

Altered heme-mediated modulation of dendritic cell function in sickle cell alloimmunization

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METHODS

Human samples

All studies were approved by the Institutional Review Boards of the New York Blood Center, Children's Hospital of Philadelphia and Montefiore Medical Center. De-identified fresh leukocyte-enriched products were obtained from healthy donors (from New York Blood Center). For SCD patients' samples, blood was obtained solely from discard apheresis waste bags collected during erythrocytapheresis procedures. Waste bags were collected from patients aged 15-34 on chronic red cell exchange therapy (every 3-4 weeks for at least two years using leukodepleted units, phenotype matched for the C, E and K red cell antigens). Informed consent was obtained from all patients prior to their participation in the study. For each patient, multiple samples (2 to 5) were analyzed on different occasions to ensure reproducibility. However, not all patient samples were tested for all experiments as the cell numbers were limited. None of the alloimmunization status changed during the study. Patients were grouped either as "non-alloimmunized" (n=6), having no history of antibody production versus "alloimmunized" (n=12), with a history of at least one alloantibody. The apheresis waste bags stripped of all identifiers except the alloimmunization state were sent to the New York Blood Center and processed within 18 hours of collection. Patients' relevant clinical information is shown in Table 1.

Antibodies

Anti-CD4 APC-Cy7 (RPA-T4), IL-6 PE (MQ2-6A3), IL-12p40 APC (C11.5), CD80 PE (L307.4), CD86 APC (2331) and HLA-DR FITC (G46-6) were from BD Pharmingen: Anti-IL-4 PE (8D4-8), TNF PerCP-Cy5.5 (MAb11), IL-17A PE-Cy7 (eBio64DEC17), IFN γ APC (4S-B3), CD11c AF700 (3.9), CD14 PerCP-Cy5.5 (61D3), TNF PE-Cy7 (Mab11) and CD83 PE-Cy7 (HD15e) were from eBioscience. Anti-HO-1 (HO-1-1) was from Thermo Scientific. Anti-TLR4 (Goat IgG) blocking antibody was from R&D Systems.

Reagents

Hemin, oxidized form of the heme moiety of hemoglobin, and Biliverdin were purchased from Frontier Scientific and were dissolved in 0.2M NaOH, neutralized to pH 7.2 with HCl and adjusted to stock concentration with distilled water.¹ CORM-3, tin protoporphyrin (SnPPiX) and

zinc protoporphyrin ZnPPiX were from Sigma-Aldrich. TLR agonists (LPS and R848) were from InvivoGen. IFN γ , IL-4 and GM-CSF were from Miltenyi Biotech.

Monocyte-Derived Dendritic Cell Preparation and T Cell Priming

Peripheral blood mononuclear cells (PBMCs) were purified and plated in complete RPMI with 5% pooled human serum. Cells were allowed to adhere for 2 hr at 37°C. Nonadherent cells were removed. The adherent monocyte-enriched fraction was supplemented with 100 IU/ml rhGM-CSF and 300 IU/ml rhIL-4 (Miltenyi Biotech) on days 0, 2, and 4. Immature DCs were harvested on day 5, treated to various concentrations of hemin (0, 5 or 20 μ M) for 2 hours and without washing out the hemin, matured/stimulated (or not for immature conditions) for 2 days using various TLR agonists: 100ng/ml LPS, 100ng/ml LPS + 500IU/ml IFN γ , or 5 μ M R848, classically known as T_H1 skewing conditions. For T cell priming studies, purified (5x10⁴) naïve (CD45RA⁺) CD4⁺ T cells (Miltenyi Biotech) from healthy donors were CFSE labeled (10 μ g/ml), added to washed allogeneic moDCs (derived from SCD patients or healthy donors, 5x10³) and incubated for 10 days in the presence of low concentration of rhIL-2 (5 IU/ml, Miltenyi Biotech), changing the media every 2-3 days. Expression of cytokines in divided (CFSE^{low}) CD4⁺ cells was detected following PMA/ionomycin in the presence of brefeldin A. T cell priming toward T_H1, T_H2 and T_H17 was defined as the frequency of CFSE^{low} CD4⁺ cells expressing IFN γ , IL-4 and IL-17, respectively.

Surface expression of CD80, CD83 and CD86 was determined at day 2 after maturation (or not). Intracellular cytokine expression (IL-12p40 and TNF α) was assessed after overnight stimulation in the presence of brefeldin A.

ELISA-based assays

IL12p70 ELISA was performed on day 2 supernatants (from 5x10³ DCs) using Ready-SET-Go! Kit (eBioscience) according to the manufacturer's instructions. NF- κ B transcription factors were detected in nuclear fractions obtained from 8x10⁶ moDCs using the Nuclear Extract Kit (Active Motif) after 2h stimulation with 5 μ M R848 in the presence or in the absence of 20 μ M hemin. Levels of the various transcription factors were assessed using TransAM technology (Active Motif), according to the manufacturer's instructions.

Statistical analysis

Separate analyses were performed for each experiment individually and shown as individual data points in most figures. In addition, for most experiments, each patient data point is an average of at least 2-5 independent experiments using samples collected on different occasions from the same patient. In some figures, data are represented as mean values \pm sem. Due to biologic variability between individuals, we performed paired t-test comparing before and after heme treatment in each individual. P values ≤ 0.05 were considered statistically significant.

REFERENCES

1. Pamplona A, Ferreira A, Balla J, et al. Heme oxygenase-1 and carbon monoxide suppress the pathogenesis of experimental cerebral malaria. *Nat Med.* 2007;13(6):703-710.

Figure S1

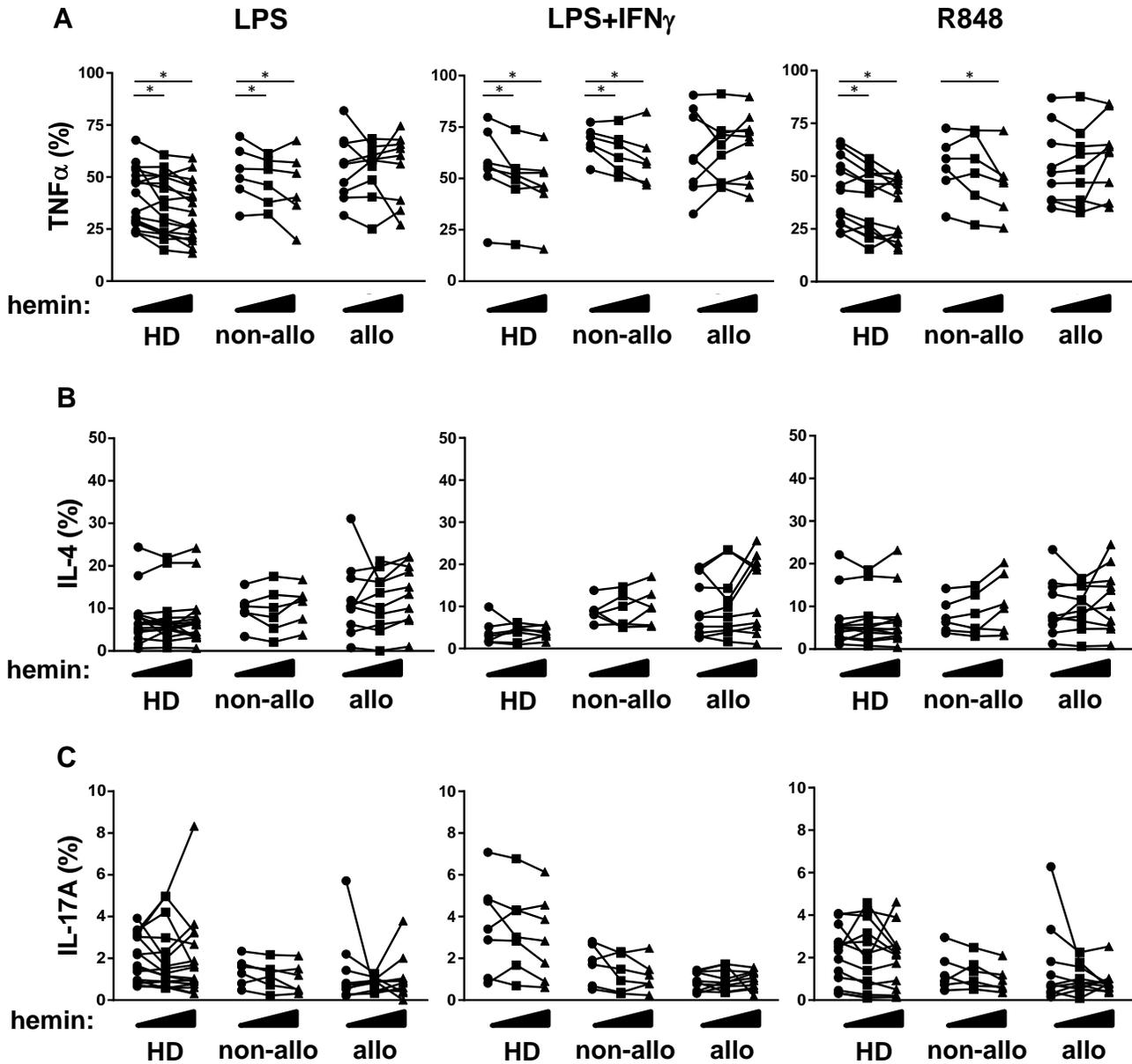


Figure S1. Effect of hemin on moDC-mediated CD4⁺ T cell priming. Immature moDCs were pre-treated or not with hemin (0, 5 or 20 μ M) at day 0. Without washing out the hemin, maturation with LPS, LPS+IFN γ or R848 was performed 2h later. After 2 days, allogeneic CFSE-stained naïve CD4⁺ T cells were stimulated by moDCs. Twelve days later, cells were stimulated with PMA/ionomycin for 5h in the presence of brefeldin A and stained for CD4 as well as TNF α (A), IL-4 (B) and IL-17A (C). * p<0.05

Figure S2

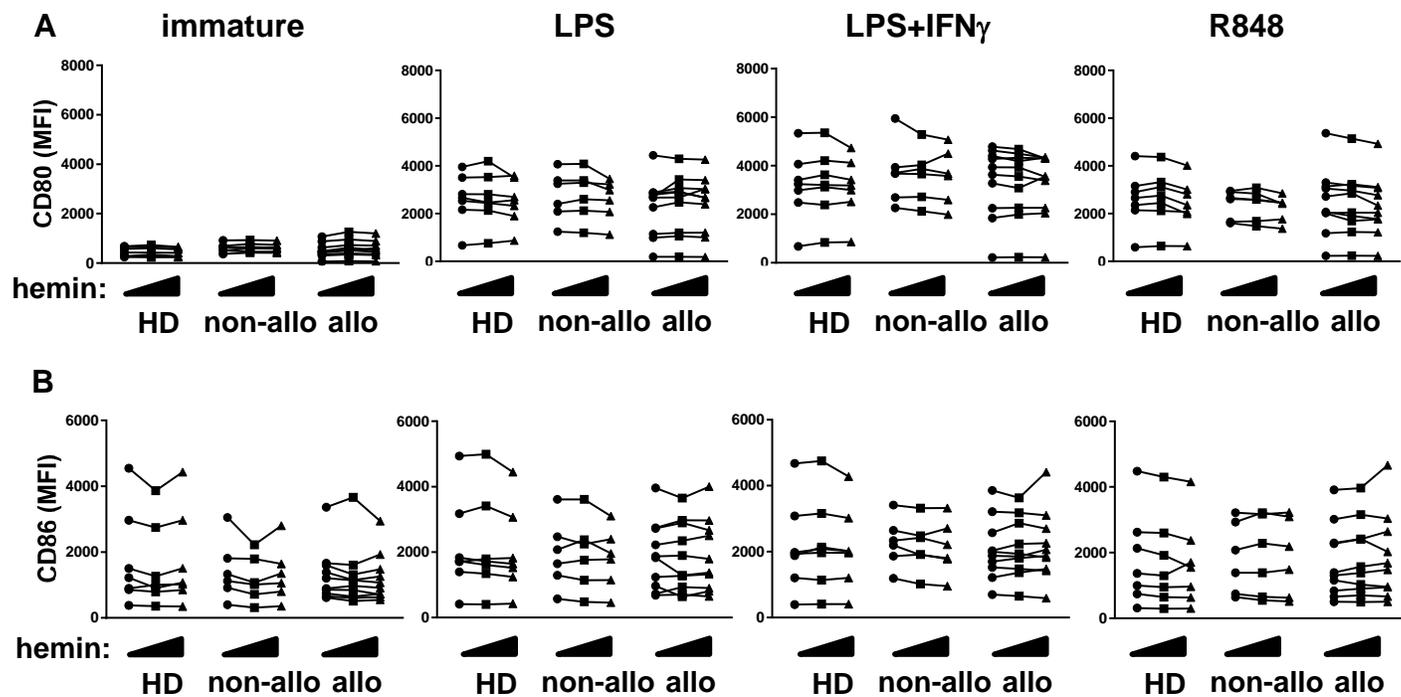


Figure S2. Effect of hemin on moDCs maturation. Immature moDCs were pre-treated or not with hemin (0, 5 or 20 μ M) at day 0. Maturation was performed 2h later. After 2 days, CD80 (A) and CD86 (B) levels were assessed by immunostaining.

Figure S3

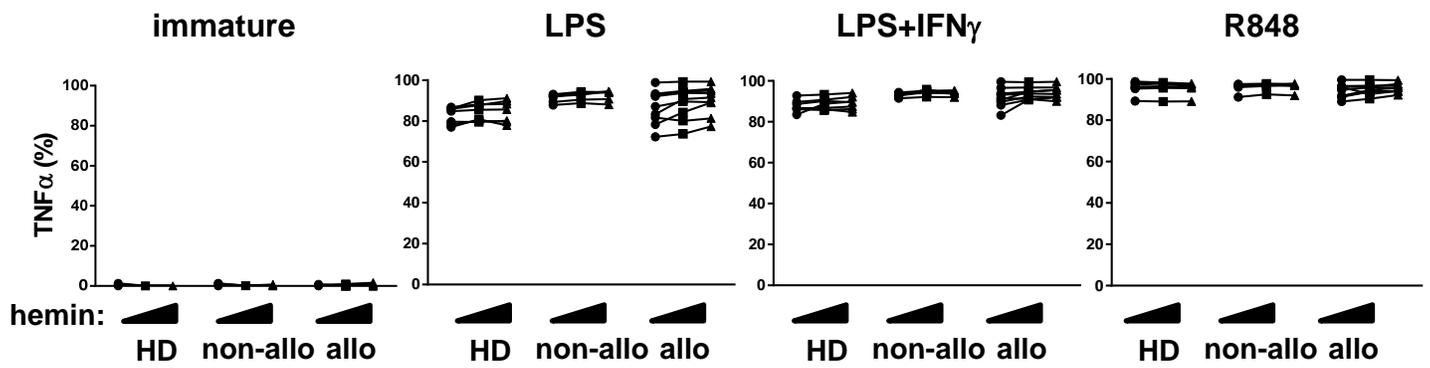


Figure S3. Effect of hemin on moDCs-derived TNF α . Immature moDCs were pre-treated or not with hemin (0, 5 or 20 μ M) at day 0. Maturation was performed 2h later. The next day, TNF α levels were assessed by immunostaining after overnight incubation with brefeldin A.

Figure S4

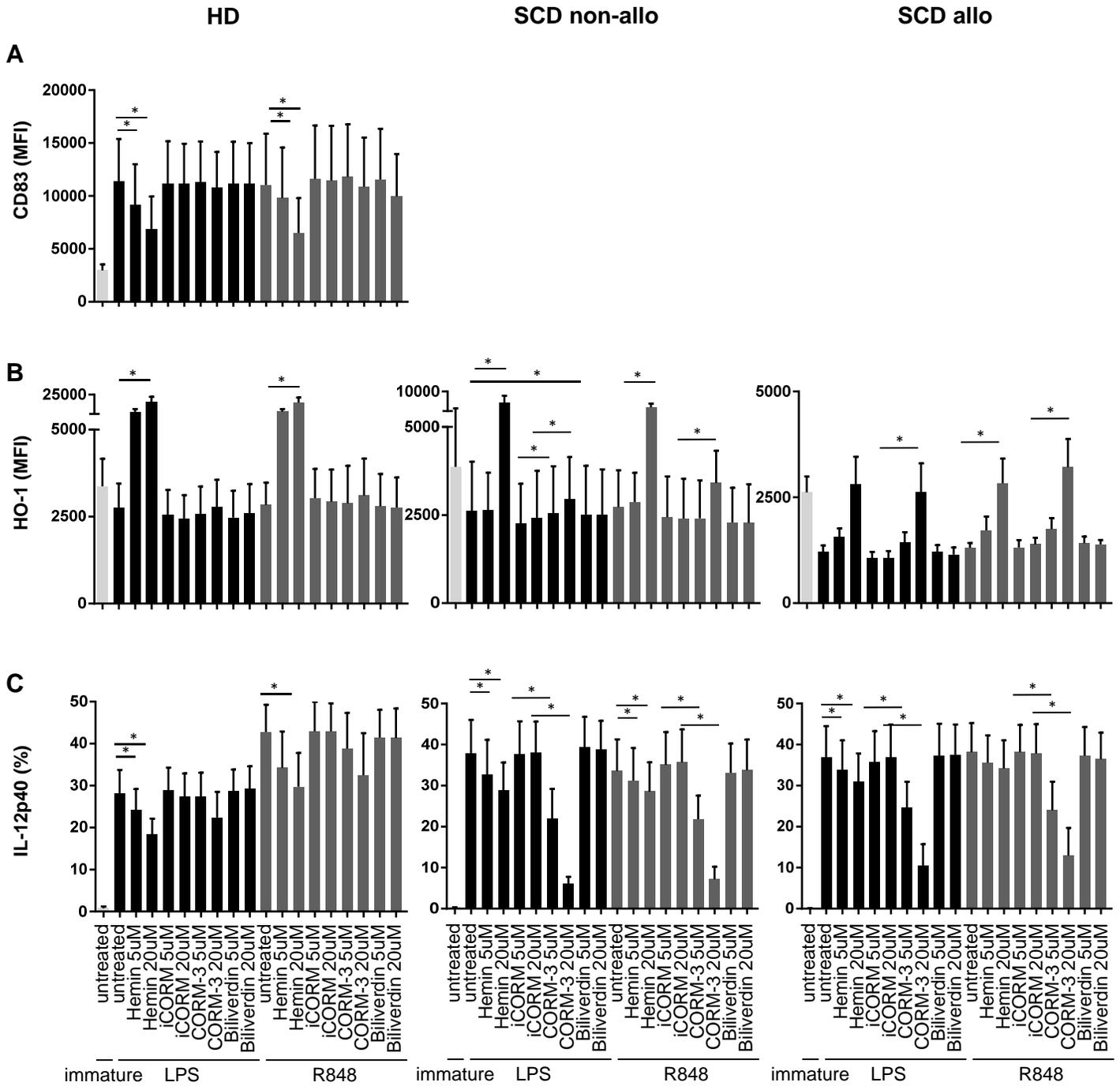


Figure S4. Effect of hemin products on moDCs. Immature moDCs were pre-treated or not with indicated doses of hemin products or with hemin at day 0. Maturation was performed 2h later. After 2 days, CD83 (A) and HO-1 (B) levels were assessed by immunostaining. CD83 levels for SCD patients are shown in Fig4C (C) IL-12p40 levels were assessed by immunostaining after overnight incubation with brefeldin A.

* p<0.05

Figure S5

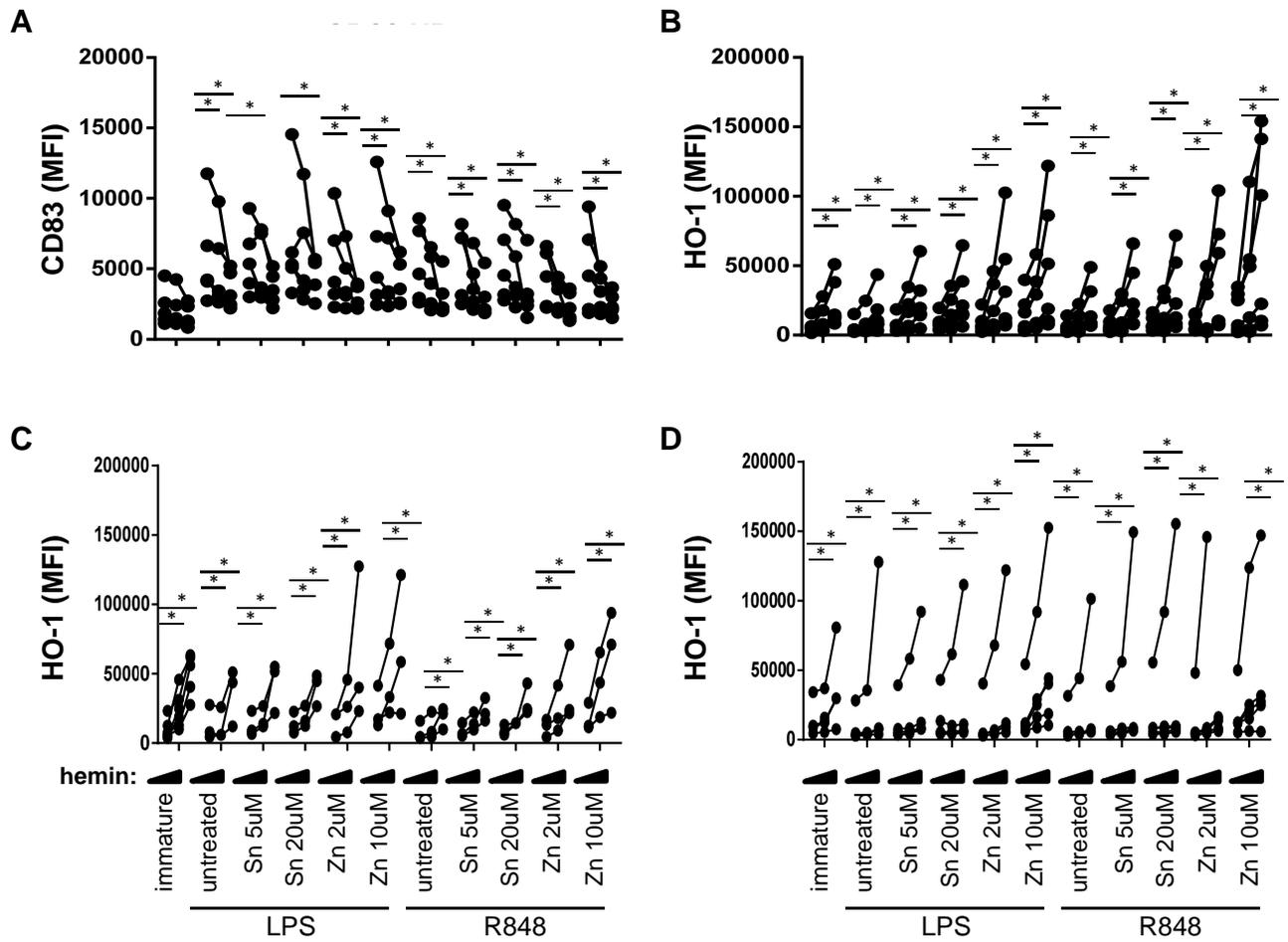


Figure S5. Effect of HO-1 inhibitors on moDCs. HO-1 inhibitors (SnPPPIX and ZNPPPIX) were used to treat immature moDCs. Hemin (0, 5 or 20 μ M) was added two hours later. After 2 additional hours, moDCs were matured or not. CD83 (A) and HO-1 (B-D) were detected at day 2 in healthy donors (A,B), non-alloimmunized (C) and alloimmunized SCD patients (D). CD83 levels for SCD patients are shown in Fig4D. * $p < 0.05$

Figure S6

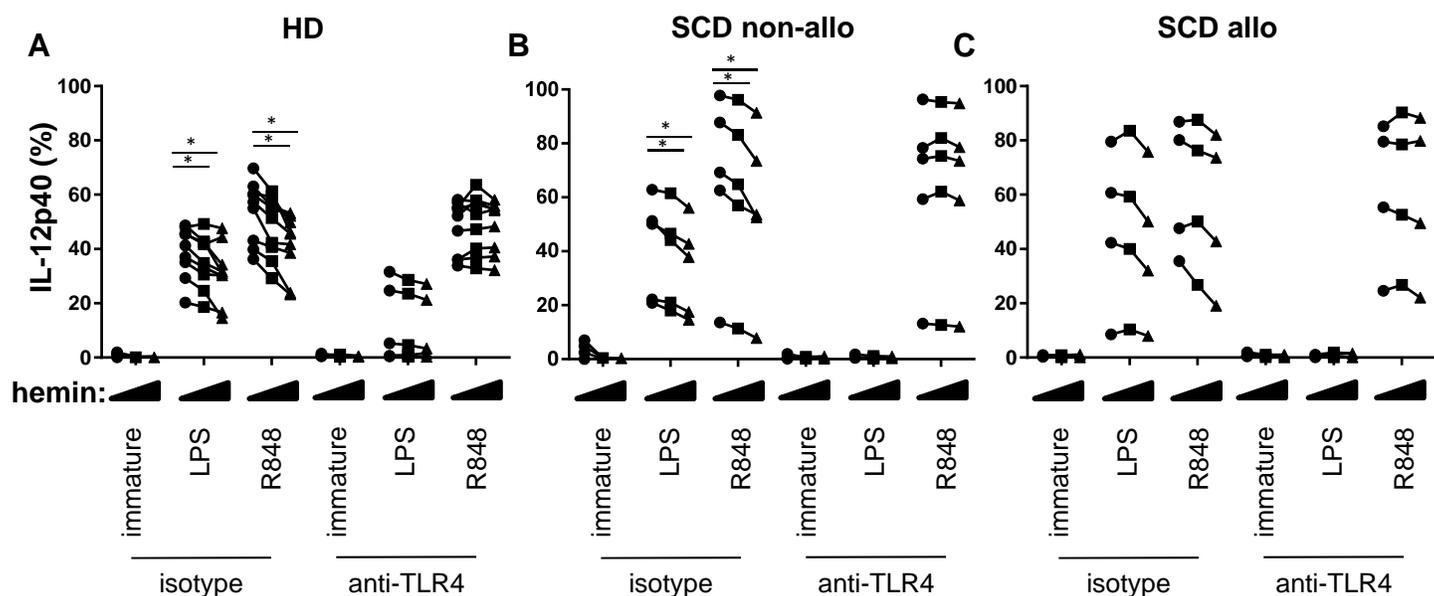


Figure S6. Hemin blocks DC maturation in a TLR4-dependent pathway. Immature moDCs from healthy donors (A), non-alloimmunized (B) or alloimmunized patients (C) were incubated for 1h in the presence of either an isotype control or a blocking antibody for TLR4 (20ug/ml). Hemin (0, 5 or 20μM) was then added for 2h before maturation. IL-12p40 was assessed after overnight incubation in the presence of brefeldin A. * p<0.05