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Human Monocytes Undergo Functional Re-programming during Sepsis Mediated by Hypoxia-Inducible Factor-1α

Irina N. Shalova,[1] Jyue Yuan Lim,[1] Manesh Chittezhath,[1] Annelies S. Zinkernagel,[2,6] Federico Beasley,[2] Enrique Hernández-Jiménez,[3] Victor Toledano,[3] Carolina Cubillos-Zapata,[3] Annamaria Rapisarda,[4] Jinmiao Chen,[1] Kaibo Duan,[1] Henry Yang,[1] Michael Poidinger,[1] Giovanni Melillo,[4] Victor Nizet,[2] Francisco Arnalich,[5] Eduardo López-Collazo,[3] and Subhra K. Biswas[1,*]

[1]Singapore Immunology Network (SIgN), Agency for Science, Technology and Research (A*STAR), Biopolis, #04-06 Immunos building, 8A Biomedical Grove, Singapore 138648, Singapore

[2]Department of Pediatrics and Skaggs School of Pharmacy and Pharmaceutical Sciences, University of California, San Diego, 9500 Gilman Drive, La Jolla, CA 92093, USA

[3]Tumor Immunology Lab and Innate Immunity Group, IdiPAZ, "La Paz" Hospital, Pseo de La Castellana 261, Madrid 28046, Spain

[4]Development Therapeutic Program, Tumor Hypoxia Laboratory, Science Applications International Corporation-Frederick, Inc., National Cancer Institute, Frederick, MD 21701, USA

[5]Department of Internal Medicine, "La Paz" Hospital, Pseo de La Castellana 261, Madrid 28046, Spain

[6]Present address: Division of Infectious Diseases and Hospital Epidemiology, University Hospital Zurich, University of Zurich, Rämistr 100, Zurich 8091, Switzerland

*Correspondence: subhra_biswas@immunol.a-star.edu.sg

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SUMMARY

Sepsis is characterized by a dysregulated inflammatory response to infection. Despite studies in mice, the cellular and molecular basis of human sepsis remains unclear and effective therapies are lacking. Blood monocytes serve as the first line of host defense and are equipped to recognize and respond to infection by triggering an immune-inflammatory response. However, the response of these cells in human sepsis and their contribution to sepsis pathogenesis is poorly understood. To investigate this, we performed a transcriptomic, functional, and mechanistic analysis of blood monocytes from patients during sepsis and after recovery. Our results revealed the functional plasticity of monocytes during human sepsis, wherein they transited from a pro-inflammatory to an immunosuppressive phenotype, while enhancing protective functions like phagocytosis, anti-microbial activity, and tissue remodeling. Mechanistically, hypoxia inducible factor-1α (HIF1α) mediated this functional re-programming of monocytes, revealing a potential mechanism for their therapeutic targeting to regulate human sepsis.

INTRODUCTION

Sepsis is a complex pathology that arises from dysregulated host inflammatory responses to systemic bacterial infection (Hotchkiss et al., 2009). Human sepsis is a leading cause of death in intensive care units (ICUs) worldwide. Yet, the immunological and molecular basis of this syndrome is poorly understood. In the last 20 years, numerous therapeutic strategies to ameliorate human sepsis have failed in clinical trials and reliable biomarkers for sepsis are still lacking (Focus on sepsis, 2012). The reason for such a dismal scenario can be partially attributed to the complex and dynamic nature of the condition, as well as important species-specific differences in innate responses between mouse model systems and human patients (Schroder et al., 2012; Seok et al., 2013).

In line with the dynamic nature of sepsis, two phases have been recognized in this disease: an early inflammatory phase and a late immunosuppressive phase (Biswas and Lopez-Collazo, 2009; Hotchkiss et al., 2009). The early phase is characterized by leukocyte activation, cytokine storm, and a systemic inflammatory response, while the later phase is characterized by immunosuppression, leukocyte deactivation, increased risks of secondary infection, and high mortality (Boomer et al., 2011; Hotchkiss et al., 2009). The scenario might be even more complicated with the overlapping co-existence of inflammatory and immunosuppressive processes, as suggested by some (Adib-Conquy and Cavaillon, 2009; Xiao et al., 2011). Further, contrasting the common view that overt inflammation drives mortality in sepsis, this response is often effectively controlled by standard ICU practices, whereas recent evidence has emphasized how immunosuppression might contribute to increasing mortality risk in most sepsis patients (Boomer et al., 2011; Hotchkiss et al., 2009; Pachot et al., 2006; Tang et al., 2010). However, the lack of infection foci in sepsis patient organs and the emerging role of immunometabolism and neurophysiological mechanisms in organ dysfunction in sepsis reveal further complexity (Deutschman and Tracey, 2014). Nonetheless, the cellular and molecular mechanisms that regulate different aspects of human sepsis pathogenesis still remain poorly understood. Moreover, the failure of numerous human clinical trials as opposed to mice preclinical studies in sepsis also emphasize the divergence in the immune mechanisms between these species as reported recently. These facts further reinforce the immediate necessity for investigating sepsis and its effects in humans.

Monocytes and macrophages are believed to play an important role in orchestrating the host immune response during

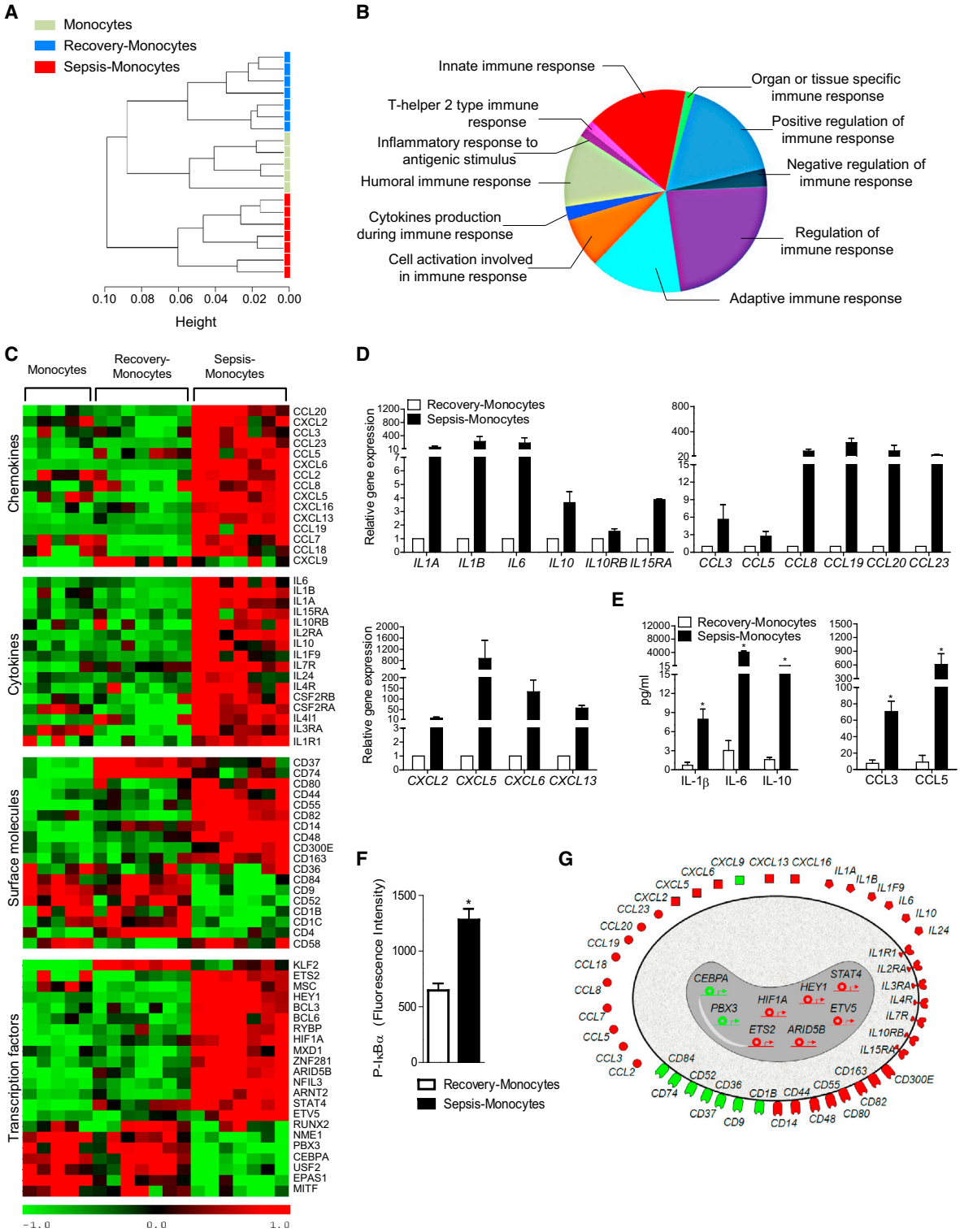

Figure 1. Transcriptome Profiling of Monocytes from Sepsis Patients

(A) Hierarchical clustering with Pearson's correlation and complete linkage of Sepsis-Monocytes (n = 7), Recovery-Monocytes (n = 7) and healthy donor Monocytes (n = 5).

(B) Gene Ontology (GO) classification of differentially expressed genes showing immune-related biological functions.

(C) Heatmap showing differential expression of genes belonging to cytokines, chemokines, selected surface molecules, and transcription factors in Sepsis- versus Recovery-Monocytes. Monocytes are shown as an additional baseline control population. Differentially expressed genes determined by Limma and row-based *Z* score were normalized.

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sepsis (Biswas and Lopez-Collazo, 2009). They can potentially participate in both phases of sepsis by releasing inflammatory cytokines that contribute to "cytokine storm" and ultimately adopting an immunosuppressive phenotype whereupon they are unable to respond to secondary infections. However, whether monocytes and macrophages can actually perform such diverse functions to contribute to the pathogenesis of sepsis in humans needs investigation. A better definition of how the gene expression and functional activities of monocyte and macrophage are regulated in human sepsis patients can provide valuable insight into fundamental disease mechanisms and their possible therapeutic targeting.

In the current study, we have applied a combination of transcriptomic and functional approaches to immune profile blood monocytes during Gram-negative sepsis in adults, including follow-on analyses of the same individuals after sepsis resolution, to clarify how these cells contribute to progression of the disorder. We demonstrate that blood monocytes from patients displayed a functional plasticity, transiting from a pro-inflammatory to an immunosuppressive state during sepsis, and identify hypoxia inducible factor-1α (HIF1α) as a key mediator of monocyte re-programming under these conditions. Our data sheds light on the molecular and cellular basis of human sepsis progression that might inform the development of targeted therapeutic interventions.

RESULTS

Monocytes from Sepsis Patients Exhibit a Distinct Genetic Signature

Blood monocytes are believed to be major players in the dysregulated inflammatory response during sepsis and characterizing their response during this pathology provides insight into the disease mechanism (Biswas and Lopez-Collazo, 2009). We performed a transcriptomic characterization of human blood monocytes isolated from patients during Gram-negative sepsis (Sepsis-Monocytes) and following their resolution or recovery (Recovery-Monocytes); monocytes from healthy donors (Monocytes) were used as an additional baseline control population. Hierarchical clustering of the monocyte transcriptomes showed a clear segregation of the two populations, Sepsis-Monocytes versus Recovery-Monocytes, indicative of their distinct gene expression profiles (Figure 1A). However, Recovery-Monocytes clustered closely with the control Monocytes suggesting that their gene-expression profile represented a return toward the baseline condition.

The transcriptome analysis of Sepsis-Monocytes identified 1,170 (561, upregulated; 609, downregulated) differentially expressed genes in Sepsis-Monocytes compared to Recovery-Monocytes. Gene Ontology (GO) analysis was performed to assess the biological processes related to these differentially expressed genes as shown in Figure S1. The top ten statistically significant GO categories showed a prominence of essentially immune response-related processes as shown in Figures S1A and S1B and Figure 1B. In particular, immune response processes mostly related to the upregulated differentially expressed genes, whereas metabolic processes related to the downregulated differentially expressed genes (Figures S1B and S1C).

Because we were interested in characterizing the immune response of monocytes in sepsis, we focused on the immune-related differentially expressed genes in Sepsis-Monocytes. The heatmap in Figure 1C shows that Sepsis-Monocytes upregulate a large number of immune-related genes encoding cytokines, chemokines, surface molecules, and transcription factors. Many of these cytokine and chemokine genes were also validated by qPCR array (Figure 1D). Analysis of Sepsis-Monocytes culture supernatants confirmed the significant upregulation of pro-inflammatory cytokines and chemokines including interlekin-1β (IL-1β), IL-6, and the chemokines CCL3 and CCL5, as compared to Recovery-Monocytes (Figure 1E). The anti-inflammatory cytokine, IL-10 was also found to be upregulated alongside pro-inflammatory cytokines and chemokines in the Sepsis-Monocytes. Consistent with these observations, NF-κB, a central transcriptional regulator of inflammatory response, was found to be activated in the Sepsis-Monocytes, as indicated by the heightened expression of phospho-IκBα (Figure 1F). Figure 1G presents a diagrammatic overview of some key immune-related genes modulated in Sepsis-Monocytes, indicating a gene-expression profile that is consistent with monocyte activation and inflammation during ongoing sepsis.

Blood Monocytes Display Altered Responses to Endotoxin in Sepsis Patients

The high risk of fatal secondary infections in sepsis is thought to be due to the immunosuppressive state of blood leukocytes in these patients (Boomer et al., 2011; Hotchkiss et al., 2009). We therefore sought to determine whether human blood monocytes displayed an altered response to endotoxin challenge in ongoing sepsis. To have a global view of this phenomenon, the response of Sepsis- and Recovery-Monocytes to ex vivo lipopolysaccharide (LPS) challenge was compared using microarray. Hierarchical clustering in Figure 2A showed a clear segregation of LPS-treated Recovery-Monocytes from their untreated counterparts (i.e., Recovery-Monocytes+LPS versus Recovery-Monocytes) suggesting a distinct transcriptomic response to LPS in these cells. In contrast, LPS-treated Sepsis-Monocytes did not segregate from their untreated counterpart (i.e., Sepsis-Monocytes+LPS versus Sepsis-Monocytes), indicating the failure to display a distinct transcriptomic response to LPS. The defective LPS response of Sepsis-Monocytes was also evident quantitatively by the markedly lesser number of differentially expressed genes (76) compared to Recovery-Monocytes (2,221 genes), in response to LPS (Figure 2B).

(D) qPCR of indicated genes in Sepsis- versus Recovery-Monocytes.
(E) Cytokine expression in culture supernatants of Sepsis- versus Recovery-Monocytes.
(F) Phospho-IκBα expression in Sepsis- versus Recovery-Monocytes. Values are mean ± SEM (D and F: n = 3; E: n = 4), *p < 0.05 versus Recovery-Monocytes.
(G) Diagrammatic overview of Sepsis-Monocytes showing selected immune-related differentially expressed genes as compared to Recovery-Monocytes. Red represents upregulated; green represents downregulated; Panels showing transcriptome analysis represent data from the number of subjects as indicated in (A). See also Figure S1.

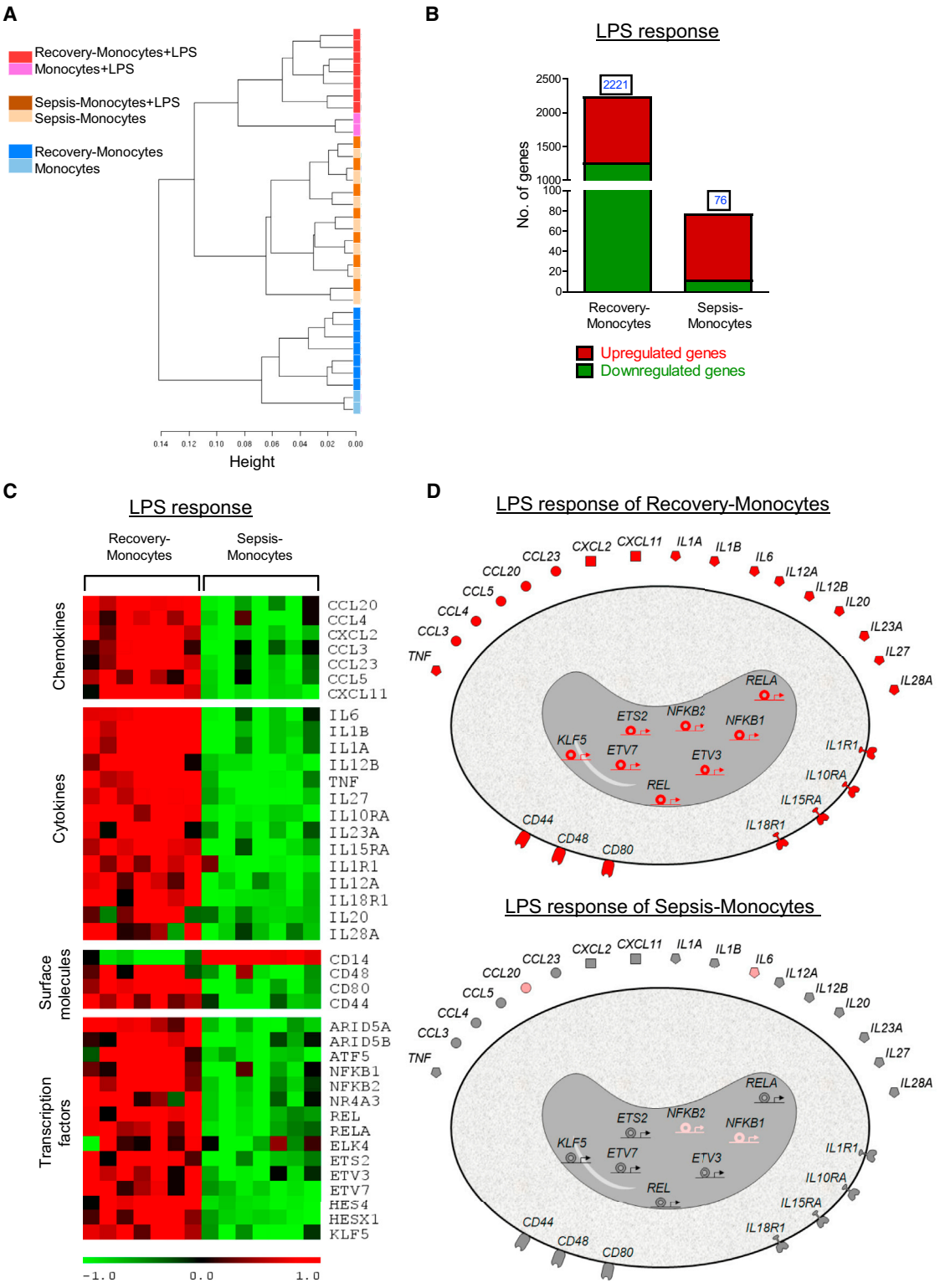

Figure 2. Blood Monocytes Display Altered Responses to Endotoxin in Sepsis Patients

(A) Hierarchical clustering with Pearson's correlation and complete linkage of Sepsis-Monocytes (n = 7), Recovery-Monocytes (n = 7), and healthy donor Monocytes (n = 2) in response to ex vivo LPS treatment (3 hr).

(B) Total number of LPS-induced differentially expressed genes in Sepsis- and Recovery-Monocytes. Box indicates the total number of differentially expressed genes.

(C) Heatmaps showing the differential modulation of indicated genes in Recovery- versus Sepsis-Monocytes, in response to LPS.

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We further analyzed the LPS response of Sepsis- versus Recovery-Monocytes. Upregulation of inflammatory cytokine and chemokine genes is a key feature of LPS response in myelomonocytic cells (Schroder et al., 2012). Accordingly, we found many cytokines (e.g., tumor necrosis factor [*TNF*], *IL1A*, *IL1B*, *IL6*, *IL12A*, *IL23A*) and chemokines (e.g., *CCL3*, *CCL4*, *CCL5*, *CCL20*, *CCL23*, *CXCL2*, *CXCL11*) to be upregulated in the LPS transcriptome of Recovery-Monocytes, but not in that of Sepsis-Monocytes, indicating a defect in the LPS-induced inflammatory response of Sepsis-Monocytes (Figure 2C). A similar defect was also noted for the expression of genes encoding several surface molecules (e.g., *CD80*, *CD44*) and transcription factors (e.g., *ATF5*, *NFKB1*, *NFKB2*, *REL*, *RELA*) related to immune activation (Figure 2C, compare Sepsis- with Recovery-Monocytes). Figure 2D shows a diagrammatic overview of the gene-expression data indicating impaired inflammatory response of Sepsis-Monocytes in response to LPS.

Distinct from the large number of immune-related genes (422) that showed downregulation in the LPS-treated Sepsis-Monocytes, an equal number of genes (504) were found to be still inducible or less repressed in the Sepsis-Monocytes than Recovery-Monocytes, in response to LPS treatment. GO analysis of these genes mapped them to miscellaneous functions including metabolism and phagocytosis-related processes (Figure S2). The anti-microbial gene *HAMP* also fell in the latter category and is investigated later in this study. Overall, these data indicate a profound gene ''re-programming'' in Sepsis-Monocytes associated with their altered response to LPS.

Monocytes Exhibit Hallmarks of Endotoxin Tolerance In Vivo in Human Sepsis

Given the altered transcriptomic response of monocytes to LPS in sepsis, we next investigated whether key monocyte functions were also disrupted under these conditions. Compared to Recovery-Monocytes, Sepsis-Monocytes exhibited a marked reduction in LPS-induced pro-inflammatory genes including *CCL3*, *4*, *5*; *CXCL2*, *11*; *IL1A*, *IL1B*, *IL6*, and *TNF*, by qPCR (Figure 3A), that mirrored our transcriptome data (Figure 2C). Reduced expression of some of these cytokines and chemokines was also confirmed in the culture supernatants of LPS-treated Sepsis-Monocytes (Figure 3B). Thus, Sepsis-Monocytes exhibit an attenuated inflammatory response to LPS. Consistent with this, LPS-treated Sepsis-Monocytes also showed reduced phospho-IκBα expression, indicating an impaired activation of NF-κB, the key transcriptional regulator of inflammatory response (Figure 3C).

We next assessed the antigen-presenting function of Sepsis-Monocytes, because it is essential for immune activation and a sustained host immune response. Sepsis-Monocytes showed decreased expression of several key co-stimulatory and major histocompatibility complex class II (MHCII) molecule genes (*CD80*, *CD40*, and *HLA-DOB*) upon LPS treatment (Figure 3D), suggesting that the antigen-presenting ability of these cells might be impaired. Indeed, a mixed lymphocyte reaction (MLR) revealed LPS-treated Sepsis-Monocytes to induce a significantly lower T cell proliferation than Recovery-Monocytes counterpart (Figure 3E).

Monocytes and macrophages also mediated direct killing of pathogens via phagocytosis. Figure 3F shows Sepsis-Monocytes displayed a higher phagocytotic ability than control monocytes (Monocytes). Supernatants of Sepsis-Monocytes also demonstrated heightened ability to restrict growth of bacteria (*E. coli*) (Figure 3G). In line with this observation, Sepsis-Monocytes displayed enhanced expression of the anti-microbial gene *HAMP* upon LPS stimulation (Figure 3H).

Defective cytokine release in response to LPS, impaired antigen presenting function, and increased expression of anti-microbial genes are features reported for in vitro endotoxin tolerance (Biswas and Lopez-Collazo, 2009; del Fresno et al., 2009; Foster et al., 2007). Therefore, the present observations on Sepsis-Monocytes strongly suggest them to be conditioned to a refractory or endotoxin tolerant phenotype in vivo during sepsis.

Sepsis-Monocytes Upregulate Tissue Re-modeling Functions

Acute inflammation during sepsis often involves tissue damage, which must be repaired to protect the host. Monocytes and macrophages release matrix metalloproteases (MMPs) and angiogenic factors such as vascular endothelial growth factors (e.g., VEGFA) and are known to contribute to tissue re-modeling and wound healing (Murray and Wynn, 2011). The heatmap in Figure 4A shows increased expression of several MMP genes in Sepsis-Monocytes compared to Recovery-Monocytes. qPCR confirmed increased *MMP9* and *MMP19* expression in the Sepsis-Monocytes (Figure 4B). MMP activity was confirmed using zymography, which showed increased gelatinase activity in the supernatants of Sepsis-Monocytes (Figure 4C). Functional consequence of this heightened MMP activity was demonstrated by the increased ability of Sepsis-Monocyte supernatants to stimulate re-epithelialization of wounded human fibroblasts (wound healing), as compared to Recovery-Monocyte (Figure 4D). Marked inhibition of this phenomenon using a pan-MMP inhibitor confirmed MMPs to mediate this event (Figure 4D).

Sepsis-Monocytes also exhibited marked upregulation of vascular endothelial growth factor-A (VEGFA) gene expression and release, compared to Recovery-Monocytes (Figures 4E and 4F). Increased VEGFA expression correlated with a significantly heightened human umbilical vein endothelial cell (HUVEC) tube formation by Sepsis-Monocyte supernatants, indicating their enhanced angiogenic activity (Figure 4G). This was mediated through VEGF as demonstrated by the ability of VEGFR2 blocking antibody to markedly inhibit this process (Figure 4G). Collectively, our above data indicate enhanced tissue re-modeling functions of Sepsis-Monocytes as compared to Recovery-Monocytes.

(D) Diagrammatic overview of the impaired LPS response of Sepsis-Monocytes. Expression of selected immune-related genes in Sepsis- and Recovery-Monocytes in response to LPS is indicated. Gene expression in response to LPS is presented relative to the respective non-LPS treated counterpart. Red-pink color coding indicates varying expression of genes that are differentially induced by LPS. Grey indicates genes not differentially induced by LPS. Transcriptome data in (B)–(D) are from n = 7 patients during Sepsis and Recovery, respectively. See also Figure S2.

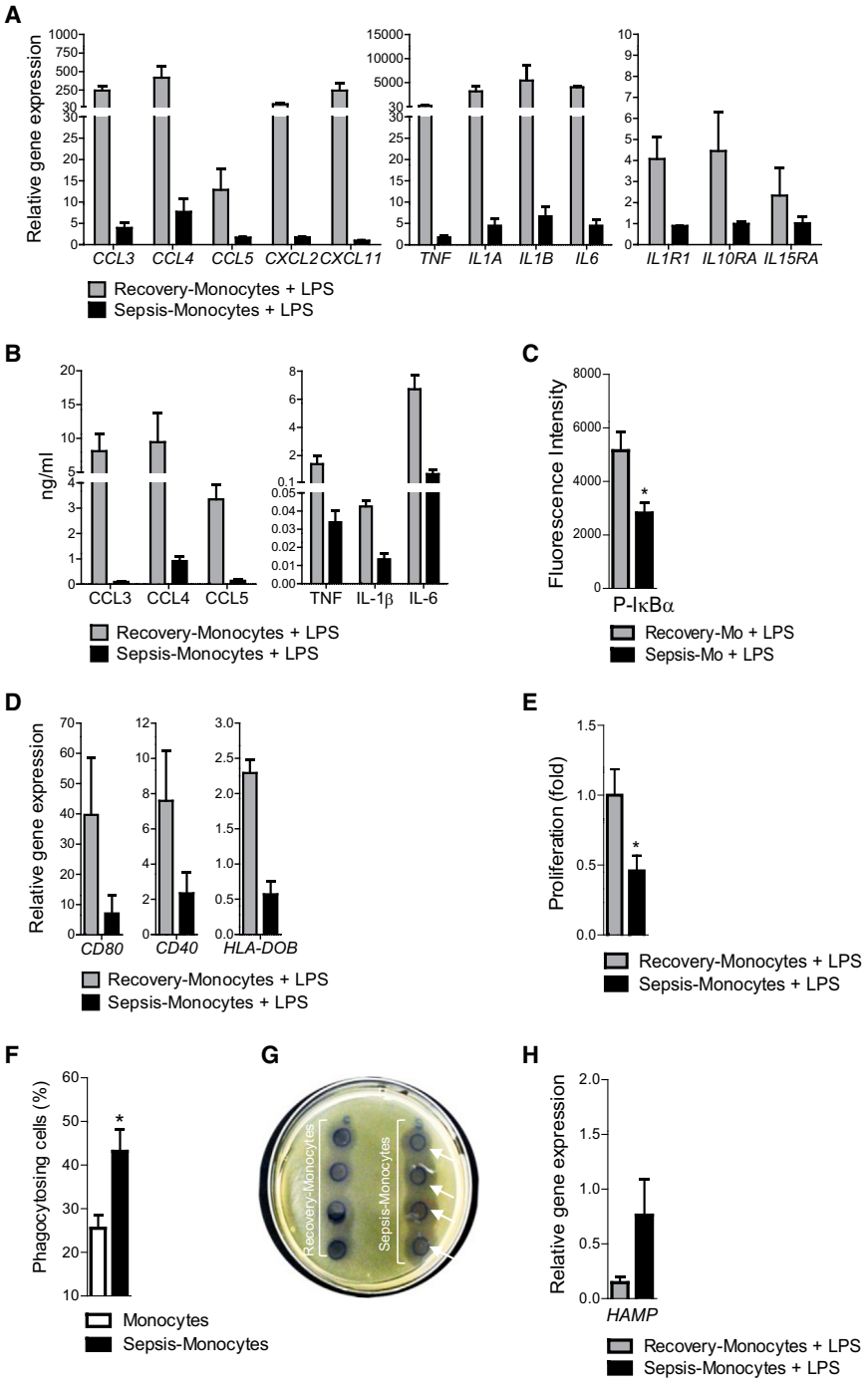

Figure 3. Monocytes Exhibit Hallmarks of Endotoxin Tolerance In Vivo in Human Sepsis

(A) qPCR of indicated genes in Sepsis- versus Recovery-Monocytes, upon LPS stimulation (3 hr).

(B) Indicated cytokine and chemokine expression in culture supernatants of Sepsis- and Recovery-Monocytes, upon LPS treatment (12 hr).

(C) Phospho-IκBα expression in response to LPS in Sepsis- versus Recovery-Monocytes, *$p < 0.05$ versus Recovery-Monocytes+LPS.

(D) qPCR of indicated genes in Sepsis- and Recovery-Monocytes upon LPS challenge.

(E) MLR assay showing proliferation of T cells from healthy donors upon incubation with LPS-treated Sepsis- or Recovery-Monocytes, *$p < 0.05$ versus Recovery-Monocytes+LPS.

(F) Ability of Sepsis- versus Control-Monocytes (Monocytes) to phagocytosize GFP-labeled *E. coli*, *$p < 0.05$ versus control Monocytes.

(G) Anti-microbial activity of supernatants from Sepsis- or Recovery-Mo showing inhibition of *E. coli* growth (dark area, see arrow), on agar plates. Each spot represents the supernatant from a particular subject.

(H) qPCR of anti-microbial gene *HAMP* in Sepsis- or Recovery-Mo following LPS stimulation. Values in all panels are mean ± SEM (A, C, D, H: n = 3; B, F, G: n = 4; E: n = 5).

HIF1α Expression and Activation Influence the Gene-Expression Profile of Sepsis-Monocytes

We investigated the potential mechanism(s) that might control the transcriptional and functional re-programming of monocytes during sepsis. The transcription factor, hypoxia inducible factor-1α (HIF1α) regulates mammalian cell response to hypoxia (low oxygen concentration) and is induced by Gram-negative endotoxin challenge in mice (Rius et al., 2008). Our microarray data indicated that *HIF1A* was differentially upregulated in Sepsis-Monocytes compared to Recovery-Monocytes (Figure 1C), also confirmed by increased *HIF1A* (not *HIF2A*) expression by qPCR and HIF1α binding assay (Figures 5A and 5B). Indeed, a gene set enrichment analysis (GSEA) revealed enrichment of hypoxia-inducible genes in the Sepsis-Monocyte transcriptome (Figure 5C). Further, hypoxia is reported to upregulate *IRAKM* (or *IRAK3*), a negative regulator of Toll-like receptor (TLR) signaling (Bosco et al., 2006; Fang et al., 2009; Kobayashi et al., 2002; López-Collazo et al., 2006). We therefore checked the expression of this gene in Sepsis-Monocytes and confirmed its upregulation by qPCR (Figure 5D).

Taken together, the upregulation of HIF1α and enrichment of hypoxia inducible genes in Sepsis-Monocytes suggested the involvement of this transcription factor in the regulation of monocyte response in sepsis, which was further investigated.

HIF1α Is a Regulator of IRAKM Expression in Monocytes

IRAKM is one of the few conserved negative regulators of TLR pathway in mice and humans (van 't Veer et al., 2007), and is implicated in endotoxin tolerance (Kobayashi et al., 2002). Hence, the upregulation of *IRAKM* in Sepsis-Monocytes (Figure 5D) is consistent with their impaired LPS response and endotoxin tolerant phenotype, as shown earlier (Figure 3). However,

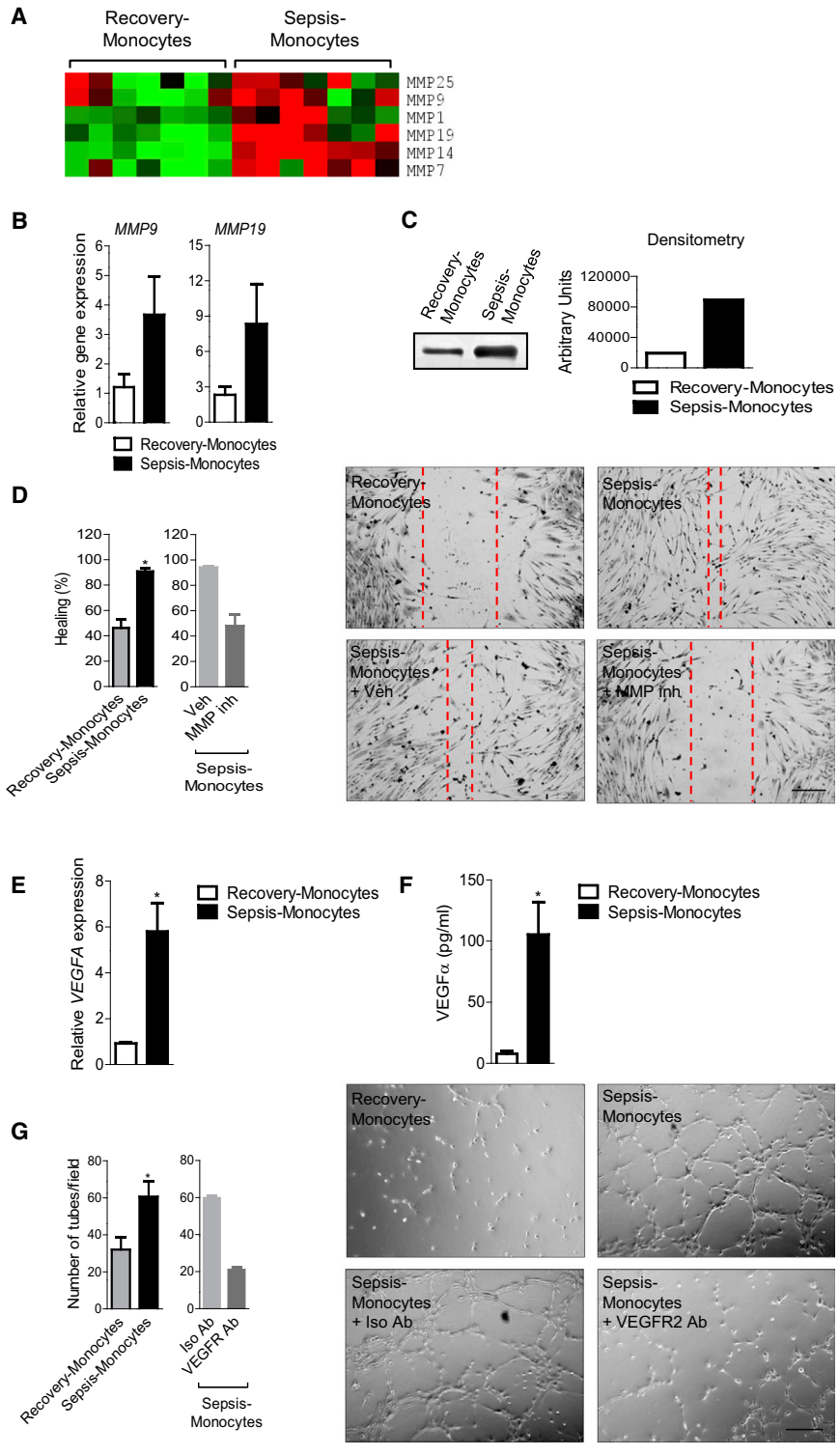

Figure 4. Sepsis-Monocytes Show Elevated Tissue Remodeling Functions

(A) Heatmap showing MMP gene modulation in Recovery- versus Sepsis-Monocytes (n = 7).

(B) qPCR for *MMP9* and *MMP19* in Sepsis- and Recovery-Mo.

(C) Zymography assay showing MMP(gelatinase) activity in culture supernatants of Sepsis- and Recovery-Monocytes.

(D) Wound-healing assay showing percent healing of wounded fibroblasts incubated with supernatant of Sepsis- or Recovery-Monocytes. Quantification and picture of wound healing (closure) is shown. Dotted line represents wound margin. Scale bar represents 0.5 mm. Wound healing in presence of a vehicle control (Veh) or MMP inhibitor is also shown. Data are representative of two independent experiments.

(E) VEGFA gene expression and (F) release by Sepsis- and Recovery-Monocytes.

(G) Enhanced angiogenesis (HUVEC tube formation) induced by supernatants of Sepsis-Monocytes. Quantification and pictures are shown. Scale bar represents 0.1 mm. The effect of VEGFR2 antibody or its isotype control (Iso Ab) on tube formation is also shown. Data are representative of two independent experiments; Values in all panels are mean ± SEM (B, E: n = 3; D, F, G: n = 4), *p < 0.05 versus Recovery-Monocytes.

the mechanism by which IRAKM is induced and regulated in these cells is not known. Based on Figure 5, we decided to investigate whether HIF1α regulates the expression of IRAKM in response to endotoxin. IRAKM expression was assessed in human monocytes treated with $CoCl_2$, an activator of HIF1 (Huang et al., 2003). $CoCl_2$-treated monocytes upregulated IRAKM gene expression and protein, indicating the regulatory role of HIF1 (Figures 6A and 6B); concurrent upregulation of *VEGFA* and *HIF1A* served as positive controls. Similar results were also obtained in hypoxia-stimulated cells (Figure S3A). Because the sepsis condition is linked to persistent exposure to endotoxins, we next checked whether HIF1 activation combined with endotoxin exposure could augment monocyte expression of IRAKM. For this purpose, monocytes were treated with or without $CoCl_2$ for 12 hr and thereafter challenged with Lipid A (LPA, a key component of gram-negative endotoxins and a specific TLR4 ligand) for 4 hr and assessed for IRAKM expression. $CoCl_2$+LPA treatment significantly increased expression of *IRAKM*, *VEGFA*, and *HIF1A*, as compared to monocytes treated with LPA alone (Figure 6C).

To directly demonstrate that HIF1α was responsible for inducing IRAKM expression in LPA-treated monocytes, we overexpressed HIF1α in monocytes and studied their response to LPA. Monocytes transfected with a HIF1α plasmid markedly upregulated *IRAKM* (and *HIF1A*) compared to control plasmid transfected cells, upon LPA challenge (Figure 6D). We also performed HIF1α small interfering RNA (siRNA)-silencing experiments in monocytes. LPA alone could induce *HIF1A*

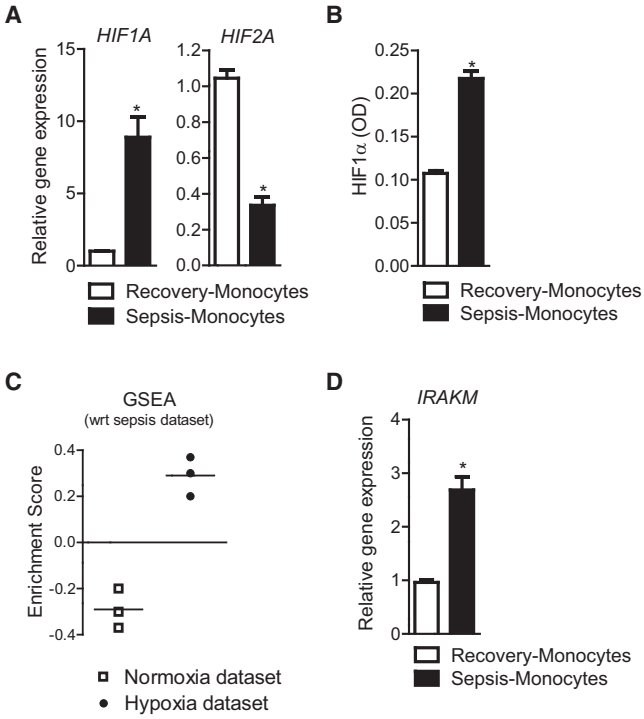

Figure 5. Sepsis-Monocytes Express Hypoxia-Inducible Genes

(A and B) HIF1α gene expression and binding assay in Sepsis- and Recovery-Monocytes.

(C) GSEA was performed between normoxia versus hypoxia datasets (from GEO and ArrayExpress) and our Sepsis- versus Recovery-Monocytes dataset. A positive score indicates positive gene enrichment between the hypoxia-inducible genes and Sepsis differentially expressed genes.

(D) Upregulated IRAKM gene expression in Sepsis-Monocytes. Values in (A), (B), and (D) are mean ± SEM (n = 3), *p < 0.05 versus Recovery-Mo.

expression in monocytes (Figure S3B). However, LPA stimulation of siHIF1α-silenced monocytes showed a significant decrease in the expression of *IRAKM* (as well as *VEGFA* and *HIF1A*) as compared to their control siRNA-treated counterparts, implicating HIF1α in mediating LPA-induced IRAKM expression (Figure 6E).

Taken together, the results from the above approaches, i.e., pharmacological activation (via $CoCl_2$), overexpression, and silencing of HIF1α, clearly establish HIF1α as an important regulator of IRAKM expression in human monocytes.

Additional support to the above findings also came from meta-analysis of GEO-deposited datasets of sepsis patients wherein a statistically significant upregulation and correlation between *HIF1A* and *IRAKM* was observed in three independent patient cohorts as compared to their respective control population (Figures S3C–S3D).

HIF1α-Induced Upregulation of IRAKM Skews Monocyte Pro-inflammatory Function

Although our results demonstrated HIF1α to regulate IRAKM expression in monocytes in response to LPA, we wondered about the functional consequence of this phenomenon. Because IRAKM is a negative regulator of TLR pathway, it is conceivable that its up-regulation (via HIF1α) should concomitantly inhibit LPA-induced pro-inflammatory response. To demonstrate this, we assessed the expression of pro-inflammatory cytokines and chemokines in all the experiments described above. Concomitant with the up-regulation of IRAKM in $CoCl_2$+LPA-treated monocytes (Figure 6C), their expression of pro-inflammatory cytokine and chemokine genes like *TNF*, *IL6*, and *CCL5* was attenuated as compared to monocytes treated with LPA alone (Figure 6F). However, this attenuation was only visible in monocytes pre-treated with $CoCl_2$ for 12 hr but not 4 hr, possibly suggesting that HIF1α activation at late time points mediated this effect (Figure S3E). Decrease in the corresponding cytokines was also detected by ELISA (Figure S3F). Further, we silenced IRAKM in these monocytes. Figure S3G, shows monocytes treated with IRAKM siRNA failed to repress the pro-inflammatory genes upon treatment with $CoCl_2$+LPA, directly implicating IRAKM in mediating the suppression of pro-inflammatory gene expression.

Mirroring the results of Figure 6F, HIF1α overexpression also showed a marked downregulation of LPA-induced pro-inflammatory cytokines (Figure 6G, Figure S3H). Taken together, the results from Figures 6C–6G clearly demonstrate that HIF1α, by inducing IRAKM expression in human monocytes, attenuates their pro-inflammatory response to endotoxin challenge.

To further demonstrate the functional impact of this attenuated pro-inflammatory response of monocytes, we also looked at the ability of these cells to polarize T cells. $CoCl_2$+LPA-treated monocytes showed downregulation of co-stimulatory molecule genes like *CD80* and *CD40* (Figure 6H). Testing their ability to polarize T cells revealed $CoCl_2$+LPA-treated monocytes to increase polarization to T regulatory (Tregs) cells (Figure 6I). This is in line with the decreased IL-6 (Figure 6F, Figure S3F) and increased TGF-β gene expression (Figure 6J) by these cells, the two key determinants for Treg cell polarization (Kimura and Kishimoto, 2010).

Collectively, the results presented in Figure 6 demonstrate that HIF1α (via IRAKM upregulation) functionally re-programs monocytes to an immunosuppressive phenotype characterized by a defective pro-inflammatory response to endotoxin and skewing of T cells to Treg cells. This immunosuppressive phenotype is reminiscent of the phenotype we observed in the Sepsis-Monocytes and in in vitro endotoxin tolerance.

HIF1α Upregulates Tissue Re-Modeling and Anti-microbial Functions of Monocytes

Since our earlier results showed sepsis monocytes to upregulate tissue re-modeling and anti-microbial functions (Figure 3 and 4), we next checked whether HIF1α regulated these functions and their genes (e.g., *MMP9*, *MMP19*, *VEGFA*, *HAMP*) in response to endotoxin. As shown in Figure 7A, $CoCl_2$+LPA-treated monocytes showed a significant upregulation of *MMP9* and *MMP19* compare to LPA-treated monocytes, suggesting an involvement of HIF1 in the regulation of these genes. Confirming this, HIF1α overexpression also upregulated *MMP9* and *MMP19* in response to LPA (Figure 7B). Conversely, HIF1α siRNA significantly downregulated LPA-induced *MMP9* and *MMP19* in monocytes (Figure 7C).

Consistent with Figure 7A, at functional level, supernatants from $CoCl_2$+LPA-treated monocytes showed significantly heightened wounded healing (Figure 7D). Inhibition of this event by a pan-MMP inhibitor proved mediation through MMPs (Figure 7D).

Angiogenesis is an important aspect of tissue remodeling that is upregulated by Sepsis-Monocytes. Pharmacological activation ($CoCl_2$) and genetic overexpression of HIF1α in monocytes was mirrored by increased *VEGFA*, a key angiogenic and well-known HIF1α target gene (Forsythe et al., 1996), whereas HIF1α siRNA abrogated this effect (Figures 6C–6E). At a functional level, supernatants from $CoCl_2$+LPA-treated monocytes markedly increase angiogenic tube formation compared to LPA-treated monocytes (Figure 7E). Inhibition of this process by a VEGFR2 blocking antibody demonstrates mediation through VEGFA (Figure 7E).

Anti-microbial activity is another vital function that is upregulated in Sepsis-Mo. These cells also upregulated the anti-microbial gene, *HAMP*. We show here that activation of HIF1α by $CoCl_2$ or its overexpression significantly upregulated *HAMP* in response to LPA (Figures 7F and 7G). Conversely, HIF1α siRNA treatment significantly downregulated *HAMP* (Figure 7H). At a functional level, $CoCl_2$-treated monocyte supernatants markedly restricted growth of *E. coli* bacteria, suggesting that HIF1 upregulates anti-microbial activity (Figure 7I). Bacterial killing also involves the generation of superoxides and phagocytosis. We showed that $CoCl_2$-treated monocytes increased their superoxide production in response to LPA (Figure S4A). Similarly, overexpression of HIF1α in monocytes upregulated their phagocytic ability toward *E. coli* (Figure S4B).

In our biochemical studies on the regulation of tissue remodeling and anti-microbial genes by HIF1α, we noted these genes to be upregulated only upon 12 hr, but not 4 hr, of $CoCl_2$ pretreatment, suggesting that HIF1α activation at late rather than early time point was responsible for this effect (Figure S4C). Further, we also noted in the $CoCl_2$+LPA-treated monocyte experiments that silencing IRAKM in these cells did not affect the expression these genes, suggesting these genes to be regulated by HIF1α, independent of IRAKM (Figure S4D).

Taken together, our above results present multiple evidence that establishes HIF1α as an important regulator of tissue remodeling and anti-microbial function in human monocytes. However, these functions are regulated by HIF1α directly, independent of IRAKM.

DISCUSSION

In the current report, we adopted a systems biology approach to reveal that blood monocytes undergo phenotypic and functional plasticity during the clinical course of Gram-negative sepsis in humans. Circulating human monocytes displayed a pro-inflammatory gene-expression profile in ongoing sepsis. These same cells also exhibited features of endotoxin tolerance such as blunted inflammatory cytokine and chemokine production and impaired antigen-presenting function when challenged with LPS ex vivo. Mechanistically, upregulated expression of HIF1α in blood monocytes mediated the expression of IRAKM, a negative regulator of TLR signaling, leading to the conversion of these cells from a pro-inflammatory to an endotoxin-tolerant phenotype in sepsis. However, the capacity to phagocytosize bacteria, express anti-microbial activity, and perform tissue re-modeling or repair functions were increased in blood monocytes from sepsis, indicating that monocyte function is re-configured rather than globally suppressed under these conditions.

Transcriptome profiling of blood monocytes sampled from patients both during and after resolution of Gram-negative sepsis revealed that a large number of pro-inflammatory cytokines, chemokines, and transcription factors were upregulated in monocytes from sepsis patients (Sepsis-Monocytes) compared with monocytes from the same subjects following resolution (Recovery-Monocytes). These data are consistent with the concept that blood monocytes (and likely tissue macrophages) directly support the induction of a ''cytokine storm'' in response to systemic bacterial infection in human sepsis. Supporting our transcriptome data, identification of soluble factors secreted by cultured Sepsis-Monocytes confirmed that blood monocytes secrete increased pro-inflammatory cytokines and chemokines including IL-1β, IL-6, CCL3, and CCL5 in ongoing sepsis. Previous microarray analyses of blood mononuclear cells from pediatric, as well as adult sepsis patients, have indicated the differential regulation of several cytokine and chemokine genes such as *IL1*, *IL8*, *CCL3*, and *CCL4* (Pachot et al., 2006; Standage and Wong, 2011; Tang et al., 2010). The objective of many of these studies was to identify a predictive gene signature or biomarker(s) for sepsis and its prognosis by comparing patients with healthy controls or between cohorts with different clinical symptoms or outcomes. However, identifying such biomarkers has been challenging due to the wide range of etiologies associated with sepsis. In contrast, our present study examined a well-defined population of sepsis patients, i.e., Gram-negative sepsis patients. In these patients, we examined the cellular and molecular basis of the aberrant immune response in sepsis, pinpointing monocytes as a key mediator of this response in humans. The importance of this cell population is heightened because apoptosis of a large number of immune subsets like lymphocytes and dendritic cells, but not blood monocytes or interstitial macrophages, has been reported in sepsis (Hotchkiss et al., 2013). Thus, characterizing Sepsis-Monocytes provides a snapshot of the immune-inflammatory response linked to sepsis. In addition, Sepsis-Monocytes also modulated anti-microbial activity, tissue re-modeling, and metabolism, suggesting other functions in sepsis.

Death in sepsis for most cases is not due to overt inflammation, which can be controlled by standard treatments such as antibiotics and steroids, but instead reflects host immunosuppression that confers high risk of fatal nosocomial infection (Hotchkiss et al., 2013). Indeed, post-mortem studies have shown unresolved opportunistic infections in a large number of patients dying of sepsis (Otto et al., 2011; Torgersen et al., 2009). In contrast, other studies report the absence of active infection in patients dying of sepsis and suggest the emerging role of immunometabolism and neurophysiology in organ dysfunction and death in sepsis (Deutschman and Tracey, 2014). It is likely that multiple events as mentioned above co-exist and contribute to this phenomenon. Among these, sepsis-related host immunosuppression has gained considerable attention recently (Hotchkiss et al., 2009; Hotchkiss et al., 2013; Pachot et al., 2006). The mechanism(s) underlying such immunosuppression (although not presently understood) might be varied such as relative changes in the abundance of different immune cell types (e.g., apoptosis of lymphocytes) (Hotchkiss et al., 2013) or change in an individual immune cell type (e.g., altered response or function). In line with the latter, a potential

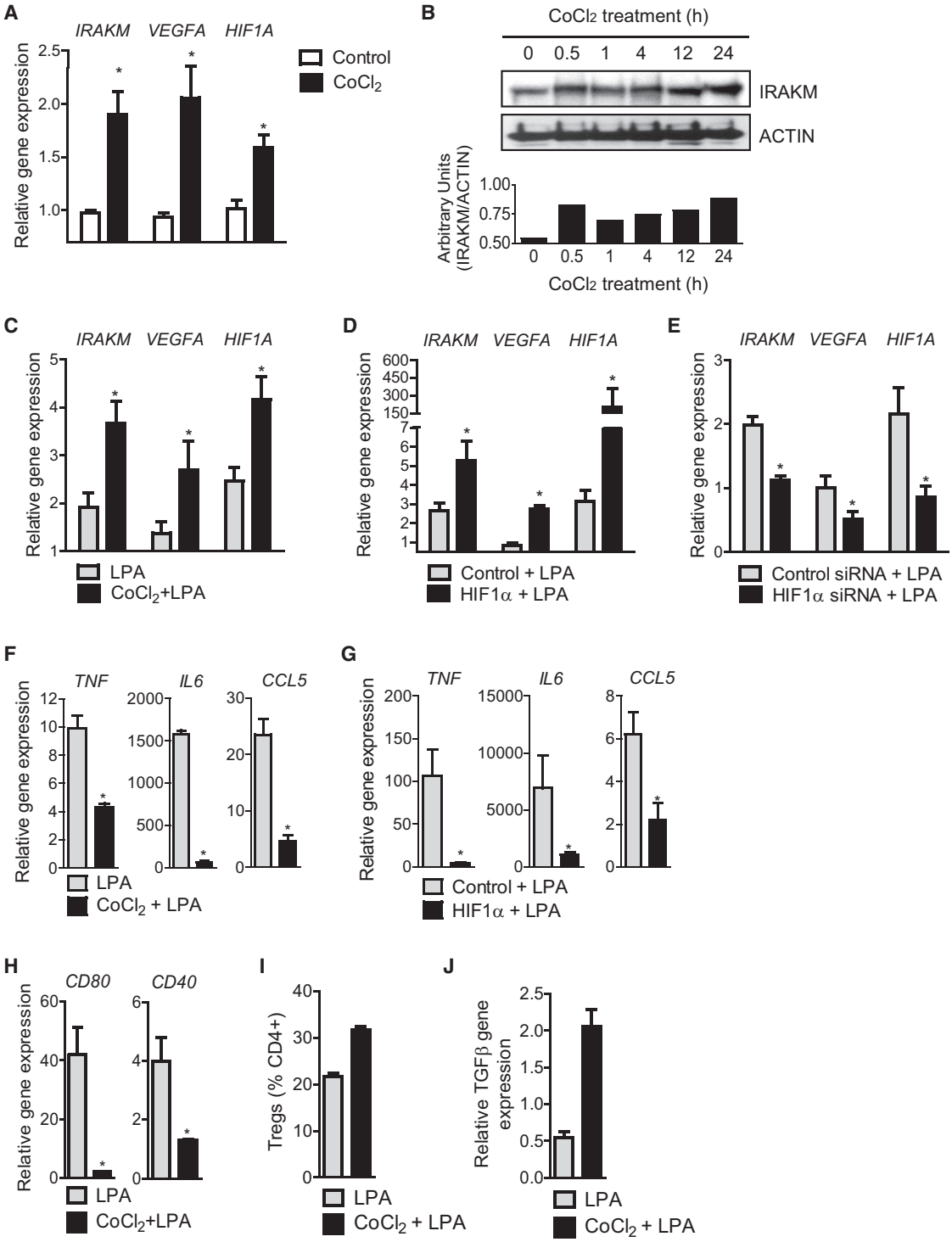

Figure 6. HIF1α Induces the Expression of IRAKM that Skews Monocyte Pro-inflammatory Function

(A) qPCR of indicated genes in monocytes from healthy donors treated or not with $CoCl_2$ (0.25mM) for 12 hr, *p < 0.05 versus Control.

(B) Immunoblot showing IRAKM expression in $CoCl_2$-treated monocytes. Actin expression indicates loading control. Inset shows densitometry analysis; qPCR of indicated genes in monocytes (C) treated or not with $CoCl_2$ (12 hr) and then exposed to LPA (100ng/ml) for 4 hr, *p < 0.05 versus LPA.

(D) transfected with a control or HIF1α expressing plasmid following by LPA challenge (4 hr), *p < 0.05 versus Control+LPA.

(E) Treated with control or HIF1α siRNA and stimulated with LPA (4 hr), *p < 0.05 versus Control siRNA$^+$LPA; qPCR analysis of indicated genes in monocytes (F) treated with LPA or $CoCl_2$+LPA, *p < 0.05 versus LPA and (G) transfected and treated as described in (D), *p < 0.05 versus Control+LPA.

(H) qPCR analysis of indicated genes in LPA or $CoCl_2$+LPA treated monocytes, *p < 0.05 versus LPA.

(legend continued on next page)

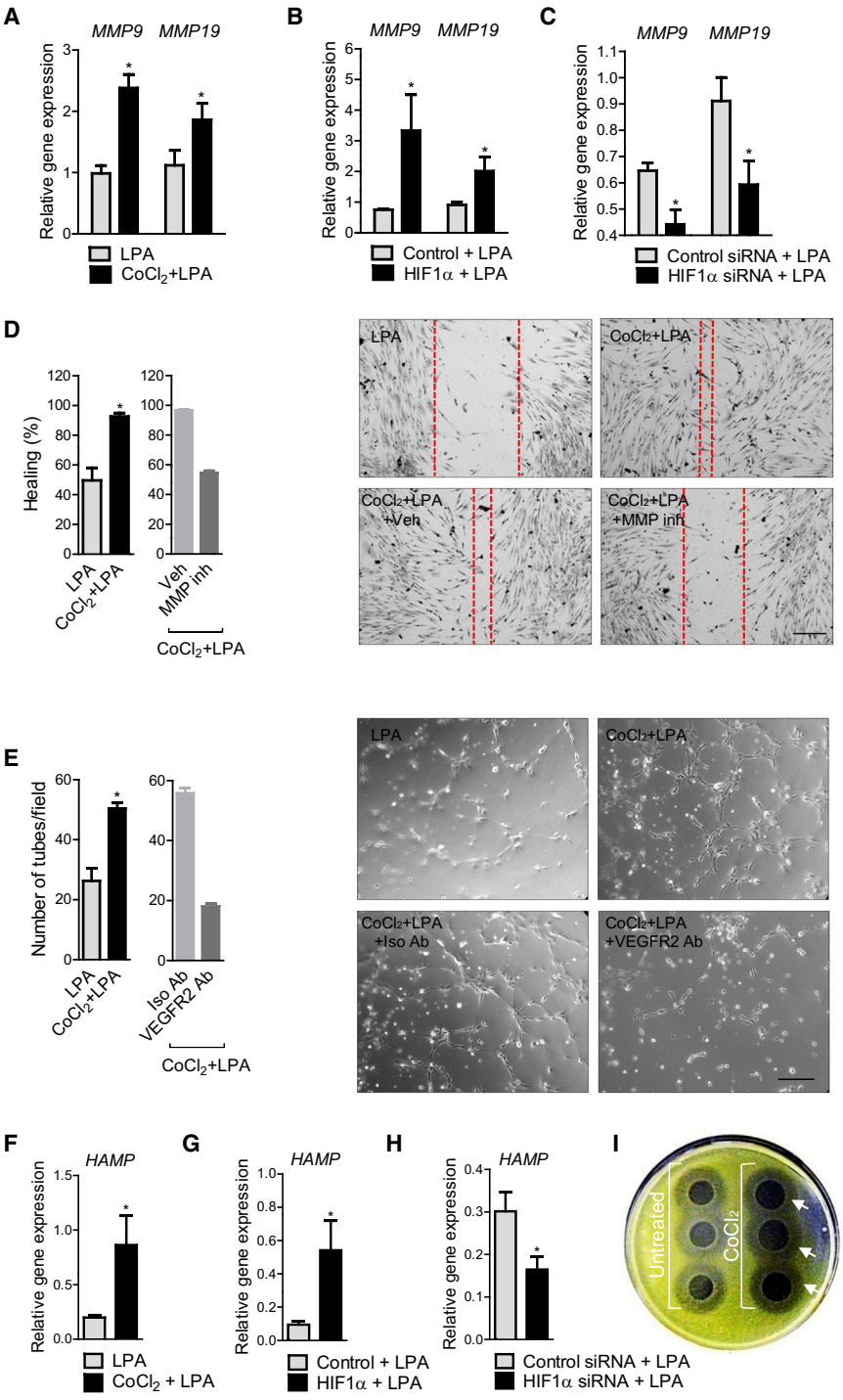

Figure 7. HIF1α Upregulates Tissue Remodeling and Anti-microbial Functions of Monocytes

(A) qPCR of indicated genes in monocytes treated with LPA or $CoCl_2$+LPA, *p < 0.05 versus LPA.

(B) qPCR of indicated genes in monocytes transfected with control or HIF1α plasmid and stimulated with LPA, *p < 0.05 versus Control+LPA.

(C) qPCR of indicated genes in monocytes treated with control or HIF1α siRNA and stimulated with LPA, *p < 0.05 versus Control siRNA+LPA.

(D) Wound-healing assay showing percent healing of wounded fibroblasts incubated with supernatant of LPA or $CoCl_2$+LPA treated monocytes (n = 3), *p < 0.05 versus LPA. Wound healing in presence of a vehicle control (Veh) or MMP inhibitor is also shown. Data are representative of two independent experiments. Dotted lines represent wound margin. Scale bar represents 0.5 mm.

(E) Angiogenesis assay enumerating HUVEC tube formation induced by supernatants from LPA or $CoCl_2$+LPA treated monocytes (n = 3). The effect of VEGFR2 antibody or its isotype control (Iso Ab) on tube formation is also shown. Data are representative of two independent experiments. Scale bar represents 0.1 mm; qPCR of *HAMP* expression in monocytes (F) treated with LPA or $CoCl_2$+LPA,*p < 0.05 versus LPA.

(G) Transfected with control or HIF1α plasmid following LPA stimulation, *p < 0.05 versus Control+LPA.

(H) Treated with control or HIF1α siRNA following LPA treatment, *p < 0.05 versus Control $siRNA^+$LPA.

(I) Anti-microbial activity of the supernatants from monocytes treated or not with $CoCl_2$ showing inhibition of bacterial (*E. coli*) growth on agar plates, as described in the legend for Figure 3G (n = 3). Values in all panels are mean ± SEM (A, F, G: n = 4; B, C, H: n = 3). See also Figure S4.

mechanism of immunosuppression in sepsis patients is the induction of endotoxin tolerance, a process by which host immune cells exposed to low doses of endotoxin over an extended period become refractory to further endotoxin challenge (Adib-Conquy and Cavaillon, 2009; Biswas and Lopez-Collazo, 2009). Studies in murine macrophages and human monocytes have demonstrated that endotoxin tolerance can be induced in vitro by prolonged exposure of these cells to low doses of LPS, leading to downregulation of inflammatory cytokines TNF, IL-6, and IL-8, and upregulation of the regulatory cytokine IL-10 (Biswas and Lopez-Collazo, 2009; Foster et al., 2007; Medvedev et al., 2000). Similar impairment in the production of TNF, IL-6, and IL-1 was also reported for monocytes from gram-negative sepsis patients, upon ex vivo LPS challenge (Munoz et al., 1991). However, whether such an effect is restricted to a few genes or a more wide-scale event is not

(I) Bar graph showing % of CD4+ T cells polarized toward Tregs (CD25+FOXP3+) in an MLR assay performed in the presence of monocytes treated with LPA or $CoCl_2$+LPA.

(J) qPCR showing TGF-β gene expression in LPA or $CoCl_2$+LPA treated monocytes. Values in (A), (C)–(J) are mean ± SEM (A and C: n = 4; D–H: n = 3; I and J: n = 2). See also Figure S3.

well-understood. To clarify this, we compared the LPS-stimulated transcriptome of Sepsis-Monocytes with that of Recovery-Monocytes to identify an impaired monocyte response to LPS during sepsis. These findings together with qPCR results showed markedly reduced gene expression of several pro-inflammatory cytokines or chemokines, inflammation-related transcription factors, and antigen presentation-related molecules, corroborating with a downregulation of monocyte activation. Our data provide transcriptomic evidence confirming the in vivo endotoxin tolerance of human blood monocytes in ongoing sepsis.

While the above observations conform to the general characteristics of an endotoxin-tolerant phenotype in Sepsis-Monocytes, several genes and functions related to anti-microbial activity and tissue remodeling remain upregulated in these cells. This suggests that monocytes undergo a transcriptomic and functional "re-programming" in sepsis rather than a general suppression of genes and functions. Supporting this, in vitro endotoxin tolerance studies in human monocytes and murine bone-marrow-derived macrophages reported the downregulation of inflammatory genes concomitant with increased expression of anti-microbial genes in these cells (Biswas and Lopez-Collazo, 2009; del Fresno et al., 2009; Foster et al., 2007). In addition, in vitro endotoxin tolerant human peripheral blood mononuclear cells (PBMCs) and macrophages were recently shown to upregulate genes related to wound healing (*VEGFA, MMP9*) and phagocytosis (*MARCO, CD23*) (Pena et al., 2011), an observation supported in vivo by the upregulation of VEGFA, MMPs, and heightened phagocytosis in Sepsis-Monocytes. Some studies also link in vitro endotoxin tolerance to M2 macrophage polarization, upregulating ARG1, YM1, and FIZZ1 genes in mice, and *CCL22*, *CCL24*, CD163, and CD206 in humans (Pena et al., 2011; Porta et al., 2009). Although our array data show *CD163* upregulation in Sepsis-Monocytes, the fact that these cells co-express pro-inflammatory signature together with immunosuppressive (e.g., impaired response to LPS ex vivo), tissue remodeling, and anti-microbial characteristics suggests a more complex profile than a simple M1 or M2 polarization state. This is conceivable considering the multitude of stimuli encountered in vivo and the dynamic nature of the disease. In line with this fact, a recent study reported that the immune response in severely ill human patients exhibits both pro-inflammatory and anti-inflammatory components that function in parallel (Xiao et al., 2011). Similarly, another study profiling PBMCs from sepsis patients also failed to demonstrate a distinctive pro- or anti-inflammatory phase at the transcriptional level (Tang et al., 2010), suggesting their co-existence, as proposed by others (Adib-Conquy and Cavaillon, 2009). The phenotype of Sepsis-Monocytes emerging from our study suggests a general adaptation response to overt inflammation, which involves an impaired capacity to support further inflammation and immune activation, while promoting a protective response through an intact phagocytotic, anti-microbial, and tissue re-modeling functions.

Our transcriptomal and functional analyses of blood monocytes demonstrates their in vivo functional re-programming in course of human sepsis. Defining the mechanisms that support monocyte functional plasticity or re-programming in human sepsis is crucial in understanding the dysregulated host immune response in sepsis progression. Several lines of evidence in the current report suggest a mechanistic role for HIF1α in guiding this functional re-programming of monocytes in human sepsis. Using gene-enrichment analysis, we found that a large proportion of the genes modulated in Sepsis-Monocytes were hypoxia inducible. Consistent with this fact, the expression and activity of HIF-1α was found to be upregulated in Sepsis-Monocytes as compared to Recovery-Monocytes. Further studies involving the modulation of HIF1α using pharmacological, genetic overexpression and siRNA silencing approach established HIF1α to regulate the expression of IRAKM, a well-known negative regulator of the TLR signaling pathway and inducer of endotoxin tolerance (Kobayashi et al., 2002; López-Collazo et al., 2006). Moreover, meta-analysis of sepsis leukocyte datasets showed a significant correlation between increased *HIF1A* and *IRAKM* expression in independent cohorts of sepsis patients. On the basis of our results, we propose that during sepsis, exposure to endotoxin triggers HIF1α activation in the monocytes, which in turn induces IRAKM expression, thereby driving these cells into an endotoxin-tolerant state. This correlates well with our observations on HIF1α activation, upregulation of IRAKM and the endotoxin tolerant phenotype of Sepsis-Monocytes. In fact, we could demonstrate that HIF1α not only upregulated IRAKM in monocytes, but concomitantly downregulated the expression of pro-inflammatory cytokines like TNF and IL-6 in response to LPA, consistent with the negative regulatory role of IRAKM. Indeed, using siIRAKM silencing in monocytes, we demonstrated that IRAKM, downstream of HIF1α, was responsible for mediating this downregulation of pro-inflammatory cytokines in response to LPA. In contrast to these observations, earlier studies in conditional genetically ablated mice have shown HIF1α to support myeloid cell-mediated inflammation and pro-inflammatory gene expression (Cramer et al., 2003; Nizet and Johnson, 2009). Recently, HIF1α was also shown to mediate IL-1β expression in LPS-treated macrophages (Tannahill et al., 2013). The apparent paradox could perhaps be explained by the fact that an initial HIF1α activation will induce a pro-inflammatory program in human monocytes, whereas chronic activation of this pathway (as occurs in sepsis) would lead to the dampening of those same inflammatory responses via induction of negative regulators such as IRAKM. Supporting such a view, we showed that HIF1α activation (by $CoCl_2$ treatment) at late, but not early time points in monocytes suppressed their pro-inflammatory cytokine expression in response to LPA. Consistent with a role for HIF1α in ameliorating ongoing inflammation, HIF1α was reported to increase the frequency and suppressive properties of naturally occurring $CD4^+CD25^+$ regulatory T cells (Ben-Shoshan et al., 2008). Tregs are increased in sepsis (Hotchkiss et al., 2013). Supporting these observations, we showed that HIF1α activation by $CoCl_2$+LPA treatment in monocytes increased their ability to induce Treg cell polarization. This was possibly driven by the skewed IL-6 versus TGF-β expression by monocytes, a hallmark for Treg cell polarization (Kimura and Kishimoto, 2010). An immunosuppressive role for HIF1α is also supported by studies in tumors, where HIF1α was instrumental in polarizing tumor associated macrophages to an M2-like phenotype and driving myeloid-derived suppressor cells to their immunosuppressive phenotype (Colegio et al., 2014; Corzo et al., 2010). In line with these, our finding that HIF1α regulates IRAKM expression in monocytes identifies

a potential mechanism for targeting the immunosuppressive response of monocytes in human sepsis.

In addition to the above data, we have presented biochemical and genetic evidence demonstrating HIF1α to regulate tissue remodeling and anti-microbial functions and their related genes (e.g., *VEGFA*, *MMP9*, *19*, and *HAMP*). However, HIF1α directly regulated these independent of IRAKM. This is conceivable because many of these molecules like VEGFA and MMPs are target genes for HIF1α and hypoxia (Forsythe et al., 1996). In line with our functional studies, mice studies have shown HIF1α to mediate the anti-microbial activity in myeloid cells (Nizet and Johnson, 2009). Taken together, our mechanistic studies suggest HIF1α as a crucial regulator of the functional plasticity of monocytes during sepsis: regulating inflammatory response on one hand and orchestrating protective responses on the other hand. However, further studies on these different aspects of HIF1α in humans would shed more light on the situation.

Although HIF1α regulates the expression of a diverse range of genes (e.g., inflammatory, negative regulator, tissue remodeling, and anti-microbial genes), it is pertinent to understand what controls the target specificity and temporal regulation of such HIF-induced genes. Recent studies indicate HIF to exert direct or indirect transcriptional regulation involving other interacting proteins, enhanceosomes, and epigenetic modifications (Schödel et al., 2011). While the relative contribution of these different modes of HIF transcriptional regulation remains unclear, future ChIP-Seq and epigenetic studies would clarify the extent of such direct versus indirect regulatory effects of HIF1α on our genes of interest (e.g., *IRAKM, VEGFA*, MMP gene), as well as gene re-programming.

Another aspect of sepsis is its long-term effect on survivors. Sepsis survivors have been reported to show significant morbidity and mortality, with 5-year mortality rates of higher than 70% (Deutschman and Tracey, 2014; Iwashyna et al., 2010; Valdés-Ferrer et al., 2013). Although the events underlying such long-term effects remain unclear, elevated serum factors like IL-6, HMGB1, and inflammatory monocytes have been suggested in human and mice studies (Valdés-Ferrer et al., 2013; Yende et al., 2008). It is possible that persistence of such subclinical inflammation might induce long-term and specific changes in these immune cells that might contribute to the altered status of these survivors. Although our study involved sepsis survivors, its main focus was to characterize the immune response of monocytes during sepsis (hence comparing Sepsis-with Recovery-Monocytes), rather than post-sepsis susceptibility in these subjects. The latter is a different issue requiring future investigation of the immune response in survivors at different time points following recovery, in relation to healthy subjects.

In conclusion, using a systems biology approach, the present study demonstrates that blood monocytes undergo a phenotypic and functional re-programming during human sepsis that allows these cells to transit from an inflammatory to an immunosuppressive state, thereby contributing to both features of sepsis progression. Mechanistically, HIF1α was identified as a key mediator of monocyte functional re-programming in sepsis, raising the possibility of pharmacologically targeting this molecule to modulate monocyte responses and human sepsis progression.

EXPERIMENTAL PROCEDURES

Human Blood Samples

All access to blood samples from patients or healthy subjects were in compliance with the guidelines approved by the local ethics committee. Further information on healthy and patient blood samples, as well as patient clinicopathological features, are provided in the Supplemental Experimental Procedures.

qPCR Analysis

Cells were lysed in Trizol (Life Technologies, Invitrogen), and total RNA prepared using the RNeasy kit (QIAGEN) as per manufacturer's instructions. For PCR Array analysis, total RNA was reverse transcribed using the RT^2 First Strand Kit (SABiosciences, QIAGEN). cDNA was utilized for qPCR using PCR array plates for human inflammatory cytokines and their receptors (SABiosciences, QIAGEN) and run on an iCycler iQ5 Real-Time PCR detection system (Bio-Rad). Gene expression was analyzed using the manufacturer's analysis software (www.sabiosciences.com/pcrarraydataanalysis.php). For normal qPCRs, total RNA was reverse transcribed using the Taqman reverse transcription kit (Applied Biosystems) and cDNA ran for qPCR on iCycler iQ5 machine. Target gene expression was normalized to the expression of a housekeeping gene, β-actin gene. Relative gene expression was calculated using the standard 2-ΔΔCt method.

siRNA Silencing and Overexpression Studies

RNA interference was performed using siRNAs targeted against human HIF1α or IRAKM and compared with a control/scrambled siRNA (Invitrogen). Similarly, HIF1α overexpression was performed by transfecting monocytes with a human HIF1α expression plasmid or a control plasmid. Monocytes were nucleoporated using Human Monocyte Nucleofector Kit (Amaxa Biosystems, Lonza) in the presence of the respective siRNAs (20 nM) or plasmids (0.5 μg) as per the manufacturer's instructions. Overexpression or silencing was assessed by qPCR analysis.

Bio-Plex Assays

Cell-free culture supernatants of human monocytes (50 μl) were assayed for the presence of the indicated cytokines, chemokines, and growth factors using the Bio-Plex Pro assay kit (Bio-Rad). For phospho-IκBα protein, monocyte protein extracts were prepared and analyzed using the Bio-Plex phospho-protein detection kit (Bio-Rad) as per the manufacturer's instructions. All data were collected using the Bio-Plex 200 array system with Luminex xMap Technology and analyzed using Bio-Plex Manager 6.0 (Bio-Rad).

ELISA

Cell-free culture supernatants of human monocytes were tested for TNF, IL-6, and CCL5 using ELISA Kits (DuoSet Kits, R&D Systems) according to the manufacturer's instructions.

HIF1α Binding Assay

Monocytes from septic and recovered patients were processed and assayed using the TransAM HIF1 kit (Active Motif) according to the manufacturer's protocol. The optical density of the samples was quantified using an Infinite M200 plate reader (Tecan).

Statistical Analysis

Statistical significance was calculated by Student's t test for comparisons between two groups, or by one-way ANOVA for comparisons of three or more groups. p values < 0.05 were considered significant.

Additional methods including microarray and bioinformatics analysis, patient details, cell isolation and culture, and biochemical and functional assays are provided in the Supplemental Experimental Procedures.

ACCESSION NUMBERS

The Gene Expression Omnibus (GEO) accession number for the microarray data reported in this paper is GSE46955.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and Supplemental Experimental Procedures and can be found with this article online at http://dx.doi.org/10.1016/j.immuni.2015.02.001.

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