr⁺ CXCL12-abundant reticular (CAR) cells were markedly reduced, whereas fibroblasts and adipo- and osteo-primed CAR cells tended to increase (Fig. 2B; Supplementary Fig. 2A). Thus, *Irf8* loss is associated with expansion of vascular-associated compartments and depletion of key hematopoietic-supportive CAR cells, consistent with early pro-fibrotic reprogramming of the bone marrow niche.

Stromal remodeling was also prominent in the spleen but followed a distinct compartmental pattern. White-pulp-associated T-cell zone reticular cells (TRCs) were significantly reduced in *Irf8*-deficient mice, consistent with the disrupted white-pulp architecture observed histologically (Fig. 2C). Additional immune-organizing stromal populations, including follicular dendritic cells (FDCs), marginal zone reticular cells (MRCs), Ly6G⁺ and Ly6G[−] adventitial reticular cells (ARCs), and pericytes, were similarly reduced, whereas red pulp reticular cells (RPRCs) were increased

(Supplementary Fig. 2B). This shift from white-pulp immune-supportive niches toward red-pulp-associated stromal compartments was accompanied by reduced white-pulp areas and thinning of the mesothelial stromal layer at the splenic capsule (Fig. 2D,E). These findings indicate that *Irf8* deficiency reorganizes the splenic niche by disrupting lymphoid stromal architecture while reinforcing red-pulp compartments associated with extramedullary hematopoiesis.

To validate this spatial remodeling *in situ*, we performed co-detection by indexing (CODEX) microscopy. *Irf8*-deficient spleens showed reduced white-pulp areas, disrupted and thickened MadCAM1⁺ marginal zones, and poorly defined B220⁺ B-cell follicles and CD3⁺ T-cell zones (Fig. 2E). In parallel, Ter119⁺ erythroid cells expanded within the red pulp, consistent with flow cytometric evidence of extramedullary hematopoiesis (Fig. 2E). Thus, stromal, and hematopoietic remodeling are spatially coupled in the *Irf8*-deficient spleen.

The bone marrow displayed a distinct but coordinated remodeling pattern. CODEX microscopy confirmed marked expansion of Ly6G⁺ neutrophils and revealed atypical, dimly stained megakaryocytes in *Irf8*-deficient mice, whereas megakaryocytes in control marrow retained normal morphology (Fig. 2F). In addition, vascular structures appeared elongated, supporting the flow cytometric evidence of vascular remodeling (Fig. 2F). These spatial changes align with the granulocytic expansion, megakaryocytic dysplasia, and early fibrotic remodeling observed in Figure 1.

Together, these data demonstrate that *Irf8* deficiency induces coordinated, but compartment-specific remodeling of hematopoietic and stromal niches. In the bone marrow, *Irf8* loss is associated with vascular expansion, CAR-cell depletion, neutrophil accumulation, and megakaryocytic dysplasia. In the spleen, it disrupts immune-supportive white-pulp stromal networks while expanding red-pulp compartments linked to extramedullary hematopoiesis. These findings establish a stromal framework for investigating how IRF8-dependent myeloid immune regulation controls inflammatory remodeling of hematopoietic tissues.

***Irf8* deficiency couples inflammatory granulopoiesis to pro-fibrotic CAR cell remodeling**

Building on the hematopoietic imbalance and compartment-specific stromal remodeling in *Irf8*^{Δ/Δ} mice, we next used scRNA sequencing to define the cellular programs associated with bone marrow niche remodeling. As *Irf8* was deleted in all compartments in this model, this analysis was used as a discovery approach to identify altered cell states and candidate immune–stromal communication pathways. Unsupervised clustering resolved major hematopoietic populations, including HSPCs, myeloid and lymphoid cells, as well as two transcriptionally distinct Cxcl12-abundant reticular (CAR) cell populations (Fig. 3A; Supplementary Fig. 3A–D).

Differential gene expression analysis revealed that *Irf8* loss most prominently remodeled the myeloid axis, affecting HSPCs, myeloid progenitors, neutrophil progenitors, and mature neutrophils, while lymphoid populations were less affected (Fig. 3B).

To connect these changes to the granulocytic expansion observed by flow cytometry, we performed pseudotime trajectory analysis of hematopoietic progenitors. This resolved erythroid, monocytic, and granulocytic differentiation trajectories and showed marked enrichment of early-to-mature neutrophil states along the granulocytic branch in *Irf8*-deficient bone marrow (Fig. 3C; Supplementary Fig. 3E-F). Thus, *Irf8* loss promotes enhanced granulopoietic flux rather than simple accumulation of mature granulocytes.

Pathway inference further identified coordinated inflammatory and stromal activation programs. PROGENy analysis⁸ showed prominently increased p53 pathway activity across myeloid compartments, whereas TNF α and NF- κ B signaling were selectively upregulated in megakaryocyte–erythroid progenitors (MEPs) and immature as well as mature neutrophils (Fig. 3D). Mature neutrophils upregulated inflammatory TNF α -associated genes whereas CAR cells 1 showed increased TGF- β pathway activity and expression of fibrosis-associated genes, including *Col1a2*, *Col6a2*, *Limch1*, *Igfbp7*, *Fbln5*, *Ltbp2*, *Pdgfra*, *Pdgfrb*, *Fap*, *Sparc*, *Bgn*, and *Adamts2* (Fig. 3D; Supplementary Fig. 3G,H). These findings indicate that *Irf8* deficiency links inflammatory myeloid activation to pro-fibrotic stromal reprogramming.

We next asked whether these transcriptional changes were associated with altered intercellular communication. Cell-cell-interaction (CCI) analysis demonstrated numerous downregulated interactions in *Irf8*^{td/d} mice. CAR cells 1 were identified as a central stromal receiver of signals from multiple hematopoietic populations, including HSPCs, GMPs, neutrophil progenitors, immature and mature neutrophils, megakaryocytic progenitors, and pDCs (Fig. 3E; Supplementary Fig. 3I). Among neutrophil-to-CAR cell interactions, TGF- β 1–integrin signaling emerged as a dominant predicted axis and was among the most frequently used ligand–receptor interactions across the dataset (Fig. 3E; Supplementary Fig. 3J–L). Consistent with this, CAR cells showed increased extracellular matrix, myofibroblast, and epithelial–mesenchymal transition signatures (Fig. 3F; Supplementary Fig. 3M).

To determine whether this early stromal response resembled disease-associated fibrosis, we compared *Irf8*-deficient CAR cell signatures with a reference bone marrow stromal dataset from classical myeloproliferative neoplasms with high-grade fibrosis⁵. Upregulated genes in *Irf8*-deficient CAR cells significantly overlapped with fibrotic MSC populations, including *S100a8*, *S100a9*, *Wisp2*, *Limch1*, *Col1a1*, *Col8a1*, *C4b*, *Igfbp7*, *Mgp*, and *Bgn* (Fig. 3G; Supplementary Fig. 3N). The strongest overlap was observed with pre-fibrotic MSC-1 and MSC-2 populations, indicating that *Irf8*-deficient CAR cells acquire early disease-associated stromal activation programs before extensive matrix deposition becomes apparent histologically.

Together, single-cell profiling of full *Irf8*-deficient bone marrow identifies a coordinated inflammatory niche program characterized by enhanced granulopoiesis, inflammatory neutrophil activation, and pro-fibrotic CAR cell remodeling. These data nominate neutrophil-to-CAR cell communication, particularly TGF- β –integrin signaling, as a candidate mechanism linking *Irf8*-dependent myeloid immune regulation to early fibrotic niche remodeling (Fig. 3H).

Hematopoietic *Irf8* loss is sufficient to activate inflammatory perivascular niche remodeling

The full *Irf8*-deficient model nominated inflammatory neutrophil–stromal communication as a driver of niche remodeling but could not distinguish whether these effects were initiated by hematopoietic or stromal *Irf8* loss. To test whether *Irf8*-deficient hematopoiesis is sufficient to remodel a wild-type stromal environment, we transplanted bone marrow from *Irf8*^{Δ/Δ} or control donors into Gli1CreERT;tdTomato reporter recipients, enabling lineage tracing of recipient-derived Gli1-lineage stromal cells after tamoxifen induction (Fig. 4A). Our previous work established that Gli1-lineage stromal cells are critical mediators of fibrotic remodeling across multiple organs and represent the major source of osteoblasts in the adult murine skeleton^{9,10}.

Following hematopoietic reconstitution, *Irf8*^{Δ/Δ}→Gli1CreERT;tdTomato chimeras developed a stable, chronic myeloid-skewed phenotype. Peripheral blood showed increased granulocyte frequencies with reduced non-granulocytic lineages, including macrophages, monocytes, DCs, T-cells, B-cells and platelets whereas total white blood cell counts, erythroid parameters and body weight remained largely preserved (Fig. 4B; Supplementary Fig. 4A-C). At 14 weeks after transplantation, bone marrow analysis confirmed expansion of Ly6G⁺ granulocytes and significant reduction of macrophages, monocytes, DCs, B- and T-cells in *Irf8*^{Δ/Δ}→Gli1CreERT;tdTomato mice. (Fig. 4C; Supplementary Fig. 4D). Histological analysis of the bone marrow showed a surprisingly normocellular bone marrow given the relative absence of stem cells and again showed enlarged or dysmorphic, small Mks with displaced or absent nuclei (Fig. 4D). Stem and progenitors populations were reduced in the bone marrow but increased in the spleen, consistent with compensatory extramedullary hematopoiesis, splenomegaly, red pulp expansion and reduced white pulp areas (Fig. 4E-G; Supplementary Fig. 4E-G). Thus, hematopoietic *Irf8* loss was sufficient to reproduce the chronic-phase-like hematopoietic and architectural phenotype observed in full *Irf8*-deficient mice.

Importantly, *Irf8*-deficient hematopoiesis also activated recipient-derived stromal cells. In control chimeras, Gli1-lineage stromal cells remained largely confined to perivascular regions, and collagen I deposition was mainly restricted to the splenic capsule and white-pulp-associated areas. In contrast, *Irf8*^{Δ/Δ} chimeras showed redistribution of Gli1-lineage stromal cells parenchyma, accompanied by aberrant collagen I deposition extending into red-pulp regions (Fig. 4G). Reticulin staining confirmed stromal remodeling in both spleen and endosteal bone marrow (Supplementary Fig. 4H). These findings demonstrate that *Irf8*-deficient hematopoiesis is sufficient to induce secondary remodeling of a wild-type stromal compartment.

To define the molecular communication programs through which *Irf8*-deficient hematopoiesis instructs the niche, we performed single-cell RNA sequencing of bone marrow from hematopoietic *Irf8*-deficient and control chimeras. Unsupervised clustering of single-cell RNA sequencing data from the bone marrow of hematopoietic *Irf8*-deficient or control mice resolved major hematopoietic and stromal populations, including HSPCs, myeloid progenitors, neutrophil-lineage cells, monocytes, B-cells,

DCs, endothelial cells, pericytes, CAR cell subsets, fibroblastic stromal cells, and nerve-associated cells (Fig. 4H, Supplementary Fig. 4I-M). Augur prioritization identified neutrophil progenitors, GMPs, HSPCs, CAR cells, and pericytes as the most transcriptionally perturbed populations in *Irf8*-deficient chimeras (Fig. 4I).

Early neutrophil progenitors drive inflammatory and structural remodeling of the vascular niche upon hematopoietic *Irf8* deficiency

Pathway analysis showed that these perturbed populations converged on inflammatory, migratory and perivascular remodeling programs. Early neutrophil progenitors showed increased TNF α , TGF- β , IL6–JAK–STAT3, and chemokine signaling, whereas HSPCs, LMPPs, and myeloid cells were enriched for chemotaxis and migration-associated pathways (Fig. 5A). CAR cells showed increased neutrophil degranulation, leukocyte and granulocyte activation, integrin–cell surface interaction, and cell motility programs, linking hematopoietic *Irf8* loss to activation of the perivascular stromal niche (Fig. 5A).

Cell–cell interaction analysis identified S100A8/A9–TLR4 signaling as a dominant inflammatory communication axis between neutrophil-lineage cells and perivascular niche populations. Neutrophils and neutrophil progenitors were predicted to signal through S100a8/a9 ligands to Tlr4-expressing pericytes and endothelial cells, with Tlr4 emerging as the most frequently used receptor for S100a8 across the dataset (Fig. 5B; Supplementary Fig. 5A-C). Consistent with activation of this inflammatory axis, inflammatory gene module scores were elevated in arteriolar endothelial cells and pericytes (Fig. 5G). Perivascular remodeling was further supported by increased blood vessel morphogenesis and angiogenesis programs in CAR cells and osteolineage cells (Fig. 5D; Supplementary Fig. 5D).

To provide orthogonal validation in a humanized three-dimensional niche model, we generated bone marrow organoids from human iPSCs and engrafted them with iPSC-derived hematopoietic progenitor cells from JAK2V617F and JAK2WT lines. Without external inflammatory stimulation, immunofluorescence revealed discrete clusters of HIF-1 α ⁺ cells that partially co-localized with S100A8/A9. A subset of HIF-1 α ⁺ S100A8/A9⁺ cells also expressed TGF- β , linking hypoxia, alarmin expression, and pro-fibrotic cytokine production within dense cellular niches (Fig. 5E, Supplementary Fig. 5E).

Together, these experiments demonstrate that hematopoietic *Irf8* loss is sufficient to induce chronic myeloid skewing, activate recipient-derived Gli1-lineage stromal cells, and remodel the perivascular niche. Mechanistically, the transplant model identifies neutrophil progenitors and perivascular niche cells as the most perturbed compartments and nominates S100A8/A9–TLR4 signaling as a central inflammatory axis linking *Irf8*-deficient hematopoiesis to vascular and stromal remodeling.

Direct regulation of S100A8 by IRF8 restrains hypoxia-associated inflammatory niche remodeling

Having identified S100A8/A9–TLR4 signaling as a candidate axis linking *Irf8*-deficient neutrophil-lineage cells to perivascular niche remodeling, we next asked whether this inflammatory circuit was associated with fibrotic activation of stromal cells. Across stromal populations in hematopoietic *Irf8*-deficient chimeras, extracellular matrix

module scoring revealed significant enrichment of NABA collagen gene sets in CAR cells, identifying them as the principal stromal population acquiring a fibrotic transcriptional program (Fig. 6A). Consistently, PROGENy pathway analysis showed strong TGF- β activation in CAR cells, whereas CAR cells also displayed a pronounced hypoxia-associated transcriptional signature (Fig. 6B). In parallel, neutrophils and their progenitors were enriched for hypoxia-, TRAIL-, NF- κ B- and Tnf α -related pathways, indicating coordinated pro-fibrotic, stress and inflammatory signaling between neutrophil populations and the perivascular stromal niche. Simultaneously, p53 signaling was upregulated in cell populations with increased hypoxia signature (Fig. 6B).

To determine whether this stromal response resembled disease-associated myelofibrosis, we compared differentially expressed genes in *Irf8*-deficient CAR cells and osteolineage cells with a reference bone marrow stromal dataset from early- and late-stage MPN fibrosis⁵. *Irf8*-deficient stromal populations shared fibrosis-associated genes with MPN stromal subsets, including *S100a8*, *S100a9*, *Mgp*, *Col1a1*, *Col8a1*, *C4b*, *Limch1*, *Cxcl14*, and *Igf1bp7* (Fig. 6C; Supplementary Fig. 6A). CAR cells further showed a shift toward myofibroblast programs, with reduced hematopoietic-supportive and vascular-signaling signatures, and upregulation of epithelial–mesenchymal transition, mesenchymal activation, extracellular matrix organization, and collagen biosynthesis programs (Fig. 6D). Gli1-lineage activation was also evident within CAR cells and osteolineage cells, particularly in the peritrabecular niche, consistent with fibrotic stromal reprogramming of the bone marrow microenvironment (Fig. 6E).

The enrichment of the S100a8, S100a9 Hif-1 α -associated programs in *Irf8*-deficient stromal populations, suggested a link between hypoxia, alarmin signaling, and fibrotic remodeling. CrossTalker analysis incorporating intracellular signaling identified Nr1h3, Sp3, Hif-1 α , and Arnt as transcriptional regulators shared across CAR cell subsets and osteolineage cells, with consistent HIF-1 α –ARNT activity across these stromal populations (Supplementary Fig. 6C,D). In cycling CAR cells, the fibrosis-associated receptor Itgb1 was linked to HIF-1 α activity, while Cxcl12 and Adipoq were among ligands associated with HIF-1 α -driven transcriptional programs (Supplementary Fig. 6E). In parallel, S100A8/A9-mediated crosstalk was enhanced between activated monocytes and neutrophil progenitors, suggesting inflammatory amplification within the myeloid compartment (Supplementary Fig. 6F). Together, these analyses support a model in which hypoxia-associated stromal activation and inflammatory myeloid alarmin signaling reinforce one another within the remodeling perivascular niche.

We next tested whether IRF8 directly regulates the S100A8/A9 alarmin axis. IRF8 chromatin immunoprecipitation sequencing in sort-purified cDC2s and GFP⁺ *Irf8*-deficient progenitors reconstituted with inducible IRF8-ERT2 revealed a prominent IRF8-binding peak at the S100A8 locus, indicating direct transcriptional regulation by IRF8 (Fig. 6F). We then assessed S100A8/A9 expression *in vivo*. Immunohistochemistry demonstrated increased S100A8/A9 protein abundance in the bone marrow and spleen of *Irf8*^{d/d} mice, with splenic S100A8/A9 predominantly localized to the red pulp, corresponding to granulocyte-rich regions (Fig. 6G–I). Intracellular flow cytometry further identified granulocytes as the principal hematopoietic source of S100A8/A9 in both bone marrow and spleen (Supplementary

399 Fig. 6G,H). These data validate S100A8/A9 as a neutrophil-associated inflammatory
400 axis activated *in vivo* upon loss of IRF8.

401 Spatial CODEX analysis of murine spleens further confirmed expansion of
402 S100A8/A9⁺ inflammatory cells in hematopoietic *Irif8*-deficient chimeras, particularly
403 near the splenic capsule, together with reduced and disorganized white-pulp areas
404 and diminished CD3⁺ T-cell zones (Supplementary Fig. 6I).

405 Collectively, these findings identify S100A8/A9 as a direct IRF8-regulated alarmin axis
406 and validate its activation at transcriptional, protein, cellular, and spatial levels. Loss
407 of IRF8 therefore unleashes a neutrophil-associated S100A8/A9 inflammatory
408 program that converges with hypoxia and TGF- β signaling to promote fibrotic
409 remodeling of the perivascular hematopoietic niche.

410 **S100A8/A9 inflammatory niche remodeling promotes BCR–ABL-driven disease** 411 **progression and fibrosis**

412 Having established that *Irif8*-deficient hematopoiesis induces S100A8/A9-associated
413 inflammatory remodeling of the bone marrow niche, we next asked whether such a
414 preconditioned microenvironment alters the course of BCR–ABL-driven
415 myeloproliferative disease. To test this, BCR–ABL-transduced c-Kit-enriched bone
416 marrow cells from wild-type donors were transplanted into lethally irradiated *Irif8*^{+/+} or
417 *Irif8*^{d/d} recipients, thereby introducing an *Irif8*-expressing leukemic clone into either a
418 control or *Irif8*-deficient niche environment (Fig. 7A). BCR–ABL-transplanted mice
419 developed rapid disease progression and were sacrificed 12 days after transplantation
420 (Fig. 7B).

421 BCR–ABL expression induced splenomegaly in both recipient genotypes, but spleen
422 weights were highest in *Irif8*^{d/d} recipients, indicating that the *Irif8*-deficient environment
423 exacerbated disease severity (Fig. 7C,D). This was accompanied by a stepwise
424 increase in circulating S100A8/A9 levels: lowest in *Irif8*^{+/+} pMIG controls, increased in
425 *Irif8*^{d/d} pMIG mice, further elevated in *Irif8*^{+/+} BCR–ABL mice, and highest in *Irif8*^{d/d} BCR–
426 ABL mice (Fig. 7E). Thus, systemic S100A8/A9 abundance paralleled inflammatory
427 niche status and BCR–ABL-driven disease severity.

428 Peripheral blood and tissue analyses confirmed progressive myeloid and
429 megakaryocytic dysregulation. BCR–ABL-transplanted mice developed leukocytosis
430 driven by granulocytic expansion, whereas erythroid parameters reflected stress
431 erythropoiesis. Platelet numbers were increased in *Irif8*-deficient recipients and further
432 increased upon BCR–ABL expression, despite reduced CD41⁺ frequencies,
433 consistent with dysregulated megakaryopoiesis (Fig. 7F; Supplementary Fig. 7A,B).
434 Histological analysis revealed that *Irif8*^{d/d} recipients transplanted with BCR–ABL-
435 expressing cells developed the most pronounced megakaryocytic atypia,
436 megakaryocyte clustering, and focal neutrophil accumulation, indicating coordinated
437 expansion of inflammatory myeloid and megakaryocytic compartments within the
438 remodeled niche (Fig. 7F).

439 Flow cytometric analysis of the bone marrow further showed increasing donor
440 chimerism and clonal dominance with disease severity accompanied by depletion of
441 stem and progenitor compartments and expansion of megakaryocyte–erythroid

progenitors. Differentiated granulocytic and monocytic populations were most strongly expanded in *Irf8^{d/d}* recipients, even when transplanted donor cells were *Irf8*-expressing, suggesting that the *Irf8*-deficient niche instructs incoming hematopoietic cells toward inflammatory myeloid differentiation. In the spleen, disease progression was associated with loss of primitive hematopoietic populations and redistribution toward committed megakaryocytic and inflammatory myeloid lineages, consistent with extramedullary hematopoiesis and conversion of the spleen into an inflammatory myelopoietic tissue (Supplementary Fig. 7C–F).

Consistent with this inflammatory remodeling, reticulin staining revealed a stepwise increase in fibrosis severity across the four conditions. *Irf8^{+/+}* pMIG controls showed no detectable fiber deposition, whereas *Irf8^{d/d}* pMIG mice displayed low-grade fibrosis. BCR–ABL expression in *Irf8^{+/+}* recipients induced intermediate fibrosis, while *Irf8^{d/d}* recipients transplanted with BCR–ABL-expressing cells developed the most severe bone marrow fibrosis, with dense and disorganized reticulin networks (Fig. 7G). A similar pattern was observed in the spleen, where BCR–ABL expression promoted reticulin extension into red-pulp and parenchymal regions, most prominently in *Irf8^{d/d}* recipients, accompanied by loss of defined white-pulp architecture (Fig. 7G).

To determine whether the S100A8/A9 inflammatory axis is linked to fibrosis in human CML, we analyzed bone marrow biopsies from patients with CML. S100A8 staining along trabecular bone enabled stratification of stromal S100A8 abundance into grades 0–3 (Fig. 7H). Stromal S100A8/A9 abundance positively correlated with reticulin fibrosis grade, supporting a relationship between alarmin-rich inflammatory niche remodeling and fibrotic disease in patients (Fig. 7I; Supplementary Fig. 7G). In addition, serum lactate dehydrogenase levels positively correlated with fibrosis grade, consistent with increased disease activity and tissue turnover in fibrotic CML (Fig. 7J; Supplementary Fig. 7G).

Together, these findings demonstrate that an *Irf8*-deficient inflammatory niche amplifies BCR–ABL-driven myeloproliferative disease, promoting S100A8/A9 production, inflammatory myeloid expansion, extramedullary hematopoiesis, and fibrotic remodeling. The correlation of S100A8/A9 abundance with fibrosis in human CML further supports the clinical relevance of this IRF8–S100A8/A9 inflammatory niche axis.

Discussion

Our data support a conceptual framework in which bone marrow fibrosis in chronic-phase CML is not solely a secondary consequence of leukemic burden, but rather a maladaptive niche response to persistent inflammatory stress. This is in line with recent clinical observations that myelofibrosis (MF) present at diagnosis in chronic myeloid leukemia (CML) patients is associated with higher failure rates of treatment-free remission (TFR), most likely due to an inflammatory response¹¹. We identify IRF8 as a regulator of myeloid homeostasis that limits the transition from transient myeloid activation to sustained stromal remodeling^{12–14}. Beyond its established role in myeloid differentiation, IRF8 therefore emerges as a suppressor of inflammation-driven niche remodeling and fibrotic transformation^{15–18}.

Mechanistically, we identify repression of S100A8 as a central component of IRF8-mediated control of inflammatory signaling, consistent with the role of IRF8 in restraining myeloid progenitor activation¹⁴. The S100A8/A9 alarmin complex has emerged as a potent amplifier of innate immune activation and tissue remodeling across inflammatory and malignant contexts^{19,20}. Loss of *Irf8* appears to release this constraint, enabling a neutrophil-driven alarmin cascade that activates TLR4-dependent inflammatory programs extending beyond the hematopoietic compartment into stromal compartments. In this context, *Irf8* deficiency initiates a multi-compartment inflammatory circuit rather than an isolated hematopoietic defect.

Our data further indicate that the vascular niche functions as an active amplifier of this process rather than a passive recipient of inflammatory stress. Endothelial and perivascular populations are known to relay signals in stress hematopoiesis^{21,22}. We demonstrate that endothelial and perivascular cells translate hematopoietic inflammation into hypoxia-associated fibrotic stromal states, characterized by increased S100A8/A9 expression. This supports a model in which the vasculature acts as a relay that stabilizes and propagates inflammatory signals, consistent with the concept of the bone marrow niche as a dynamic, inflammation-sensitive ecosystem²¹.

Importantly, the association of diagnostic myelofibrosis with poor TKI responses and reduced treatment-free remission suggests that fibrotic niche remodeling may persist despite effective suppression of the malignant clone. This supports a model in which inflammatory signaling within non-hematopoietic compartments sustains a self-reinforcing activation state, potentially explaining the persistence of bone marrow abnormalities despite effective BCR-ABL1 inhibition. Together, these findings support a two-component model of CML in which leukemic clearance does not necessarily restore niche homeostasis once vascular-stromal inflammatory circuits are established. In this setting, IRF8 deficiency recapitulates key features of CML-associated vascular inflammation²³ and stromal activation, indicating that dysregulated immune differentiation may be sufficient to drive microenvironmental remodeling even in the absence of oncogenic BCR-ABL1 signaling^{11,24,25}. Related concepts have been proposed in the context of myeloid-driven inflammation^{7,25}.

Comparison with Philadelphia chromosome-negative MPNs further suggests that similar stromal outputs can arise across distinct myeloid stress states, despite differences in upstream oncogenic and inflammatory triggers⁵. In human CP-CML, reduced IRF8 expression is associated with stromal S100A8 accumulation and fibrosis severity, supporting a model in which IRF8 may act as a proximal regulator of this axis. These observations point to convergence on a shared pathological endpoint with divergence at the level of initiating signals and transcriptional control, positioning IRF8 as one entry point into a broader conserved program.

Collectively, our findings nominate the IRF8–S100A8/A9–TLR4 axis as a targetable inflammatory circuit in CML-associated niche remodeling. Therapeutic disruption of this pathway may complement BCR–ABL1-directed therapy by addressing the microenvironmental component of disease persistence, particularly in patients with residual fibrosis or inflammatory marrow remodeling despite molecular response. More broadly, our work positions IRF8 as a molecular brake on maladaptive myeloid inflammation, preventing inflammatory hematopoiesis from becoming self-sustaining stromal pathology.

531

532 **Methods**

533 **Animals and mouse models**

534 *Irf8*^{td/d} mice (IRF8^{tm1Hor}) aged 4 to 12 months were bred and maintained in the central
535 animal facility of the RWTH Aachen University Hospital and previously described¹².

536 Gli1^{tm3}(cre/ERT2)Alj/J(Gli1CreERT) and B6.Cg-Gt(ROSA)26Sortm9(CAG-
537 tdTomato)Hze/J(tdTomato) mice were obtained from The Jackson Laboratory (strains
538 #007913 and #007909) and crossed to generate Gli1CreERT;tdTomato reporter mice.
539 Offspring were genotyped by PCR.

540 B6.SJL-PtprcaPepcb/BoyCrl (CD45.1) were purchased from Charles River (strain
541 #564) and C57BL/6 wild-type mice (The Jackson Laboratory; strain #000664) served
542 as controls.

543 All animal experiments were approved by State Agency for Consumer Protection and
544 Nutrition North-Rhine-Westphalia (Landesamt für Verbraucherschutz und Ernährung
545 Nordrhein-Westfalen), protocol reference number [M2026-154, formerly:
546 2020.A128_40091G]. All procedures were conducted in strict accordance with the
547 approved protocols, including defined parameters for donor and recipient age,
548 treatment schedules, blood collection, phenotypic assessment, and experimental
549 endpoints, as well as housing and welfare conditions. All mice were maintained on a
550 12-hour light/dark cycle with ad libitum access to food and water. At the experimental
551 endpoint, bones (femur, tibia, sternum, and spine) and spleens were used for 10x
552 single cell sequencing, flow cytometry, immunofluorescence analysis or histology.

553 *Retroviral transduction*

554 HEK293T cells were maintained in DMEM (Gibco) supplemented with 10% fetal calf
555 serum (FCS) and 1% penicillin-streptomycin (Thermo Fisher Scientific). Cells were
556 seeded at 6x10⁶ cells and transfected with pMIG-BCR-ABL or pMIG control vectors
557 together with pCLEco using Lipofectamine 3000 (Thermo Fisher Scientific) or TransIT-
558 LT1 (MoBiTec) according to the manufacturer's instructions. For IRF8 expression,
559 cells were transfected with IRF8-ERT2 in MIEG3²⁶, pVpack-Eco (Stratagene) or pV-
560 pack-GP (Stratagene) using CaCl₂ precipitation according to the manufacturer's
561 instructions. Viral supernatants were collected after 48 hours, filtered through 0.45 µm
562 filters, and used for transduction.

563 *Murine bone marrow transplantation*

564 For bone marrow isolation, single-cell suspensions were prepared as described below.
565 c-Kit⁺ cells were enriched using magnetic separation with LS columns, following the
566 manufacturer's protocol except that 2% FCS in 1x PBS was used as a separation
567 buffer. Typically, 6-10% c-Kit⁺ cell yield relative to viable cells after RBC lysis was
568 targeted. If this threshold was not reached, enrichment was repeated. Enriched cells
569 (1x10⁶/mL) were cultured in 3 mL StemSpan supplemented with 50 ng/mL
570 recombinant murine stem cell factor (mSCF; PeproTech), 50 ng/mL recombinant
571 murine thrombopoietin (mTPO; PeproTech) and 1% penicillin-streptomycin.

For RetroNectin-mediated transduction, 6-well plates were coated with RetroNectin (Takara Bio) for 2 hours at room temperature or overnight at 4°C. Filtered viral supernatants (4 mL per well) were added and centrifuged at 2700 x g for 1 hour at 32°C. The supernatant was then removed, and c-Kit-enriched cells (3×10^6 cells/well) were plated directly onto the RetroNectin-coated surface. After 24 hours of incubation, cells were harvested, analyzed for transduction efficiency by flow cytometry, and prepared for transplantation.

Transduced cells were gently detached, washed, and resuspended in 1x PBS (600 μ L per well). Cell suspensions were filtered through pre-wetted cell strainers into cryotubes and used immediately for transplantation.

Irf8^{d/d} bone marrow culture and retroviral transduction

Bone marrow cells were isolated from femur and tibia of *Irf8^{d/d}* mice by flushing the bones with pre-warmed BMC medium (RPMI 1640 supplemented with 10% FCS, 1% L-glutamine, 1% penicillin/streptomycin, 0.1% β -Mercaptoethanol; Gibco). Single-cell suspensions were cultured at 1.5×10^6 cells/mL in BMC medium supplemented with stem cell factor (30 U/mL), IL-6/soluble IL-6 hyper fusion (hyper-IL-6, 25 ng/mL), Flt3 ligand (25 ng/mL) and long-range insulin-like growth factor (IGF-1, 40 ng/mL) at 37°C with 5% CO₂ as described²⁷.

After 72 h, viable mononuclear cells were enriched by Ficoll Hypaque density-gradient centrifugation using lymphocyte separation medium (LSM 1077; PAA). Cells were reseeded at 1.5×10^6 cells/mL in BMC medium, with medium replacement every 36 hours to maintain a density of $1.5\text{--}2 \times 10^6$ cells/mL.

Retroviral supernatant containing MIEG-IRF8-ERT2 was generated in HEK293T cells as described above. Viral supernatant was concentrated with Hexadimethrin bromide [polybrene] and chondroitin sulfate salt from shark cartilage polymers [CSC] (80 μ g/mL each; both Sigma-Aldrich)²⁸; and added to *Irf8^{d/d}* progenitor bone marrow cultures on day 4. Cells were incubated for 24 hours, and transduction was repeated once. Twenty-four hours later, polybrene-CSC complexes were removed by Ficoll Hypaque density-gradient centrifugation. Transduction efficiencies were determined by measuring GFP expression by flow cytometry and fluorescence microscopy.

For induction IRF8-ERT2 activity, cells were seeded at 1.5×10^6 cells/mL and stimulated with 1 μ M 4-hydroxytamoxifen for 24 hours on day 9-10 of culture.

Blood Collection and Processing

Mouse blood processing

Blood was collected via the facial vein into EDTA-coated tubes for flow cytometry, blood smears, plasma isolation, and complete blood counts. For blood smears, 1 μ L of blood was applied to one end of a glass slide and spread using a second glass slide held at an angle. For flow cytometry, 20 μ L blood was processed following red blood cell lysis with 1 mL 1x RBC lysis buffer (BD Biosciences) as described below. Plasma was isolated by centrifugation at 2,000 x g for 5 minutes, and the supernatant was stored at -80°C.

613 *Human blood processing*

614 Whole blood was collected in EDTA tubes, and peripheral blood mononuclear cells
615 (PBMCs) were isolated by density gradient centrifugation using lymphocyte separation
616 medium (PromoCell). PBMCs were either used immediately or cryopreserved at -80°C
617 in RPMI-1640 supplemented with 20% FCS and 10% DMSO. All patients and healthy
618 blood donors provided written informed consent (EK206/09, EK127/12, EK099/14).

619 *Mouse S100A8/A9 Heterodimer DuoSet ELISA*

620 Murine S100A8/A9 serum levels were measured using the DuoSet ELISA kit for
621 mouse S100A8/A9 (R&D Systems) according to the manufacturer's instructions.
622 Deviations from the protocol included the use of a custom reagent diluent comprising
623 50 mM Tris, 10 mM CaCl₂, 150 mM NaCl and 0.05% Tween20. Plasma samples were
624 diluted 1:25 in this reagent prior to analysis.

625 **Isolation of cells**

626 *Isolation of single bone marrow cells*

627 Bones were crushed with pestle and mortar and flushed with a cell isolation buffer (2%
628 FCS in 1x PBS) until bone fragments were devoid of visible marrow. The resulting cell
629 suspension was filtered through a 70 µm strainer and kept on ice, while remaining
630 bone fragments were digested in 10 mL MEM Alpha Modification (Cytiva)
631 supplemented with 10% FCS, 1% penicillin-streptomycin, and 1 mg/mL Collagenase
632 II (Thermo Fisher Scientific) for 90 min at 37°C. Digested fragments were washed
633 twice with 5 mL FACS buffer and combined with the flushed fraction through the same
634 70 µm cell strainer.

635 Red blood cells were lysed by incubation with 10 mL 1x RBC lysis buffer per bone set
636 for 10 min at room temperature, followed by quenching with 10 mL FACS buffer. Cells
637 were centrifuged for 5 minutes at 450 x g, resuspended in 5 mL FACS buffer, filtered
638 through a fresh 70 µm cell strainer and washed with an additional 5 mL FACS buffer
639 to yield a final suspension of 10 mL. Cell counts were determined, and suspensions
640 were allocated for downstream analysis, including flow cytometry analysis (stromal,
641 stem cell or immune panel) or 10x single-cell RNA sequencing.

642 For stromal enrichment, lineage-positive cells were depleted using a biotinylated CD45
643 antibody (clone 30-F11, BioLegend) or a combination of CD3 (clone 145-2C11,
644 eBioScience), CD19 (clone 6D5, BioLegend), Nk1.1 (clone PK136, BioLegend), Gr1
645 (clone RB6-8C5, BioLegend) and Ter119 (clone TER-119, eBioscience) antibodies
646 and anti-biotin MicroBeads (Miltenyi Biotec) with magnetic separation. Cells were
647 incubated with biotinylated antibodies for 30 min at 4°C, washed, and incubated with
648 MicroBeads with volumes scaled to cell number. Labelled cells were processed using
649 the "deplete" program of the AutoMACS Pro Separator (Miltenyi Biotec) according to
650 the manufacturer's instructions, and the negative fraction was collected for
651 downstream analysis.

652 *Isolation of single spleen cells*

653 Spleens were harvested, weighed, and a single-cell suspension prepared following
654 the STAR protocol²⁹, with a 30-minute incubation at 37°C during enzymatic digestion.
655 Tissues were gently triturated using a cut 1 mL pipette tip to achieve a uniform single-
656 cell suspension and filtered through a 70 µm cell strainer. Red blood cells were lysed,
657 and stromal enrichment was performed as described for bone marrow (10 mL 1x RBC
658 lysis buffer per spleen). Cell counts were determined, and suspensions were allocated
659 to flow cytometric analysis of stromal, immune or stem and progenitor cell populations.
660 Splenic stromal subtypes were identified using the gating strategy of Alexandre et al³⁰.

661 **Flow cytometry**

662 Fluorescence compensation was performed using single-stained UltraComp
663 eBeads™ Plus Compensation beads (Thermo Fisher Scientific). Following cell
664 isolation and red blood cell lysis, single-cell suspensions were stained with
665 fluorochrome-conjugated antibodies against extracellular antigens for 30 minutes at
666 4°C in the dark. When indicated, intracellular staining was performed after fixation and
667 permeabilization using the True-Nuclear Transcription factor Buffer Set (BioLegend)
668 according to the manufacturer's instructions. Cell viability was assessed using fixable
669 viability dyes (Zombie-NIR; Biolegend, Fixable Viability Stain 700; BD Biosciences) or
670 DAPI. Data was acquired on BD LSRFortessa™ equipped with four lasers (405, 488,
671 561, 640 nm) and analyzed using FlowJo software (FlowJo Engine version 5.0000,
672 BD Biosciences).

673 Hematopoietic stem and progenitor cells were defined as nucleated, viable, lineage
674 negative (CD11b, CD3e, B220, Ly6C) single cells. Within this compartment, LK cells
675 were identified as c-Kit⁺ Sca-1⁻ and LSKs cells as c-Kit⁺ Sca1⁺. LK cells were further
676 subdivided into megakaryocyte-erythroid progenitors (MEPs; CD34⁻, CD16/32⁻),
677 granulocyte-monocyte progenitors (GMPs; CD16/32⁺) and common myeloid
678 progenitors (CMPs; CD16/32⁻, CD34⁺). LSKs were further classified as multipotent
679 progenitors (MPPs; CD48⁺, CD150⁻), short-term HSCs (ST-HSCs; CD48⁻, CD150⁻)
680 and long-term HSCs (LT-HSCs; CD150⁺, CD48⁻). In transplantation experiments,
681 donor chimerism within each population was quantified based on the relative
682 contribution of CD45.1⁺ and CD45.2⁺ cells.

683 Immune cells were defined as nucleated, viable, Ter119⁻, (CD45⁺) single cells. Within
684 this compartment, granulocytes were defined as Ly6G⁺ CD11b⁺ cells, Mks as CD41⁺
685 cells, T-cells as CD3⁺ cells, B-cells as B220⁺ cells, dendritic cells as MHCII⁺ cells, and
686 monocytes as CCR2⁺ or CD115⁺ cells. Donor chimerism in transplantation
687 experiments was assessed as described above. Erythroid subpopulations were
688 defined according to Pronk et al³¹.

689 Bone marrow stromal cells were defined as nucleated, viable, lineage negative (CD41,
690 CD45, Ter119, c-Kit) single cells. Endothelial cells were identified as CD31⁺ and
691 further subdivided into PDPN⁺ lymphatic vessels, LEPR⁺ peri-sinusoidal endothelial
692 cells, Sca1⁻ sinusoidal endothelial cells and Sca-1⁺ arteriolar endothelial cells. LEPR⁺
693 Sca-1⁻ stromal cells were further subdivided into NG2⁺ pericytes, ALPL⁺ CD63⁺ osteo-
694 primed cells, CD51⁺ osteo-lineage cells and CD63⁻ CD106⁺ adipocyte-biased cells.

695 Fibroblasts were defined as LEPR^{+/+} Sca1⁺ cells and further characterized as PDPN⁺
696 PDGFRβ⁺ arteriolar fibroblasts.

697 **Molecular gene expression analyses**

698 *cDNA synthesis and quantitative PCR*

699 Complementary DNA (cDNA) was generated from 1 µg of RNA using the High-
700 Capacity cDNA Reverse Transcription Kit with Rnase Inhibitor (Thermo Fisher
701 Scientific), following the manufacturer's instructions. For quantitative PCR, cDNA was
702 diluted 1:5 and mixed with Applied Biosystems Fast SYBR Green Master Mix and
703 gene-specific-primers. Eight microliters of master mix and 2 µL of diluted cDNA were
704 loaded per well, centrifuged briefly, and analyzed on a CFX Opus 96 Real-Time PCR
705 System (Bio-Rad) according to the manufacturer's protocol.

706 *Restriction digestion*

707 Plasmids (1 µg) were digested with 1 µL of the appropriate restriction enzyme in 1 x
708 CutSmart buffer (New England Biolabs) in a total restriction volume of 50 µL and
709 incubated for 1 h at 37°C. Digested samples were mixed with 1x TriTrack DNA loading
710 dye and separated by agarose gel electrophoresis alongside a GeneRuler 1 kb DNA
711 Ladder. Electrophoresis was performed on 0.8-1% agarose gels containing ethidium
712 bromide at 90 V. DNA fragments were visualized under UV illumination.

713 *DH5a Escherichia coli transformation and plasmid purification*

714 Plasmid DNA was added to 100 µL aliquots of CaCl₂-competent *Escherichia coli* DH5α
715 cells. Cells were placed on ice for 15 min, heat-shocked at 42°C for 45 s, and
716 subsequently placed on ice for 2 min. LB medium (0.9 mL) was added, and cells were
717 incubated for 1 hour at 37°C with shaking to allow recovery.

718 Following recovery, 20 µL of the transformation mixture was plated onto agar plates
719 supplemented with 0.1 mg/mL ampicillin and incubated at 37°C overnight. A single
720 colony was then inoculated into 3 mL medium containing 0.1 mg/µL ampicillin and
721 cultured for approximately 6 h at 37°C with shaking.

722 The starter culture was subsequently transferred into 300 mL LB medium
723 supplemented with 0.1 mg/µL ampicillin and incubated overnight at 37°C with shaking.
724 Bacterial cells were harvested by centrifugation for 10 min at 4000 x g at 4°C.

725 Plasmid DNA was purified using the NucleoBond Xtra Maxi Plus 50 (Takara Bio)
726 according to the manufacturer's instructions. DNA was eluted in 1 mL TE-Buffer, and
727 concentration and purity were assessed using a NanoDrop instrument.

728 **Chromatin immunoprecipitation (ChIP)**

729 *ChIP sample preparation*

730 On day 10 of bone marrow culture, cells were harvested and resuspended in PBS
731 (500 µL/10⁶ cells). Chromatin was crosslinked with 1% formaldehyde (37% stock,
732 Sigma-Aldrich) for 6 minutes at room temperature and quenched with one-third volume
733 of 1.25 M glycine for 5 min. Cells were washed with cold PBS, resuspended in FACS

734 staining buffer (PBS with 1 mM EDTA and 0.5% heat-inactivated FCS) at 1×10^8
735 cells/mL, filtered through 35 μ m cell strainers and eGFP-positive cells were sorted on
736 a BD FACS Aria I. Sorted cells were centrifuged, washed with PBS, transferred to low-
737 binding tubes and centrifuged again. Supernatant was discarded leaving ~ 20 μ L per
738 tube and cell pellets were snap frozen.

739 For chromatin preparation, frozen pellets (6×10^6 cells) were thawed, spun down and
740 lysed in 200 μ L ChIP lysis buffer (50 mM Tris-HCl (pH 8.0), 10 mM EDTA, 1 % SDS)
741 supplemented with Proteinase K Inhibitor cocktail (1:100, Sigma-Aldrich) essentially
742 as described³². Briefly, lysates were transferred to Diagenode shearing tubes and
743 sonicated using a Bioruptor Pico (Diagenode) at 4°C for eight cycles (30 sec on, 30
744 sec off). Chromatin fragmentation was monitored by agarose gel electrophoresis
745 following Proteinase K digestion and if required sonication was repeated until a
746 fragment size of approximately 300-500 bp was achieved. Sheared chromatin was
747 clarified by centrifugation (12,000 x g, 10 min, 4°C) and supernatants were transferred
748 to low-binding tubes and either processed immediately or stored at - 80°C.

749 *Chromatin immunoprecipitation*

750 Chromatin immunoprecipitation with an anti-IRF8 antibody (Bethyl Laboratories,
751 A304-027A, polyclonal rabbit IgG, 1 mg/mL) was performed in duplicates for each
752 sample including IgG control (rabbit IgG, kch-504-250, Diagenode). Dynabeads
753 Protein G (Thermo Fisher Scientific) were equilibrated in RIPA buffer (10 mM Tris-HCl
754 (pH 7.5), 140 mM NaCl, 0.5 mM EDTA, 1% TritonX-100, 0.1% SDS, 0.1% Na-
755 deoxycholate) and then incubated with 10 μ g anti-IRF8 antibody or IgG control for 2
756 hours at 4°C with rotation to form antibody-bead complexes.

757 Sheared chromatin (45 μ g) was diluted 1:10 in RIPA buffer supplemented with a
758 Protease Inhibitor cocktail (1:100, Sigma-Aldrich). Ten percent of chromatin was
759 reserved as input reference. Diluted chromatin was incubated with IRF8-antibody-
760 bead complexes overnight at 4°C with rotation. Beads with IRF8-bound chromatin
761 were placed in a magnetic rack and washed three times with RIPA buffer and treated
762 with RNase A (50 μ g/mL, Roche) for 30 minutes to remove RNA contaminations.

763 *Chromatin elution and ChIP analysis*

764 Chromatin was eluted from beads in ChIP elution buffer (20 mM Tris-HCl (pH 7.5), 5
765 mM EDTA, 50 mM NaCl) supplemented with 1% SDS and Proteinase K and incubated
766 for 2 hours at 68°C with agitation. A second elution step was performed under the
767 same conditions and eluates were combined. DNA was purified using the QIAquick
768 PCR Purification Kit (Qiagen) according to the manufacturer's instructions and eluted
769 in 50 μ L TE-Buffer (10 mM Tris-HCl (pH 8.0), 10 mM EDTA).

770 Input DNA was diluted 1:10 in TE-Buffer for qPCR analysis. ChIP enrichment was
771 quantified by qPCR as described above. Signals were normalized to input chromatin
772 by calculating the percentage of input (% input) from averaged CT values using the
773 formula: $\% \text{ input} = 2^{(Ct(\text{input}) - 6.5 - Ct(\text{sample}))}$. Normalization to input corrects for
774 variation in chromatin quantity between samples and allows comparison of enrichment
775 between conditions.

Following PCR amplification, DNA was concentrated by SpeedVac centrifugation until completely dry. DNA was dissolved in 15 μ L nuclease-free water, and 1 μ L was used for DNA quantification using the Qubit fluorometric assay. Samples with DNA concentrations below the sequencing threshold were excluded and samples meeting the required concentration were subjected to ChIP-seq analysis on the Illumina HiSeq2000 platform using 50-bp single-end reads with a median insert size of 250 bp. Sequencing reads were demultiplexed and aligned to the mouse reference genome (mm9). Visualization and inspection of alignments were performed using the Integrative Genomics Viewer (v2.19.7).

Histology and immunostaining

Processing of tissue for OCT and paraffin embedding

Bones and spleens were fixed in 2% paraformaldehyde (PFA) overnight at room temperature and washed 3 x 5 minutes with 1x PBS with gentle agitation. Bones were subsequently decalcified for 6-8 days at 4°C with Entkalker Soft Solvagreen (Carl Roth), while soft tissues were processed directly. Spleens, livers, and bones were cryoprotected in 30% sucrose at 4°C overnight, blotted dry, and embedded in optimal cutting temperature compound (OCT; Sakura Finetek) in appropriately sized molds. Even freezing was achieved on a metal plate resting on dry ice, and blocks stored at -80°C until sectioning.

For paraffin embedding, tissues were transferred to 70% ethanol, dehydrated using an automated tissue processor, and embedded in paraffin. Sections (3-8 μ m) were cut using a microtome or cryostat and mounted onto slides.

Histological stainings

Reticulin fibers were visualized using the Reticulin Silver Plating Kit (Sigma-Aldrich) according to Gordon & Sweets following the manufacturer's instructions, with all washes performed using Milli-Q water. Sections were counterstained with eosin for three minutes and briefly passed through an ascending ethanol series (70%, 90%, 96% ethanol). Slides were subsequently incubated three times in 100% ethanol for 2 minutes each and cleared in xylene (3x5 minutes) before mounting under coverslips with Entellan.

Hematoxylin and eosin (H&E) staining was performed on OCT-embedded cryosections which were rehydrated in 1x PBS for 4 minutes at room temperature. Sections were incubated in hemalum for 1 minute, rinsed in warm, running tap water for 10-15 minutes, counterstained with eosin for 3 minutes, enhanced with 1% acetic acid for 4 minutes and dehydrated and cleared as above. Paraffin-embedded sections were deparaffinized and rehydrated prior to H&E staining following the same protocol.

Closest neighbor analysis of Mks

For closest-neighbor analysis, the coordinates of individual Mks were extracted from H&E-stained bone marrow and spleen sections using ImageJ, with biological triplicates analyzed per bone or spleen from one mouse. Two rectangular regions were defined for each image: an outer rectangle encompassing all detected xy coordinates, and an inner, centrally placed rectangle covering 70% of the larger area. The resulting smaller region was used to exclude cells that were located at the periphery of images.

819 After scaling the coordinates to physical dimensions, Euclidean distances between
820 neighboring cell pairs were calculated and clusters were visualized by connecting
821 neighboring cells (blue dots) with lines using matplotlib³³. The intercellular distance
822 data were used for quantification of cluster characteristics and cell-to-cell spacing.

823 *Immunohistochemistry*

824 For immunohistochemistry, paraffin sections were deparaffinized in xylene (3 x 5
825 minutes), rehydrated through a graded ethanol series (3x 100%, 1x 96%, 1x 90%, 1x
826 70%; 2 minutes each) and subjected to antigen retrieval using a 1:100 dilution of
827 antigen unmasking solution (Vector Laboratories) in a steam cooker (Philips HD9140)
828 for 25 minutes. After boiling, sections were cooled to room temperature for 45 minutes
829 and washed 3x5 minutes with 1x PBS. Endogenous peroxidase activity was quenched
830 with 3% hydrogen peroxide for 10 minutes, followed by three washes in 1x PBS.
831 Tissue areas were demarcated using a DAKO hydrophobic barrier pen (Biozol) and
832 blocked with the Avidin/Biotin Blocking Kit (Vector Laboratories) for 10 minutes each,
833 with 1x PBS wash in between. Primary antibodies diluted in Antibody Diluent (Zytomed
834 Systems) were incubated overnight at 4°C.

835 The following day, sections were washed and incubated with a biotinylated secondary
836 antibody for 30 minutes followed by application of avidin-biotin complex
837 (VECTASTAIN ELITE ABC HRP Detection Kit; Vector Laboratories) for 30 minutes.
838 Signal was developed using the DAB (3,3' diaminobenzidine) Peroxidase (HRP)
839 Substrate Kit with Nickel (Vector Laboratories) for 4-10 minutes, and sections were
840 rinsed, counterstained with hemalum, differentiated under running warm water for 10
841 minutes, dehydrated, cleared in xylene, and mounted in Entellan as described above.

842 *Immunofluorescence staining of OCT-embedded tissue*

843 Cryosections were thawed at room temperature for 30 minutes, and tissue areas were
844 demarcated using a DAKO hydrophobic barrier pen. Sections were rehydrated for 5
845 minutes in a PBS-TS buffer and placed in a plastic chamber positioned between two
846 LED light sources for bleaching. Bleaching was performed twice for 10 minutes each,
847 with replacement of the bleaching solution between cycles. Sections were then
848 washed three times for 3 minutes and incubated in a staining buffer for 1 hour at room
849 temperature.

850 After blocking, primary antibodies were diluted in a staining buffer (1% BSA in 1x PBS)
851 was applied and incubated overnight at 4°C in a humidified chamber. The following
852 day, sections were washed three times in staining buffer for 5 minutes each and
853 incubated with a mixture of secondary antibodies and DAPI diluted in staining buffer
854 for 30 minutes at room temperature in the dark.

855 Finally, slides were washed three times without incubation, followed by three additional
856 5-minute washes in the staining buffer, and mounted using ProLong Diamond Antifade
857 Mountant (Thermo Fisher Scientific).

858 *Bone marrow organoids*

859 Bone marrow organoids (BMOs) were generated from the HD353.6 human induced
860 pluripotent stem cell (iPSC) line using an optimized protocol based on previously
861 described methods^{34,35}. On day 14, mature BMOs were engrafted with iPSC-derived

hematopoietic progenitor cells (day 14 spinEB cultures) from JAK2^{V617F} (RFP-labeled) and JAK2^{WT} (GFP-labeled) iPSC lines, using adapted protocols^{36,37}. Hematopoietic cells were harvested on day 14, and CD34⁺ cells were enriched using the CD34 MicroBead Kit UltraPure (Miltenyi Biotec) according to the manufacturer's instructions before engraftment.

iPSCs were maintained on growth factor-reduced basement membrane matrix (GeltrexTM; Gibco) in StemFlexTM Medium (Gibco) at 37°C and 5% CO₂, with daily medium changes, and passaged at 60–80% confluency either as single cells using Accutase Cell Detachment Solution (Pan Biotech) or as small aggregates using EDTA-based dissociation (0.5 mM EDTA in PBS).

For BMO generation, iPSCs were dissociated as small aggregates and cultured in StemFlex Medium supplemented with 1% RevitaCell Supplement (Gibco). Mesoderm induction (phase I) was performed in STEMDiff APEL2 Medium (Stemcell Technologies) supplemented with 50 ng/mL Human FGF basic (PeproTech), 50 ng/mL Human BMP-4 (Miltenyi Biotec), 3 mM CHIR99021 (Merck), and 25 ng/mL Human VEGF (165) IS (Miltenyi Biotec). Hematopoietic and vascular specification (phase II) was induced with 10 ng/mL Human SCF (Miltenyi Biotec), and 25 ng/mL Human Flt-3 Ligand (FLT3L) Recombinant Protein (PeproTech). From day 5, organoids were embedded in a hydrogel consisting of 60% Collagen I (Corning) and Human Collagen Type IV (Cell Systems) and 40% Matrigel Basement Membrane Matrix (Corning). Subsequently, vascular sprouting (phase III) was induced using medium supplemented with 5% KnockOut Serum Replacement (Gibco), 5 U/mL Heparin sodium salt from porcine intestinal mucosa (Merck), 50 ng/mL Human FLT3L (PeproTech), 20 ng/mL Recombinant Human IL-3 (ImmunoTools), 20 ng/mL Human Recombinant IL-6 (STEMCELL Technologies), 50 ng/mL Human M-CSF Recombinant Protein (PeproTech), 50 ng/mL Recombinant Murine TPO (PeproTech), and 50 ng/mL Recombinant Human EPO-α (ImmunoTools).

From day 13 onward (phase IV), BMOs were maintained in the same cytokine-containing maturation medium, with medium changes three times per week.

Hematopoietic progenitor cells were generated from patient-specific iPSCs using a modified and optimized SpinEB protocol^{36,37}. Briefly, single-cell-dissociated iPSCs were aggregated by centrifugation in serum-free differentiation medium composed of IMDM and Ham's F-12 Nutrient Mix (1:1; both Gibco), supplemented with 0.4% bovine serum albumin, Chemically Defined Lipid Concentrate (Gibco), 2 mM GlutaMAX Supplement (Gibco), 0.4 mM 1-Thioglycerin (Sigma-Aldrich), 50 µg/mL Ascorbic Acid (Stemcell Technologies), and 6 µg/mL Human holo-Transferrin (Sigma-Aldrich). Mesoderm induction was initiated with 10 ng/mL human FGF basic (PeproTech), 10 ng/mL Human BMP-4 (Miltenyi Biotec), and 10 µM Y-27632 dihydrochloride (Abcam). From day 2 to day 14, cultures were maintained with daily partial medium exchange and supplementation with human SCF (Miltenyi Biotec), 25 ng/mL Human VEGF (Miltenyi Biotec), 10 ng/mL Human FLT3L (PeproTech), 30 ng/mL Recombinant Human IL-3 (ImmunoTools), 25 ng/mL Human Recombinant IL-6 (Stemcell Technologies), and 50 ng/mL Human M-CSF Recombinant Protein (Thermo Fisher).

Immunofluorescence staining of paraffin-embedded tissue

Paraffin-embedded sections were deparaffinized and re-hydrated through xylene (2 x 5 min), followed by a descending ethanol series (96%, 90% and 70% ethanol for 30

seconds each). Sections were rinsed with tap water for 3 minutes and then washed in 1x PBS for 10 minutes under gentle agitation.

For antigen retrieval, a steam boiler was filled to the maximum water level, and a staining vessel containing 25 mL 1x Tris EDTA antigen retrieval buffer diluted in tap water placed inside. The boiler was then pre-heated for 20 minutes after which sections were incubated in the buffer at boiling temperature for 20 minutes, then cooled to room temperature over 45 minutes with gentle agitation on ice. Sections were washed in 1x PBS for 5 minutes under gentle agitation and a primary antibody mix was prepared in 1% BSA/1x PBS. Sections were outlined using a DAKO hydrophobic barrier pen and incubated with primary antibodies in a humidified chamber overnight at 4°C. The following day, sections were washed 3 x 5 minutes each with 1x PBS and incubated with secondary antibodies diluted in 1% BSA/1x PBS for 1 hour at room temperature. Sections were washed 3 x 5 minutes with slow rotation, excess PBS was removed, and samples were mounted using ProLong Diamond Antifade Mountant.

Statistical analysis for bar plots

Data are presented as mean ± standard error of the mean (SEM), with individual mice or patients, respectively, per data point shown in bar graphs. All statistical analyses were performed in R (v4.3.2). For comparisons between two groups (*Irf8^{d/d}* vs. *Irf8^{+/+}* in Supplementary Fig. 1E-F, Main and Supplementary Fig. 2, Main and Supplementary Fig. 4), statistical significance was assessed using an unpaired two-tailed Student's t-test.

For comparisons among three (*Irf8^{+/+}*, *Irf8^{+/d}*, and *Irf8^{d/d}*) or four groups (*Irf8^{+/+}* + pMIG, *Irf8^{d/d}* + pMIG, *Irf8^{+/+}* + BCR-ABL and *Irf8^{d/d}* + BCR-ABL) differences were calculated using one-way analysis of variance (ANOVA). To correct for multiple testing, post hoc pairwise comparisons were performed using Tukey's Honestly Significant Difference (HSD) test. Figures containing three or four-group comparisons include Main Figure 1, Supplementary Figure 1A-D, G, H, K, L, Main Figure 7, and Supplementary Figure 7.

Statistical significance for both tests was defined as not significant for $p > 0.05$, $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***). Only statistically significant comparisons are annotated in figures.

Codex multiplexed imaging

Antibody conjugation, tissue staining, and imaging were performed following the "CODEX multiplexed tissue imaging with DNA-conjugated antibodies" protocol 14, adhering to the manufacturer's recommendations for fresh-frozen tissues.

Fresh-frozen spleen sections (8 µm) and bone sections (4 µm) were used, and stained tissues were imaged on a PhenoCycler-Fusion (Akoya Biosciences).

946 **Mouse single-cell RNA sequencing and computational analysis**

947 *Library preparation*

948 3 mice per library carrying both hematopoietic and stromal *Irf8* deletion were analyzed
949 alongside littermate controls (dataset 1), as were three mice with hematopoietic *Irf8*
950 deletion only (dataset 2).

951 For both datasets, bone immune cells were isolated as described above and bone
952 marrow stroma cells were enriched prior to sorting.

953 For dataset 1, cells were sort-purified using a BD Biosciences FACSMelody into
954 stromal (CD45⁻, lineage⁻, lineage markers: CD3, CD19, NK1.1, Gr1, Ter119), immune
955 cells (lineage⁻, CD45⁺) and endothelial cells (CD31⁺) and pooled at defined ratios prior
956 to library preparation.

957 For dataset 2, cells were sort-purified using a BD Biosciences FACSMelody into
958 stromal cells (CD45⁻, lineage⁻, lineage markers: CD3, CD71, Ter119, CD19),
959 neutrophils (lineage⁻, Ly6G⁺) and stem/progenitor cells (lineage⁻, Sca-1⁺), and pooled
960 at defined ratios prior to library preparation.

961 Single-cell libraries were generated using the 10x Genomics Chromium Next GEM
962 Single Cell 3' v3.3 (dataset 1) or 3' v3.4 chemistry (dataset 2) according to the
963 manufacturer's protocol. Libraries were sequenced to a minimum depth of 20,000
964 reads per cell using recommended read parameters (28-10-10-90 cycles).

965 *Preprocessing and quality control*

966 Single-cell RNA sequencing of stromal and hematopoietic *Irf8* knockout or littermate
967 control marrow dataset (dataset 1) or the hematopoietic *Irf8* knockout or littermate
968 control dataset (dataset 2) was performed using the 10x Genomics platform. Reads
969 were aligned to the mm10 reference genome with Cell Ranger³⁸ (dataset 1: v7.1.0,
970 dataset 2: v9.0.1). Downstream processing was carried out in R (v4.3.2) using Seurat
971 (dataset 1: v5.3.0, dataset 2: v3.2.3). For quality control, cells with fewer than 400
972 features detected, more than 40,000 UMIs or >20% mitochondrial (dataset 1) or >10%
973 mitochondrial gene expression (dataset 2) were excluded and ambient RNA
974 contamination exceeding 50% was removed using DecontX³⁹ (v1.6.0). Doublet
975 removal was implemented based on DoubletFinder⁴⁰ (v2.0.3) classification.

976 Data were log-normalized, and mitochondrial genes as well as cell cycle scores were
977 regressed out during scaling. Principal component analysis was performed, and
978 datasets were integrated using Harmony⁴¹ (v1.2.4) on the first 25 (dataset 1, dataset
979 2: 30) principal components.

980 A shared nearest neighbor graph was built using 25 PCA dimensions (dataset 1,
981 dataset 2: 50), followed by unsupervised clustering at a resolution of 0.6 (dataset 1,
982 dataset 2: 0.5). After quality control, 22,593 cells (dataset 1, dataset 2: 42,343) were
983 retained, with enrichment in megakaryocyte progenitors and mature neutrophils for
984 dataset 1.

985 To enhance representation of mature neutrophils and megakaryocyte progenitors,
986 wild-type (WT) cells of the dataset were enriched with empty vector (EV) control cells

from an independent dataset. 6,254 EV-derived neutrophils and 1,451 EV-derived megakaryocyte progenitors were merged with the WT cells after simplification of the annotation. As the cells of the transferred populations also clustered with other populations, three purely EV-derived clusters were excluded from further analysis. Cluster-specific marker genes were identified using the FindAllMarkers function in Seurat with adjusted $p < 0.05$.

Cell type prioritization

Cell type prioritization was performed using R package Augur⁴² (v1.0.3), based on gene-level count data, with cell type annotation and condition metadata as input. This machine learning approach assigns an area under the curve (AUC) score to each cell type, reflecting the extent of transcriptional perturbations between conditions. Cell types with an AUC of > 0.5 were considered to exhibit substantial transcriptional divergence and were included in the plots.

Differential expression analysis

Differentially expressed genes (DEGs) were identified between the *lrf8^{d/d}* and *lrf8^{+/+}* mouse models at the single-cell level using MAST⁴³ (v1.16.0), applying the parameters specified in the following Nextflow pipeline: https://github.com/mms100/scRNA-seq_nextflow_DEG_pipeline. DEGs per cluster are listed in the Supplementary Tables.

To assess transcriptional overlap with fibrotic bone marrow states, *lrf8*-dependent differentially expressed genes from stromal cell populations (CAR subsets and OLCs) were compared with a published single-cell RNA-sequencing dataset of MPN-associated bone marrow fibrosis⁵. This reference dataset was stratified into mesenchymal stromal cell populations MSC1-4 and disease stages pre-fibrotic and fibrotic. Gene-level overlap between datasets was evaluated separately for each MSC subset and disease stage combination.

Statistical significance of overlapping differentially expressed genes was assessed using Fisher's exact test (alternative = greater) based on 2x2 contingency tables defining differentially expressed or non-differentially expressed genes in each dataset. Odds ratios were calculated to quantify enrichment strength. P-values were adjusted to multiple testing using the Benjamini-Hochberg method implemented in R. For each pairwise comparison, results were summarized as overlap size, odds ratio, and adjusted p-value.

Pathway analysis

Pathway enrichment scores of Hallmark⁴⁴, GO⁴⁵, Reactome⁴⁶, KEGG⁴⁷ and PROGENy⁸ databases were derived from the differential gene expression analysis performed with MAST comparing *lrf8^{d/d}* with *lrf8^{+/+}* samples. Enrichment analysis was conducted using decoupleR⁴⁸ (v2.9.7) with the ULM (Univariate Linear Model) method specified. p values were adjusted using the FDR Benjamini-Hochberg method implemented in R.

1027 *Pseudotime trajectory analysis*

1028 Clusters “Ly6c_low_monocytes”, “Ly6c_high_monocytes” and “CoMoPs” were
1029 aggregated in one single “Monocytes” cluster; and “Pre_neutrophils”,
1030 “Immature_neutrophils” and “Mature_neutrophils” were aggregated as well in a
1031 “Neutrophils” cluster. Then cells with a number of RNA counts lower than 3000 and T-
1032 cell, B-cell and CAR cell clusters were excluded. The principal component analysis
1033 (PCA) embeddings were recalculated on this data subset and the first 30 principal
1034 components were employed for recalculating the UMAP embedding using the scanpy
1035 (v1.11.1)⁴⁹ implementation with default parameters. Afterwards, we used the Palantir⁵⁰
1036 framework in Python (v1.3.6) to reconstruct cell state trajectories.

1037 First, the data was log-scaled using the palantir.preprocess.log_transform function,
1038 which adds a pseudocount of 0.1. Then, we performed diffusion map dimensionality
1039 reduction using 30 principal components (palantir.utils.run_diffusion_maps). To
1040 account for sparsity in the gene expression matrix, gene expression data was imputed
1041 via the MAGIC⁵¹ implementation from Palantir (palantir.utils.run_magic_imputation).

1042 Terminal cell states were identified using palantir.utils.find_terminal_states, specifying
1043 'Neutrophils', 'Monocytes', and 'Erythroid_progenitors' as final cell states. Finally, we
1044 inferred cell state trajectories using palantir.core.run_palantir, using the cell with the
1045 highest expression of the HSPC marker Cd34 as starting point.

1046 *CrossTalker analysis considering intracellular signaling*

1047 Differential cell–cell communication analysis incorporating both intercellular and
1048 intracellular signaling was performed using CrossTalker⁵² (v2.0). To connect ligand–
1049 receptor signaling to downstream transcriptional responses, transcription factor (TF)
1050 activities were inferred from normalized gene expression using the univariate linear
1051 model (ULM) implemented in the decoupleR⁴⁸ (v2.0.6) package, based on the
1052 DoRothEA⁵³ regulon (confidence levels A and B). Condition-specific TF activity
1053 differences at the cluster level were identified using a t-test implemented in the scan
1054 package⁵⁴ (v1.34.0) with default parameters, and the results were filtered based on
1055 log-fold-change and adjusted p-value thresholds (pval < 0.05 and |logfc| > 0.5). TFs
1056 passing these criteria were connected to their regulated target genes using the
1057 corresponding DoRothEA regulon, retaining only expressed targets.

1058 Receptor–TF connections were derived from protein–protein interactions in
1059 OmniPath⁵⁵ by identifying the shortest paths between receptors and transcription
1060 factors. The resulting intracellular network was restricted to receptors and target genes
1061 associated with ligand–receptor pairs predicted to be active in the ligand–receptor
1062 analysis.

1063 Differential cell–cell communication between *lrf8*^{d/d} and *lrf8*^{+/+} conditions was then
1064 evaluated with CrossTalker. Cytokine-mediated cell-cell interactions were filtered
1065 using the NicheSphere cytokine database
1066 (https://github.com/CostaLab/NicheSphere/blob/main/db/nichesphereDB_pmid.csv).

1067 Intracellular signaling within the cycling CAR cell cluster was visualized using Sankey
1068 diagrams, in which edges were weighted by the average ligand–receptor interaction
1069 score representing incoming and outgoing signaling through receptors and ligands.

1070 *Module scores*

1071 To quantify the transcriptional program activity of curated gene sets at a single-cell
1072 resolution, module scores were calculated separately for each dataset using the
1073 AddModuleScore function of Seurat (v5.3.0). For each cell, the score represents the
1074 average expression of the genes in each signature normalized against expression-
1075 matched control genes.

1076 To assess differences in module scores between the stages *lrf8^{+/+}* and *lrf8^{d/d}*, we
1077 performed a non-parametric Wilcoxon rank-sum test (two-sided) using R (v4.3.2).
1078 Unless otherwise specified, p values were not adjusted for multiple comparisons.
1079 Statistical significance was defined as not significant $p > 0.05$, $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***).

1081 *Myofibroblast program module score*

1082 Defined by the genes *Limch1*, *Igfbp7* and *Wisp2* as previously described⁵.

1083 *Vascular signaling module score*

1084 Defined by the genes *Foxc2*, *Hes1* and *Vcam1* as previously described⁵.

1085 *Hematopoietic support score*

1086 Defined by the genes *Kitl*, *Il7*, *Igf1*, *Cxcl12*, *Csf1*, *Bmp4*, *Tlx1* as previously
1087 described^{5,56}.

1088 *Endothelial inflammation module score*

1089 The endothelial inflammation module score was defined using the following genes:
1090 *Abi1*, *Acvr1b*, *Akt1*, *Akt3*, *Alox5ap*, *Ano6*, *Ash1l*, *Ass1*, *Atp2b1*, *Atp2c1*, *B4galt1*, *Birc2*,
1091 *Bst2*, *Btg1*, *Btg2*, *Btg3*, *Btk*, *C3*, *Calclrl*, *Cbl*, *Cblb*, *Ccnd2*, *Ccnd3*, *Ccnl1*, *Cd44*, *Cd48*,
1092 *Cd69*, *Cd82*, *Cdkn1a*, *Cebpb*, *Cebpd*, *Cflar*, *Cish*, *Clec5a*, *Crff2*, *Crebbp*, *Cxcl1*,
1093 *Cxcl10*, *Cybb*, *Dennd5a*, *Dnajb4*, *Dusp1*, *Ebi3*, *Egr1*, *Ehd1*, *Eif1*, *Eif2ak1*, *Eif2ak2*,
1094 *Elane*, *Emp3*, *Ep300*, *Ets2*, *Ext1*, *Ffar2*, *Fosl2*, *Fos*, *Fosb*, *Fut4*, *Fut7*, *G0s2*, *Gadd45a*,
1095 *Gadd45b*, *Ghr*, *Gna15*, *Gnai3*, *Grb2*, *H2-T23*, *Hif1a*, *Hp*, *Icam1*, *Id2*, *Ifih1*, *Ifit2*, *Ifnar1*,
1096 *Ifngr1*, *Ier2*, *Ier3*, *Ier5*, *Il10*, *Il10rb*, *Il15*, *Il15ra*, *Il18rap*, *Il1b*, *Il3ra*, *Il31ra*, *Il6*, *Il6st*, *Il7*,
1097 *Irak2*, *Irf1*, *Irs2*, *Itga5*, *Jak1*, *Jak2*, *Jun*, *Junb*, *Kcnj2*, *Kdm6b*, *Kif1b*, *Klf10*, *Klf6*, *Klf9*,
1098 *Lamb3*, *Lbp*, *Lcp2*, *Ldlr*, *Lepr*, *Litaf*, *Lifr*, *Lrp1*, *Lyn*, *Ly6e*, *Map2k3*, *Map3k8*, *Marcks*,
1099 *Mcl1*, *Mefv*, *Mxd1*, *Myc*, *Nampt*, *Nfat5*, *Nfe2l2*, *Nfil3*, *Nfkb1*, *Nfkb2*, *Nfkbia*, *Ninj1*, *Nmi*,
1100 *Nr4a2*, *Olr1*, *Panx1*, *Park7*, *Per1*, *Pias1*, *Pias2*, *Pik3ca*, *Pik3cg*, *Pik3r1*, *Pik3r5*, *Pim1*,
1101 *Plaur*, *Plek*, *Pp1r15a*, *Prcp*, *Psen1*, *Ptger2*, *Ptger4*, *Ptges*, *Ptpre*, *Ptpn11*, *Raf1*, *Rcan1*,
1102 *Rela*, *Rhob*, *Rhbdd3*, *Rhog*, *Ripk2*, *Rnf144b*, *Sat1*, *Sema4d*, *Sele*, *Sell*, *Selp*, *Selenos*,
1103 *Sigirr*, *Slc31a1*, *Slc31a2*, *Slc7a1*, *Sgms2*, *Smad3*, *Socs1*, *Socs2*, *Socs3*, *Socs4*,
1104 *Socs5*, *Sod2*, *Sos1*, *Spred1*, *Spred2*, *Spry1*, *Spry2*, *Spry4*, *Sqstm1*, *Sri*, *Stam*, *Stam2*,
1105 *Stat5a*, *Stat5b*, *Tank*, *Tfrc*, *Tgif1*, *Timp1*, *Tnf*, *Tnfaip3*, *Tnfrsf1b*, *Tnfsf10*, *Trib1*, *Trip10*,
1106 *Vcam1*, *Yrdc*, *Zc3h12a* and *Zfp36*. This score was derived from the GO term Acute
1107 Inflammatory Response, the Hallmark Inflammatory Response and TNFa signaling via

1108 NF- κ B gene sets, as well as the KEGG JAK-STAT signaling pathway (KEGG),
1109 together with published references⁵⁷⁻⁵⁹.

1110 Main figure legends

1111 **Figure 1: Loss of *Irf8* Recapitulates a Chronic-Phase CML–Like Phenotype with** 1112 **Pro-Fibrotic Remodeling.**

1113 Data show mean $\pm$ SEM.; statistical testing was performed as described in the
1114 Methods section.

1115 **(A)** PCR analysis of *Irf8* expression in control, polycythemia vera and chronic myeloid
1116 leukemia patient samples. **(B, C)** Frequencies of Ly6G⁺ granulocytes and long-term
1117 HSCs (LT-HSCs) in the bone marrow **(B)** and spleen **(C)** of *Irf8*^{+/+}, *Irf8*^{+/-} and *Irf8*^{-/-}
1118 mice. **(D)** Gross spleen morphology demonstrating splenomegaly in *Irf8*^{-/-} mice. Scale
1119 bar: 1cm. **(E, F)** H&E–stained spleen sections with quantification of white pulp area;
1120 white pulp regions are highlighted by dashed lines. Scale bar: 300 μ m. **(G)** H&E–
1121 stained bone marrow and spleen sections as a basis for megakaryocyte
1122 characterization. Scale bar: 60 μ m. **(H)** Nearest–neighbor analysis of Mks in bone
1123 marrow and spleen derived from H&E–stained sections. 200 μ m = 395 pixels. **(I–K)**
1124 Quantification of megakaryocyte size **(I)** and absolute number **(J)** and quantification of
1125 Mks cluster numbers derived from nearest–neighbor analysis **(K)**, normalized to tissue
1126 area. **(L)** Immunofluorescence of Ly6G⁺ neutrophils (white), CD61⁺ Mks (yellow),
1127 CD31⁺ vessels (red) and DAPI (blue), illustrating emperipolesis between low CD61–
1128 expressing Mks and Ly6G⁺ neutrophils in *Irf8*^{-/-} mice. Scale bar: 10 μ m. **(M)** Reticulin–
1129 stained bone marrow and spleen sections showing mild bone marrow fibrosis and
1130 dysplastic white pulp architecture. Scale bar bone: 60 μ m. Scale bar spleen: 200 μ m.

1131 **Figure 2: *Irf8* deficiency drives compartment-specific stromal remodeling in** 1132 **bone marrow and spleen.**

1133 Data show mean $\pm$ SEM.; statistical testing was performed as described in the
1134 Methods section. All flow cytometry data were normalized to *Irf8*^{+/+} (set to 1).

1135 **(A)** Flow cytometric analysis of bone marrow CD31⁺ endothelial cells of *Irf8*^{+/+} and
1136 *Irf8*^{-/-} mice, showing significantly increased frequencies of arteriolar, peri-sinusoidal
1137 and lymphatic endothelial cells within viable, lineage–negative cells. **(B)** Flow
1138 cytometric determination of LEPR⁺ CXCL12-abundant reticular (CAR) stromal cell
1139 frequencies among viable, lineage–negative cells in the bone marrow in *Irf8*^{-/-} mice
1140 relative to *Irf8*^{+/+} controls. **(C)** Flow cytometric analysis of T-cell zone reticular cells
1141 (TRCs) in splenic white pulp among viable, lineage–negative cells. **(D)** Flow cytometric
1142 measurement of mesothelial cell frequency in the spleen in *Irf8*^{+/+} and *Irf8*^{-/-} mice. **(E)**
1143 Representative CODEX microscopy pictures of *Irf8*^{+/+} and *Irf8*^{-/-} spleens. Scale bar:
1144 200 μ m. **(F)** Representative CODEX microscopy pictures of *Irf8*^{+/+} and *Irf8*^{-/-} bones
1145 showing extensive neutrophil expansion and atypical Mks. Scale bar: 50 μ m.

1146 **Figure 3: *Irf8* deficiency couples inflammatory granulopoiesis to pro-fibrotic CAR** 1147 **cell remodeling.**

1148 **(A)** UMAP visualization of single-cell transcriptomes from *Irf8*^{+/+} and *Irf8*^{-/-} mouse bone
1149 marrow, colored by major hematopoietic lineages and two transcriptionally distinct
1150 Cxcl12-abundant reticular (CAR) cell clusters. **(B)** Differentially expressed gene
1151 analysis between *Irf8*^{-/-} and *Irf8*^{+/+} bone marrow (FDR = 0.05, $|\log_2\text{FC}| > 0.25$). **(C)**
1152 Pseudotime trajectory analysis of hematopoietic progenitors, comparing *Irf8*^{-/-} and
1153 *Irf8*^{+/+} bone marrow cell populations. **(D)** PROGENy-based pathway activity inference

across bone marrow populations, comparing *lrf8^{d/d}* and *lrf8^{+/+}* bone marrow cell populations. **(E)** Global cell-cell-interaction (CCI) analysis. **(F)** Module score-based enrichment of extracellular matrix production (NABA) collagens, myofibroblast differentiation and vascular signaling. **(G)** Volcano plot of selected, differentially expressed fibrosis-associated genes in CAR cells 1 (n = 11,319 total genes) comparing *lrf8^{d/d}* vs. *lrf8^{+/+}* bone marrow, highlighting overlap with a reference high-grade fibrosis MPN dataset. Fisher's exact test showed significant enrichment for overlap of CAR cells 1 with pre-fibrotic MSC-1 (29 overlapping genes, odds ratios = 10.93, p-value = 4.46×10^{-16}) and MSC-2 (16 overlapping genes, odds ratio = 12.93, p-value = 3.76×10^{-10}), as well as fibrotic MSC-1 (9 overlapping genes, odds ratio = 6.19, p-value = 1.41×10^{-4}), while overlap with fibrotic MSC-2 was not significant (6 overlapping genes, odds ratio = 2.21, p-value = 0.08). **(H)** Cytokine-filtered CCI analysis of bone marrow populations.

Figure 4: Hematopoietic *lrf8* loss is sufficient to activate inflammatory perivascular niche remodeling.

Data show mean $\pm$ SEM.; statistical testing was performed as described in the Methods section. **(A)** Experimental design: Lethally irradiated wild-type Gli1CreERT;tdTomato reporter recipients were reconstituted with c-Kit-enriched bone marrow cells from *lrf8^{+/+}* and *lrf8^{d/d}* donors and analyzed 14 weeks after transplantation, enabling lineage tracing of recipient-derived Gli1⁺ stromal cells. **(B, C)** Flow cytometric analysis of immune cell populations in peripheral blood **(B)** and bone marrow **(C)** of transplanted *lrf8^{d/d}*→Gli1CreERT;tdTomato and control mice. **(D)** Representative H&E staining of bone marrow sections from transplanted mice. Scale bar: 200 μ m. **(E, F)** Flow cytometric analysis of long-term HSC frequencies in bone marrow and spleen. **(G)** Quantification of spleen weight. **(H)** Immunofluorescence staining of collagen fibers (white), Gli1⁺ cells (red) and DAPI (blue). Scale bar: 200 μ m. **(I)** Single-cell transcriptomic analysis of bone marrow from Gli1CreERT;tdTomato recipient mice reconstituted with *lrf8^{+/+}* or *lrf8^{d/d}* c-Kit-enriched hematopoietic cells. UMAP visualization of single-cell transcriptomes from the bone marrow of *lrf8^{+/+}* and *lrf8^{d/d}*→Gli1CreERT;tdTomato chimeras, colored by major hematopoietic and stromal populations. **(J)** Augur-based prioritization analysis identifies cell populations most transcriptionally perturbed by hematopoietic *lrf8* deficiency (AUC threshold = 0.5).

Figure 5: Early neutrophil progenitors drive inflammatory and structural remodeling of the vascular niche upon hematopoietic *lrf8* deficiency.

(A) Heatmaps of motility-, cytoskeleton and inflammation-associated pathway activity across selected cell populations comparing *lrf8^{d/d}*→Gli1CreERT;tdTomato to *lrf8^{+/+}* chimeras. **(B)** Sankey plot depicting inferred receptor-ligand interactions with a focus on Tlr4 as a central receptor on pericytes. **(C)** Inflammation module scores for arteriolar ECs, smooth muscle cells, sinusoidal ECs and pericytes. **(D)** Heatmap of blood vessel morphogenesis and angiogenesis-related pathways across selected cell populations of bone marrow of *lrf8^{d/d}* and *lrf8^{+/+}* chimeras. **(E)** Hypoxia (turquoise, HIF-1 α)-associated S100A8/9 (white) expression and pro-fibrotic signaling (orange, TGF- β) in bone marrow organoids. Scale bar: 50 μ m.

Figure 6: Direct regulation of S100A8 by IRF8 restrains hypoxia-associated inflammatory niche remodeling.

Single-cell transcriptomic analysis of bone marrow from Gli1CreERT;tdTomato recipient mice reconstituted with *Irfl8*^{+/+} or *Irfl8*^{d/d} c-Kit-enriched hematopoietic cells. **(A)** Module scores for extracellular matrix-associated NABA collagen gene sets across stromal populations. **(B)** PROGENy-based pathway activity inference across stromal and myeloid populations comparing *Irfl8*^{d/d} vs. *Irfl8*^{+/+} bone marrow populations. **(C)** Volcano plot of selected, differentially expressed fibrosis-associated genes in adipogenic CAR cells (n = 7,884 total genes) comparing *Irfl8*^{d/d} vs. *Irfl8*^{+/+} bone marrow, highlighting overlap between *Irfl8*^{d/d} adipogenic CAR cells and fibrotic MSC-1 (29 overlapping genes (odds ratio = 29.0, p-value = 1.02x10⁻²⁸). **(D)** Module scores for hematopoiesis support, myofibroblast programs and vascular signaling across stromal populations in *Irfl8*^{d/d} and *Irfl8*^{+/+} chimeras. **(E)** Immunofluorescence staining of Gli1⁺ cells (yellow), collagen I (white) and DAPI (blue) in bone marrow. Scale bar: 100 μm. **(F)** IRF8 chromatin immunoprecipitation (ChIP-seq) in sort-purified cDC2s and GFP⁺ *Irfl8*^{d/d} progenitors reconstituted with inducible IRF8-ERT2. **(G)** DAB-based immunohistochemical staining of S100A8 in bone marrow and spleen. Scale bar bone: 200 μm. Scale bar spleen: 300 μm. **(H,I)** Quantification of S100A8 DAB staining in bone marrow **(H)** and spleen **(I)** from *Irfl8*^{+/+} and *Irfl8*^{d/d} mice, demonstrating significantly increased alarmin levels in *Irfl8*^{d/d} tissues.

Figure 7: S100A8/A9 inflammatory niche remodeling promotes BCR–ABL-driven disease progression and fibrosis.

Data show mean ± SEM.; statistical testing was performed as described in the Methods.

(A) Experimental design: Lethally irradiated *Irfl8*^{+/+} or *Irfl8*^{d/d} recipients were reconstituted with pMIG- or BCR-ABL-transduced c-Kit-enriched hematopoietic cells and analyzed 12 days post-transplantation; panels. Panels **(B-F)** correspond to data from this experiment. **(B)** Body weight curves of all four experimental groups. **(C)** Representative gross spleen images. Scale bar: 1 cm. **(D)** Spleen-to-body weight ratios in all four experimental groups. **(E)** S100A8/A9 plasma levels measured by ELISA. **(F)** H&E staining of sternum of *Irfl8*^{+/+} + pMIG, *Irfl8*^{d/d} + pMIG, *Irfl8*^{+/+} + BCR-ABL and *Irfl8*^{d/d} + BCR-ABL mice. Scale bar: sternum 50 μm, scale bar spleen: 100 μm. **(G)** Reticulin staining in spleen and sternum of *Irfl8*^{+/+} + pMIG, *Irfl8*^{d/d} + pMIG, *Irfl8*^{+/+} + BCR-ABL and *Irfl8*^{d/d} + BCR-ABL mice. Scale bar: sternum 50 μm, scale bar spleen: 100 μm. **(H)** DAB-based immunohistochemical staining of stromal S100A8 along trabecular bone in human CML bone marrow biopsies, enabling stratification into stromal S100A8 grades 0-3. Scale bar: 200 μm. **(I)** Correlation between stromal S100A8 abundance (DAB grade) and fibrosis grade, demonstrating a positive association between stromal alarmin levels and fibrosis grade. **(J)** Correlation between serum lactate dehydrogenase (LDH) levels and fibrosis grade in chronic myeloid leukemia patients, indicating higher LDH with increasing fibrosis.

Data availability

Mouse single-cell RNA sequencing data, CODEX data, and results from the decoupleR analysis are available as processed objects in the following Zenodo repository: doi: **10.5281/zenodo.19816973**

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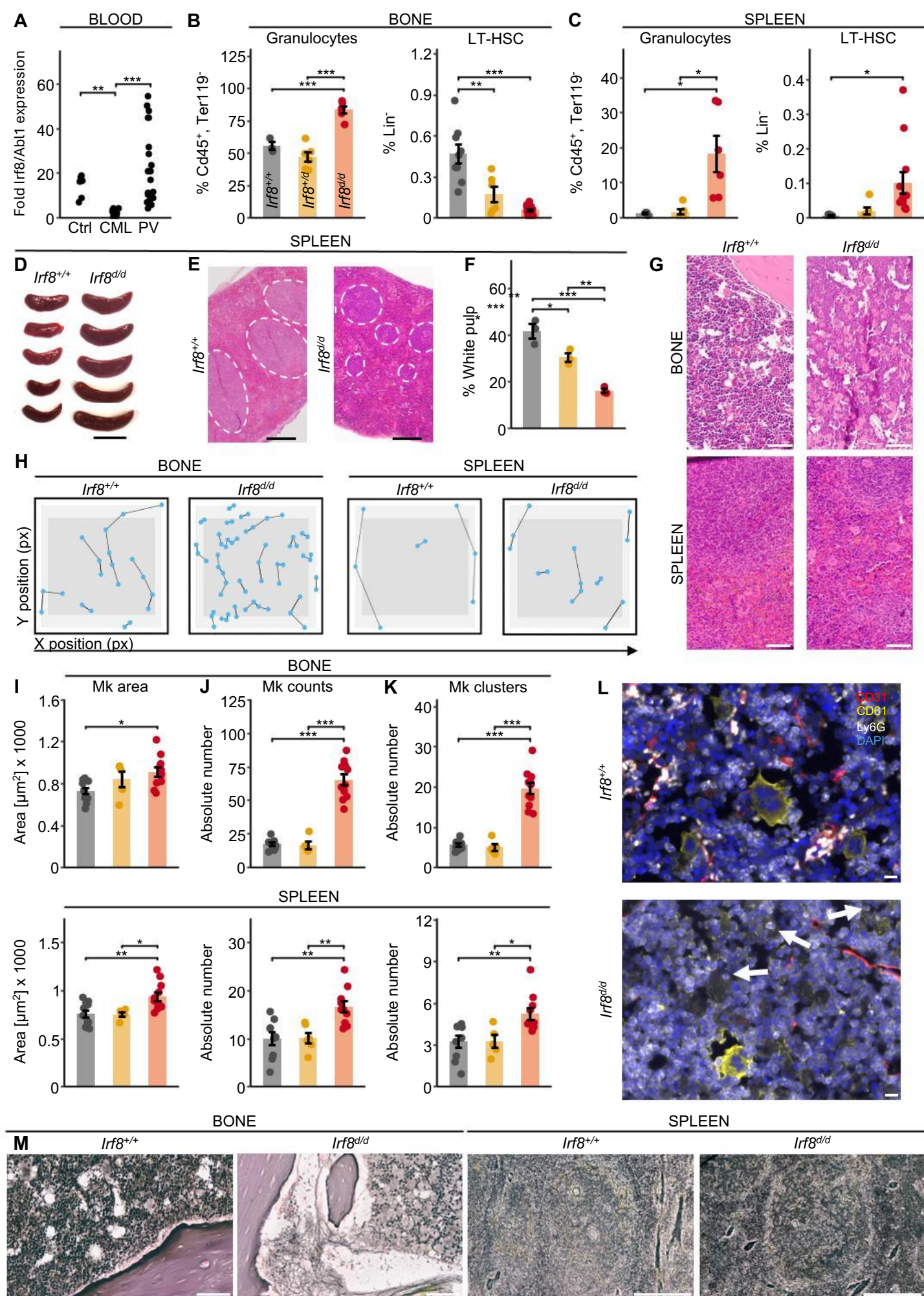
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Author contributions

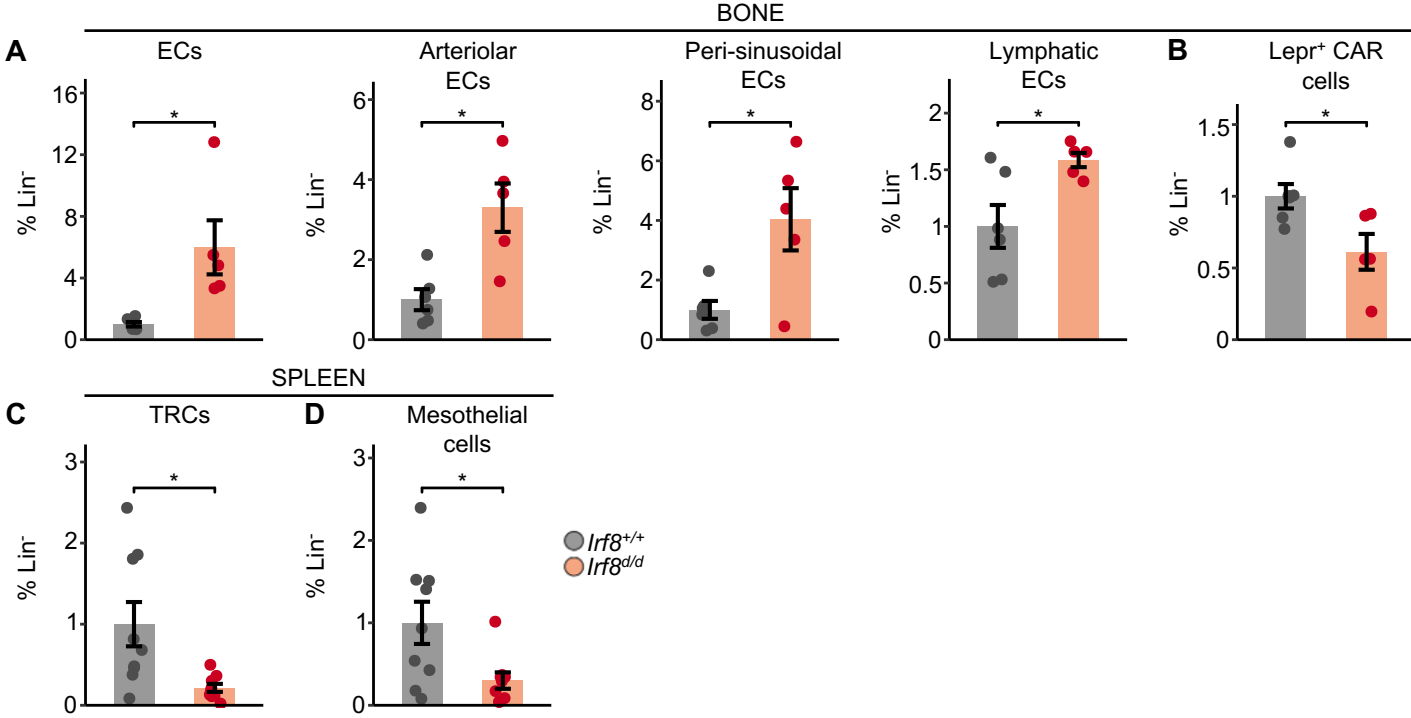
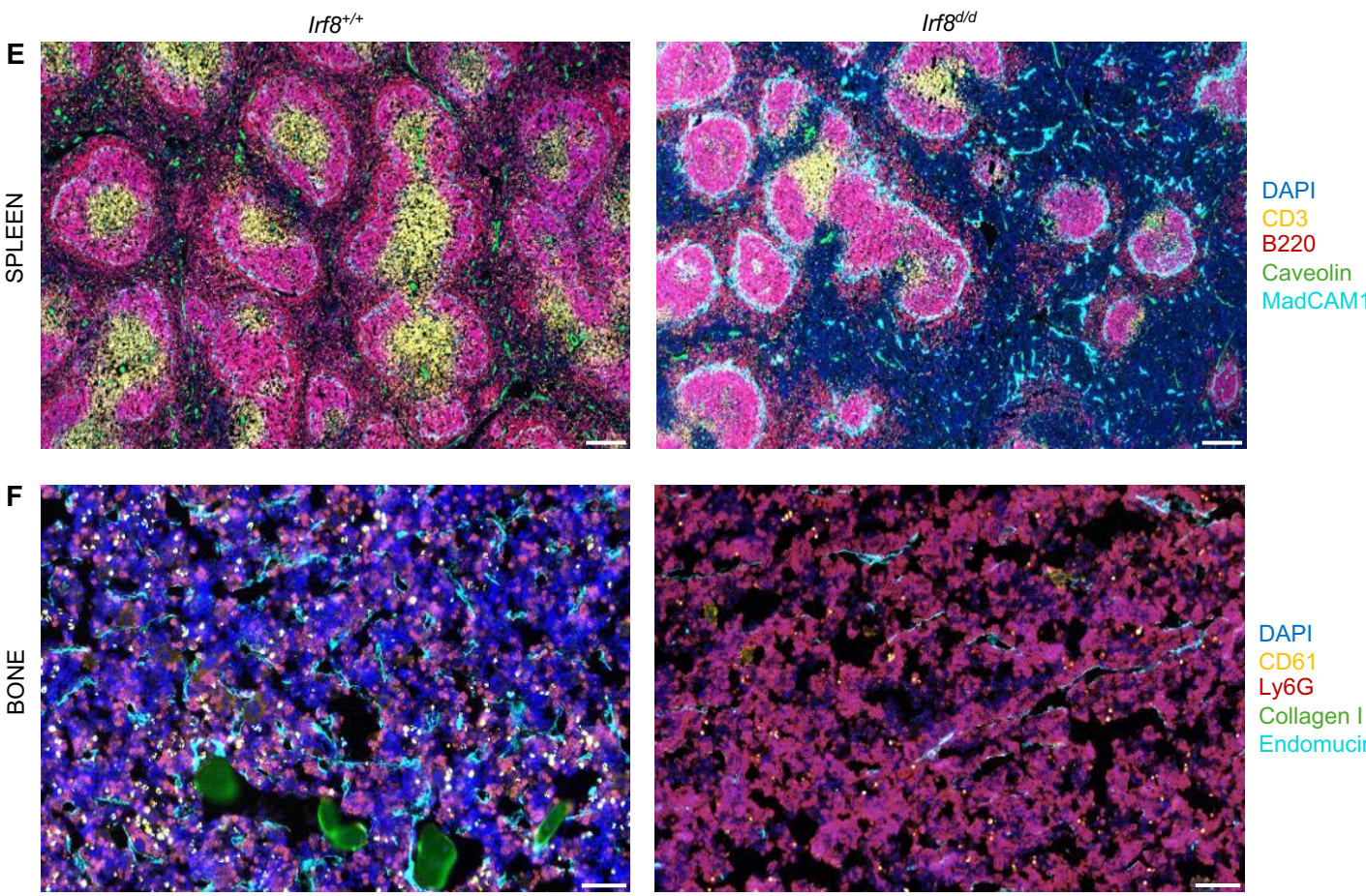
A.K.G. designed and performed experiments, analyzed, and interpreted results of human and murine samples, conducted bioinformatics analyses on murine single cell RNA-sequencing data, and wrote the manuscript; M.S. conducted bioinformatics analyses; C.S. performed CODEX stainings; P. W. was involved in mouse experiments, analyzed and interpreted data; M.L.R.T.S. conducted bioinformatics analyses; V.K. conducted bioinformatics analyses; L.G. performed bone marrow organoid culture and staining; J.E.P. conducted bioinformatics analyses; L.S. performed segmentation of CODEX data; K.G. was involved in mouse experiments. Pa.W. performed CODEX stainings; N.L. was involved in mouse experiments; P.B. provided access to and operated the scanner used for H&E and immunohistochemical stainings; A.S. contributed to data generation and analysis; M.M. provided IRF8-ERT2-pMIEG3 plasmid; S.R.J. provided hyper-IL-6; M.Z. performed ChIP sequencing experiments; S.K. provided patient data and RT-PCR results and contributed to data interpretation; T.B. provided patient data and RT-PCR results and contributed to data interpretation; M.C. provided patient sample information; A.B. participated in project planning and contributed to data interpretation; I.G.C. interpreted results and conducted bioinformatics analyses; K.S. performed ChIP sequencing experiments, participated in project planning, experimental design and contributed to data interpretation; R.K.S. supervised the project, organized funding, designed and performed experiments, analyzed and interpreted results, and wrote the manuscript.

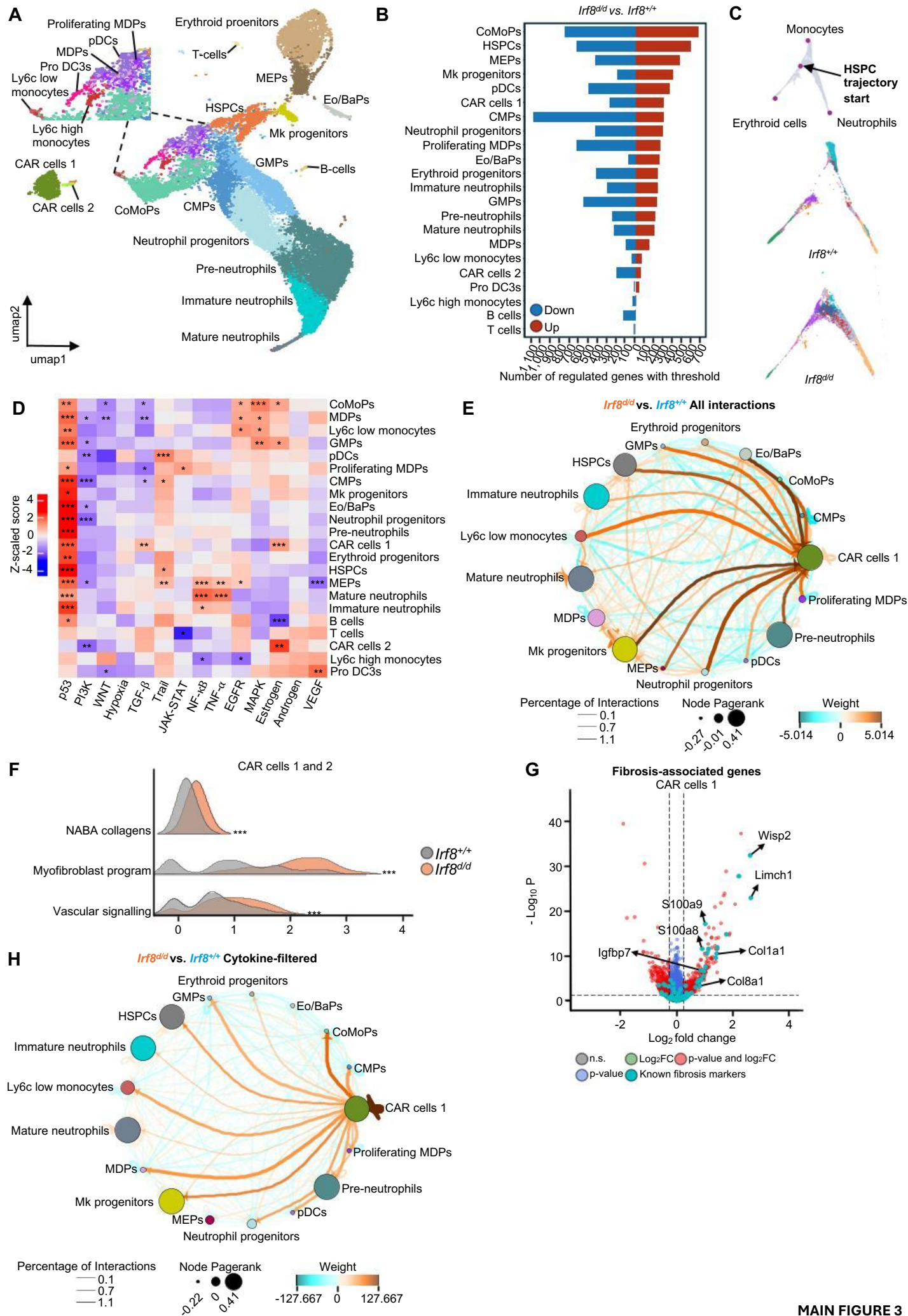
Conflict of interest disclosure: The authors declare no competing interests.

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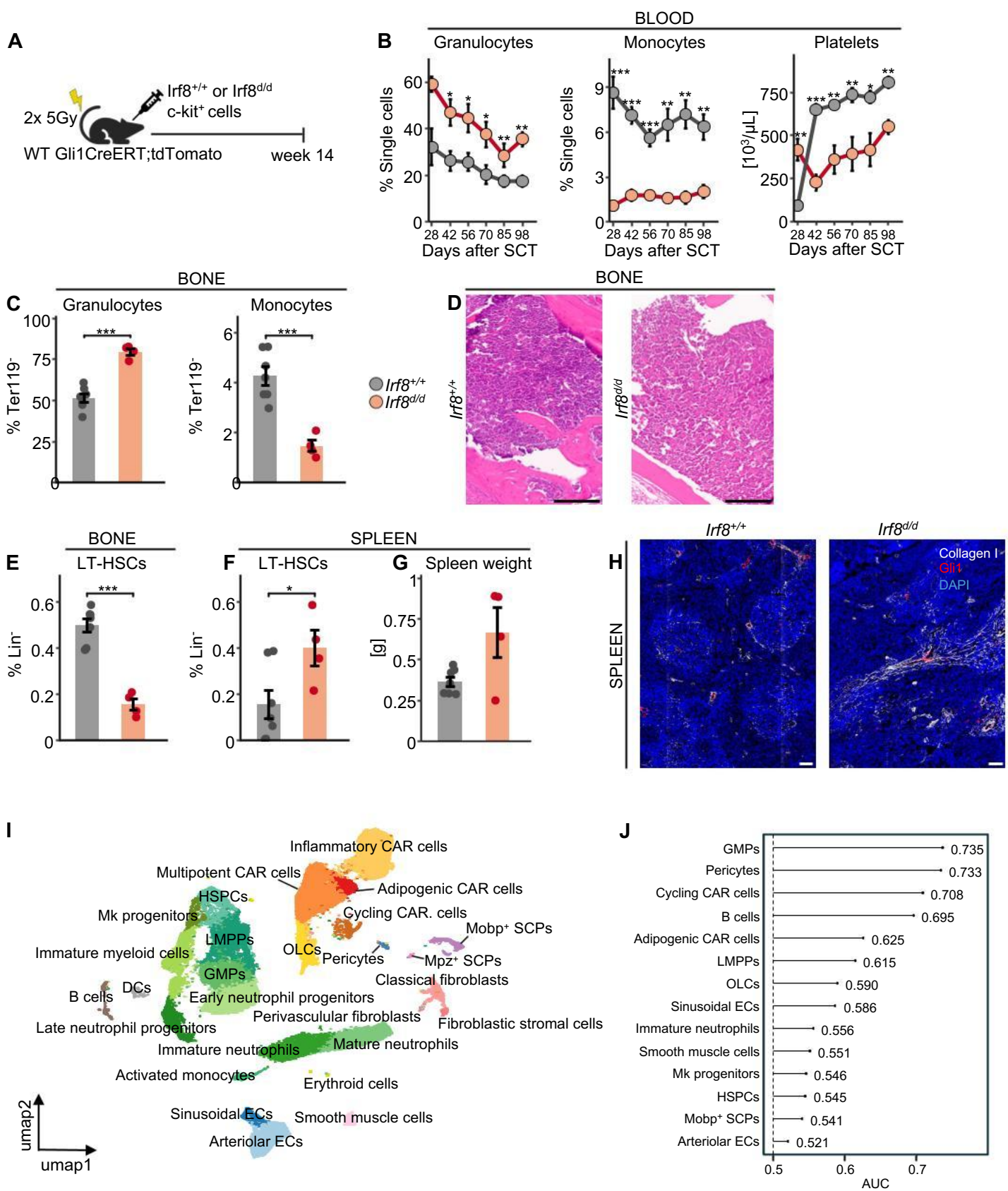


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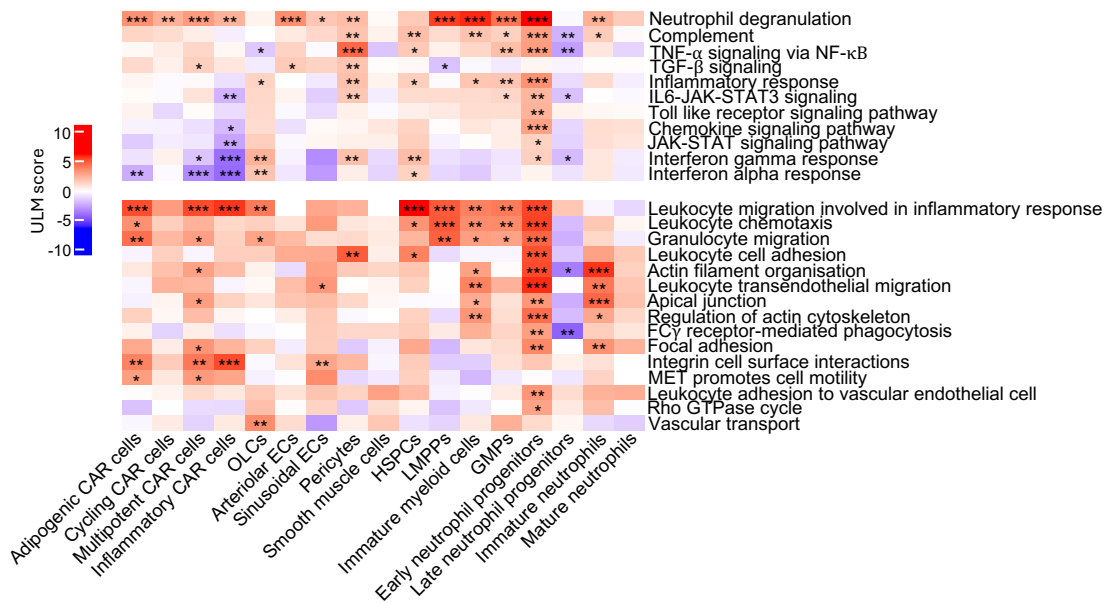




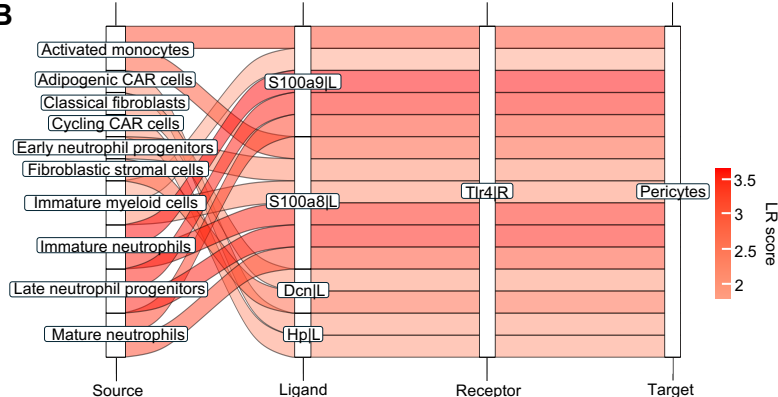
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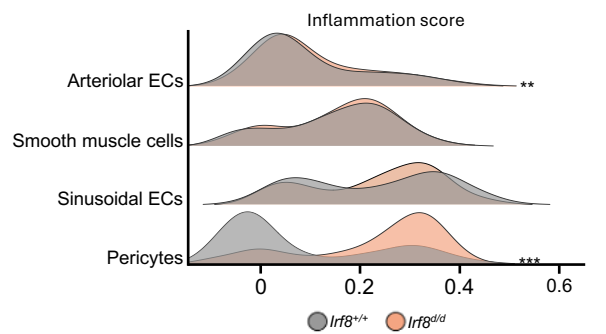
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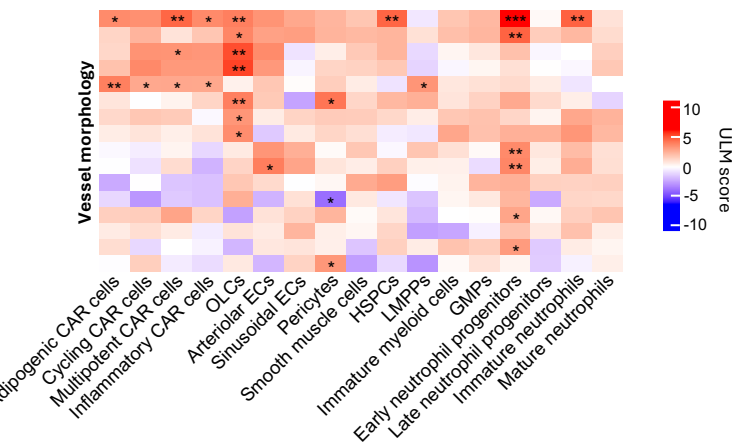
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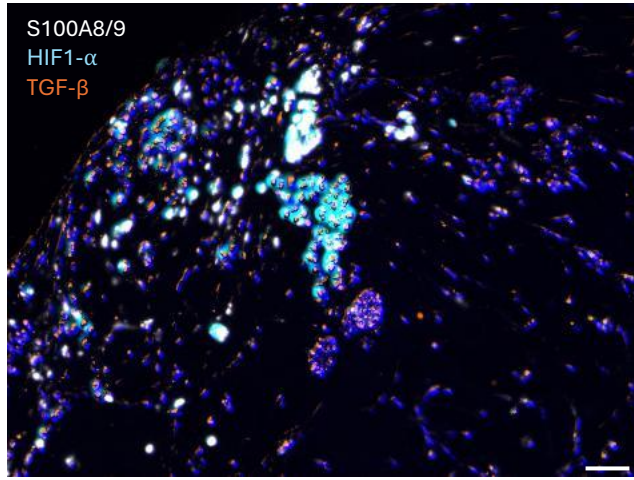
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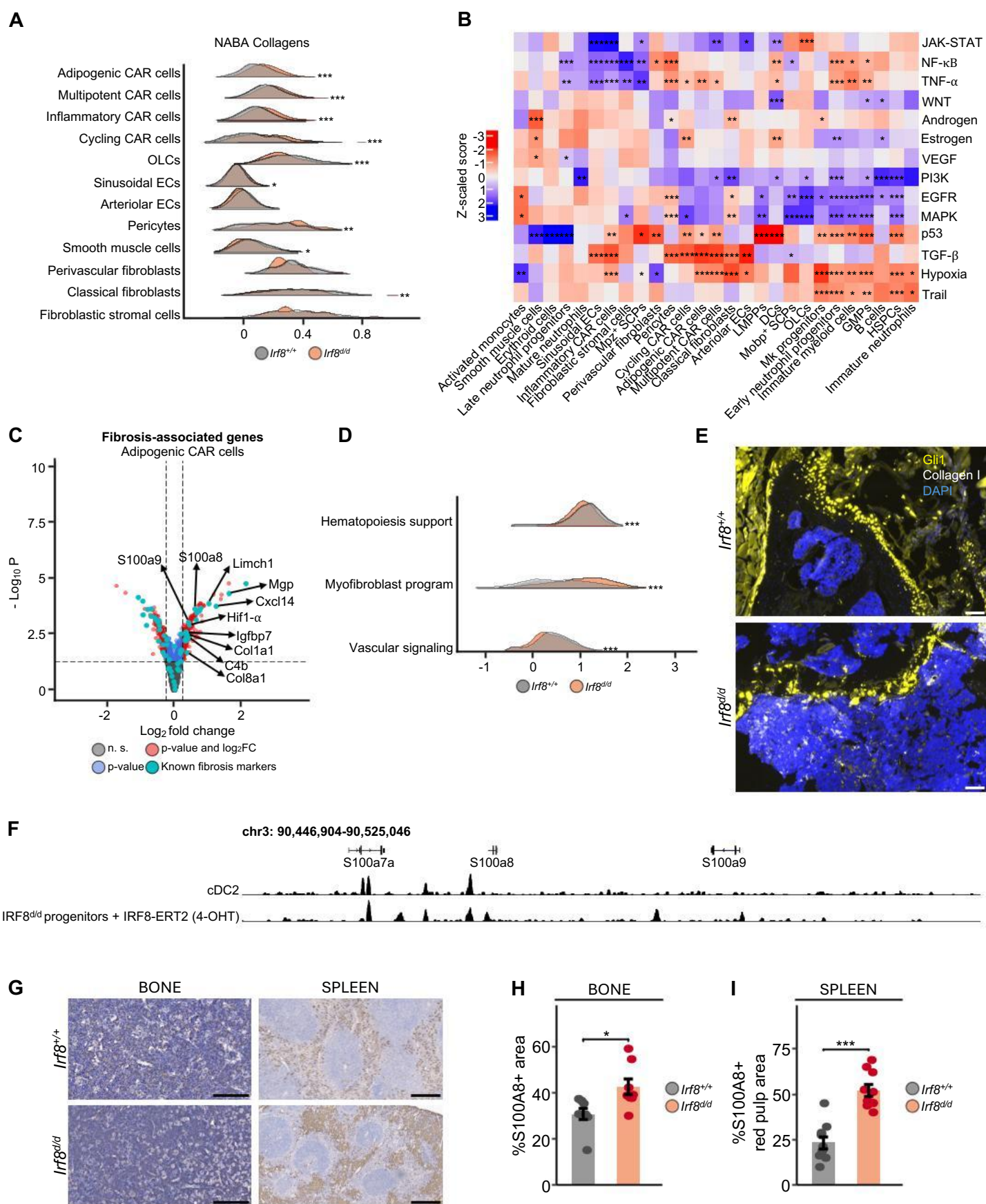


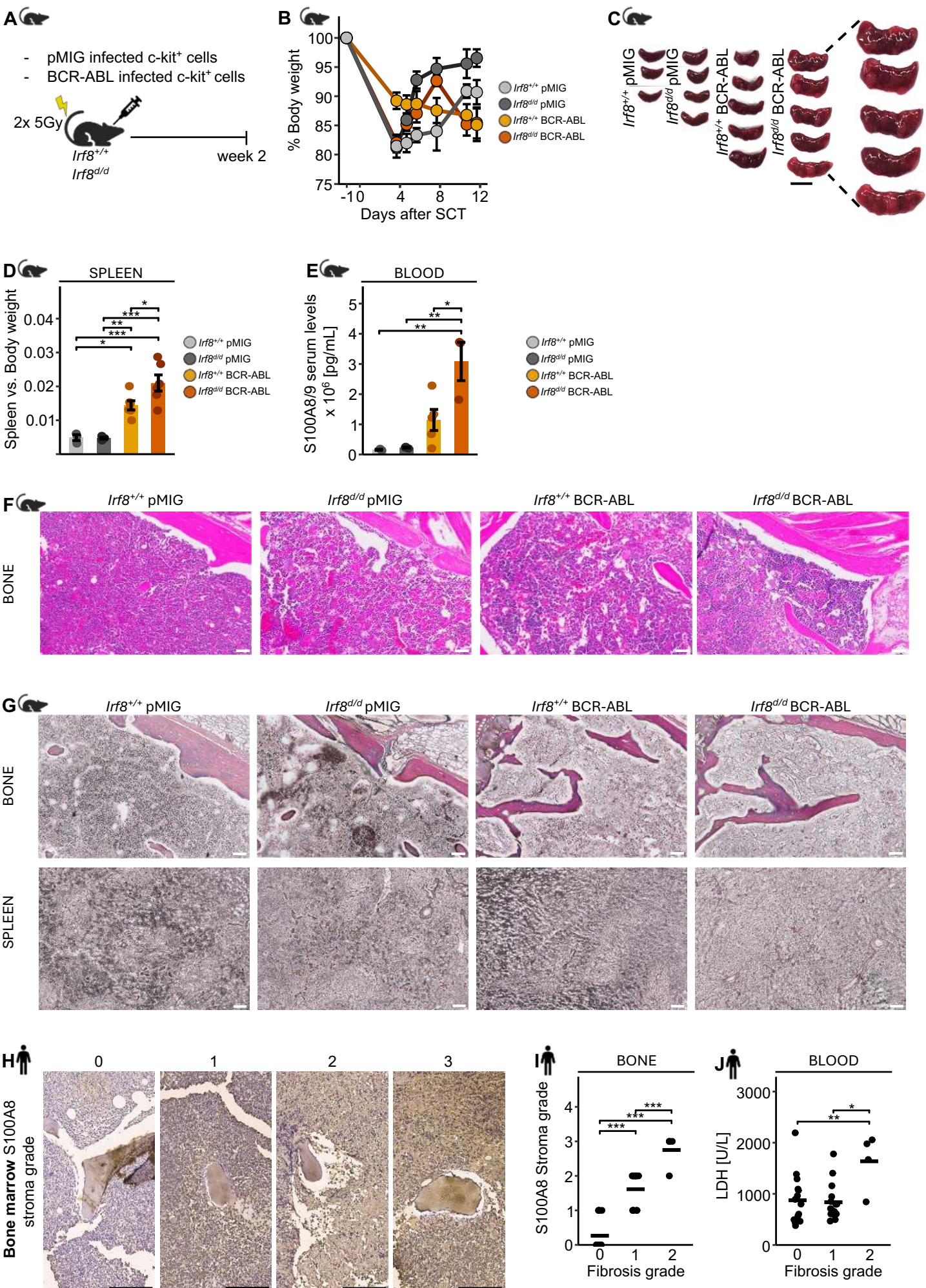
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MAIN FIGURE 7

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