Macrophages induce inflammation by efferocytosis of apoptotic prostate cancer cells via HIF-1α stabilization

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Abstract

Clearance of apoptotic cancer cells by macrophages, known as efferocytosis, fuels the bone-metastatic growth of prostate cancer cells via pro-inflammatory and immunosuppressive processes. However, the exact molecular mechanisms remain unclear. In this study, single-cell transcriptomics of bone marrow macrophages undergoing efferocytosis of apoptotic prostate cancer cells revealed a significant enrichment of a cellular response to hypoxia. Here we show that efferocytic macrophages promote HIF-1α stabilization under normoxic conditions through interaction with phosphorylated STAT3. Inflammatory cytokine gene expression analysis of efferocytic HIF-1α-mutant macrophages revealed a reduced expression of the pro-tumorigenic *Mif*. Furthermore, stabilization of HIF-1α using the HIF-prolyl-hydroxylase inhibitor, Roxadustat, enhanced MIF expression in macrophages. Finally,

macrophages treated with recombinant MIF protein activated NF-κB (p65) signaling and increased the expression of pro-inflammatory cytokines. Altogether, these findings suggest that the clearance of apoptotic cancer cells by tumor-associated macrophages triggers p-STAT3/HIF-1α/MIF signaling to enhance tumor-promoting inflammation in bone, suggesting this axis as a target for metastatic prostate cancer.

Introduction

The process of clearing of apoptotic cancer cells by macrophages, known as efferocytosis, commonly occurs during tumor progression and fuels the bone-metastatic growth of cancer cells, via subsequent pro-inflammatory and immunosuppressive activity (Graham *et al*, 2014; Lecoultre *et al*, 2020; Roca & McCauley, 2018; Stanford *et al*, 2014). Our previous published work reported that bone marrow macrophage-dependent efferocytosis of apoptotic prostate cancer cells supported skeletal tumor growth through the secretion of pro-inflammatory cytokines resulting in an immunosuppressive response (Mendoza-Reinoso *et al*, 2020; Roca *et al*, 2018). Recently, a single-cell RNA sequencing study reported that peritoneal macrophage efferocytosis of apoptotic T cells displayed a heterogeneous transcriptional activity including genes associated with predisposition for efferocytosis, macrophage differentiation, locomotion and inflammation (Lantz *et al*, 2020). However, the precise molecular mechanisms involved in bone marrow macrophage response to efferocytosis of apoptotic cancer cells remains to be elucidated.

The majority of solid tumors present areas of permanent or transient hypoxia due to poor vascularization and blood supply (Pouyssegur *et al*, 2006). Hypoxic conditions activate hypoxia inducible factor (HIF) signaling which has a crucial role in pro-tumorigenic inflammatory processes via cytokine secretion, reactive oxygen species (ROS) production and angiogenesis (Triner & Shah, 2016). HIFs are heterodimers consisting of an oxygen labile alpha (α) subunit and a stable beta (β) subunit. There are three isoforms of HIF-α, including HIF-1α, HIF-2α (EPAS1) and HIF-3α (IPAS) (Kaelin & Ratcliffe, 2008). HIF-1α upregulates glycolytic genes such as phosphoglycerate kinase (PGK) and lactate dehydrogenase A (LDHA) whereas HIF-2α induces the expression of genes related to oxygen supply improvement in hypoxic regions such as erythropoietin (EPO) (Hu *et al*, 2003). HIF-1α has been identified as a key regulator of proliferative, invasive and immunosuppressive mechanisms that favor tumor progression (Engel *et al*, 2017; Hatfield *et al*, 2019). Under hypoxic conditions, HIF-1α hydroxylation by prolyl hydroxylase is reduced. This inhibits the HIF-1α/Von Hippel-Lindau (VHL) interaction and consequent HIF-1α degradation by ubiquitin E3 ligase complex (Jaakkola *et al*, 2001). Therefore, HIF-1α is stabilized in the cytosol and translocated to the nucleus to promote the transcription of multiple target genes (Semenza, 2011). HIF-1α is strikingly upregulated under hypoxic conditions, however, HIF-1α can also be regulated at transcriptional, translational and post-translational levels under normoxic conditions (Hayashi *et al*, 2019). HIF-1α is expressed and stabilized in immune cells via hypoxia or other factors such as inflammation, cancer and infectious microorganisms (Blouin *et al*, 2004; Hartmann *et al*, 2008; Peyssonnaux *et al*, 2005). HIF-1α is crucial for myeloid cell-mediated inflammation (Cramer *et al*, 2003) and it has been demonstrated that tumor-associated macrophages (TAMs) also

express HIF-2α under hypoxic conditions (Imtiyaz *et al*, 2010; Talks *et al*, 2000). Various studies have shown a relationship between HIF-1α induction and STAT3 activation at post-translational and transcriptional levels (Gray *et al*, 2005; Jung *et al*, 2008; Niu *et al*, 2008; Xu *et al*, 2005).

Previous reports have shown that low oxygen concentrations in tumors promote the secretion of cytokines and chemokines that recruit pro-tumorigenic Tregs, tumor-associated macrophages, neutrophils, B cells, and myeloid-derived suppressor cells (MDSCs) to support tumor growth (Blaisdell *et al*, 2015; Du *et al*, 2008; Facciabene *et al*, 2011; Zhu *et al*, 2014). One of these cytokines is macrophage migration factor (MIF), which is a direct target gene of HIF-1α (Winner *et al*, 2007) and a hypoxia-induced gene in colon and breast cancer cells (Larsen *et al*, 2008; Yao *et al*, 2005). MIF acts as an autocrine or paracrine cytokine, it is upregulated in several types of cancer (Mawhinney *et al*, 2015; Tomiyasu *et al*, 2002; Wilson *et al*, 2005) and its expression correlates with disease malignancy and invasiveness (Lippitz, 2013). Studies consistently demonstrated that MIF signals primarily through CD74 in association with CD44, CXCR2, CXCR4, and CXCR7 to activate the ERK MAP kinase cascade (Leng *et al*, 2003; Shi *et al*, 2006; Tarnowski *et al*, 2010). Finally, MIF signaling induces the activation and secretion of pro-tumorigenic cytokines to support tumor growth (Bach *et al*, 2008; Bucala & Donnelly, 2007) .

Using single-cell transcriptomic sequencing, we investigated the signature changes in bone marrow macrophage gene expression during efferocytosis of apoptotic prostate cancer cells. We found that macrophages engulfing apoptotic prostate cancer cells promoted HIF-1α stability by its interaction with phosphorylated STAT3 and induced the expression of the pro-inflammatory cytokine MIF. Thus, p-STAT3/HIF1α/MIF signaling in tumor-associated macrophages may have a pro-tumorigenic effect in the bone marrow tumor microenvironment that contributes to skeletal metastasis and can be used to target preventive and therapeutic approaches.

Results

Single cell analyses of macrophages engulfing apoptotic prostate cancer cells show a distinct transcriptional signature and the activation of hypoxia-related genes.

Published findings suggest that macrophages induce distinctive tumor-promoting signaling in response to efferocytosis of apoptotic cancer cells (Mendoza-Reinoso *et al.*, 2020; Roca *et al.*, 2018; Soki *et al*, 2014; Stanford *et al.*, 2014). However, the mechanisms that govern these specific responses in connection to tumor acceleration are not completely understood. To further investigate the efferocytosis-mediated signaling in bone macrophages, primary bone marrow macrophages from immunocompetent C57BL/6J mice were co-cultured with $CFSE^{+}$ (pre-labeled) apoptotic prostate cancer RM1 cells. Macrophages engulfing these apoptotic cancer cells (efferocytic) were compared to non-engulfing (non-efferocytic) macrophages by single-cell RNA sequencing upon sorting by flow cytometry (Figure 1A). After identification of high quality sequenced single cells (Table 1,

Supplementary data), UMAP (Becht *et al*, 2018) was applied for dimension reduction of single cell analysis and visualization of the transcriptional data for efferocytic and non-efferocytic macrophages. As shown in Figure 1B the cell distribution in two UMAP projections depicts different cluster enrichment in efferocytic vs. non-efferocytic macrophages from two independent experiments (Exp. 1 and Exp. 2). For example, efferocytic cells demonstrate enriched in cluster in the direction of increased UMAP-2-projection while the opposite is observed in non-efferocytic macrophages as shown in the split visualization of these cells (Figure 1B). These results correlate with distinct transcriptional heatmaps in efferocytic relative to non-efferocytic macrophages (Figure 1C) where the great majority of differentially expressed genes (DEG's) significantly changed in the same direction (3277 vs 482) in both experiments as analyzed in the Venn diagram in Figure 1D. DEG's commonly upregulated in efferocytic macrophages in both experiments were further processed using the PANTHER analysis (Mi *et al*, 2013; Thomas *et al*, 2003) and gene ontology database (Ashburner *et al*, 2000; Gene Ontology, 2021) to identify the relevant biological pathways. Among the significantly enriched GO- biological processes identified we found pathways related to the innate immune system and wound healing responses which are related to phagocytosis of apoptotic cells, inflammation, and regeneration (Figure 1E, list of enriched GO terms in Table 1, Supplementary data) (Koh & DiPietro, 2011; Minutti *et al*, 2017). Intriguingly, biological processes related to hypoxia were identified even though these experiments were performed under normoxia (normal oxygen conditions), which suggests that efferocytosis mediated activation and upregulation of factors directly related to cellular hypoxia is independent of the oxygen concentration (Figure 1E and Figure 1F).

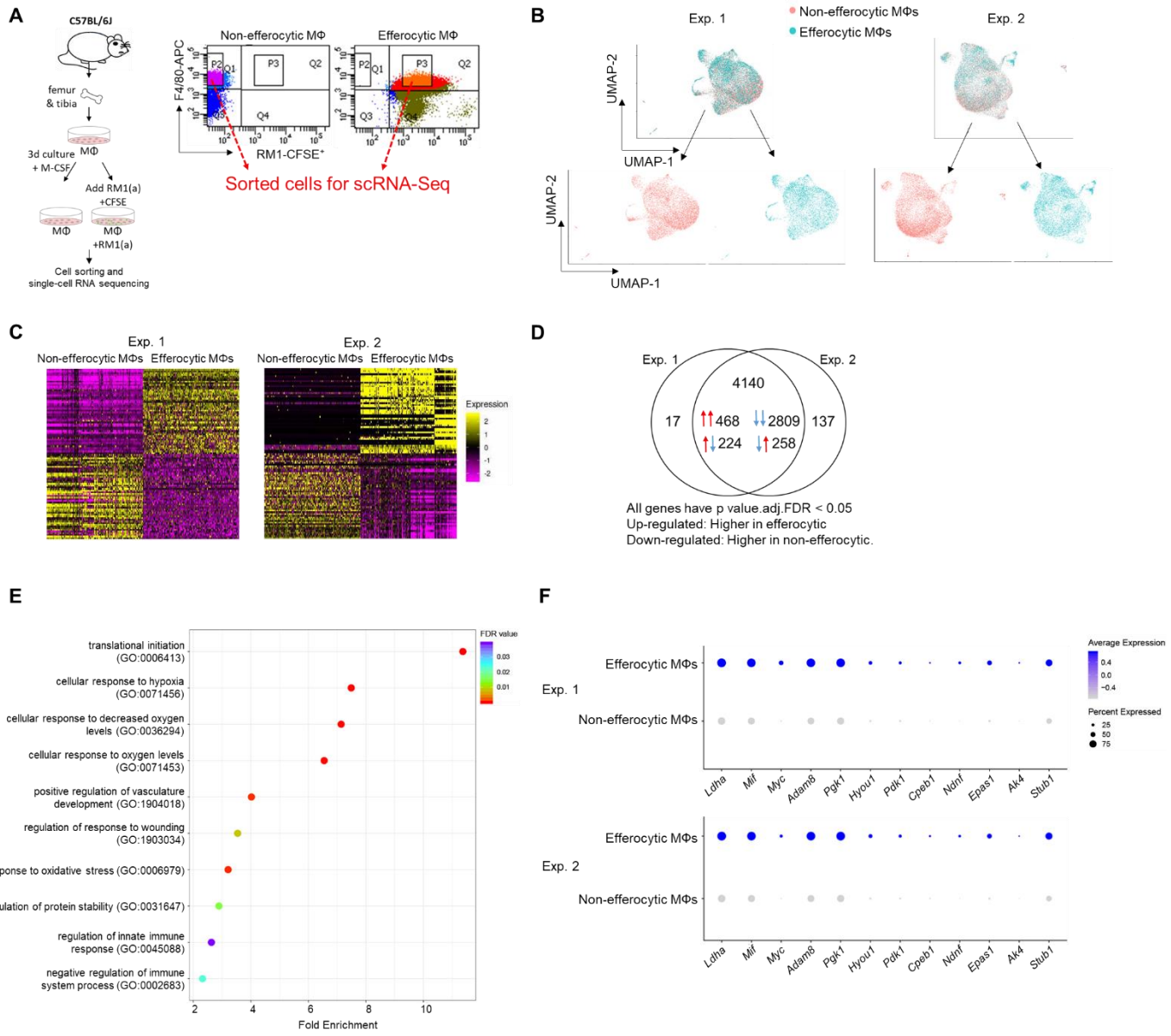


Figure 1. Single-cell experiments comparing efferocytic macrophages (engulfing apoptotic cancer cells) vs control (non-engulfing macrophages)

A. Bone marrow-derived macrophages were isolated from 4 week old C57BL/6J mice and co-cultured with apoptotic RM1(a) cells for 16-18 hours. Efferocytic ($F4/80^+CFSE^+$) and control macrophages ($F4/80^+$) were sorted and used for single-cell libraries followed by single-cell RNA sequencing. **B.** UMAP of all cells, blue represents efferocytic macrophages and red represents non-efferocytic macrophages. **C.** Heatmap of top differential expressed genes in either experiment. **D.** Venn Diagram of overlap of all differentially expressed genes between both experiments. **E.** Cleveland plot of top enrichment pathways in efferocytic macrophages. **F.** Dot plots of hypoxia-related genes in both experiments. Size relates to the percentage of macrophage cells that expressed each gene. Color denotes the average expression for each gene across all expressing cells. Additional results are shown in Supplementary data – Table 1, 2 and 3.

Efferocytosis of apoptotic cancer cells stabilizes HIF-1α in bone macrophages which is mediated by the activation of STAT3.

Single cell analysis of efferocytic macrophages identified upregulated molecules associated with gene ontology (GO) term of cellular hypoxia (Figure 1). To investigate these findings in the overall macrophage population, co-cultures of bone macrophages with apoptotic prostate cancer RM1 cells (workflow Figure 1A) were analyzed. Selected hypoxia-GO associated genes showing significant upregulation by sc-RNAseq were investigated by RT-qPCR from total RNA isolated from co-cultures of independent bone macrophage populations. Fold changes in efferocytic relative to control macrophages were calculated and plotted in Figure 2A. The majority of analyzed genes showed a significant mRNA increase in efferocytic macrophages relative to control in correlation with the single cell results. Some of these molecules are crucial components of glycolysis including *Pdk1*, *Pgk1*, and *Ldha* and are known targets of hypoxia inducible factor 1a (HIF-1α), a master transcriptional regulator of cellular response to hypoxia that promotes metabolic switch to glycolysis (Niu *et al.*, 2008; Palazon *et al*, 2014). *Hif1a* was upregulated in the overall population of efferocytic macrophages (although it was not identified as an upregulated gene in the single-cell experiments). Contrary to the results observed by single-cell experiments, the hypoxia-inducible transcription factor *Epas1* (HIF-2α) (Imtiyaz *et al.*, 2010) showed a small but significant decrease in mRNA expression by efferocytosis.

Because HIF-1α is largely regulated post-transcriptionally resulting in a protein targeted for degradation under normoxic conditions (Ivan *et al*, 2001; Jaakkola *et al.*, 2001), HIF-1α protein was further evaluated by Western blot. As shown in Figure 2B, Western blot analysis of efferocytic macrophages (co-cultured for 16-18 hours with apoptotic RM1 cells) evidenced a significant increase of HIF-1α induced by efferocytosis. These findings were further corroborated in efferocytic bone marrow derived macrophages co-cultured with murine prostate cancer Myc-CaP cells, which share several molecular characteristics of human prostate cancer (Dudzinski *et al*, 2019; Ellwood-Yen *et al*, 2003). As Myc-CaP cancer cells were obtained from FVB/NJ mice, the primary macrophages were obtained from the bone marrow of the same strain. Similar results were observed using this model (Figure 2C). These results suggest that HIF-1α is stabilized in macrophages engulfing apoptotic cancer cells.

The potential mechanism inducing HIF-1α stabilization was further investigated. Previous findings suggested that one potential mechanism leading to HIF-1α stabilization is via interaction with activated (phosphorylated) STAT3 (Gray *et al.*, 2005; Jung *et al.*, 2008; Li *et al*, 2019; Xu *et al.*, 2005). Since STAT3 activation is sustained in efferocytic macrophages and considered a hallmark macrophage response to engulfing apoptotic cancer cells it was hypothesized that STAT3 activation is critical in HIF-1α stabilization by efferocytosis. We investigated this using two well characterized STAT3 inhibitors: Stattic and S3I-201 (Schust *et al*, 2006; Siddiquee *et al*, 2007). Both inhibitors significantly reduced the activation of STAT3 analyzed after incubation with apoptotic cancer cells for two hours. This treatment also impacted the stabilization of HIF-1α (Figure 2D-2G and Figure 2–Figure supplement 1). Similarly, these findings were observed in macrophages efferocytosing apoptotic Myc-CaP cells (Figure 2–Figure supplement 2). These findings strongly support the hypothesis that STAT3 activation is a critical signal that mediates the stabilization of HIF-1α by efferocytosis.

To further investigate these findings, a direct interaction between HIF-1α and p-STAT3 was evaluated in immunoprecipitation assays. Protein lysates collected from macrophages co-cultured with apoptotic prostate cancer RM1 cells were used to perform immunoprecipitation with HIF-1α-specific antibodies and the pull-down of p-STAT3 was evaluated by Western Blot. The blot showed a p-STAT3 band in the macrophage lysates from efferocytic samples which were immunoprecipitated with HIF-1α (Figure 2H). The p-STAT3 band was undetected in immunoprecipitated protein samples from non-efferocytic macrophages. These results strongly suggest the interaction between HIF-1α and p-STAT3 in efferocytic macrophages which support the hypothesis of STAT3 activation as a mechanism mediating the stabilization of HIF-1α.

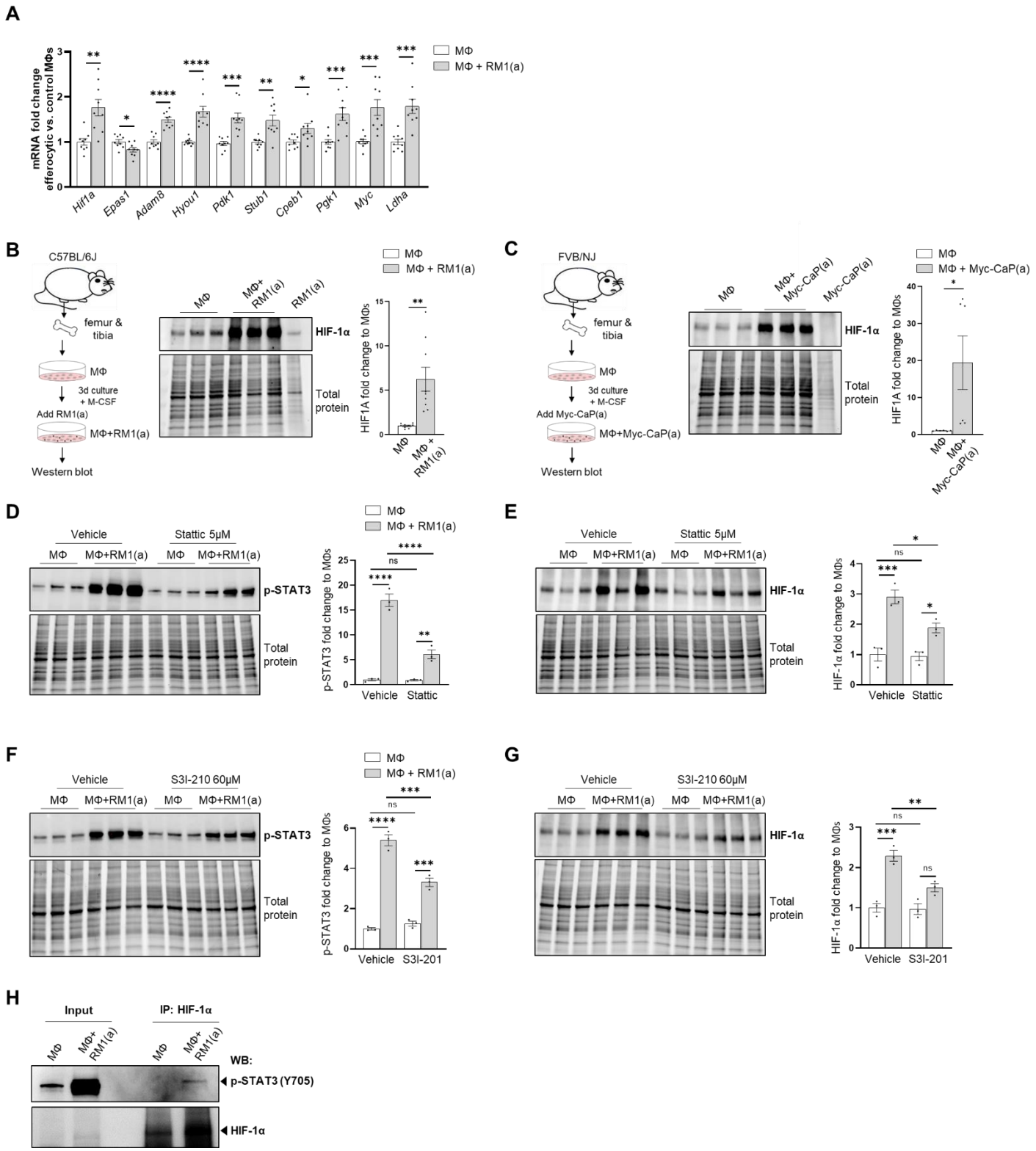


Figure 2. Macrophage efferocytosis of apoptotic cancer cells promotes HIF-1α stabilization through STAT3 activation.

Bone marrow-derived macrophages were isolated from 4 week old C57BL/6J or FVB/NJ mice and co-cultured with apoptotic RM1(a) or Myc-CaP(a) cells for 16-18 hours.

A. mRNAs isolated from efferocytic and control macrophages were analyzed by quantitative PCR (qPCR) for a set of genes involved in the cellular response to hypoxia (n=9). **B. & C.** Protein lysates from C57BL/6J (n=9) and FVB/NJ (n=6) efferocytic macrophages were analyzed by western blot using HIF-1α antibody. Protein lysates from C57BL/6J efferocytic and control macrophages (n=3) treated with 5μM Stattic and 60μM S3I-201 STAT3 inhibitors for 2 hours were analyzed by western blot using **D. & F.** Phospho-STAT3 antibody and **E. & G.** HIF-1α antibody. **H.** Cell lysates from efferocytic and non-efferocytic macrophages were immunoprecipitated with anti-HIF-1α antibody and immunoblotted with phospho-STAT3 antibody. Data plotted are mean ± SEM, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, ns=not significant (Ordinary one-way ANOVA; Tukey's multiple-comparisons test and unpaired t-test). Additional results are shown in Figure 2–Figure supplement 1 and 2.

Efferocytosis stimulates the expression of pro-inflammatory MIF cytokine in macrophages

Accumulating experimental evidence suggests that efferocytosis of apoptotic cancer cells accelerates tumor progression and metastatic growth by fostering an inflammatory and immunosuppressive microenvironment (Elliott *et al*, 2017; Werfel & Cook, 2018). Single-cell data identified the negative regulation of the immune system process (GO: 0002683; related to the immunosuppressive response) as one of the GO terms upregulated in efferocytic macrophages. Using STRING, a database of known and predicted protein-protein interactions, we identified strong network association between this immune response and the biological process of hypoxia (GO: 0071456) (Figure 3A, GO gene list Table 2 and Table 3 in Supplementary data). Although not identified by single cell STAT3 was added because of its key role in the stabilization of HIF-1α as shown in Figure 2. Central nodes identified in this network are HIF-1α, Myc and STAT3 and the findings show direct or indirect interactions between hypoxia and the negative immune regulation processes. Furthermore, single cell analysis identified the cytokine macrophage migration inhibitory factor *Mif* as upregulated in efferocytic macrophages ($p<10^{-6}$) (Figure 1F, Figure 3B and Figure 3–Figure supplement 1). *Mif* belongs to both GO: 0002683 and GO: 0071456 (Gene Ontology, 2021) and mediates both immunosuppression and inflammation and has been associated with increased tumorigenesis and disease progression in different cancer types including prostate cancer (Penticuff *et al*, 2019).

MIF changes were investigated in the overall macrophage population co-cultured with apoptotic RM1 prostate cancer cells by RT-qPCR and Western blot. In correlation with single cell results both MIF mRNA (Figure 3C) and protein increased in bone macrophages efferocytosing apoptotic RM1 cells (Figure 3D). Similarly bone marrow macrophages isolated from FVB mice upregulated MIF protein upon efferocytosis of Myc-CaP prostate cancer cells (Figure 3E).

These findings indicate MIF is part of the signaling response of macrophages to the engulfing of apoptotic prostate cancer cells and suggests a network connection with the activation of hypoxia-related molecules by efferocytosis.

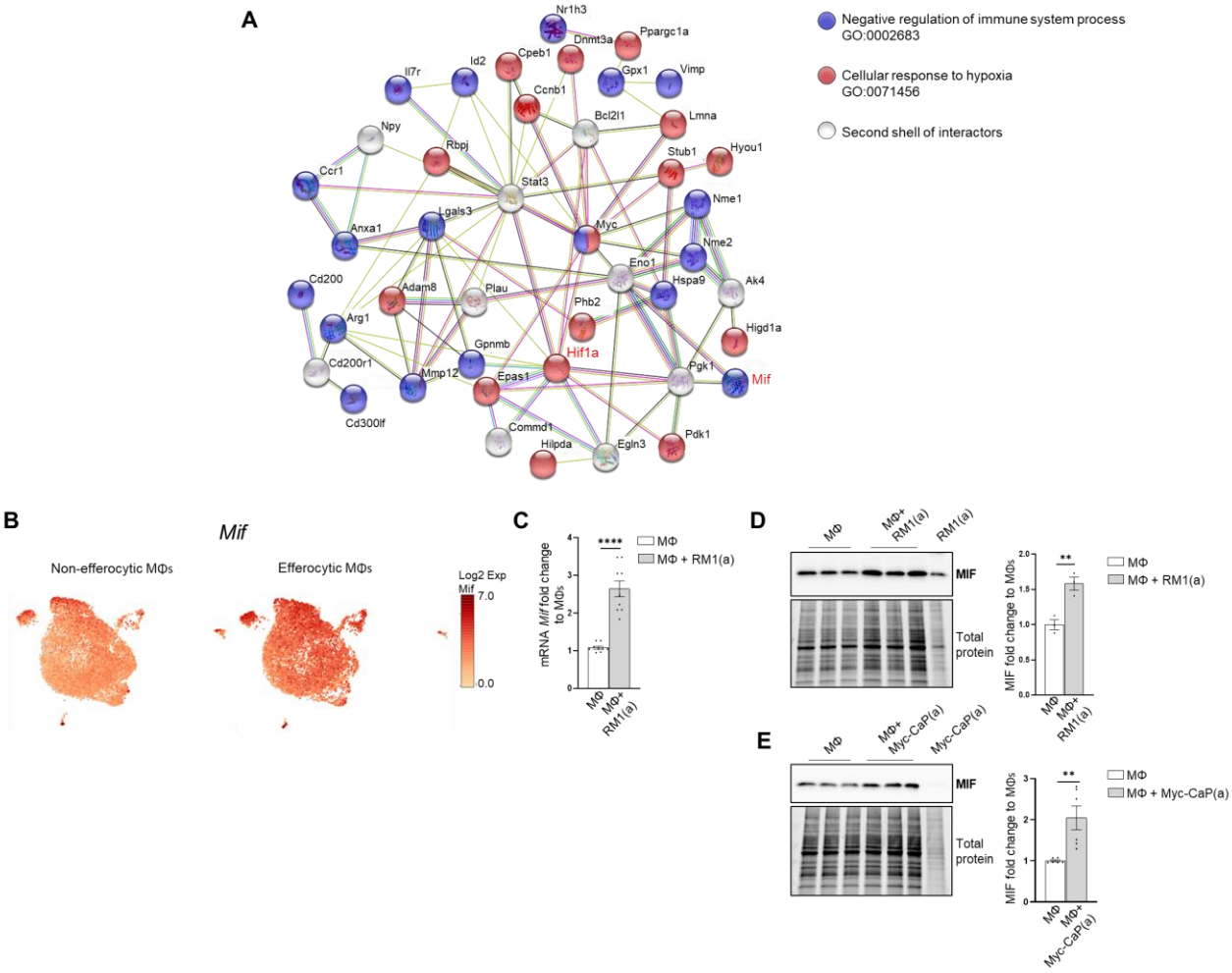


Figure 3. Macrophage efferocytosis induces MIF expression.

A. Protein-protein interaction network between the immune pathway regulation and the hypoxia related pathway. Biological Process GO terms of selected genes upregulated by efferocytosis (GO:0002683, all genes; GO:0071456, 25 out of 33 genes). Bone marrow-derived macrophages were isolated from 4 week old C57BL/6J or FVB/NJ mice and co-cultured with apoptotic RM1(a) or Myc-CaP(a) cells for 16-18 hours. **B.** scRNA-Seq analysis plot shows *Mif* distribution in efferocytic and non-efferocytic clusters of macrophages. **C.** *Mif* mRNA expression in efferocytic macrophages assessed by RT-qPCR (n=9). Western blot analysis of protein lysates from **D.** C57BL/6J macrophages co-cultured with apoptotic RM1 prostate cancer cells (n=3) and **E.** FVB/NJ efferocytic and control macrophages using MIF antibody (n=6). Data plotted are mean ± SEM, $^{**}p < 0.01$, $^{****}p < 0.0001$ (unpaired t-test). Additional results are shown in Figure 3–Figure supplement 1.

HIF-1α mediates the expression of MIF cytokine in bone efferocytic macrophages

The implication of HIF-1α in tumor promoting inflammation, immunosuppression and metastasis has been documented in different cancer models in relation to hypoxia (Triner & Shah, 2016). We hypothesized that the stabilization of HIF-1α in bone macrophages by the clearance of apoptotic cancer cells induces the expression of key pro-inflammatory cytokines. This was investigated by crossing LysM-Cre mice with the HIF-1α $^{flox/flox}$ mice to obtain mutated HIF-1α myeloid lineage mutant mice (*Hif1a*^{mut}). These mice create a null allele in the Cre-expressing cells (myeloid) lacking the exon 2 of *Hif1a* and were used to obtain *Hif1a*^{mut} bone macrophages. Relative *Hif1a* specific mRNAs were quantified with a primer/probe set corresponding to exon 2 of the mRNA. The qPCR analysis showed the upregulation of *Hif1a* mRNA in the efferocytic macrophages relative to control (Figure 4A). In support of the model a significant decrease in the *Hif1a* mRNA containing the exon 2 was observed in *Hif1a*^{mut} macrophages corresponding to control and efferocytic samples (Figure 4A). In addition, characterization of *Hif1a*^{mut} bone marrow derived macrophages by Western blot demonstrated a lower molecular weight in *Hif1a*^{mut} which corresponds with the deletion in the DNA binding domain encoded by the exon 2 (Figure 4C) by Cre-induced recombination which renders a non-functional *Hif1a*^{mut} protein. However even this mutant protein was stabilized by efferocytosis as shown by quantitative Western blot analyses, which suggests that STAT3-mediated stabilization is independent of HIF-1α binding to the chromatin (Figure 4C). Of note STAT3 activation remained unaffected in mutant macrophages (Figure 4–Figure supplement 1). Previous studies suggest a link between HIF-1α and MIF in different cell models, including macrophages (Alonso *et al*, 2019; Baugh *et al*, 2006). We investigated the expression of MIF and other pro-inflammatory factors as potential targets of HIF-1α. These included critical inflammatory cytokines previously found upregulated in efferocytic macrophages: CXCL1, CXCL5 and IL6 and CXCL4 (also known as platelet factor 4, *Pf4*) (Vaught *et al*, 2015). From the selected cytokines it was found that *Mif* and *Cxcl4* expression was significantly reduced in *Hif1a*^{mut} efferocytic macrophages relative to wild type (WT) after normalization to their specific control macrophages (Figure 4B).

Further analysis of MIF protein indicated a significant reduction in MIF protein expression and no upregulation was observed by efferocytosis in the *Hif1a*^{mut} macrophages relative to WT (Figure 4D). In contrast upregulation of MIF by efferocytosis was found in WT macrophages as previously shown in (Figure 3D-3E).

To address the specificity of HIF-1α in the control of MIF regulation, similar experiments were performed with *Epas1*-myeloid lineage-mutant (*Epas1*^{mut}) mice. These mice were obtained by crossing LysM-Cre mice with the Epas1$^{flox/flox}$ mice. The mutant mice expressed significantly lower levels of *Epas1* mRNA by RT-qPCR using the primer/probe set corresponding to the deleted exon2 (Figure 4–Figure supplement 2A). In addition, qPCR analysis of pro-inflammatory cytokines indicated a significant decrease in *Cxcl1* in the mutant mice, while no changes in *Mif* and *Cxcl4* (Figure 4–Figure supplement 2B), suggesting Epas1- specific regulation of pro-inflammatory cytokines, which is different than the observed for HIF-1α-mediated regulation in macrophages (Figure 4B). Quantitative protein analysis of mutant macrophages indicated no change by efferocytosis in HIF-1α stabilization in the *Epas1*^{mut} macrophages relative to WT control (Figure 4–Figure supplement 2C) and no change by efferocytosis in MIF expression when compared mutant versus WT

(Figure 4–Figure supplement 2D) different from the results for efferocytic *Hif1a*^{mut} macrophages (Figure 4D). Furthermore, when analyzed relative to the control macrophages, the efferocytic *Epas1*^{mut} macrophages showed a significant increase in MIF (Figure 4–Figure supplement 2D), while no change was observed in efferocytic *Hif1a*^{mut} macrophages (Figure 4D). These results highlight the specificity in the regulation of MIF expression by HIF-1α relative to EPAS1, a similar hypoxia-inducible transcription factor.

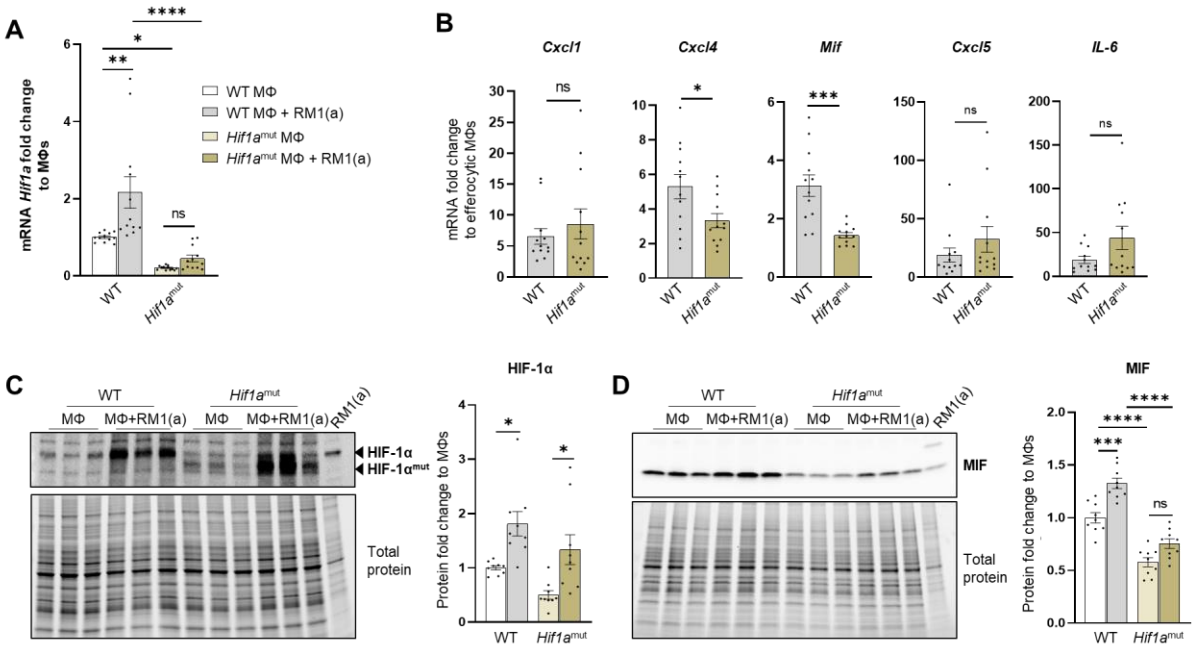


Figure 4. HIF-1α depletion in efferocytic macrophages reduces MIF expression.

A. *Hif1a* mRNA expression levels in *Hif1a*$^{flox/flox}$ and *Hif1a*^{mut} macrophages. **B.** mRNA from efferocytic and control macrophages from *Hif1a*$^{flox/flox}$ and *Hif1a*^{mut} mice were analyzed by RT-qPCR for the specified inflammatory cytokine genes (n=12). **C. & D.** Protein lysates from efferocytic and control macrophages from *Hif1a*$^{flox/flox}$ (WT) and *Hif1a*^{mut} mice were analyzed by Western blot with HIF-1α and MIF antibodies (n=9). Data plotted are mean ± SEM. *p < 0.05, ***p < 0.001, ****p < 0.0001, ns=not significant (Ordinary one-way ANOVA; Tukey's multiple-comparisons test and unpaired t-test). Additional results are shown in Figure 4–Figure supplement 1 and 2.

HIF-1α-mediated MIF regulation was further investigated by using a HIF α-prolyl-hydroxylase inhibitor FG-4592 (also known as Roxadustat) (Hsieh *et al*, 2007). FG-4592 was used in efferocytosis assays, where a strong correlation was observed between the stabilization of HIF-1α and MIF protein expression. FG-4592 alone stabilized HIF-1α which correlated with up-regulation of MIF protein in non-efferocytic macrophages. Intriguingly, when the inhibitor was used in efferocytic macrophages a further increase in HIF-1α and MIF protein was observed (Figure 5A and 5B). RT-qPCR analysis showed a significant increase in *Mif* mRNA induced by FG-4592 relative to control however no further increase was observed in efferocytic macrophages (Figure 5C).

Altogether, these findings suggest that HIF-1α mediates the expression of MIF, where HIF-1α stabilization by efferocytosis or prolyl-hydroxylase inhibitor significantly upregulates MIF expression in bone macrophages.

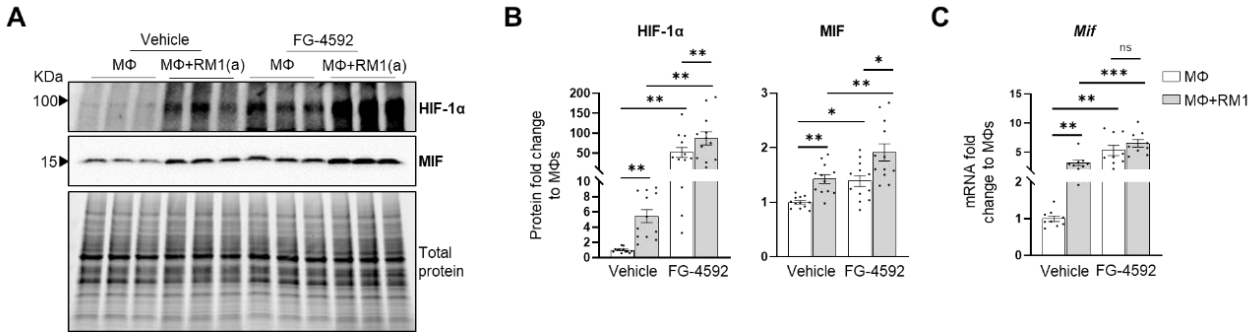


Figure 5. Efferocytic macrophages stabilize HIF-1α and induce MIF expression.

Bone marrow-derived macrophages were isolated from C57BL/6J mice and co-cultured alone or with apoptotic RM1(a) cells and treated with FG-4592, HIF prolyl-hydroxylase inhibitor (10μM) or vehicle control for 16-18 hours. **A. & B.** Protein lysates from co-cultures were analyzed by Western blot with HIF-1α and MIF antibodies (n=12). **C.** mRNAs were isolated from co-cultures and analyzed by RT-qPCR for *Mif* expression (n=9). Graphs show the fold change relative to macrophage control for each group. Data plotted are mean ± SEM; *P<0.05, **P < 0.01, ***p < 0.001, ns=not significant (Repeated measures one-way ANOVA; Tukey's multiple-comparisons test).

MIF activates inflammation in bone macrophages

CD74 is a critical receptor for MIF signal transduction in cells; however, CD74 lacks kinase activity and requires a complex formation with other co-receptors including CD44 and CXCR4 (Bernhagen *et al*, 2007; Leng *et al.*, 2003; Shi *et al.*, 2006). Results from single-cell data analyses identified a significant downregulation of CD74 in efferocytic macrophages as compared with non-efferocytic (Figure 6A, Figure 6–Figure supplement 1), while no significant differences were detected in the co-receptors CD44 or CXCR4. The downregulation of CD74 was also evident in the overall efferocytic macrophages relative to control by RT-qPCR analyses (Figure 6B).

Although these results do not rule out a potential endocrine signaling, they suggest that MIF could induce a potent paracrine signaling in non-efferocytic macrophages and other cells. To evaluate the MIF-induced signaling in macrophages a purified recombinant MIF protein expressed in mammalian cells was used. Macrophages were incubated for 2 hours with MIF and signaling activation was analyzed by Western blot with specific phospho-peptide antibodies. A hallmark MIF transducing signal resulting in the activation (sustained phosphorylation) of the extracellular signal related kinase ERK1/2 MAPK (Calandra & Roger, 2003; Penticuff *et al.*, 2019) was found highly upregulated in macrophages treated with recombinant MIF protein relative to control (Figure 6C and Figure 6–Figure supplement 2A). Furthermore, an increase in the critical inflammatory NF-kB signaling (phospho-p65) was observed in macrophages treated with MIF (Figure 6C and Figure 6–Figure supplement 2B).

This potent inflammation-transduced signaling pathway was further correlated with the increased expression in MIF activated macrophages of several pro-inflammatory cytokines including: *Ccl5*, *Cxcl5*, *Il6*, *Cxcl1* and *Cxcl4* (Figure 6D and Figure 6–Figure

supplement 2B). Similar results were observed in FVB macrophages where MIF activated the expression of these cytokines, except for *Cxcl4* (Figure 6E). The activation of these cytokines has also been detected in macrophages engulfing prostate cancer cells and function to accelerate tumor growth in bone as previously demonstrated (Roca & McCauley, 2018). Altogether, these results suggest that the STAT3-HIF-1α-MIF is a potent signaling axis induced by efferocytosis of apoptotic cancer cells which may act via paracrine signaling to perpetuate inflammation.

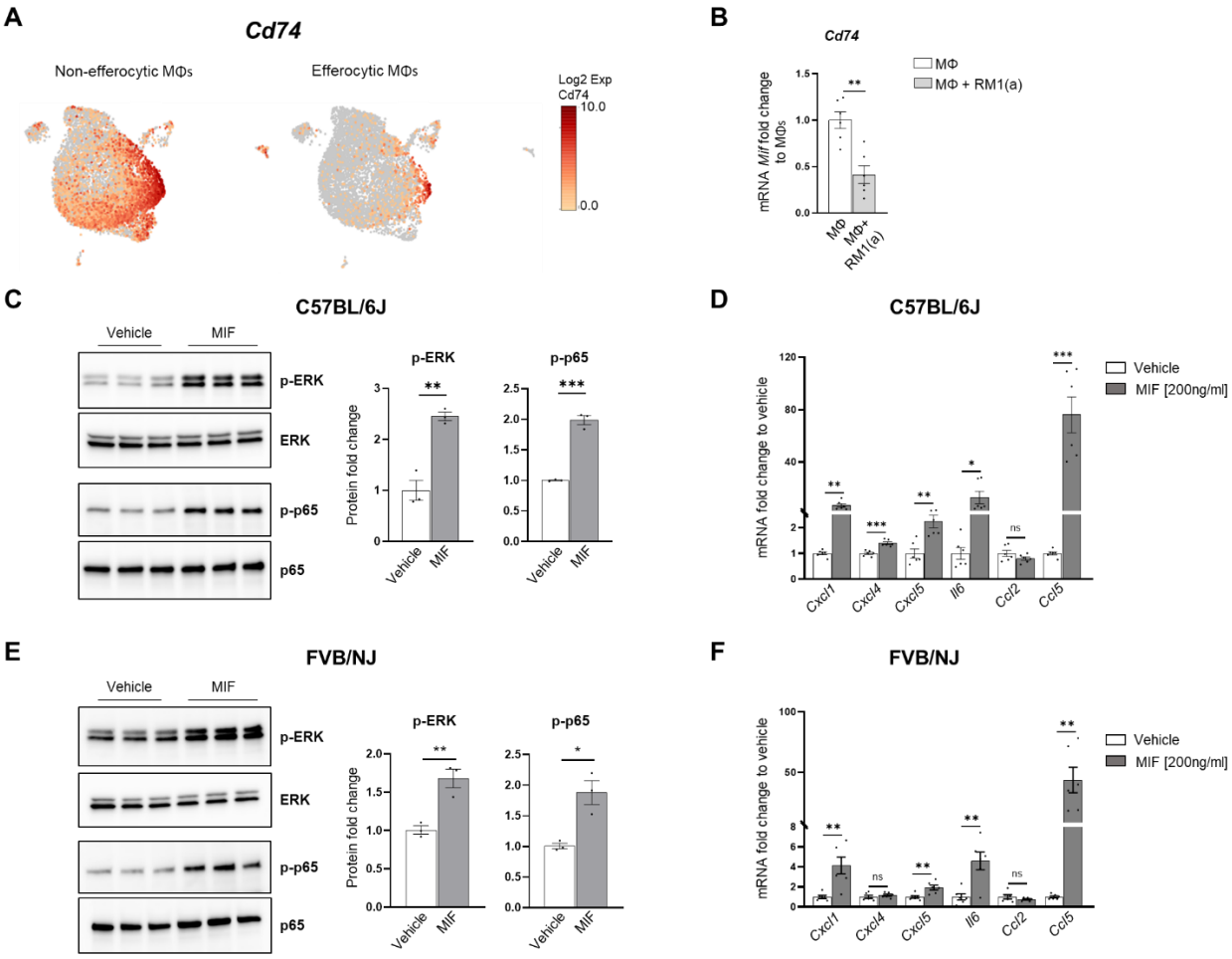


Figure 6. MIF induces a pro-inflammatory response in macrophages

Bone marrow-derived macrophages were isolated from C57BL/6J mice and treated with 200ng/ml of MIF protein or vehicle control during 2 hours for Western blot analysis and 8 hours for mRNA analysis. **A.** scRNA-Seq analysis plot shows *Cd74* distribution in efferocytic and non-efferocytic clusters of macrophages. **B.** *Cd74* mRNA expression in efferocytic macrophages assessed by RT-qPCR (n=6). **C.** Protein lysates from macrophages treated with MIF and vehicle control were analyzed by Western blot with total ERK, p-ERK, total p65 and p-p65 antibodies (n=3). **D.** mRNAs were isolated from co-cultures and analyzed by qPCR for the specified genes (n=6). Data are mean ± SEM; *p < 0.05, **p < 0.01, ***p < 0.001, ns=not significant (unpaired t-test). Additional results are shown in Figure 6–Figure supplement 1 and 2.

Discussion

Chronic inflammation has a major impact on cancer progression and metastasis in various organs (Coussens & Werb, 2002; Mantovani *et al*, 2008; Trinchieri, 2011). One of the inflammatory mechanisms that promotes tumor growth is the secretion of cytokines and chemokines by cancer and immune cells in the tumor microenvironment (de Visser *et al*, 2006; Quail & Joyce, 2013). Tumor-associated macrophages play a critical role in accelerating tumor progression in different cancer types (Aras & Zaidi, 2017; Duan & Luo, 2021). In bone, apoptotic cancer cell efferocytosis by macrophages generates a unique inflammatory milieu rich in cytokines that promote and support tumor progression (Mendoza-Reinoso *et al.*, 2020; Roca *et al.*, 2018). However, the molecular mechanisms that induce this tumor-promoting inflammatory response in bone marrow macrophages during the efferocytosis of apoptotic prostate cancer cells are still unclear.

Analysis of single-cell transcriptomics of sorted macrophages engulfing apoptotic prostate cancer RM1 cells (efferocytic, F4/80^{+}CFSE^{+}) versus sorted macrophages alone (non-efferocytic, F4/80^{+}) by flow cytometry (Figure 1A) revealed two distinctive clusters of cells, each of them with a unique gene expression profile. Gene ontology term enrichment analysis of the upregulated genes in efferocytic macrophages identified several biological pathways including those directly related to macrophage functions such as response to wounding, innate immune response and regulation of vasculature development (Okabe & Medzhitov, 2016). Although the efferocytosis experiments in vitro were conducted under normal oxygen levels, the analysis identified GO-terms related to cell response to hypoxia or decreased oxygen levels in efferocytic macrophages, these terms included genes that are known targets of HIF-1α, a master transcriptional regulator of cell response to hypoxia (Cui *et al*, 2017) . Similarly, hypoxia-independent HIF-1α expression has been observed in prostate cancer tumors, which correlates with recurrence following surgery or therapy, increased chemoresistance and accelerated metastatic progression, suggesting that alternative mechanisms of post-translational stabilization could lead to its accumulation and transcriptional activity in non-hypoxic environments (Ranasinghe *et al*, 2015; Ranasinghe *et al*, 2014). Here we found that efferocytic bone marrow macrophages promoted HIF-1α stabilization and induced a strong and sustained phosphorylation of STAT3 under normoxic conditions. Moreover, co-inmunoprecipitation experiments performed in this study demonstrated p-STAT3/HIF-1α interaction in efferocytic macrophages, which correlates with previous studies (Gray *et al.*, 2005; Jung *et al.*, 2008; Jung *et al*, 2005).

Previous studies have associated the expression of HIF-1α and its target genes with immunosuppressive functions in the tumor microenvironment (Chiavarina *et al*, 2010; Palazon *et al*, 2017). Here we identified a strong protein network association between the genes related to cellular response to hypoxia and negative regulation of the immune response in bone marrow efferocytic macrophages, suggesting that HIF-1α signaling in efferocytic macrophages may exert immunosuppressive functions in the tumor microenvironment. It has been reported that HIF-1α promotes the secretion of cytokines and chemokines such as CXC motif chemokine ligand 5 (CXCL5), CXC motif chemokine ligand 12 (CXCL12), chemokine ligand 28 (CCL28), and macrophage migration inhibitory factor

(MIF) (Blaisdell *et al.*, 2015; Du *et al.*, 2008; Facciabene *et al.*, 2011; Zhu *et al.*, 2014). One of the genes included in the network analysis was *Mif*. MIF is a pro-inflammatory cytokine expressed by monocytes, macrophages, blood dendritic cells, B cells, neutrophils, eosinophils, mast cells, and basophils; and its expression is involved in both innate and adaptive immune processes, as well as in response to hypoxia. Several studies have shown that MIF mediates inflammatory processes such as sepsis and cancer (Bernhagen *et al*, 1993; O'Reilly *et al*, 2016).We demonstrated that MIF mRNA and protein levels were increased in bone marrow macrophages upon efferocytosis of apoptotic cancer cells. MIF is highly expressed in prostate cancer patients and its expression has been associated with higher severity and poor outcome (Meyer-Siegler *et al*, 2005). Also, it has been reported that HIF-1α regulates MIF secretion in breast cancer cells to promote tumor proliferation, angiogenesis and metastasis (Larsen *et al.*, 2008). Interestingly, we found that HIF-1α but not HIF-2α depletion in bone marrow macrophages reduced the expression of the pro-tumorigenic inflammatory cytokines *Mif* and *Cxcl4* after being exposed to apoptotic prostate cancer cells when compared to wildtype bone marrow macrophages. Furthermore, HIF stabilization by a prolyl-hydroxylase inhibitor further stabilized HIF-1α and induced MIF mRNA and protein expression in efferocytic bone marrow macrophages. These results suggest that HIF-1α specifically regulates MIF expression in efferocytic macrophages, which may induce a chronic inflammatory response in the bone tumor microenvironment.

MIF signals through its main receptor CD74 and its co-receptors CD44, CXCR2, or CXCR4 (Bernhagen *et al.*, 2007; Leng *et al.*, 2003; Shi *et al.*, 2006). MIF/CD74 activity promotes immunosuppressive signaling in macrophages and dendritic cells and inhibition of this signaling reestablishes the antitumor immune response in metastatic melanoma (Figueiredo *et al*, 2018; Tanese *et al*, 2015). Moreover, MIF/CD74 signaling also activates the NF-κB signaling pathway in chronic lymphocytic leukemia (Binsky *et al*, 2007; Gil-Yarom *et al*, 2017). CD74/CD44 activation by MIF is followed by phosphorylation of the proto-oncogene tyrosine-protein kinase (SRC), extracellular signal-related kinase 1/2 (ERK1/2), phosphoinositide 3-kinase (PI3K), and protein kinase B (AKT) (Gore *et al*, 2008; Leng *et al.*, 2003; Lue *et al*, 2007; Shi *et al.*, 2006). These kinases promote the activation of transcription factors such as nuclear factor-kappa B (NF-κB, p65) which induces the secretion of pro-inflammatory cytokines such as IL-6, IL-8, CCL2 and CCL5 (Abdul-Aziz *et al*, 2017; Binsky *et al.*, 2007; Gregory *et al*, 2006; Johnson *et al*, 2018; Xu *et al*, 2008). Here we found that *Cd74* expression is downregulated in efferocytic bone marrow macrophages, suggesting a paracrine signaling in non-efferocytic macrophages. Recombinant MIF protein treatment of non-efferocytic macrophages activated the ERK1/2 and the NF-κB (p65) pathways and increased the expression of pro-inflammatory cytokines such as CXCL1, CXCL5, IL-6 and CCL5.

Altogether, these findings reveal a new regulatory mechanism of HIF-1α in macrophages during efferocytosis of apoptotic cancer cells where the p-STAT3/HIF-1α/MIF signaling pathway induces chronic inflammation in the tumor bone microenvironment and promotes the expression of pro-inflammatory cytokines (Figure 7). Since enhanced cell death inevitably occurs during tumor growth and is increased with many cancer therapies our results suggest this pathway becomes highly activated in bone metastatic patients undergoing chemo or radiation therapies and may contribute to increased inflammation and accelerated cancer growth.

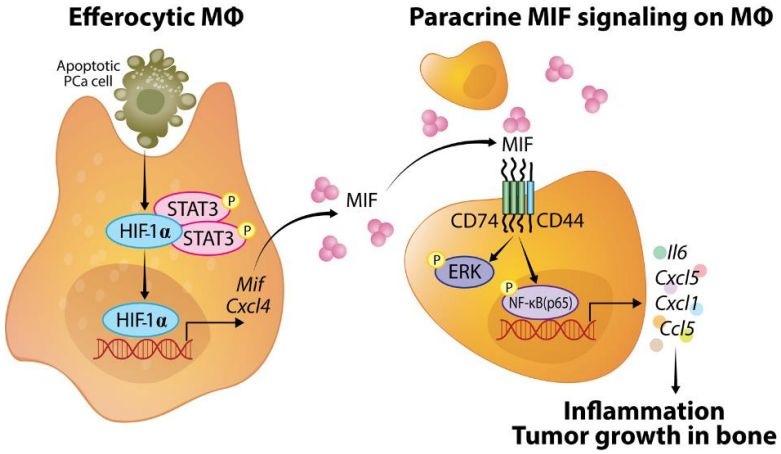


Figure 7. HIF-1α signaling during bone macrophage efferocytosis of apoptotic cancer cells. Bone macrophage engulfment of apoptotic prostate cancer cells promotes HIF-1α stability by its interaction with p-STAT3. Once HIF-1α is stabilized it is translocated to the nucleus to initiate the transcription and secretion of *Mif* and *Cxcl4*. Secreted MIF binds CD74/CD44 receptor complex of neighboring macrophages and activates ERK1/2 and p65 to induce the production of pro-inflammatory cytokines in the tumor microenvironment to support tumor growth in bone.

Material and Methods

Key resources table

Species/reagent type	Designation	Source of reference	Identifiers	Aditional information
Mouse line	C57BL/6J	Jackson Laboratory	C57BL/6J (Stock# 000664)	
Mouse line	FVB/NJ	Jackson Laboratory	FVB/NJ (Stock# 001800)	
Mouse line	*Hif1a*^{mut}	Jackson Laboratory	B6.129-*Hif1a*^{tm3Rsjo}/J (Stock# 007561)	Contains loxP sites flanking exon 2 of *Hif1a* (HIF-1α). Exposure to Cre recombinase removes the floxed sequence - creating a null allele.
Mouse line	*Epas1*^{mut}	Jackson Laboratory	STOCK *Epas1*^{tm1Mcs}/J (Stock# 008407)	Contains loxP sites flanking exon 2 of *Epas1* (HIF-2α). Exposure to Cre recombinase results in exon 2 deletion.
Mouse line	LysMCre	Jackson Laboratory	B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J (Stock# 004781)	
Cell line (*Mus musculus*)	Prostate cancer, fibroblast-like (C57BL/6J)	ATCC	RM1 (ATCC, Cat.# CRL-3310)	Ras+Myc-induced prostate cancer that developed from a urogenital sinus mouse prostate reconstitution.
Cell line (*Mus musculus*)	Prostate cancer, epithelial-like (FVB/NJ)	ATCC	Myc-CaP (ATCC, Cat.# CRL-3255)	Derived from a genetically engineered mouse prostate cancer removed from an animal that was never exposed to hormone ablation.
Probe	*18S* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm03928990_g1	
Probe	*Hif1a* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00468869_m1	
Probe	*Epas1* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm01236108_m1	
Probe	*Adam8* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm01163449_g1	
Probe	*Hyou1* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00491279_m1	
Probe	*Pdk1* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00554300_m1	
Probe	*Stub1* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00490634_m1	
Probe	*Cpbe1* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm01314928_m1	
Probe	*Pgk1* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00435617_m1	
Probe	*Myc* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00487804_m1	
Probe	*Ldha* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm01612132_g1	
Probe	*Mif* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm01611157_gH	
Probe	*Cd74* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00658576_m1	
Probe	*Cxcl1* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm04207460_m1	
Probe	*Pf4* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00451315_g1	
Probe	*Cxcl5* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00436451_g1	
Probe	*Il6* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00446190_m1	
Probe	*Ccl2* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm00441242_m1	
Probe	*Ccl5* (*Mus musculus*)	ThermoFisher Scientific	TaqMan , assay ID Mm01302427_m1	
Primers	B6.129-*Hif1a*^{tm3Rsjo}/J (genotyping)			Forward: 5'-TGCATGTGTATGGGTGTTTTG-3' Reverse: 5'-GAAAACTGTCTGTAACTTCATTTCC-3'
Primers	STOCK *Epas1*^{tm1Mcs}/J (genotyping)			Forward: 5'-GAGAGCAGCTTCTCCTGGAA-3' Reverse: 5'-TGTAGGCAAGGAAACCAAGG-3'
Primers	B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J (genotyping)			Mutant: 5'- CCCAGAAATGCCAGATTACG-3' Common: 5'-CTTGGGCTGCCAGAATTTCTC-3' Wildtype:5'-TTACAGTCGGCCAGGCTGAC-3'
Antibody	Anti HIF-1α (D1S7W) XP, Rabbit monoclonal	Cell Signaling Technology	Cat.# 36169	WB (1:2000); IP (1:50)
Antibody	Anti MIF, Rabbit polyclonal	Cell Signaling Technology	Cat.# 88186	WB (1:3000)
Antibody	Anti-phospho Stat3 (Tyr705) (D3A7) XP®, Rabbit monoclonal	Cell Signaling Technology	Cat.# 9145	WB (1:3000)
Antibody	Anti Stat3 (124H6), Mouse monoclonal	Cell Signaling Technology	Cat.# 9139	WB (1:2000)
Antibody	Anti-phospho-Stat3 (Tyr705) (3E2), Mouse monoclonal	Cell Signaling Technology	Cat.# 9138	WB (1:2000)
Antibody	Anti p44/42 MAPK (Erk1/2) (137F5), Rabbit monoclonal	Cell Signaling Technology	Cat.# 4695	WB (1:3000)
Antibody	Anti-phospho p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (D13.14.4E) XP®, Rabbit monoclonal	Cell Signaling Technology	Cat.# 4370	WB (1:3000)
Antibody	Anti NFκB p65 (D14E12) XP®, Rabbit monoclonal	Cell Signaling Technology	Cat.# 8242	WB (1:3000)
Antibody	Anti-phospho NF-κB p65 (Ser536) (93H1), Rabbit monoclonal	Cell Signaling Technology	Cat.# 3033	WB (1:3000)
Antibody	APC Anti-F4/80 antibody [CI:A3-1] (Allophycocyanin)	Abcam	Cat.# ab105080	FC (1:100)
Antibody	APC Rat IgG2b, kappa monoclonal [KLH/G2b-1-2] - Isotype control (Allophycocyanin)	Abcam	Cat.# ab154434	FC (1:100)

Animals and cell lines

All animal experiments were performed with approval from the University of Michigan Institutional Animal Care and Use Committee.

Immunocompetent C57BL/6J, FVB/NJ, B6.129P2-*Lyz2*$^{tm1(cre)Ifo}$/J (LysMCre), B6.129-*Hif1a*^{tm3Rsjo}/J (HIF-1α^{flox}) (Ryan *et al*, 2000) and

Epas1^{tm1Mcs}/J (HIF-2α^{flox}) (Gruber *et al*, 2007) mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The HIF-1α^{flox} and HIF-2α^{flox} mice were crossed consecutively with LysMCre mice to achieve the Hif1a$^{flox/flox}$-LysMCre$^{+/-}$ (*Hif1a*^{mut}) and Epas1$^{flox/flox}$-LysMCre$^{+/-}$ (*Epas1*^{mut}) mice that exhibit HIF-1α and HIF-2α inactivation in myeloid cells including macrophages. HIF-1α$^{flox/flox}$ and HIF-2α$^{flox/flox}$ mice were used as experimental controls.

RM1 is a (Ras+Myc)-induced prostate cancer cell line developed in C57BL/6J mice and was a gift from Timothy C. Thompson (Baylor College of Medicine, Houston, TX, USA) (Baley *et al*, 1995; Thompson *et al*, 1989). Myc-CaP prostate cancer cell line is derived from a prostate carcinoma from a Hi-Myc FVB/NJ mice and was donated by Russell Taichman and Frank Cackowski (University of Michigan, Ann Arbor, MI, USA) (Watson *et al*, 2005). Both cell lines were cultured on RPMI 1640 media containing 10% fetal bovine serum (FBS) and grown at 37ºC with ambient O_2 and 5% CO_2.

Murine efferocytosis in vitro model

Bone marrow-derived macrophages (MΦs) were isolated from 4–6 week old male C57BL/6J, FVB/NJ, *Hif1a*^{mut}, *Epas1*^{mut}, HIF-1α^{flox} and HIF-2α^{flox} mice by flushing the femur and tibia with minimum essential medium eagle - alpha modification (αMEM) supplemented with L-glutamine, antibiotic-antimycotic 1× and 10% fetal bovine serum (FBS). Macrophages were cultured in αMEM (L-glutamine, antibiotic-antimycotic 1×, 10% FBS) in the presence of macrophage colony stimulating factor (M-CSF) (30 ng/mL, #315-02, Peprotech, Rocky Hill, NJ, USA). After three days in culture, macrophages were plated independently at 2 × 10^6 cells/well in αMEM (L-glutamine, antibiotic-antimycotic 1×, 0.25% FBS) for co-culture experiments. RM1 and Myc-CaP cells were exposed to UV light for 30 min to induce apoptosis. Apoptotic (a) cells (>90% trypan blue incorporation) were co-cultured with macrophages at a 1:1 ratio in αMEM (L-glutamine, 0.25% FBS) for 16–18 h. Macrophages from mutant mice were compared with those from respective littermate controls.

Prolyl hydroxylase inhibition in efferocytic and non-efferocytic macrophages was performed using 10μM Roxadustat (FG-4592) (Cayman Chemical, 15294) for 16–18 hours. STAT3 inhibition in efferocytic and non-efferocytic macrophages was performed using 5μM Stattic (Cayman Chemical, 14590) or 60μM S3I-201 (Millipore Sigma, SML0330) for 10 minutes, then media was replaced and incubated for an additional 2 hours. Macrophages alone were treated with 200ng/ml of recombinant MIF protein (SinoBiological, 50066-M08H) during 2 hours for Western blot analysis and 8 hours for RNA expression analysis.

Single-cell library preparation and RNA sequencing

A modified murine efferocytosis *in vitro* model was used. Apoptotic RM1 cells were labeled with CellTrace™ CFSE (ThermoFisher Scientific, C34554), and then co-cultured with macrophages for 16–18 h. Efferocytic and non-efferocytic macrophages were collected and incubated in fluorescence-activated cell sorter (FACS) staining buffer (phosphate buffered saline-1X, 0.2% bovine serum albumin). F4/80 antibody and isotype control were added and incubated for 1 h at 4 °C. F4/80$^+$ only (non-efferocytic macrophages) and F4/80$^+$CFSE$^+$ (efferocytic macrophages) were sorted using a BD FACSAriaTM III (BD biosciences, San Jose, CA, USA). Antibody information is available in Key resources table.

The single cell scRNA-seq libraries were prepared at the University of Michigan Advanced Genomics Core using the 10X Genomics Chromium Next GEM Single Cell 3' Kit v3.1 (part number 1000268) following the manufacturer's protocol. Cell suspensions were diluted to target a recovery of 10,000 cells per sample. The libraries were run on an Agilent TapeStation 4200 (part number G2991BA) for library quality control before sequencing. Libraries were sequenced at a depth of 50,000 reads/cell on a NovaSeq6000 with the following run configuration: Read 1 - 150 cycles; i7 index read - 8 cycles; Read 2 - 150 cycles.

Single-cell RNA-sequencing analysis and visualization

The sequenced data was processed using the 10X Genomics CellRanger software suite v3.0.0. Briefly, fastq files from each of the samples were mapped to the Mouse genome mm10 and genes were counted using CellRanger software and the STAR aligner (Dobin *et al*, 2013). The barcode-gene matrices were further analyzed using the Seurat R package (v3.1) (Butler *et al*, 2018). Following standard practices to remove low-quality cells, cells that expressed less than 200 genes or less than 1,000 transcripts, or had greater than 10% mitochondrial genes were filtered from the datasets (Supplementary Table 1). For genes, only the top 5,000 variable genes were included for downstream analysis. Samples were then normalized and integrated according to the Seurat suggested pipeline. To reduce the dimensionality of the samples, we first performed a principal component analysis (PCA). The number of principle components for further downstream applications were 20, and UMAP was employed for final dimensionality reduction and visualization of the data.

Differential Expression and Gene Ontology Analysis

Differential expression analysis was conducted using the DESingle R package (Miao *et al*, 2018). Genes with a false discovery rate adjusted p-value < 0.05 were considered differentially expressed. For pathway analysis, we used PANTHER analysis (Mi *et al.*, 2013; Thomas *et al.*, 2003) with the gene ontology database (Ashburner *et al.*, 2000; Gene Ontology, 2021). Only genes differentially expressed and up-regulated in the efferocytic macrophages in both experiments were included in the GSEA analysis.

Western blot analysis and co-immunoprecipitation assay

Whole cell lysate was extracted in Cell Lysis Buffer 1X (Cell signaling, 9803) containing 1X protease and phosphatase inhibitor cocktail (ThermoFisher Scientific, 78440). Estimation of protein concentration was done using Bradford assay (BioRad, 5000006). Samples were diluted using 1X Laemmli Sample Buffer (4X stock, BioRad, 1610747) with 10% β-Mercaptoethanol (Millipore Sigma, M3148). Protein lysates were separated using 4-20% Mini-PROTEAN® TGX Stain-Free™ gels (BioRad, 4568096) and transferred to PVDF membrane using the Trans-Blot Turbo RTA kit (BioRad, 1704272). The membrane was blocked with 5% milk in 1X TBS- 0.1% Tween for 1 hour at room temperature, then incubated with primary antibodies in 5% BSA during overnight at 4ºC. Secondary antibody was diluted in 5% milk in 1X TBS- 0.1% Tween. For co-immunoprecipitation assays macrophage and apoptotic prostate cancer cell co-cultures were lysed on ice with 1% Triton X-100 in 1X PBS with 1X protease and phosphatase inhibitor cocktail. Whole cell lysates were

immunoprecipitated using HIF-1α antibody and protein A-magnetic beads (Cell Signaling Technology, 73778) during overnight incubation at 4°C. Binding and washing were performed in the same lysis buffer followed by immunoblotting with appropriate antibodies. Blots were developed using SuperSignal™ West Femto Maximum Sensitivity Substrate (ThermoFisher Scientific, 34095). Protein gels used for protein normalization and blots were imaged using the ChemiDoc™ MP Imaging System (BioRad, 12003154). Antibody information is available in Methods - Key resources table.

RT-qPCR

Cells were harvested using RNeasy Mini Kit (Qiagen, 74106) RNA was eluted with nuclease-free water. The RNA was quantified using a NanoDrop 2000 (Thermo Scientific) and cDNA was synthetized 1µg of RNA per 20 µl reaction mixture using High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, 4368814). RT-qPCR was performed using TaqMan® probes and Gene Expression qPCR Assays TaqMan Gene Express (ThermoFisher Scientific, 4369016) with 40 cycles on an ABI PRISM 7700 (Applied Biosystems, Foster City, CA, USA). The analysis was performed using 2-ΔΔCT method (Schmittgen & Livak, 2008). TaqMan® probes information is available in Key resources table.

Statistics

Statistical analyses were performed using GraphPad Prism 9 (GraphPad Software, version 9.1.0, San Diego, CA, USA) using ordinary and repeated measures one-way analysis of variance (ANOVA) with Tukey's multiple-comparisons, and unpaired t-tests analyses with significance of $p < 0.05$.

Data availability

Raw sequencing data for both experiments: Experiment 1 (GSM5466517/roca4, non-efferocytic and GSM5466518/roca5, efferocytic). Experiment 2 (GSM5466519/roca6, non-efferocytic and GSM5466520/roca7, efferocytic) are deposited in the NCBI Sequence Read Archive (SRA) and can be accessed from the NCBI Gene Expression Omnibus (GEO, Series Accession: GSE180638).

Funding

This work was supported by NIH award P01-CA093900 to L.K.M and E.T.K. DoD award EIRA-W81XWH-21-1-0122 log#PC200058 to V.M.-R.

Acknowledgements

The authors would like to thank the University of Michigan Advanced Genomics Core for the single-cell processing and sequencing of

the samples, and the Rogel Cancer Center Single Cell Analysis Core for further analysis of the single-cell data included is this study.

Conflicts of Interest

The authors declare no conflict of interest.

References

Abdul-Aziz AM, Shafat MS, Mehta TK, Di Palma F, Lawes MJ, Rushworth SA, Bowles KM (2017) MIF-Induced Stromal PKCbeta/IL8 Is Essential in Human Acute Myeloid Leukemia. Cancer Res 77: 303-311. https://doi.org/10.1158/0008-5472.CAN-16-1095

Alonso D, Serrano E, Bermejo FJ, Corral RS (2019) HIF-1alpha-regulated MIF activation and Nox2-dependent ROS generation promote Leishmania amazonensis killing by macrophages under hypoxia. Cell Immunol 335: 15-21. https://doi.org/10.1016/j.cellimm.2018.10.007

Aras S, Zaidi MR (2017) TAMeless traitors: macrophages in cancer progression and metastasis. Br J Cancer 117: 1583-1591. https://doi.org/10.1038/bjc.2017.356

Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, Davis AP, Dolinski K, Dwight SS, Eppig JT *et al* (2000) Gene ontology: tool for the unification of biology. The Gene Ontology Consortium. Nat Genet 25: 25-29. https://doi.org/10.1038/75556

Bach JP, Rinn B, Meyer B, Dodel R, Bacher M (2008) Role of MIF in inflammation and tumorigenesis. Oncology 75: 127-133. https://doi.org/10.1159/000155223

Baley PA, Yoshida K, Qian W, Sehgal I, Thompson TC (1995) Progression to androgen insensitivity in a novel in vitro mouse model for prostate cancer. J Steroid Biochem Mol Biol 52: 403-413. https://doi.org/10.1016/0960-0760(95)00001-q

Baugh JA, Gantier M, Li L, Byrne A, Buckley A, Donnelly SC (2006) Dual regulation of macrophage migration inhibitory factor (MIF) expression in hypoxia by CREB and HIF-1. Biochem Biophys Res Commun 347: 895-903. https://doi.org/10.1016/j.bbrc.2006.06.148

Becht E, McInnes L, Healy J, Dutertre CA, Kwok IWH, Ng LG, Ginhoux F, Newell EW (2018) Dimensionality reduction for visualizing single-cell data using UMAP. Nat Biotechnolhttps://doi.org/10.1038/nbt.4314

Bernhagen J, Calandra T, Mitchell RA, Martin SB, Tracey KJ, Voelter W, Manogue KR, Cerami A, Bucala R (1993) MIF is a pituitary-derived cytokine that potentiates lethal endotoxaemia. Nature 365: 756-759. https://doi.org/10.1038/365756a0

Bernhagen J, Krohn R, Lue H, Gregory JL, Zernecke A, Koenen RR, Dewor M, Georgiev I, Schober A, Leng L *et al* (2007) MIF is a noncognate ligand of CXC chemokine receptors in inflammatory and atherogenic cell recruitment. Nat Med 13: 587-596. https://doi.org/10.1038/nm1567

Binsky I, Haran M, Starlets D, Gore Y, Lantner F, Harpaz N, Leng L, Goldenberg DM, Shvidel L, Berrebi A *et al* (2007) IL-8 secreted in a macrophage migration-inhibitory factor- and CD74-dependent manner regulates B cell chronic lymphocytic leukemia survival. Proc Natl Acad Sci U S A 104: 13408-13413. https://doi.org/10.1073/pnas.0701553104

Blaisdell A, Crequer A, Columbus D, Daikoku T, Mittal K, Dey SK, Erlebacher A (2015) Neutrophils Oppose Uterine Epithelial Carcinogenesis

via Debridement of Hypoxic Tumor Cells. Cancer Cell 28: 785-799. https://doi.org/10.1016/j.ccell.2015.11.005

Blouin CC, Page EL, Soucy GM, Richard DE (2004) Hypoxic gene activation by lipopolysaccharide in macrophages: implication of hypoxia-inducible factor 1alpha. Blood 103: 1124-1130. https://doi.org/10.1182/blood-2003-07-2427

Bucala R, Donnelly SC (2007) Macrophage migration inhibitory factor: a probable link between inflammation and cancer. Immunity 26: 281-285. https://doi.org/10.1016/j.immuni.2007.03.005

Butler A, Hoffman P, Smibert P, Papalexi E, Satija R (2018) Integrating single-cell transcriptomic data across different conditions, technologies, and species. Nat Biotechnol 36: 411-420. https://doi.org/10.1038/nbt.4096

Calandra T, Roger T (2003) Macrophage migration inhibitory factor: a regulator of innate immunity. Nat Rev Immunol 3: 791-800. https://doi.org/10.1038/nri1200

Chiavarina B, Whitaker-Menezes D, Migneco G, Martinez-Outschoorn UE, Pavlides S, Howell A, Tanowitz HB, Casimiro MC, Wang C, Pestell RG *et al* (2010) HIF1-alpha functions as a tumor promoter in cancer associated fibroblasts, and as a tumor suppressor in breast cancer cells: Autophagy drives compartment-specific oncogenesis. Cell Cycle 9: 3534-3551. https://doi.org/10.4161/cc.9.17.12908

Coussens LM, Werb Z (2002) Inflammation and cancer. Nature 420: 860-867. https://doi.org/10.1038/nature01322

Cramer T, Yamanishi Y, Clausen BE, Forster I, Pawlinski R, Mackman N, Haase VH, Jaenisch R, Corr M, Nizet V *et al* (2003) HIF-1alpha is essential for myeloid cell-mediated inflammation. Cell 112: 645-657. https://doi.org/10.1016/s0092-8674(03)00154-5

Cui XG, Han ZT, He SH, Wu XD, Chen TR, Shao CH, Chen DL, Su N, Chen YM, Wang T *et al* (2017) HIF1/2alpha mediates hypoxia-induced LDHA expression in human pancreatic cancer cells. Oncotarget 8: 24840-24852. https://doi.org/10.18632/oncotarget.15266

de Visser KE, Eichten A, Coussens LM (2006) Paradoxical roles of the immune system during cancer development. Nat Rev Cancer 6: 24-37. https://doi.org/10.1038/nrc1782

Dobin A, Davis CA, Schlesinger F, Drenkow J, Zaleski C, Jha S, Batut P, Chaisson M, Gingeras TR (2013) STAR: ultrafast universal RNA-seq aligner. Bioinformatics 29: 15-21. https://doi.org/10.1093/bioinformatics/bts635

Du R, Lu KV, Petritsch C, Liu P, Ganss R, Passegue E, Song H, Vandenberg S, Johnson RS, Werb Z *et al* (2008) HIF1alpha induces the recruitment of bone marrow-derived vascular modulatory cells to regulate tumor angiogenesis and invasion. Cancer Cell 13: 206-220. https://doi.org/10.1016/j.ccr.2008.01.034

Duan Z, Luo Y (2021) Targeting macrophages in cancer immunotherapy. Signal Transduct Target Ther 6: 127. https://doi.org/10.1038/s41392-021-00506-6

Dudzinski SO, Cameron BD, Wang J, Rathmell JC, Giorgio TD, Kirschner AN (2019) Combination immunotherapy and radiotherapy causes an abscopal treatment response in a mouse model of castration resistant prostate cancer. J Immunother Cancer 7: 218. https://doi.org/10.1186/s40425-019-0704-z

Elliott MR, Koster KM, Murphy PS (2017) Efferocytosis Signaling in the Regulation of Macrophage Inflammatory Responses. J Immunol 198: 1387-1394. https://doi.org/10.4049/jimmunol.1601520

Ellwood-Yen K, Graeber TG, Wongvipat J, Iruela-Arispe ML, Zhang J, Matusik R, Thomas GV, Sawyers CL (2003) Myc-driven murine prostate cancer shares molecular features with human prostate tumors. Cancer Cell 4: 223-238. https://doi.org/10.1016/s1535-

6108(03)00197-1

Engel C, Brugmann G, Lambing S, Muhlenbeck LH, Marx S, Hagen C, Horvath D, Goldeck M, Ludwig J, Herzner AM *et al* (2017) RIG-I Resists Hypoxia-Induced Immunosuppression and Dedifferentiation. Cancer Immunol Res 5: 455-467. https://doi.org/10.1158/2326-6066.CIR-16-0129-T

Facciabene A, Peng X, Hagemann IS, Balint K, Barchetti A, Wang LP, Gimotty PA, Gilks CB, Lal P, Zhang L *et al* (2011) Tumour hypoxia promotes tolerance and angiogenesis via CCL28 and T(reg) cells. Nature 475: 226-230. https://doi.org/10.1038/nature10169

Figueiredo CR, Azevedo RA, Mousdell S, Resende-Lara PT, Ireland L, Santos A, Girola N, Cunha R, Schmid MC, Polonelli L *et al* (2018) Blockade of MIF-CD74 Signalling on Macrophages and Dendritic Cells Restores the Antitumour Immune Response Against Metastatic Melanoma. Front Immunol 9: 1132. https://doi.org/10.3389/fimmu.2018.01132

Gene Ontology C (2021) The Gene Ontology resource: enriching a GOld mine. Nucleic Acids Res 49: D325-D334. https://doi.org/10.1093/nar/gkaa1113

Gil-Yarom N, Radomir L, Sever L, Kramer MP, Lewinsky H, Bornstein C, Blecher-Gonen R, Barnett-Itzhaki Z, Mirkin V, Friedlander G *et al* (2017) CD74 is a novel transcription regulator. Proc Natl Acad Sci U S A 114: 562-567. https://doi.org/10.1073/pnas.1612195114

Gore Y, Starlets D, Maharshak N, Becker-Herman S, Kaneyuki U, Leng L, Bucala R, Shachar I (2008) Macrophage migration inhibitory factor induces B cell survival by activation of a CD74-CD44 receptor complex. J Biol Chem 283: 2784-2792. https://doi.org/10.1074/jbc.M703265200

Graham DK, DeRyckere D, Davies KD, Earp HS (2014) The TAM family: phosphatidylserine sensing receptor tyrosine kinases gone awry in cancer. Nat Rev Cancer 14: 769-785. https://doi.org/10.1038/nrc3847

Gray MJ, Zhang J, Ellis LM, Semenza GL, Evans DB, Watowich SS, Gallick GE (2005) HIF-1alpha, STAT3, CBP/p300 and Ref-1/APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas. Oncogene 24: 3110-3120. https://doi.org/10.1038/sj.onc.1208513

Gregory JL, Morand EF, McKeown SJ, Ralph JA, Hall P, Yang YH, McColl SR, Hickey MJ (2006) Macrophage migration inhibitory factor induces macrophage recruitment via CC chemokine ligand 2. J Immunol 177: 8072-8079. https://doi.org/10.4049/jimmunol.177.11.8072

Gruber M, Hu CJ, Johnson RS, Brown EJ, Keith B, Simon MC (2007) Acute postnatal ablation of Hif-2alpha results in anemia. Proc Natl Acad Sci U S A 104: 2301-2306. https://doi.org/10.1073/pnas.0608382104

Hartmann H, Eltzschig HK, Wurz H, Hantke K, Rakin A, Yazdi AS, Matteoli G, Bohn E, Autenrieth IB, Karhausen J *et al* (2008) Hypoxia-independent activation of HIF-1 by enterobacteriaceae and their siderophores. Gastroenterology 134: 756-767. https://doi.org/10.1053/j.gastro.2007.12.008

Hatfield S, Veszeleiova K, Steingold J, Sethuraman J, Sitkovsky M (2019) Mechanistic Justifications of Systemic Therapeutic Oxygenation of Tumors to Weaken the Hypoxia Inducible Factor 1alpha-Mediated Immunosuppression. Adv Exp Med Biol 1136: 113-121. https://doi.org/10.1007/978-3-030-12734-3_8

Hayashi Y, Yokota A, Harada H, Huang G (2019) Hypoxia/pseudohypoxia-mediated activation of hypoxia-inducible factor-1alpha in cancer. Cancer Sci 110: 1510-1517. https://doi.org/10.1111/cas.13990

Hsieh MM, Linde NS, Wynter A, Metzger M, Wong C, Langsetmo I, Lin A, Smith R, Rodgers GP, Donahue RE *et al* (2007) HIF prolyl hydroxylase inhibition results in endogenous erythropoietin induction, erythrocytosis, and modest fetal hemoglobin expression in rhesus macaques. Blood 110: 2140-2147. https://doi.org/10.1182/blood-2007-02-073254

Hu CJ, Wang LY, Chodosh LA, Keith B, Simon MC (2003) Differential roles of hypoxia-inducible factor 1alpha (HIF-1alpha) and HIF-2alpha in hypoxic gene regulation. Mol Cell Biol 23: 9361-9374. https://doi.org/10.1128/MCB.23.24.9361-9374.2003

Imtiyaz HZ, Williams EP, Hickey MM, Patel SA, Durham AC, Yuan LJ, Hammond R, Gimotty PA, Keith B, Simon MC (2010) Hypoxia-inducible factor 2alpha regulates macrophage function in mouse models of acute and tumor inflammation. J Clin Invest 120: 2699-2714. https://doi.org/10.1172/JCI39506

Ivan M, Kondo K, Yang H, Kim W, Valiando J, Ohh M, Salic A, Asara JM, Lane WS, Kaelin WG, Jr. (2001) HIFalpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. Science 292: 464-468. https://doi.org/10.1126/science.1059817

Jaakkola P, Mole DR, Tian YM, Wilson MI, Gielbert J, Gaskell SJ, von Kriegsheim A, Hebestreit HF, Mukherji M, Schofield CJ *et al* (2001) Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. Science 292: 468-472. https://doi.org/10.1126/science.1059796

Johnson DE, O'Keefe RA, Grandis JR (2018) Targeting the IL-6/JAK/STAT3 signalling axis in cancer. Nat Rev Clin Oncol 15: 234-248. https://doi.org/10.1038/nrclinonc.2018.8

Jung JE, Kim HS, Lee CS, Shin YJ, Kim YN, Kang GH, Kim TY, Juhnn YS, Kim SJ, Park JW *et al* (2008) STAT3 inhibits the degradation of HIF-1alpha by pVHL-mediated ubiquitination. Exp Mol Med 40: 479-485. https://doi.org/10.3858/emm.2008.40.5.479

Jung JE, Lee HG, Cho IH, Chung DH, Yoon SH, Yang YM, Lee JW, Choi S, Park JW, Ye SK *et al* (2005) STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells. FASEB J 19: 1296-1298. https://doi.org/10.1096/fj.04-3099fje

Kaelin WG, Jr., Ratcliffe PJ (2008) Oxygen sensing by metazoans: the central role of the HIF hydroxylase pathway. Mol Cell 30: 393-402. https://doi.org/10.1016/j.molcel.2008.04.009

Koh TJ, DiPietro LA (2011) Inflammation and wound healing: the role of the macrophage. Expert Rev Mol Med 13: e23. https://doi.org/10.1017/S1462399411001943

Lantz C, Radmanesh B, Liu E, Thorp EB, Lin J (2020) Single-cell RNA sequencing uncovers heterogenous transcriptional signatures in macrophages during efferocytosis. Sci Rep 10: 14333. https://doi.org/10.1038/s41598-020-70353-y

Larsen M, Tazzyman S, Lund EL, Junker N, Lewis CE, Kristjansen PE, Murdoch C (2008) Hypoxia-induced secretion of macrophage migration-inhibitory factor from MCF-7 breast cancer cells is regulated in a hypoxia-inducible factor-independent manner. Cancer Lett 265: 239-249. https://doi.org/10.1016/j.canlet.2008.02.012

Lecoultre M, Dutoit V, Walker PR (2020) Phagocytic function of tumor-associated macrophages as a key determinant of tumor progression control: a review. J Immunother Cancer 8https://doi.org/10.1136/jitc-2020-001408

Leng L, Metz CN, Fang Y, Xu J, Donnelly S, Baugh J, Delohery T, Chen Y, Mitchell RA, Bucala R (2003) MIF signal transduction initiated by binding to CD74. J Exp Med 197: 1467-1476. https://doi.org/10.1084/jem.20030286

Li J, Shen J, Wang Z, Xu H, Wang Q, Chai S, Fu P, Huang T, Anas O, Zhao H *et al* (2019) ELTD1 facilitates glioma proliferation, migration and invasion by activating JAK/STAT3/HIF-1alpha signaling axis. Sci Rep 9: 13904. https://doi.org/10.1038/s41598-019-50375-x

Lippitz BE (2013) Cytokine patterns in patients with cancer: a systematic review. Lancet Oncol 14: e218-228. https://doi.org/10.1016/S1470-2045(12)70582-X

Lue H, Thiele M, Franz J, Dahl E, Speckgens S, Leng L, Fingerle-Rowson G, Bucala R, Luscher B, Bernhagen J (2007) Macrophage migration inhibitory factor (MIF) promotes cell survival by activation of the Akt pathway and role for CSN5/JAB1 in the control of autocrine MIF activity. Oncogene 26: 5046-5059. https://doi.org/10.1038/sj.onc.1210318

Mantovani A, Allavena P, Sica A, Balkwill F (2008) Cancer-related inflammation. Nature 454: 436-444. https://doi.org/10.1038/nature07205

Mawhinney L, Armstrong ME, C OR, Bucala R, Leng L, Fingerle-Rowson G, Fayne D, Keane MP, Tynan A, Maher L *et al* (2015) Macrophage migration inhibitory factor (MIF) enzymatic activity and lung cancer. Mol Med 20: 729-735. https://doi.org/10.2119/molmed.2014.00136

Mendoza-Reinoso V, Baek DY, Kurutz A, Rubin JR, Koh AJ, McCauley LK, Roca H (2020) Unique Pro-Inflammatory Response of Macrophages during Apoptotic Cancer Cell Clearance. Cells 9https://doi.org/10.3390/cells9020429

Meyer-Siegler KL, Iczkowski KA, Vera PL (2005) Further evidence for increased macrophage migration inhibitory factor expression in prostate cancer. BMC Cancer 5: 73. https://doi.org/10.1186/1471-2407-5-73

Mi H, Muruganujan A, Thomas PD (2013) PANTHER in 2013: modeling the evolution of gene function, and other gene attributes, in the context of phylogenetic trees. Nucleic Acids Res 41: D377-386. https://doi.org/10.1093/nar/gks1118

Miao Z, Deng K, Wang X, Zhang X (2018) DEsingle for detecting three types of differential expression in single-cell RNA-seq data. Bioinformatics 34: 3223-3224. https://doi.org/10.1093/bioinformatics/bty332

Minutti CM, Knipper JA, Allen JE, Zaiss DM (2017) Tissue-specific contribution of macrophages to wound healing. Semin Cell Dev Biol 61: 3-11. https://doi.org/10.1016/j.semcdb.2016.08.006

Niu G, Briggs J, Deng J, Ma Y, Lee H, Kortylewski M, Kujawski M, Kay H, Cress WD, Jove R *et al* (2008) Signal transducer and activator of transcription 3 is required for hypoxia-inducible factor-1alpha RNA expression in both tumor cells and tumor-associated myeloid cells. Mol Cancer Res 6: 1099-1105. https://doi.org/10.1158/1541-7786.MCR-07-2177

O'Reilly C, Doroudian M, Mawhinney L, Donnelly SC (2016) Targeting MIF in Cancer: Therapeutic Strategies, Current Developments, and Future Opportunities. Med Res Rev 36: 440-460. https://doi.org/10.1002/med.21385

Okabe Y, Medzhitov R (2016) Tissue biology perspective on macrophages. Nat Immunol 17: 9-17. https://doi.org/10.1038/ni.3320

Palazon A, Goldrath AW, Nizet V, Johnson RS (2014) HIF transcription factors, inflammation, and immunity. Immunity 41: 518-528. https://doi.org/10.1016/j.immuni.2014.09.008

Palazon A, Tyrakis PA, Macias D, Velica P, Rundqvist H, Fitzpatrick S, Vojnovic N, Phan AT, Loman N, Hedenfalk I *et al* (2017) An HIF-1alpha/VEGF-A Axis in Cytotoxic T Cells Regulates Tumor Progression. Cancer Cell 32: 669-683 e665. https://doi.org/10.1016/j.ccell.2017.10.003

Penticuff JC, Woolbright BL, Sielecki TM, Weir SJ, Taylor JA, 3rd (2019) MIF family proteins in genitourinary cancer: tumorigenic roles

and therapeutic potential. Nat Rev Urol 16: 318-328. https://doi.org/10.1038/s41585-019-0171-9

Peyssonnaux C, Datta V, Cramer T, Doedens A, Theodorakis EA, Gallo RL, Hurtado-Ziola N, Nizet V, Johnson RS (2005) HIF-1alpha expression regulates the bactericidal capacity of phagocytes. J Clin Invest 115: 1806-1815. https://doi.org/10.1172/JCI23865

Pouyssegur J, Dayan F, Mazure NM (2006) Hypoxia signalling in cancer and approaches to enforce tumour regression. Nature 441: 437-443. https://doi.org/10.1038/nature04871

Quail DF, Joyce JA (2013) Microenvironmental regulation of tumor progression and metastasis. Nat Med 19: 1423-1437. https://doi.org/10.1038/nm.3394

Ranasinghe WK, Baldwin GS, Bolton D, Shulkes A, Ischia J, Patel O (2015) HIF1alpha expression under normoxia in prostate cancer--which pathways to target? J Urol 193: 763-770. https://doi.org/10.1016/j.juro.2014.10.085

Ranasinghe WK, Baldwin GS, Shulkes A, Bolton D, Patel O (2014) Normoxic regulation of HIF-1alpha in prostate cancer. Nat Rev Urol 11: 419. https://doi.org/10.1038/nrurol.2013.110-c2

Roca H, Jones JD, Purica MC, Weidner S, Koh AJ, Kuo R, Wilkinson JE, Wang Y, Daignault-Newton S, Pienta KJ *et al* (2018) Apoptosis-induced CXCL5 accelerates inflammation and growth of prostate tumor metastases in bone. J Clin Invest 128: 248-266. https://doi.org/10.1172/JCI92466

Roca H, McCauley LK (2018) Efferocytosis and prostate cancer skeletal metastasis: implications for intervention. Oncoscience 5: 174-176. https://doi.org/10.18632/oncoscience.440

Ryan HE, Poloni M, McNulty W, Elson D, Gassmann M, Arbeit JM, Johnson RS (2000) Hypoxia-inducible factor-1alpha is a positive factor in solid tumor growth. Cancer Res 60: 4010-4015.

Schmittgen TD, Livak KJ (2008) Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 3: 1101-1108. https://doi.org/10.1038/nprot.2008.73

Schust J, Sperl B, Hollis A, Mayer TU, Berg T (2006) Stattic: a small-molecule inhibitor of STAT3 activation and dimerization. Chem Biol 13: 1235-1242. https://doi.org/10.1016/j.chembiol.2006.09.018

Semenza GL (2011) Oxygen sensing, homeostasis, and disease. N Engl J Med 365: 537-547. https://doi.org/10.1056/NEJMra1011165

Shi X, Leng L, Wang T, Wang W, Du X, Li J, McDonald C, Chen Z, Murphy JW, Lolis E *et al* (2006) CD44 is the signaling component of the macrophage migration inhibitory factor-CD74 receptor complex. Immunity 25: 595-606. https://doi.org/10.1016/j.immuni.2006.08.020

Siddiquee K, Zhang S, Guida WC, Blaskovich MA, Greedy B, Lawrence HR, Yip ML, Jove R, McLaughlin MM, Lawrence NJ *et al* (2007) Selective chemical probe inhibitor of Stat3, identified through structure-based virtual screening, induces antitumor activity. Proc Natl Acad Sci U S A 104: 7391-7396. https://doi.org/10.1073/pnas.0609757104

Soki FN, Koh AJ, Jones JD, Kim YW, Dai J, Keller ET, Pienta KJ, Atabai K, Roca H, McCauley LK (2014) Polarization of prostate cancer-associated macrophages is induced by milk fat globule-EGF factor 8 (MFG-E8)-mediated efferocytosis. J Biol Chem 289: 24560-24572. https://doi.org/10.1074/jbc.M114.571620

Stanford JC, Young C, Hicks D, Owens P, Williams A, Vaught DB, Morrison MM, Lim J, Williams M, Brantley-Sieders DM *et al* (2014)

718 Efferocytosis produces a prometastatic landscape during postpartum mammary gland involution. J Clin Invest 124: 4737-4752.
719 https://doi.org/10.1172/JCI76375

720 Talks KL, Turley H, Gatter KC, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL (2000) The expression and distribution of the hypoxia-
721 inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. Am J Pathol 157:
722 411-421. https://doi.org/10.1016/s0002-9440(10)64554-3

723 Tanese K, Hashimoto Y, Berkova Z, Wang Y, Samaniego F, Lee JE, Ekmekcioglu S, Grimm EA (2015) Cell Surface CD74-MIF Interactions
724 Drive Melanoma Survival in Response to Interferon-gamma. J Invest Dermatol 135: 2901. https://doi.org/10.1038/jid.2015.259

725 Tarnowski M, Grymula K, Liu R, Tarnowska J, Drukala J, Ratajczak J, Mitchell RA, Ratajczak MZ, Kucia M (2010) Macrophage migration
726 inhibitory factor is secreted by rhabdomyosarcoma cells, modulates tumor metastasis by binding to CXCR4 and CXCR7 receptors and
727 inhibits recruitment of cancer-associated fibroblasts. Mol Cancer Res 8: 1328-1343. https://doi.org/10.1158/1541-7786.MCR-10-0288

728 Thomas PD, Campbell MJ, Kejariwal A, Mi H, Karlak B, Daverman R, Diemer K, Muruganujan A, Narechania A (2003) PANTHER: a library
729 of protein families and subfamilies indexed by function. Genome Res 13: 2129-2141. https://doi.org/10.1101/gr.772403

730 Thompson TC, Southgate J, Kitchener G, Land H (1989) Multistage carcinogenesis induced by ras and myc oncogenes in a reconstituted
731 organ. Cell 56: 917-930. https://doi.org/10.1016/0092-8674(89)90625-9

732 Tomiyasu M, Yoshino I, Suemitsu R, Okamoto T, Sugimachi K (2002) Quantification of macrophage migration inhibitory factor mRNA
733 expression in non-small cell lung cancer tissues and its clinical significance. Clin Cancer Res 8: 3755-3760.

734 Trinchieri G (2011) Inflammation in cancer: a therapeutic target? Oncology (Williston Park) 25: 418-420.

735 Triner D, Shah YM (2016) Hypoxia-inducible factors: a central link between inflammation and cancer. J Clin Invest 126: 3689-3698.
736 https://doi.org/10.1172/JCI84430

737 Vaught DB, Stanford JC, Cook RS (2015) Efferocytosis creates a tumor microenvironment supportive of tumor survival and metastasis.
738 Cancer Cell Microenviron 2https://doi.org/10.14800/ccm.666

739 Watson PA, Ellwood-Yen K, King JC, Wongvipat J, Lebeau MM, Sawyers CL (2005) Context-dependent hormone-refractory progression
740 revealed through characterization of a novel murine prostate cancer cell line. Cancer Res 65: 11565-11571. https://doi.org/10.1158/0008-
741 5472.CAN-05-3441

742 Werfel TA, Cook RS (2018) Efferocytosis in the tumor microenvironment. Semin Immunopathol 40: 545-554.
743 https://doi.org/10.1007/s00281-018-0698-5

744 Wilson JM, Coletta PL, Cuthbert RJ, Scott N, MacLennan K, Hawcroft G, Leng L, Lubetsky JB, Jin KK, Lolis E *et al* (2005) Macrophage
745 migration inhibitory factor promotes intestinal tumorigenesis. Gastroenterology 129: 1485-1503.
746 https://doi.org/10.1053/j.gastro.2005.07.061

747 Winner M, Koong AC, Rendon BE, Zundel W, Mitchell RA (2007) Amplification of tumor hypoxic responses by macrophage migration
748 inhibitory factor-dependent hypoxia-inducible factor stabilization. Cancer Res 67: 186-193. https://doi.org/10.1158/0008-5472.CAN-06-
749 3292

750 Xu Q, Briggs J, Park S, Niu G, Kortylewski M, Zhang S, Gritsko T, Turkson J, Kay H, Semenza GL *et al* (2005) Targeting Stat3 blocks both

HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways. Oncogene 24: 5552-5560. https://doi.org/10.1038/sj.onc.1208719

Xu X, Wang B, Ye C, Yao C, Lin Y, Huang X, Zhang Y, Wang S (2008) Overexpression of macrophage migration inhibitory factor induces angiogenesis in human breast cancer. Cancer Lett 261: 147-157. https://doi.org/10.1016/j.canlet.2007.11.028

Yao K, Shida S, Selvakumaran M, Zimmerman R, Simon E, Schick J, Haas NB, Balke M, Ross H, Johnson SW *et al* (2005) Macrophage migration inhibitory factor is a determinant of hypoxia-induced apoptosis in colon cancer cell lines. Clin Cancer Res 11: 7264-7272. https://doi.org/10.1158/1078-0432.CCR-05-0135

Zhu G, Tang Y, Geng N, Zheng M, Jiang J, Li L, Li K, Lei Z, Chen W, Fan Y *et al* (2014) HIF-alpha/MIF and NF-kappaB/IL-6 axes contribute to the recruitment of CD11b+Gr-1+ myeloid cells in hypoxic microenvironment of HNSCC. Neoplasia 16: 168-179. https://doi.org/10.1593/neo.132034

Supplementary data

Table 1. Features of the data included in the single-cell RNA sequencing analysis.

Sample	# of cells	# of unique genes per cell	# of transcripts per cell	Ave. mitochondrial %
Non-efferocytic MΦ (1)	7,813	3,029	12,837	3.39
Efferocytic MΦ (1)	6,262	3,726	19,768	3.18
Non-efferocytic MΦ (2)	10,612	2,909	11,326	2.89
Efferocytic MΦ (2)	7,984	3,800	17,984	3.27

Table 2. Selected GO terms (Figure 1E) enriched in efferocytic bone marrow macrophages.

GO term biological process	# of Genes in Term	# of GO term genes in upload list	Expected	Fold enrichment	Raw P-value	FDR value
Regulation of innate immune response (GO:0045088)	287	16	6.08	2.63	6.38E-04	3.89E-02
Cellular response to hypoxia (GO:0071456)	82	13	1.74	7.49	8.25E-08	2.90E-05
Cellular response to decreased oxygen levels (GO:0036294)	86	13	1.82	7.14	1.36E-07	4.20E-05
Cellular response to oxygen levels (GO:0071453)	101	14	2.14	6.55	1.18E-07	3.80E-05
Translational initiation (GO:0006413)	54	13	1.14	11.37	1.02E-09	9.44E-07
Regulation of protein stability (GO:0031647)	278	17	5.89	2.89	1.58E-04	1.44E-02
Positive regulation of vasculature development (GO:1904018)	200	17	4.23	4.02	3.13E-06	5.38E-04
Regulation of response to wounding (GO:1903034)	187	14	3.96	3.54	8.34E-05	8.73E-03
Response to oxidative stress (GO:0006979)	338	23	7.16	3.21	2.22E-06	4.27E-04
Negative regulation of immune system process (GO:0002683)	469	23	9.93	2.32	3.04E-04	2.35E-02

Table 3. List of upregulated genes from GO terms shown in Figure 1E and Table 2.

Regulation of innate immune response (GO:0045088)	Cellular response to hypoxia (GO:0071456)	Cellular response to decreased oxygen levels (GO:0036294)	Cellular response to oxygen levels (GO:0071453)	Translational initiation (GO:0006413)	Regulation of protein stability (GO:0031647)	Positive regulation of vasculature development (GO:1904018)	Regulation of response to wounding (GO:1903034)	Response to oxidative stress (GO:0006979)	Negative regulation of immune system process (GO:0002683)
Cadm1	*Egln3*	*Egln3*	*Egln3*	*Eif4a1*	*Hypk*	*Kdr*	*Plau*	*Ndufa12*	*Cb1b*
Ccr1	*Hyou1*	*Hyou1*	*Hyou1*	*Denr*	*Rnf128*	*Mydgf*	*Cd109*	*Prdx1*	*Nme2*
Ube2k	*Ak4*	*Ak4*	*Ak4*	*Eif3b*	*Stub1*	*Itgax*	*Anxa1*	*Sod2*	*Anxa1*
Adam8	*Ppargc1a*	*Ppargc1a*	*Ppargc1a*	*Eif4e2*	*Commd1*	*Fgf2*	*Cd9*	*Gsr*	*Selenos*
Hsp90aa1	*Eno1*	*Eno1*	*Eno1*	*Eif4ebp1*	*Hip1*	*Hyal1*	*Fgf2*	*Srxn1*	*Ccr1*
Rala	*Pgk1*	*Pgk1*	*Pgk1*	*Eif5a*	*Hsp90aa1*	*Ago2*	*Cd36*	*Ppargc1a*	*Gpnmb*
Arg1	*Ndnf*	*Ndnf*	*Ndnf*	*Eif6*	*Phb2*	*Sphk1*	*Anxa2*	*Nme2*	*Cd200r1*
Mmp12	*Epas1*	*Epas1*	*Epas1*	*Eif1a*	*Rnf149*	*Il1a*	*Rtn4r*	*Apex1*	*Mif*
Psmd14	*Rbpj*	*Rbpj*	*Rbpj*	*Eif4g1*	*Plpp3*	*Pkm*	*Flna*	*Anxa1*	*Il7r*
Psmd1	*Stub1*	*Stub1*	*Stub1*	*Eif4e*	*Cct8*	*Lgals3*	*Pdgfa*	*Epas1*	*Nr1h3*
Psmd7	*Commd1*	*Commd1*	*Commd1*	*Eif1ax*	*Hspd1*	*Hspa4*	*Spp1*	*Selenos*	*Hspa9*
Psmd12	*Adam8*	*Adam8*	*Adam8*	*Ago2*	*B4galt5*	*Angpt2*	*Mif*	*Gpx1*	*Rala*
Psmc5	*Pdk1*	*Pdk1*	*Pdk1*	*Eif2s1*	*Lmna*	*Nrp1*	*F7*	*Atf4*	*Gpx1*
Psmb5	*Lmna*	*Lmna*	*Lmna*	*Atf4*	*Ptges3*	*Hmga2*	*Plpp3*	*Cd36*	*Arg1*
Psmb6	*Eif4eb1*	*Eif4eb1*	*Eif4eb1*	*Atf3*	*Flna*		*Plaur*	*Hyal1*	*Cd200*
Nr1h3	*Myc*	*Myc*	*Myc*		*Cct3*			*Rwdd1*	*Mmp12*
Psmd2	*Plau*	*Plau*	*Plau*		*Ank2*			*Arl6ip5*	*Myc*
	Cpeb1	*Cpeb1*	*Cpeb1*		*Ppargc1a*			*Eif2s1*	*Nme1*
	Mif	*Mif*	*Mif*					*Pdk1*	*Cd300lf*
	Dnmt3a	*Dnmt3a*	*Dnmt3a*					*Ndufs8*	*Lgals3*
	Hilpda	*Hilpda*	*Hilpda*					*Sphk1*	*Npy*
	Phb2	*Phb2*	*Phb2*					*Psmb5*	*Id2*
	Higd1a	*Higd1a*	*Higd1a*					*Pon2*	*Iglc1*
	Ccnb1	*Ccnb1*	*Ccnb1*					*Cygb*	
	Bcl2l1	*Bcl2l1*	*Bcl2l1*					*Pcna*	
	Psmd14	*Psmd14*	*Psmd14*					*Hspd1*	
	Psmd7	*Psmd7*	*Psmd7*					*Mif*	
	Psmd12	*Psmd12*	*Psmd12*					*Il1a*	
	Psmc5	*Psmc5*	*Psmc5*					*Hk3*	
	Psmb5	*Psmb5*	*Psmb5*					*Ldha*	
	Psm6	*Psm6*	*Psm6*					*Bcl2l1*	
	Psm2	*Psm2*	*Psm2*					*Arg1*	
	Psmd1	*Psmd1*	*Psmd1*					*Cycs*	
		Atf4	*Atf4*					*G6pdx*	
			Atp6v1a					*Atf1*	
								Scara3	
								Por	

Figure 2–Figure supplement 1. STAT3 inhibition (Stattic and S3I-201) in C57BL/6J in bone marrow macrophages efferocytosing apoptotic prostate cancer RM1 cells (n=3 per group).

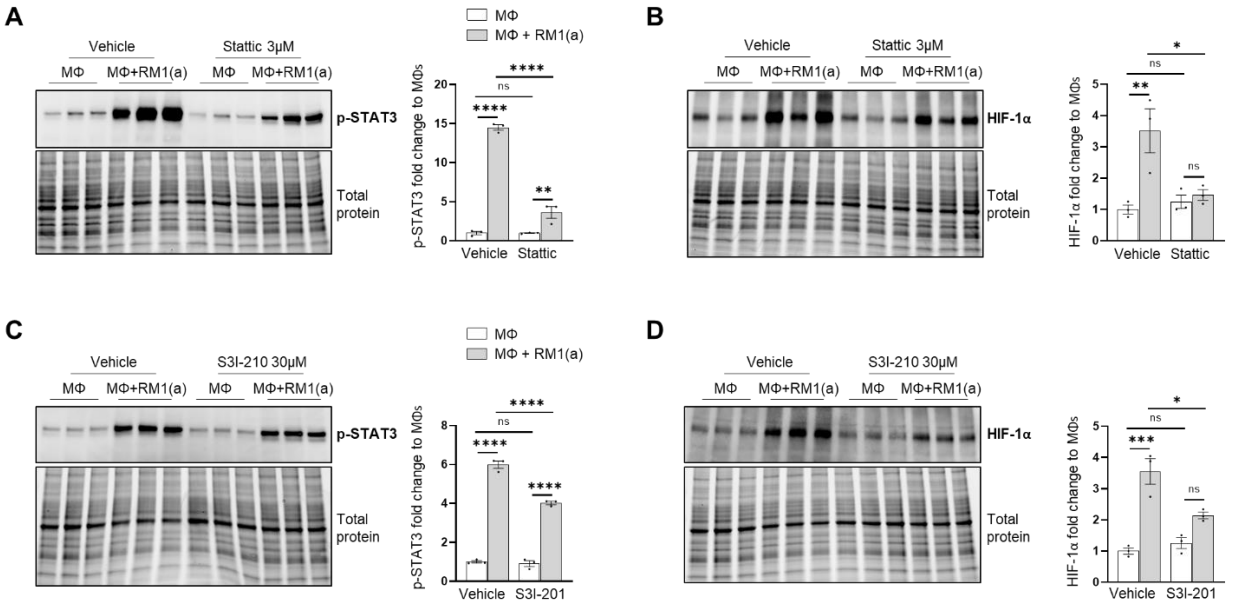


Figure 2–Figure supplement 2. STAT3 inhibition (S3I-201) in FVB/NJ bone marrow macrophages efferocytosing apoptotic prostate cancer Myc-CaP cells (n=3 per group).

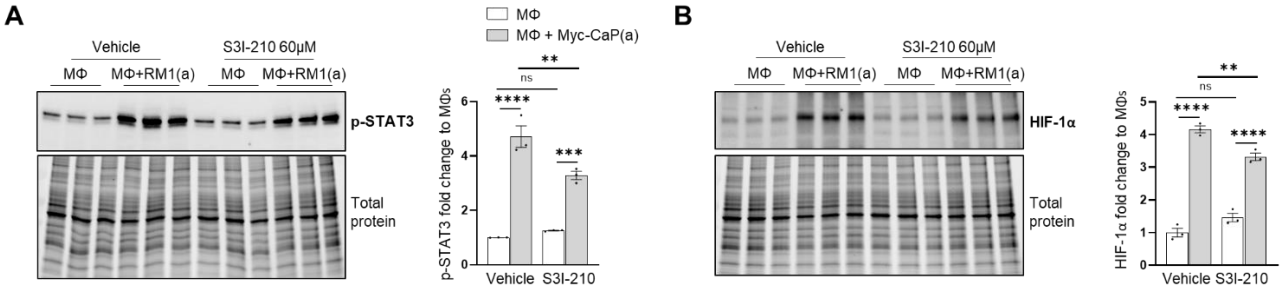


Figure 3–Figure supplement 1. Single-cell plots of *Mif* expression in efferocytic macrophages (corresponding to Exp. 1)

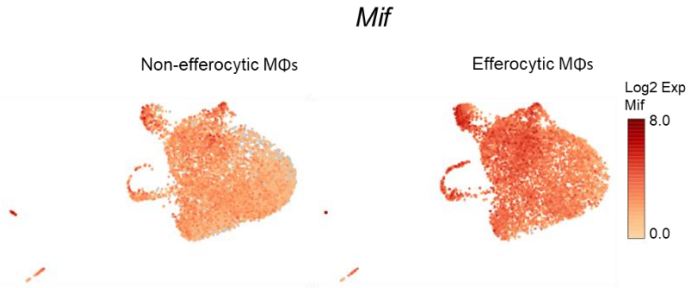


Figure 4–Figure supplement 1. p-STAT3 expression in efferocytic *Hif1a*^{mut} macrophages compared to control macrophages (n=3).

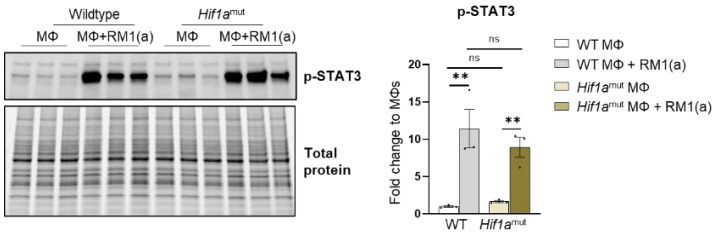


Figure 4–Figure supplement 2. HIF-2α depletion (*Epas1*^{mut}) effect in efferocytic macrophages.

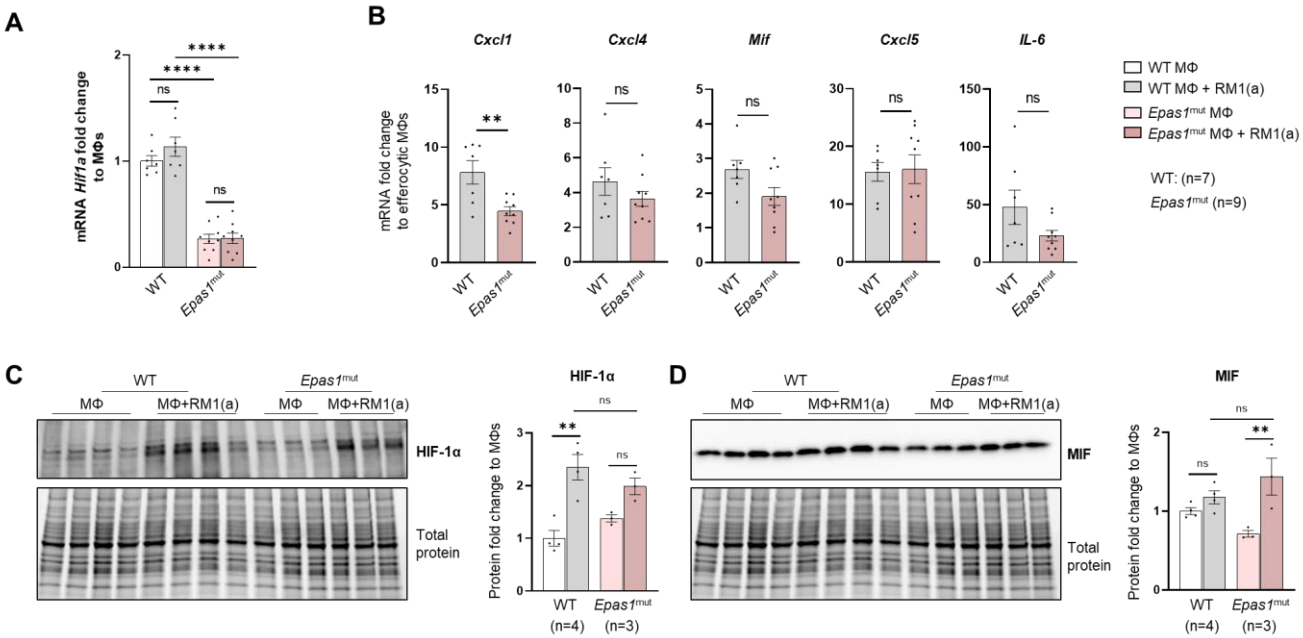
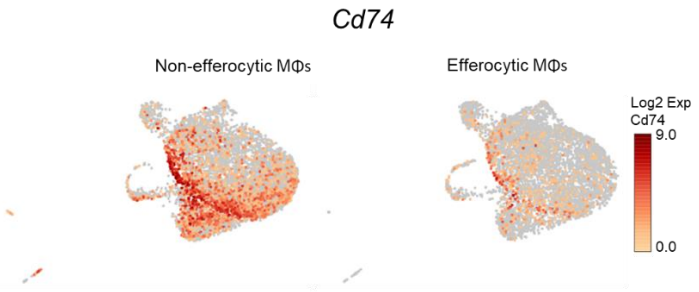


Figure 6–Figure supplement 1. Single-cell plots of *Cd74* expression in efferocytic macrophages (corresponding to Exp. 1).



822 **Figure 6–Figure supplement 2.** C57BL/6J bone marrow macrophages treatment with MIF (100ng/ml, n=3 per group).

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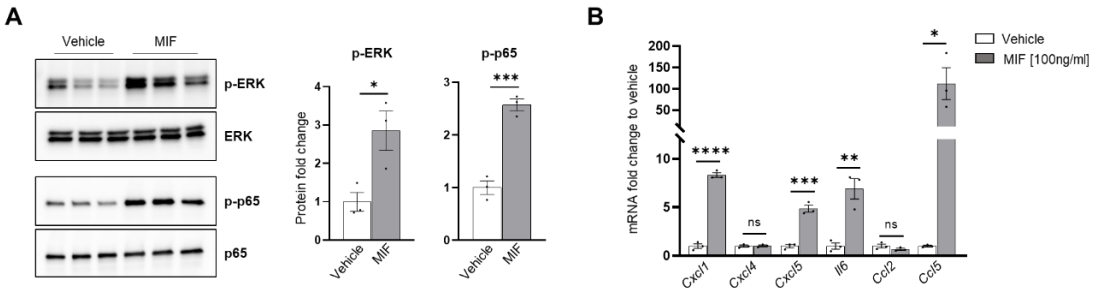

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