D. & Hari, P. (2016) Heavy/light chain ratio normalization prior to transplant is of independent prognostic significance in multiple myeloma: a BMT CTN 0102 correlative study. *British Journal of Haematology*, **178**, 816.

Durie, B.G., Harousseau, J.L., Miguel, J.S., Bladé, J., Barlogie, B., Anderson, K., Gertz, M., Dimopoulos, M., Westin, J., Sonneveld, P., Ludwig, H., Gahrton, G., Beksac, M., Crowley, J., Belch, A., Boccadaro, M., Cavo, M., Turesson, I., Joshua, D., Vesole, D., Kyle, R., Alexanian, R., Tricot, G., Attal, M., Merlini, G., Powles, R., Richardson, P., Shimizu, K., Tosi, P., Morgan, G. & Rajkumar, S.V.; for the International Myeloma Working Group. (2006) International uniform response criteria for multiple myeloma. *Leukemia*, **20**, 1467–1473.

Kumar, S., Paiva, B., Anderson, K.C., Durie, B., Landgren, O., Moreau, P., Munshi, N., Lonial, S., Bladé, J., Mateos, M.V., Dimopoulos, M., Kastritis, E., Boccadoro, M., Orlowski, R., Goldschmidt, H., Spencer, A., Hou, J., Chng, W.J., Usmani, S.Z., Zamagni, E., Shimizu, K., Jagannath, S., Johnsen, H.E., Terpos, E., Reiman, A., Kyle, R.A., Sonneveld, P., Richardson, P.G., McCarthy, P., Ludwig, H., Chen, W., Cavo, M., Harousseau, J.L., Lentzsch, S., Hillengass, J., Palumbo, A., Orfao, A., Rajkumar, S.V., San Miguel, J. & Avet-Loiseau, H. (2016) International Myeloma Working Group consensus criteria for response and minimal residual disease assessment in multiple myeloma. *The Lancet Oncology*, **17**, e328–e346.

Ludwig, H., Milosavljevic, D., Zojer, N., Zojer, N., Faint, J.M., Bradwell, A.R., Hübl, W. & Harding, S.J. (2013) Immunoglobulin heavy/light chain ratios improve paraprotein detection and monitoring, identify residual disease and correlate with survival in multiple myeloma patients. *Leukemia*, **27**, 213–219.

Martínez-López, J., Paiva, B., López-Anglada, L., Mateos, M.V., Cedena, T., Vidríales, M.B., Sáez-Gómez, M.A., Contreras, T., Oriol, A., Rapado, I., Teruel, A.I., Cordón, L., Blanchard, M.J., Bengoechea, E., Palomera, L., de Arriba, F., Cueto-Felgueroso, C., Orfao, A., Bladé, J., San Miguel, J.F. & Lahuerta, J.J.; Spanish Multiple Myeloma Group/Program for the Study of Malignant Blood Diseases Therapeutics (GEM/PETHEMA) Cooperative Study Group. (2015) Critical analysis of the stringent complete response in multiple myeloma: contribution of sFLC and bone marrow clonality. *Blood*, **126**, 858–862.

Paolini, L., Di Noto, G., Maffina, F., Martellosio, G., Radeghieri, A., Luigi, C. & Ricotta, D. (2015) Comparison of Hevylite™ IgA and IgG assay with conventional techniques for the diagnosis and follow-up of plasma cell dyscrasia. *Annals of Clinical Biochemistry*, **52**, 337–345.

Suehara, Y., Takamatsu, H., Fukumoto, K., Fujisawa, M., Narita, K., Usui, Y., Takeuchi, M., Endean, K. & Matsue, K. (2017) Abnormal heavy/light chain ratio after treatment is associated with shorter survival in patients with IgA myeloma. *Cancer Science*, **108**, 187–192.

Tacchetti, P., Pezzi, A., Zamagni, E., Pantani, L., Rocchi, S., Zannetti, B.A., Mancuso, K., Rizzello, I. & Cavo, M. (2017) Role of serum free light chain assay in the detection of early relapse and prediction of prognosis after relapse in multiple myeloma patients treated upfront with novel agents. *Haematologica*, **102**, e104–e107.

Haem augments and iron chelation decreases toll-like receptor 4 mediated inflammation in monocytes from sickle cell patients

A central role of inflammation in the pathophysiology of sickle cell disease (SCD) is supported by clinical observations. Both an elevated leucocyte count and C-reactive protein (CRP) are associated with early death in SCD (Platt *et al*, 1994; van Beers *et al*, 2015). We have shown that iron-regulated gene expression is associated with striking upregulation of inflammasome pathway gene expression, including a 200-fold increase in Toll-like receptor 4 (TLR4) expression in peripheral blood mononuclear cells, suggesting a cross-talk between iron and inflammation pathways (van Beers *et al*, 2015). Haem, a form of iron, augments pro-inflammatory TLR4 signalling in sickle cell mice, with subsequent inflammation, vaso-occlusion, organ damage and death (Ghosh *et al*, 2013; Belcher *et al*, 2014; Vinchi *et al*, 2016). TLR4 is highly expressed on macrophages and peripheral blood monocytes, and its ligand, lipopolysaccharide (LPS), induces expression of the pro-inflammatory cytokine interleukin 6 (IL6). We hypothesized that intracellular iron is involved in this LPS induction of IL6.

Subjects were recruited under a protocol approved by the National Institutes of Health (NIH) Institutional Review Board (ClinicalTrials.gov identifier NCT00542230). Blood was obtained from 18 patients with homozygous sickle cell anaemia (HbSS) in steady state and from 10 healthy controls. See Supplemental Table SI for baseline characteristics.

We aliquoted 1 ml of heparinized fresh whole blood from SCD patients or controls, blocked cytokine secretion with Brefeldin A, added iron chelator [0·1 mmol/l deferasirox, haem (20 μmol/l) and/or LPS (1, 10 or 100 ng/ml)] and incubated the tubes at 37°C. After a 3-h incubation, samples were put on ice and the percentage monocytes expressing intracellular IL6 were quantified using intracellular staining by flow cytometry.

Surprisingly, monocytes of patients and controls had a comparable number of IL6 positive monocytes after stimulation with LPS, haem or iron chelation and comparable intracellular iron. This report therefore presents the pooled analysis of these experiments.

After 3 h of stimulation with LPS, the median percentage of monocytes expressing IL6 was 83·4% (81·0–89·6). Iron chelation diminished this percentage significantly, to 62·0 (54·9–71·8, $P = 0·004$; Fig 1C). The TLR4 inhibitor TAK-242 inhibited the response to LPS. In the absence of LPS, haem

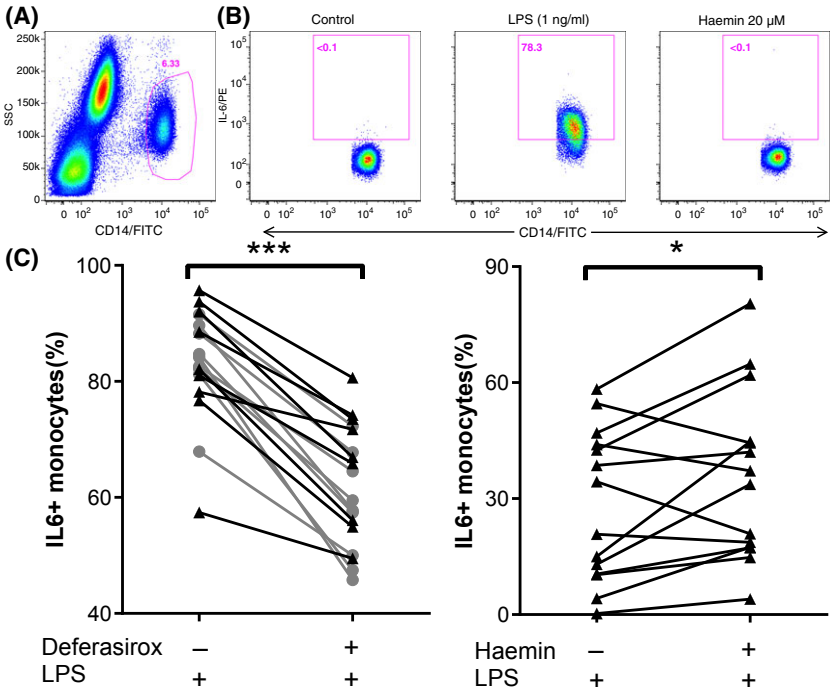

Fig 1. Haem increases and iron chelation decreases TLR4 signaling. Fresh whole blood from patients (n = 10) and controls (n = 10) was incubated with combinations of vehicle, a Toll-like receptor-4 (TLR4) agonist [lipopolysaccharide (LPS)] or 20 μmol/l haem. After 3 h, the percentage of monocytes with detectable levels of intracellular interleukin 6 (IL6) was quantified by flowcytometry. (A) Monocytes were identified using side scatter (SSC) and CD14-positivity. (B) Representative results of monocytes from sickle cell disease patients that had been treated with vehicle, LPS or Haem. (C) Compared to incubation with high dose LPS (100 ng/ml) alone, co-incubation with the iron chelator deferasirox significantly decreased the absolute percentage of IL6-producing monocytes by 20·4% (15·2–26·3) (P = 0·004) (D) In contrast, compared to incubation with low dose LPS (1 ng/ml or 10 ng/ml) alone, co-incubation with LPS and haem increased the absolute percentage of monocytes producing IL6 with a median 5·7% (interquartile range −3·3 to 19·7, P = 0·046). ***P < 0·005, *P < 0·05. Black triangles denote patients. Grey spheres denote healthy controls. [Colour figure can be viewed at wileyonlinelibrary.com]

was insufficient to induce IL6 producing monocytes (Fig 1B). In contrast, haem potentiated the effect of LPS (1 ng/ml) with a median of 5·7% (−3·3 to 19·7, P = 0·046) compared to LPS alone (Fig 1D).

Finally, we evaluated the intracellular free or chelatable iron content of the monocytes (as quantified by the calcein assay; Epsztejn *et al*, 1997) and the plasma level of CRP, a marker of *in vivo* inflammation. The monocyte intracellular chelatable iron [expressed as delta mean fluorescence intensity (ΔMFI) (arbitrary units)] in patients and controls was comparable [median (interquartile range) 192 (160–333) vs. 218 (161–271)]. The individual monocyte chelatable iron pool correlated positively with individual plasma levels of CRP (Spearman R = 0·454, P = 0·044), supporting a relationship of intracellular iron to inflammation.

Our results in samples from SCD patients and healthy controls are consistent with previous results obtained in animals (Fernandez *et al*, 2010; Belcher *et al*, 2014). Figueiredo *et al* (2007) had previously shown that the addition of haem but not protoporphyrin IX (porphyrin without iron) to thioglycollate-activated macrophages increased TLR4-mediated inflammation, and that haem alone did not induce inflammation (Fernandez *et al*, 2010). Importantly, Fernandez *et al* (2010) showed that this non-specific pro-inflammatory effect of haem can be mimicked by the addition of paraquat, an strong oxidant, and can be inhibited either by iron chelation or anti-oxidants. All of these results suggest that haem alone cannot induce TLR4 signalling, but rather that the pro-oxidant effect of haem-bound iron amplifies the activity of TLR4 ligands. Although this pro-inflammatory effect of haem is thus not specific to SCD monocytes, its downstream effect in SCD pathophysiology is profound in SCD mice, resulting in cell adhesion, organ damage and death (Ghosh *et al*, 2013; Belcher *et al*, 2014).

Given that SCD monocytes are exposed to intravascular haemolysis and free haem *in vivo*, we expected a higher intracellular iron and higher response to LPS in monocytes from SCD patients compared to healthy controls. We speculate that we did not find this because chronic exposure of monocytes to haem leads to its rapid export, involving upregulation of the iron exporter ferroportin (Theurl *et al*, 2016), probably maintaining normal levels of intracellular free iron in monocytes and subsequent normal response to LPS. It is also possible that intracellular iron level is mitigated via the induction of ferroxidase activity by ferritin heavy chain, as documented in SCD mice (Vercellotti *et al*,

2014). IL6 induction might also be dampened by anti-inflammatory IL10, which we found to be associated with iron-regulated genes in monocytes of SCD patients (van Beers *et al*, 2015).

In the light of present literature, our human data strongly support animal data indicating that haem iron is able to augment ligand (LPS)-induced TLR4 signalling. There is increasing evidence that inflammation plays a central role in SCD pathophysiology. Our results show that the iron chelator deferasirox ameliorates the proinflammatory effect of haem iron *ex vivo*. Deferasirox, widely used in patients with transfusional iron overload, readily achieves plasma levels comparable to our assay concentration of 0·1 mmol/l. Therefore, iron chelation could provide an interesting, readily available alternative therapeutic option to reduce inflammatory burden in SCD. This hypothesis merits further evaluation.

In conclusion, we suggest that haem-bound iron, which is released during intravascular haemolysis and scavenged by monocytes, can contribute to activation and pro-inflammatory state in human SCD monocytes, by augmenting TLR4 signalling, consistent with SCD mice models.

Author contributions

All authors critically reviewed and approved the submitted and final versions of the paper. P.D., E.B. and L.M. performed the research. J.N., L.M. and C.S. contributed to acquisition of data and the inclusion of patients. P.D., E.B. J.M. and G.K. designed the study and interpreted the data. P.D. and E.B. analysed the data and drafted the paper.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Baseline characteristics of patients and controls.

References

van Beers, E.J., Yang, Y., Raghavachari, N., Tian, X., Allen, D.T., Nichols, J.S., Mendelsohn, L., Nekhai, S., Gordeuk, V.R., Taylor, J.G. & Kato, G.J. (2015) Iron, inflammation, and early death in adults with sickle cell disease. *Circulation Research*, **116**, 298–306.

Belcher, J.D., Chen, C., Nguyen, J., Milbauer, L., Abdulla, F., Alayash, A.I., Smith, A., Nath, K.A., Hebbel, R.P. & Vercellotti, G.M. (2014) Heme triggers TLR4 signaling leading to endothelial cell activation and vaso-occlusion in murine sickle cell disease. *Blood*, **123**, 377–390.

Epsztejn, S., Kakhlon, O., Glickstein, H., Breuer, W. & Cabantchik, I. (1997) Fluorescence analysis of the labile iron pool of mammalian cells. *Analytical Biochemistry*, **248**, 31–40.

Fernandez, P.L., Dutra, F.F., Alves, L., Figueiredo, R.T., Mourão-Sa, D., Fortes, G.B., Bergstrand, S., Lönn, D., Cevallos, R.R., Pereira, R.M.S., Lopes, U.G., Travassos, L.H., Paiva, C.N. & Bozza, M.T. (2010) Heme amplifies the innate immune response to microbial molecules through Syk-dependent ROS generation. *The Journal of Biological Chemistry*, **285**, 32844–32851.

Figueiredo, R.T., Fernandez, P.L., Mourao-Sa, D.S., Porto, B.N., Dutra, F.F., Alves, L.S., Oliveira, M.F., Oliveira, P.L., Graça-Souza, A.V. & Bozza, M.T. (2007) Characterization of heme as activator of Toll-like receptor 4. *The Journal of Biological Chemistry*, **282**, 20221–20229.

Ghosh, S., Adisa, O.A., Chappa, P., Tan, F., Jackson, K.A., Archer, D.R. & Ofori-Acquah, S.F. (2013) Extracellular hemin crisis triggers acute chest syndrome in sickle mice. *The Journal of Clinical Investigation*, **123**, 4809–4820.

Platt, O.S., Brambilla, D.J., Rosse, W.F., Milner, P.F., Castro, O., Steinberg, M.H. & Klug, P.P. (1994) Mortality in sickle cell disease. Life expectancy and risk factors for early death. *The New England Journal of Medicine*, **330**, 1639–1644.

Theurl, I., Hilgendorf, I., Nairz, M., Tymoszuk, P., Haschka, D., Asshoff, M., He, S., Gerhardt, L.M.S., Holderried, T.A.W., Seifert, M., Sopper, S., Fenn, A.M., Anzai, A., Rattik, S., McAlpine, C., Theurl, M., Wieghofer, P., Iwamoto, Y., Weber, G.F., Harder, N.K., Chousterman, B.G., Arvedson, T.L., McKee, M., Wang, F., Lutz, O.M.D., Rezoagli, E., Babitt, J.L., Berra, L., Prinz, M., Nahrendorf, M., Weiss, G., Weissleder, R., Lin, H.Y. & Swirski, F.K. (2016) On-demand erythrocyte disposal and iron recycling requires transient macrophages in the liver. *Nature Medicine*, **22**, 945–951.

Vercellotti, G.M., Khan, F.B., Nguyen, J., Chen, C., Bruzzone, C.M., Bechtel, H., Brown, G., Nath, K.A., Steer, C.J., Hebbel, R.P. & Belcher, J.D. (2014) H-ferritin ferroxidase induces cytoprotective pathways and inhibits microvascular stasis in transgenic sickle mice. *Frontiers in Pharmacology*, **5**, 79.

Vinchi, F., Costa da Silva, M., Ingoglia, G., Petrillo, S., Brinkman, N., Zuercher, A., Cerwenka, A., Tolosano, E. & Muckenthaler, M.U. (2016) Hemopexin therapy reverts heme-induced proinflammatory phenotypic switching of macrophages in a mouse model of sickle cell disease. *Blood*, **127**, 473–486.