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Letter

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A Unique Monocyte Transcriptome Discriminates Sickle Cell Disease From Other Hereditary Hemolytic Anemias and Shows the Particular Importance of Lipid and Interferon Signaling

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Sickle cell disease (SCD) is a relatively common, hereditary hemolytic anemia characterized by complex pathophysiology, including chronic inflammation and oxidative stress.[1] Ischemia-reperfusion injury and hemolysis have been recognized as potent triggers of inflammation, and the inflammatory marker C-reactive protein was identified as an independent predictor of early mortality.[2] Emerging studies highlight the importance of pro-inflammatory Toll-like receptor 4 (TLR4) signaling in acute and chronic SCD complications.[3–5]

In general, TLR signaling is dependent on reactive oxygen species (ROS) production by nicotinamide-adenine dinucleotide phosphate oxidase highly expressed on monocyte membranes. Chronic intravascular hemolysis leads to release of damage-associated molecular pattern (DAMP) molecules like high mobility group box 1 (HMGB1) protein.[4,6] HMGB1 plays a predominant role in TLR4 activity in SCD and synchronization with heme attenuates the pro-inflammatory TLR4 response.[4] Therefore, heme is currently designated as an erythroid-derived (e-)DAMP molecule.[7,8] The contribution of heme seems to be highly dependent on the pro-oxidant effect of hemin-bound iron, and iron is known to catalyze redox signaling by participating in the Fenton reaction. ROS production by heme has shown to indirectly activate the nuclear factor kappa B (NF-κB) pathway.[6,9]

Free heme, product of intravascular hemolysis, therefore, serves as a potent modulator of TLR4 signaling in SCD, and TLR4$^+$ cells, monocytes, play an important role in the pathophysiology of SCD.[10] The aim of our study was to identify the main activated pathways in monocytes in response to intravascular hemolysis in SCD patients. In our analyses, we compared SCD to a group of other hemolytic diseases characterized almost exclusively by extravascular hemolysis and thus not subjected to large amounts of intravascular cell-free heme. We investigated gene expression profiles of TLR4$^+$ cells, by positive selection of its co-receptor CD14, from patients with SCD and other hereditary hemolytic anemias to identify differential regulated genes and pathophysiological pathways. Materials and methods are described in the Section S1, http://links.lww.com/HS/A129. Our results illustrate the importance of the relative contribution of pro- and anti-inflammatory signaling in SCD monocytes, which may contribute to the SCD phenotype.

Data were available from 14 individual SCD patients (ie, 11 HbSS, 3 HbSC), obtained during steady-state disease defined as no acute SCD complications in the preceding month. Table S1 (http://links.lww.com/HS/A129) highlights the main clinical characteristics of the SCD patients without (n = 11) or on deferasirox (DFX) therapy (n = 3), healthy controls (n = 10), and patients with various forms of hereditary hemolytic anemia (n = 46). The latter group consists of patients diagnosed with pyruvate kinase deficiency (n = 14), β-thalassemia (n = 2), hereditary xerocytosis (n = 7), and hereditary spherocytosis (n = 23). None of these patients was treated with DFX at the time of blood sampling. In the SCD patient group, 5 patients required regular exchange red cell transfusions, including 3 patients treated with hydroxyurea.

For visualization of the data, a plot was generated based on a principal component analysis of the 3000 most variable genes in the dataset (Figure 1). The cluster of CD14$^+$ cells of SCD patients not treated with DFX clustered apart from both CD14$^+$ cells derived from healthy controls and cells from patients with various hemolytic anemias. Whereas CD14$^+$ cells of healthy controls and patients with other hemolytic anemias overlapped in the principal component analysis. DFX treatment seemed to correct the transcriptome alterations of SCD patients towards normal.

Next, we analyzed the differentially expressed genes (DEGs) from CD14$^+$ cells of non-DFX SCD patients when compared with either healthy controls or patients with other hemolytic anemias. The analysis rendered 744 genes differentially expressed in the comparison of non-DFX SCD and healthy controls, of which 505 were upregulated in SCD. In the comparison of non-DFX SCD and other hereditary hemolytic anemias, 593 genes were differentially expressed, including 248 genes higher expressed in SCD.

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Supplemental digital content is available for this article.

HemaSphere (2021) 5:2(e531). http://dx.doi.org/10.1097/HS9.0000000000000531.
Received: 27 March 2020 / Accepted: 17 December 2020

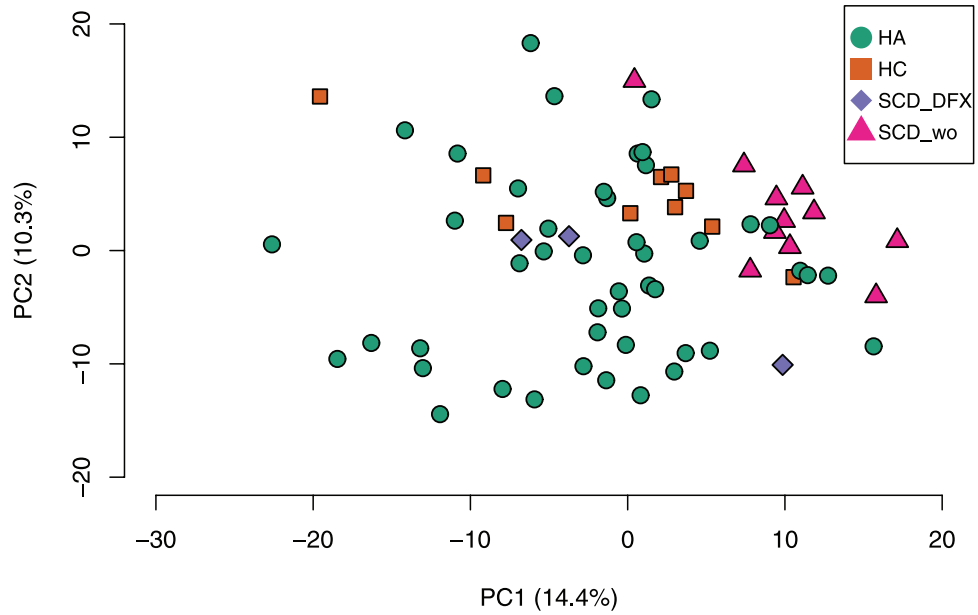

Figure 1. Principle component analysis (PCA). PCA is a technique to reduce dimensionality and emphasize variation in a dataset. New variables are constructed as weighted averages of the original variables. These new variables are called the principal components. The plot of the 2 most important principal components (explaining the variance or dimensionality of gene expression) illustrates distinct patterns in our dataset. The percentages provided on x-axis and y-axis (…) are relative but display the variance that the principal component accounts for in the analysis. Gene expression profiles of patients that are more closely correlated cluster together. Input data is regularized log-transformed. Each dot represents 1 patient, and the location of a dot is determined from the top 3000 most variable genes in the dataset. The dots are colored according to disease category. HA = hemolytic anemia; HC = healthy control; SCD_DFX = deferasirox-treated sickle cell disease; SCD_wo = sickle cell disease without deferasirox treatment.

Pathway enrichment analysis showed significant enrichment of genes involved in interferon (IFN) type I and II signaling in the set of individual genes that were differentially expressed in non-DFX SCD when compared with healthy controls (adjusted $P = 4.4 \times 10^{-16}$; Table S2A, http://links.lww.com/HS/A129). We observed a profound upregulation of all components of IFN signaling, including IFN receptors (eg, *IFNGR1*, *IFNGR2*), signal transduction molecules (eg, *JAK2*, *STAT1*, *STAT2*, *IRF7*), and a broad range of IFN-stimulated genes (eg, *IFI27*, *IFIT3*, *OAS1*, *ISG15*). Furthermore, pathway enrichment analysis showed significant enrichment of pathways involved in chemokine signaling (adjusted $P = 0.06$) and TLR2/4 signaling (adjusted $P = 0.06$) with profound upregulation of the individual genes in CD14$^+$ cells derived from SCD patients.

In addition, pathway enrichment analysis in the set of individual genes differentially expressed between non-DFX SCD and other hereditary hemolytic anemias underlined the importance of chemokine signaling (adjusted $P = 0.01$; Table S2B, http://links.lww.com/HS/A129) with the most profound upregulation of IFNγ-inducible genes *CXCL11* and *CXCL9*. Our analysis also showed enrichment of genes involved in cholesterol biosynthesis (the mevalonate pathway; adjusted $P = 0.09$), which were all upregulated in SCD, as well as upregulation of genes involved in immune interactions between lymphoid and nonlymphoid cells (adjusted $P = 0.09$).

Next, we aimed to define a core list of protein-encoding genes that represents the specific features of CD14$^+$ cells in SCD. For this purpose, we made a more stringent selection of the individual DEGs (see Statistical analysis in Section S1, http://links.lww.com/HS/A129) and selected those protein-encoding genes that were differentially expressed when comparing CD14$^+$ cells of SCD patients with CD14$^+$ cells of healthy controls and patients with other hereditary hemolytic anemias. This analysis rendered 29 genes, as presented in Figure 2. Several of the encoded proteins in the selection have previously been related to SCD. For the other proteins, an important role in SCD could be presumed based on protein characteristics and actions in other disease models. Again, the list highlights the importance of 2 processes related to immune signaling: CXCR3 (chemokine) signaling by CXCL9 and CXCL11 and lipid metabolism (*STARD4*, *DLC1*, *SQLE*, *ME1*). Thereby, a role for CD14$^+$ cells in development of vasculopathy in SCD is supported by upregulation of *PPARG*, *GUCY1A1*, *KLF5*, *CTSL*, and CXCR3 signaling (*CXCL9* and *CXCL11*), which all have previously been associated with vascular remodeling and development of pulmonary hypertension.

Interestingly, but not unexpected, heme oxygenase-1 (*HMOX1*) was one of these genes (versus healthy controls adjusted $P = 5.6 \times 10^{-13}$; versus other hemolytic anemias adjusted $P = 3.3 \times 10^{-15}$). Profound upregulation of *HMOX1* in CD14$^+$ cells of SCD patients is in line with the hypothesis that intravascular free heme is an important effector of gene regulation in monocytes.[11] Heme oxygenase-1 (HO-1) mediates heme detoxification. Transcriptional regulation is highly complex and upregulation is mediated by multiple pathways involved in stress and inflammation, as extensively reviewed by others.[12] Monocyte HO-1 has known to be important in prevention of vasculopathic injury in SCD. HO-1 induction required crosstalk with endothelial cells damaged by toxic heme.[10] A large proportion of the immunomodulatory effectivity of HO-1 is contributed to one of the end products of heme degradation, carbon monoxide (CO).[13] So, the strong upregulation of HO-1 in SCD monocytes suggests an essential preventive response to counterbalance continuous pro-inflammatory signaling initiated by a broad range of e-DAMPs.

The regulation of gene expression of many pro-inflammatory genes relies on integration of signals from TLR4 and IFN signaling pathways. Combined action of STAT1-containing transcription factor complexes and NF-κB provides a robust platform for transcriptional activation of a broad range of pro-inflammatory genes.[14] These processes are tightly controlled by HO-1. HO-1 seemed to be required for early activation of the type I IFN-inducing pathway. However, CO suppressed the capacity to

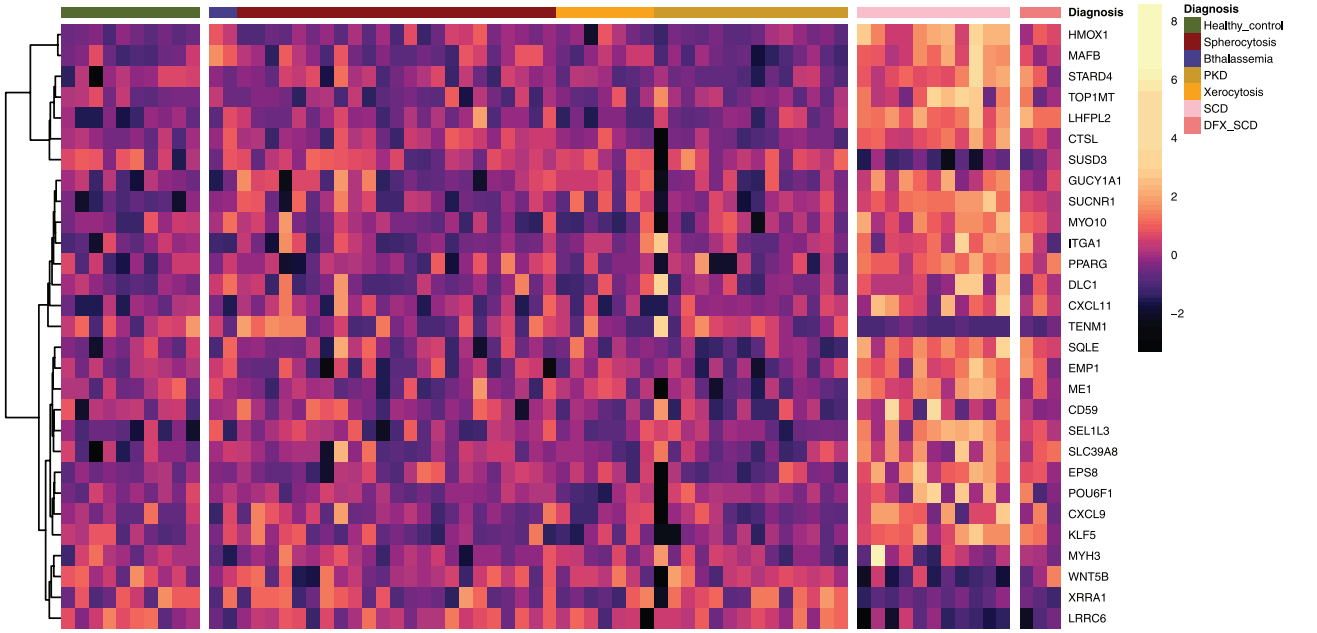

Figure 2. Heatmap of top 29 differentially expressed genes in SCD not treated with DFX compared with other hemolytic anemias and healthy controls. The heatmap is constructed with *Z* scores of quantile normalized, log2 transformed data. Hierarchical, unsupervised clustering of the genes (rows) was applied by the complete linkage method. This mathematical method defines the distance between 2 classes as the maximum (Euclidean) distance between 2 elements from each class. The pair of elements that have the smallest distance between each other are aggregated in a new element. The dendrogram provides a graphical representation of the aggregations of the clustering analysis. The columns of the heatmap are colored by diagnosis. DFX_SCD = deferasirox-treated sickle cell disease; PKD = pyruvate kinase deficiency; SCD = sickle cell disease.

secrete pro-inflammatory cytokines, including IFN-stimulated genes, in response to TLR4 activation.[15]

Moreover, we identified a profound upregulation of IFNγ-inducible cytokines *CXCL9-CXCL11* messenger RNA expression in SCD patients not on DFX, which are associated with Th1 polarization and activation. Monocytes do have a pivotal role in directing T cell fate: heme induced a relative regulatory T cell proliferation via monocyte HO-1, and subsequent CO production, and inhibited antigen-independent activation, proliferation, and maturation of naïve Th1 and $CD8^+$ T cells in SCD.[16]

Hence, the extent of pro-inflammatory signaling in SCD depends on crosstalk between various pathways, including type I and II IFN, TLR4, and the inhibitory effects mediated by HO-1.

Disruption of cellular cholesterol homeostasis occurs as part of the innate immune response.[17] Of particular interest is the upregulation of various enzymes involved in the mevalonate pathway (*SQLE*, *DHCR24*, *MSMO1*, *SC5D*, *HMGCS1*) and of genes presumably involved in lipid raft formation or preservation (*ABCG1*, *STARD4*).[18] Mevalonate is crucial for induction of trained immunity in monocytes, a feature characterized by increased expression of cytokines (tumor necrosis factor-α, interleukin [IL]-6, and IL-1β) and genes in the glycolytic pathway, and for a hyperinflammatory phenotype in specific diseases.[19] Inhibition of cholesterol synthesis with statins (3-hydroxy-3-methylglutaryl-coenzyme A [HMG-CoA] reductase inhibitors, which prevent conversion of HMG-CoA into mevalonate) could be an effective therapy for hyperinflammatory disorders in which trained immunity plays a role.[19] Upregulation of expression of the enzymes of mevalonate pathway in SCD suggests that this pathway contributes to the hyperinflammatory profile in SCD. Various reports on the efficacy of statins in prevention of vascular complications in SCD support this hypothesis.[20]

Generally, the acute inflammatory response inhibits reverse cholesterol transport back to the liver as a way to amplify inflammation. In our cohort, we observed in SCD monocytes a reduction of *ABCG1* transcript expression, encoding a cholesterol exporter. TLR4 signaling itself is known to suppress *ABCG1* expression in oxidized low-density lipoprotein-induced inflammation (one of the most identifiable toxic end products of intravascular heme release). Expression of the cholesterol exporters ABCA1 and ABCG1 was further decreased in response to trained immunity induction and could be counteracted with statin therapy.[19] Moreover, our data hints towards a previously suggested role for lipid rafts or caveolae formation in TLR4 signaling.[21] Remarkably, CO both inhibited translocation of TLR4 to lipid rafts,[22] and attenuated interaction between Caveolin-1 and TLR4 which dampens TLR4 signaling.[23]

In summary, both the mevalonate pathway and lipid rafts are known enhancers of TLR4 signaling. Our data shows the importance of both processes in pro-inflammatory signaling in SCD monocytes.

In conclusion, our analysis of the $CD14^+$ cell transcriptome in patients with hereditary hemolysis shows that patients with SCD have a characteristic gene expression pattern. This pattern includes upregulation of *HMOX1*, a signature of high intracellular iron and oxidative stress, and thereby underlines previous observations on the importance of e-DAMP molecules (including heme) in initiating pro-inflammatory signaling in SCD. Moreover, it shows that lipid metabolism and IFN signaling are important differentiating pro-immune signaling pathways. The unique SCD monocyte transcriptome also underlines the importance of both pro- and anti-inflammatory pathways. Coexistence of anti- and pro-inflammatory transcriptional activity has previously been shown in monocytes from sepsis patients.[24] And, in line with sepsis and sepsis recovery, the balance might shift in response to, for example, vaso-occlusive crises. We hypothesize that the relative upregulation of both pathways is associated with disease complications, especially vasculopathic complications, in SCD and that HO-1 has an important role in determining this balance. Importantly, all discussed pathways yield potentially druggable targets that possibly could reduce the pro-inflammatory phenotype in SCD and related (vasculopathic) complications.

Disclosures

The authors have no conflicts of interest to disclose.

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