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

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ABSTRACT

Transfusions are the main treatment for patients with sickle cell disease. However, alloimmunization remains a major life-threatening complication for these patients, but the mechanism underlying pathogenesis of alloimmunization is not known. Given the chronic hemolytic state characteristic of sickle cell disease, resulting in release of free heme and activation of inflammatory cascades, we tested the hypothesis that anti-inflammatory response to heme is compromised in alloimmunized sickle patients, increasing their risk of alloimmunization. Heme-exposed monocyte-derived dendritic cells from both non-alloimmunized sickle patients and healthy donors inhibited priming of pro-inflammatory CD4$^+$ type 1 T cells, and exhibited significantly reduced levels of the maturation marker CD83. In contrast, in alloimmunized patients, heme did not reverse priming of pro-inflammatory CD4$^+$ cells by monocyte-derived dendritic cells or their maturation. Furthermore, heme dampened NF-κB activation in non-alloimmunized, but not in alloimmunized monocyte-derived dendritic cells. Heme-mediated CD83 inhibition depended on Toll-like receptor 4 but not heme oxygenase 1. These data suggest that extracellular heme limits CD83 expression on dendritic cells in non-alloimmunized sickle patients through a Toll-like receptor 4-mediated pathway, involving NF-κB, resulting in dampening of pro-inflammatory responses, but that in alloimmunized patients this pathway is defective. This opens up the possibility of developing new therapeutic strategies to prevent sickle cell alloimmunization.

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Introduction

Sickle cell disease (SCD) results from a mutation in the β-globin gene causing hemoglobin to polymerize when deoxygenated to form rigid polymers within red blood cells (RBC), which leads to complications including chronic hemolytic anemia.[1] Transfusion therapy remains an important treatment modality for patients with SCD. Despite its therapeutic benefits, 20%-60% patients with SCD develop alloantibodies with specificities against disparate antigens on transfused RBC, causing complications ranging from life-threatening hemolytic transfusion reactions, to logistical problems in finding compatible RBC for transfusion.[2] The immunological basis for SCD alloimmunization remains ill-defined. Consistent with the importance of CD4$^+$ helper T cells (T_H) in driving B-cell responses, several studies have identified altered T_H cell phenotypes and/or activity in alloimmunized patients with SCD.[3-7] Given the ongoing hemolysis in SCD,[8] we had previously investigated the effects of RBC breakdown product heme on immune responses of patients, with and without alloantibodies, undergoing chronic transfusion therapy, and found altered anti-inflammatory response to exogenous heme by monocytes from alloimmunized patients with SCD, resulting in a T-cell profile with heightened pro-

inflammatory (T_H1), but lower anti-inflammatory (T_{REG}) T-cell subsets.[9] These data suggested aberrant innate immune control of T-cell polarization in SCD alloimmunization, although the exact nature of the innate immune cell type or underlying molecular mechanism for these alterations remains elusive.

Dendritic cells (DCs) are key antigen presenting cells in initiating/shaping T-cell immune responses.[10] During an inflammatory response, they can be activated/matured by toll-like receptor (TLR) ligands. Once activated, they migrate to the lymphoid organs to activate/prime naïve T cells into effector cells.[11] The DC maturation process which is key to initiate T-cell responses, involves upregulation of co-stimulation molecules, e.g. CD80, CD86, and *de novo* expression of CD83, as well as cytokine secretion.[12] In response to a homolog of heme, TLR-matured human monocyte derived DCs (moDCs), in a non-SCD setting, were shown to display less immunogenic properties, including lower expression of DC maturation markers and proinflammatory cytokines than untreated DCs.[13] Although this has not yet been tested, less immunogenic DCs are likely to dampen proinflammatory T-cell polarization profiles, thereby reducing the risk of mounting immune responses, including humoral responses. In this study, we tested the hypothesis that, in response to exogenous heme, DCs differentially shape T-cell polarization toward pro-inflammatory (T_H1) phenotype in alloimmunized compared to non-alloimmunized SCD patients.

Methods

Human samples

All studies were approved by the Institutional Review Boards of the New York Blood Center (NYBC), the Children's Hospital of Philadelphia, and the Montefiore Medical Center. De-identified fresh leukocyte-enriched products were obtained from NYBC's healthy donors. For SCD patient samples, blood was obtained solely from discarded apheresis waste bags collected during erythrocytapheresis procedures from patients aged 15-34 years on chronic red cell exchange therapy (every 3-4 weeks for at least 2 years using leuko-depleted units, phenotype-matched for the C, E and K red cell antigens; see *Online Supplementary Appendix*).

T-cell priming and DC analysis

Monocyte-derived DCs (moDCs) were prepared from peripheral blood mononuclear cells (PBMC) (see *Online Supplementary Appendix*). CFSE labeled purified ($5x10^4$) naïve ($CD45RA^+$) $CD4^+$ T cells from healthy donors were added to allogeneic moDCs (derived from SCD patients or healthy donors; $5x10^3$). T-cell priming toward T_H1, T_H2 and T_H17 detected after ten days was defined as the frequency of $CFSE^{low}$ (divided) $CD4^+$ cells expressing IFNγ, 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 TNFα) was assessed after overnight stimulation in the presence of brefeldin A while IL12p70 ELISA was performed on day 2 supernatants, and NF-κB transcription factors in nuclear fractions were analyzed using TransAM technology (Active Motif), as described in the *Online Supplementary Appendix*.

Statistical analysis

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. Due to biological variability between individuals, we performed a paired *t*-test comparing before and after heme treatment in each individual. $P \leq 0.05$ was considered statistically significant. [Data are represented as mean values±standard error of the mean (SEM) where indicated].

Results

Hemin-treated moDCs down-regulate priming of IFNγ-producing CD4+ T cells in non-alloimmunized, but not in alloimmunized SCD patients

Because of ongoing hemolysis in SCD, we studied the effect of exogenously added hemin, as a surrogate for hemoglobin breakdown product,[8,14-18] on moDCs in driving naïve T-cell differentiation, known as T-cell priming. Three different, but classical, DC maturation agents were used (LPS, LPS+ IFNγ or R848) and the effects of heme exposure during DC maturation on priming of allogeneic naïve T cells from healthy donors were studied. Baseline (without hemin) frequency of primed IFNγ-producing $CD4^+$ cells (T_H1) by moDCs from healthy donors and SCD patients were comparable ($P>0.1$). Hemin treatment (5 μM or 20 μM) resulted in significant inhibition of T_H1 priming by moDCs from healthy donors as well as non-alloimmunized SCD patients irrespective of the DC maturation method used: approximately 25% less IFNγ-producing cells were primed by DCs treated with 20 μM hemin as compared to untreated cells ($n>7$, $P<0.002$) (Figure 1A-D). In contrast, hemin had no effect on T_H1 priming by moDCs from alloimmunized SCD patients: IFNγ was not down-regulated in $CD4^+$ T cells primed by hemin-exposed moDCs from alloimmunized patients using any of the DC maturation methods ($P>0.21$, $n=9$) (Figure 1B-D). Similarly, TNFα- (T_H1) producing $CD4^+$ T cells (*Online Supplementary Figure S1A*) were significantly lower when primed with heme-exposed DCs from healthy donor and non-alloimmunized SCD patients but not from the alloimmunized group. IL-4 production was not affected by hemin-treated moDCs in this system (*Online Supplementary Figure S1B*). Only low levels of IL-17A were detected, precluding the assessment of the effects of hemin on these cells (*Online Supplementary Figure S1C*). These results show that while hemin dampens the ability of moDCs to prime T_H1 cells in non-alloimmunized SCD patients, it has little or no effect in alloimmunized patients.

Hemin down-regulates CD83 on moDCs derived from non-alloimmunized, but not from alloimmunized SCD patients

To investigate the mechanism of altered heme-regulation of immunogenicity in alloimmunized SCD patients, we first assessed the effect of hemin on the expression of co-stimulation molecules CD80 (*Online Supplementary Figure S2A*) and CD86 (*Online Supplementary Figure S2B*) on moDCs. Both molecules were up-regulated upon maturation, but neither was affected by hemin irrespective of whether moDCs were derived from healthy donors or SCD patients, (*Online Supplementary Figure S2A and B*). We next tested the expression of CD83 maturation marker before and after hemin treatment (Figure 2). As expected, immature DCs expressed only very low CD83 levels (Figure 2A and B) which were up-regulated upon maturation, albeit to the same extent in both healthy donor and patient groups in the absence of heme (Figure 2A, C-E). Upon hemin treatment, CD83 expression was inhibited

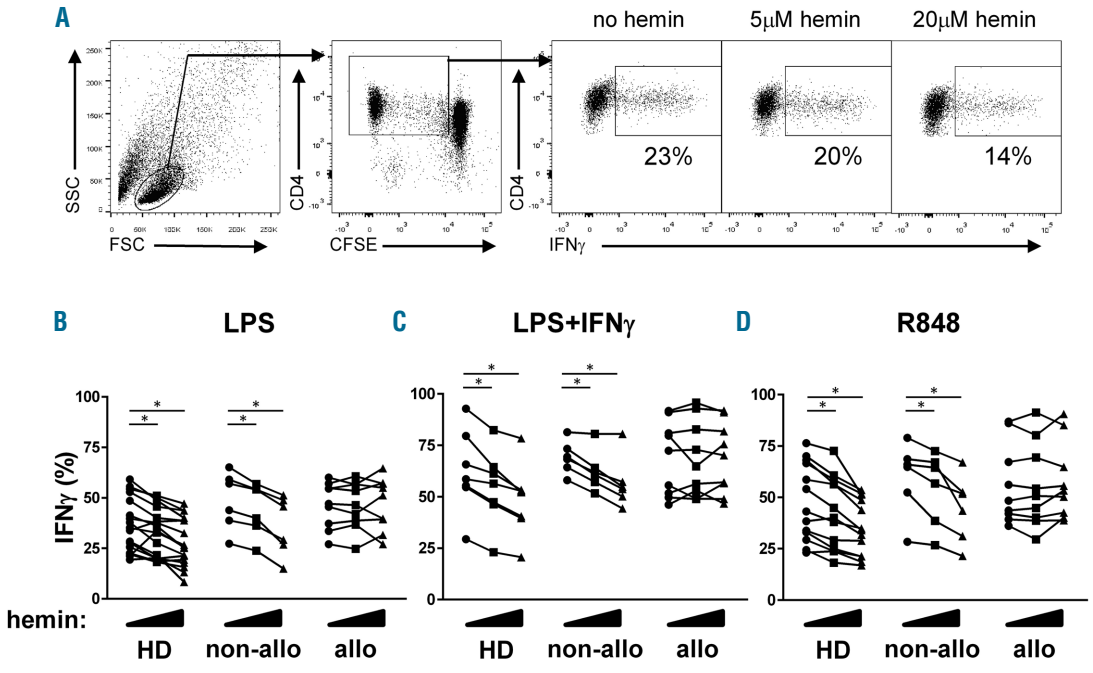

Figure 1. Production of IFNγ by CD4⁺ T cells primed with hemin-treated human monocyte derived dendritic cells (moDCs). CFSE-stained naïve CD4⁺ T cells (50x10³/well) were cultured with allogeneic mature moDCs (5x10³/well) pre-treated or not with hemin (0, 5, or 20 μM) in the presence of 5 UI/mL IL-2. MoDCs had been matured using 100 ng/mL lipopolysaccharide (LPS) (A and B), 100 ng/mL LPS + 500 UI/mL IFNγ (C), or 5 μM R848 (D). Twelve days later, cells were stimulated with PMA/ionomycin for 5 h in the presence of brefeldin A and stained for CD4 and IFNγ. (A) Representative example for non-alloimmunized patients. (B-D) The amount of cytokine-producing T cell is represented as a percentage in $CFSE^{low}/CD4^{+}$ cells. The middle data point refers to 5 μM hemin and the 3rd data point to 20 μM hemin. Two-tailed paired Student's *t*-tests were used to compare the production of cytokines by T cells stimulated with hemin-treated moDCs *versus* untreated moDCs. **P*<0.05.

on moDCs from healthy donors (*P*<0.02) and non-alloimmunized patients (*P*<0.025). In contrast, heme had no effect on CD83 expression levels on moDCs from alloimmunized SCD patients regardless of maturation method (*P*>0.25) (Figure 2C-E). These data indicate that downregulation of CD83 maturation marker in response to heme is defective in alloimmunized SCD patients.

IL-12 production is comparable in hemin-treated moDCs derived from alloimmunized and non-alloimmunized SCD patients

We next investigated whether altered cytokine expression was involved in the differential effects of heme on moDC immunogenicity in the 2 patient groups. Because IL-12 is crucial for skewing towards T_H1 IFNγ-secreting cells,[19] we first examined the effect of hemin on IL-12p40 production by moDCs. As expected, immature DCs did not produce IL-12p40 without or with heme treatment (Figure 3A and B). Hemin inhibited IL-12p40 production by moDCs from healthy donors matured with LPS, LPS+ IFNγ and R848 (*P*<0.04 for 20 μM hemin, n=7) (Figure 3A and B). Similarly, IL-12p40 was down-regulated in response to hemin in moDCs not only from non-alloimmunized SCD patients, but also from alloimmunized SCD patients (Figure 3A and B).

We also tested the levels of bioactive IL-12p70, which is composed of the 2 subunits IL-12p35 and IL-12-p40. Immature moDCs produced no IL-12p70. As previously reported in human settings,[20,21] moDCs matured with LPS alone produced significantly less IL-12p70 relative to LPS+ IFNγ and R848-matured DCs (Figure 3C; note scale difference). Overall, levels of IL-12p70 were significantly higher in matured moDCs from patients with SCD as compared to healthy donors (Figure 3C). As with IL-12p40, IL-12p70 was inhibited by hemin in moDCs from SCD patients regardless of alloimmunization status (LPS+ IFNγ: non-alloimmunized, *P*=0.07, and alloimmunized, *P*=0.004; R848: non-alloimmunized, *P*=0.04, and alloimmunized, *P*=0.04) (Figure 3C).

We also tested TNFα typically expressed by activated moDCs. Similar levels of TNFα were produced by moDCs in the absence or presence of heme, indicating lack of toxic effects of heme on moDCs (*Online Supplementary Figure S3*). Altogether, these data indicate that heme down-regulates IL-12 expression by moDCs regardless of alloimmunization status, suggesting that IL-12 inhibition is not involved in the differential heme-mediated regulation of DC immunogenicity. Furthermore, the data clearly show that moDCs from alloimmunized SCD patients are still responsive to heme at least in their ability to down-regulate IL-12 production.

HO-1, CO and biliverdin are not involved in CD83 regulation of hemin-treated moDCs

HO-1 is induced by hemin/heme and is known for its immunoregulatory functions.[13,22] We have previously reported that altered monocyte control of T-cell responses in alloimmunized SCD patients following exposure to exogenous heme was in part due to monocyte HO-1 levels.[9] In contrast to monocytes,[9] baseline HO-1 levels (in the

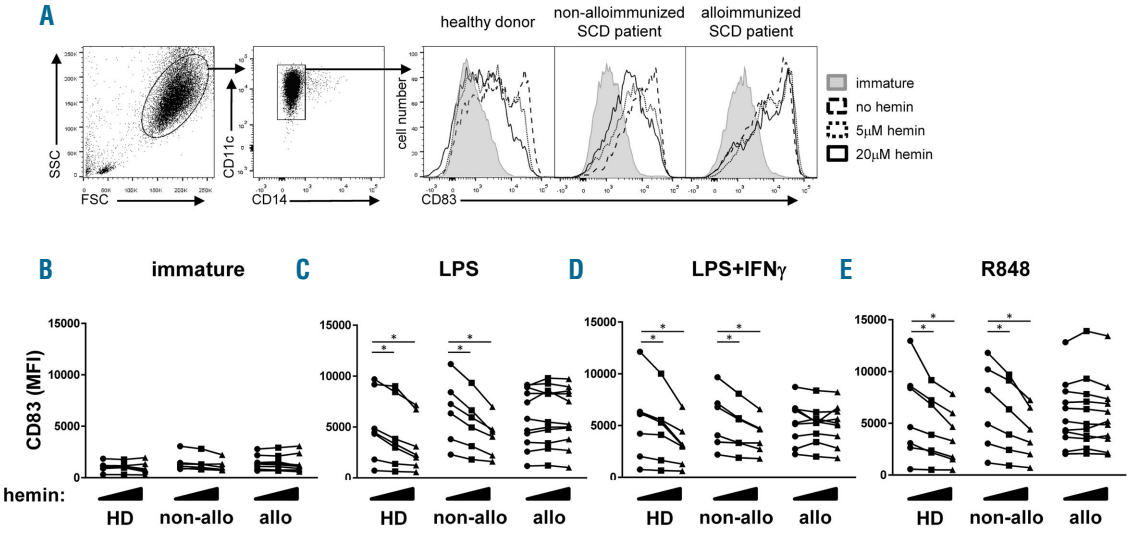

Figure 2. Expression of CD83 by hemin-treated human monocyte derived dendritic cells (moDCs). Immature moDCs were pre-treated or not with hemin (0, 5, or 20 μM) at day 0. After 2 h, maturation was performed or not (B) using 100 ng/mL lipopolysaccharide (LPS) (A and C), 100 ng/mL LPS + 500 UI/mL IFNγ (D), or 5 μM R848 (E). At day 2, CD83 expression was assessed by immunostaining. (A) Representative example for non-alloimmunized patients. Two-tailed paired Student's *t*-tests were used to compare the expression of co-stimulation markers on hemin-treated moDCs *versus* untreated moDCs. **P*<0.05.

absence of hemin) in moDCs from alloimmunized SCD patients were not statistically different from those of non-alloimmunized and healthy donors (Figure 4A and B). Interestingly, and as already reported,[22] upregulation of HO-1 in response to hemin was lower in matured moDCs than in immature moDCs (Figure 4B), and this trend was similar in both healthy donors and patients.

We found higher levels of HO-1 induction following hemin treatment in immature moDCs from non-alloimmunized than from alloimmunized SCD patients (12±2-fold increase *vs.* 6±1-fold increase; *P*=0.02), in LPS-matured moDCs (5±0.6-fold increase *vs.* 3±0.3-fold increase; *P*=0.02), and to some extent in LPS+ IFNγ-matured moDCs (6±1-fold increase *vs.* 3±0-fold increase; *P*=0.05), but not in R848-matured moDCs (4±0.4-fold increase *vs.* 3±0.4-fold increase; *P*=0.3).

To further explore the role of HO-1 induction in CD83 regulation, we first examined the direct effects of HO-1 enzymatic products. HO-1 catabolizes heme into equimolar amounts of labile Fe, biliverdin (BV) and carbon monoxide (CO). Its products, especially BV and CO, are thought to drive HO-1 immunomodulatory effects.[23-27] We therefore tested whether HO-1 could down-regulate CD83 through BV or CO. MoDCs were cultured in the presence of BV or the CO-release molecule CORM-3[28] before being matured with TLR agonists. Whereas hemin-mediated downregulation of CD83 was observed in both healthy donors (*Online Supplementary Figure S4A*) and non-alloimmunized SCD patients (Figure 4C, left panel), HO-1 products had no effect in inhibiting CD83 expression (Figure 4C, see CORM-3 or Biliverdin lanes). As expected, CD83 was not down-regulated by hemin in alloimmunized patients (Figure 4D, right panel). Furthermore, CD83 expression was unaffected by HO-1 products in alloimmunized patients (Figure 4D, right panel). Efficacy of the hemin treatment was controlled by measuring induction of HO-1 levels (*Online Supplementary Figure S4B*) and CORM-3 treatment was controlled by IL-12 inhibition (*Online Supplementary Figure S4C*).[22]

As a second approach to examine the potential role of HO-1 in CD83 regulation, we blocked HO-1 activity using 2 inhibitors, namely SnPPIX and ZnPPIX.[29] MoDCs treated with these inhibitors were not capable of blocking CD83 downregulation in the presence of hemin either in healthy donors (*Online Supplementary Figure S5A*) or in non-alloimmunized patients (Figure 4D, left panel). As expected, CD83 was not down-regulated by hemin in alloimmunized patients and was unaffected by HO-1 inhibitors (Figure 4D, right panel). Efficacy of the hemin treatment was controlled by measuring HO-1 levels (*Online Supplementary Figure S5B-D*). Altogether, these results show that CD83 downregulation is HO-1-independent.

Hemin blocks CD83 through TLR4 triggering

Heme can trigger TLR4.[14,30-32] We therefore examined whether hemin down-regulates CD83 through a TLR-4-mediated pathway. TLR4 also binds LPS and, as expected, TLR4 blockade using a blocking polyclonal antibody against TLR4[33] strongly prevented TLR4 activation by LPS in the absence of hemin, as demonstrated by inhibition of CD83 and IL-12 (Figure 5 and *Online Supplementary Figure S6*, respectively; compare isotype control *vs.* anti-TLR4). Pre-treatment of mature moDCs derived from healthy donors (Figure 5A) or non-alloimmunized SCD patients (Figure 5B) with anti-TLR4 no longer resulted in downregulation of CD83 in response to hemin, whereas the isotype control effectively inhibited CD83 expression in hemin-treated moDCs. TLR4 blockade did not further affect CD83 on the moDCs from the alloimmunized group (Figure 5C). Anti-TLR4 reversed the inhibition of IL-12p40 by hemin in all groups, confirming the efficacy of TLR4 blockade (*Online Supplementary Figure S6A-C*). These data strongly suggest that hemin down-regulates DC maturation by signaling through TLR4.

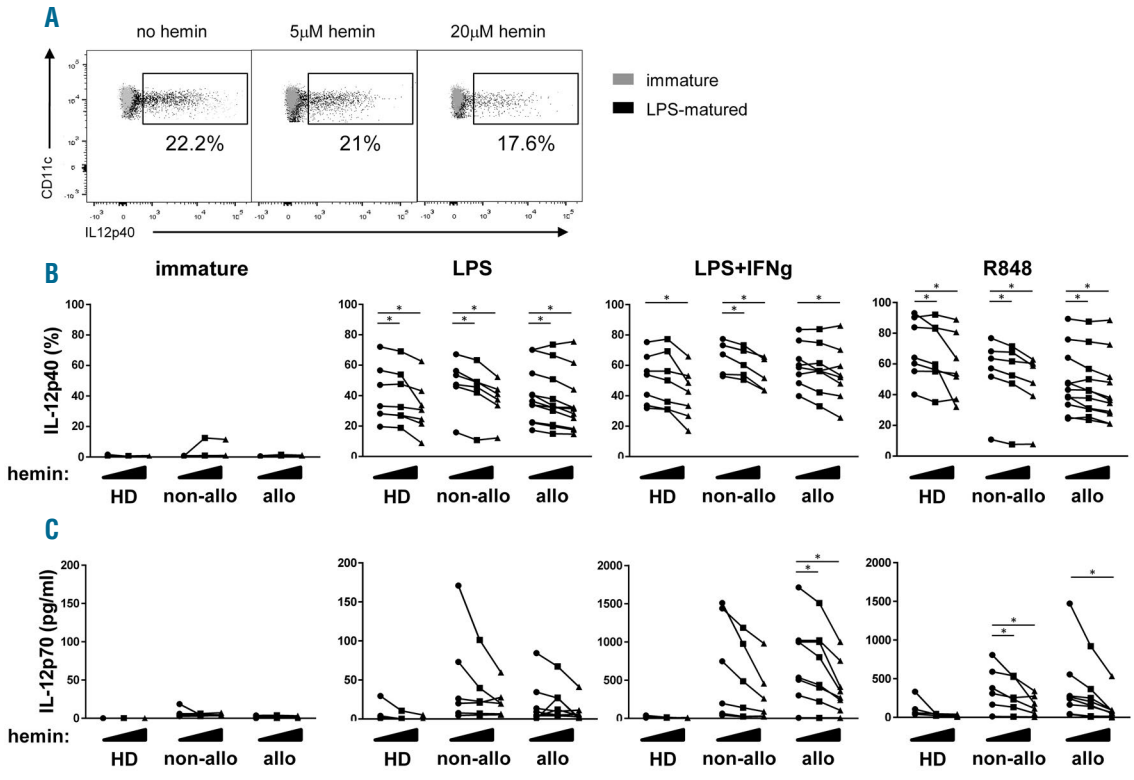

Figure 3. Production of IL-12 by hemin-treated human monocyte derived dendritic cells (moDCs). Immature moDCs were pre-treated or not with indicated doses of hemin (0, 5, or 20 μM) at day 0. When maturation was performed, 100 ng/mL lipopolysaccharide (LPS), 100ng/mL LPS + 500UI/mL IFNγ, or 5 μM R848 was added 2 h later. For intracellular staining, all conditions were cultured in the presence of brefeldin A. The next day, cells were fixed and stained intracellularly for IL-12p40 (A and B). (C) For IL-12p70 detection, supernatants (without brefeldin A) were harvested two days after maturation. (A) Representative example for non-alloimmunized patients. In the case of LPS+ IFNγ matured moDCs, at lower concentrations of hemin, we found a more robust downregulation of IL-12p40 in non-alloimmunized moDCs (no hemin *vs.* 5 μM hemin; $P<0.002$) as compared to alloimmunized ($P>0.1$), similar to our published data in LPS/ IFNγ treated monocytes.[9] Two-tailed paired Student's *t*-tests were used to compare the expression of cytokines produced by hemin-treated moDCs *versus* untreated moDCs. *$P<0.05$.

Hemin inhibits NF-κB responses in moDCs from non-alloimmunized, but not alloimmunized SCD patients

The *cd83* promoter contains NF-κB binding sites and NF-κB transcription factors are involved in regulating maturation-specific CD83 expression in DCs.[34] To test whether heme alters NF-κB-mediated maturation of DCs, we first examined the activation levels of NF-κB transcription factors in hemin-exposed moDCs from healthy donors. Immature moDCs were stimulated for 2 h with R848 or LPS+ IFNγ in the presence or absence of hemin, and NF-κB transcription factors, namely p50, p52, p65, RelB and c-Rel, were tested in nuclear fractions (Figure 6). We found a robust and consistent downregulation of p50 activation (Figure 6A) and RelB (Figure 6D), but not of the other NF-κB subunits in non-alloimmunized hemin-treated mature moDCs.

These results suggest a model whereby hemin blocks p50 and RelB activation in non-alloimmunized, but not in alloimmunized SCD patients.

Discussion

In this study, we identified striking differences in innate immune response to exogenous heme in alloimmunized and non-alloimmunized SCD patients. Hemin-exposed moDCs derived from non-alloimmunized SCD patients inhibited priming of pro-inflammatory $CD4^+$ T cells expressing IFNγ (T_H1) (Figure 1) and TNFα (T_H1-like) (*Online Supplementary Figure S1A*) and exhibited a significant decrease in the levels of the maturation marker CD83 expression (Figure 2), similar to those of healthy donors. Strikingly, in alloimmunized patients, hemin did not affect DC maturation or their ability to prime T_H1 cells. The underlying mechanism for CD83 regulation depended on TLR4 (Figure 7), but not on HO-1. The prototypical pro-inflammatory signaling pathway NF-κB transcription factor, specifically p50 and RelB subunits, were down-regulated in response to hemin in moDCs from non-alloimmunized, but not alloimmunized patients (Figure 6). These data suggest that hemin dampens DC immunogenicity through downregulation of CD83/p50/RelB and that this pathway is defective in alloimmunized SCD patients.

There was no statistical difference in baseline levels of the key immune parameters tested in this study, including CD83, IL-12, HO-1, p50 and RelB levels, between alloimmunized and non-alloimmunized SCD patients, probably due to person to person variations. On the other hand, differences were observed upon treatment with exogenous heme. Specifically, DC immunogenicity was dampened in non-alloimmunized and healthy donors, but not in alloim-

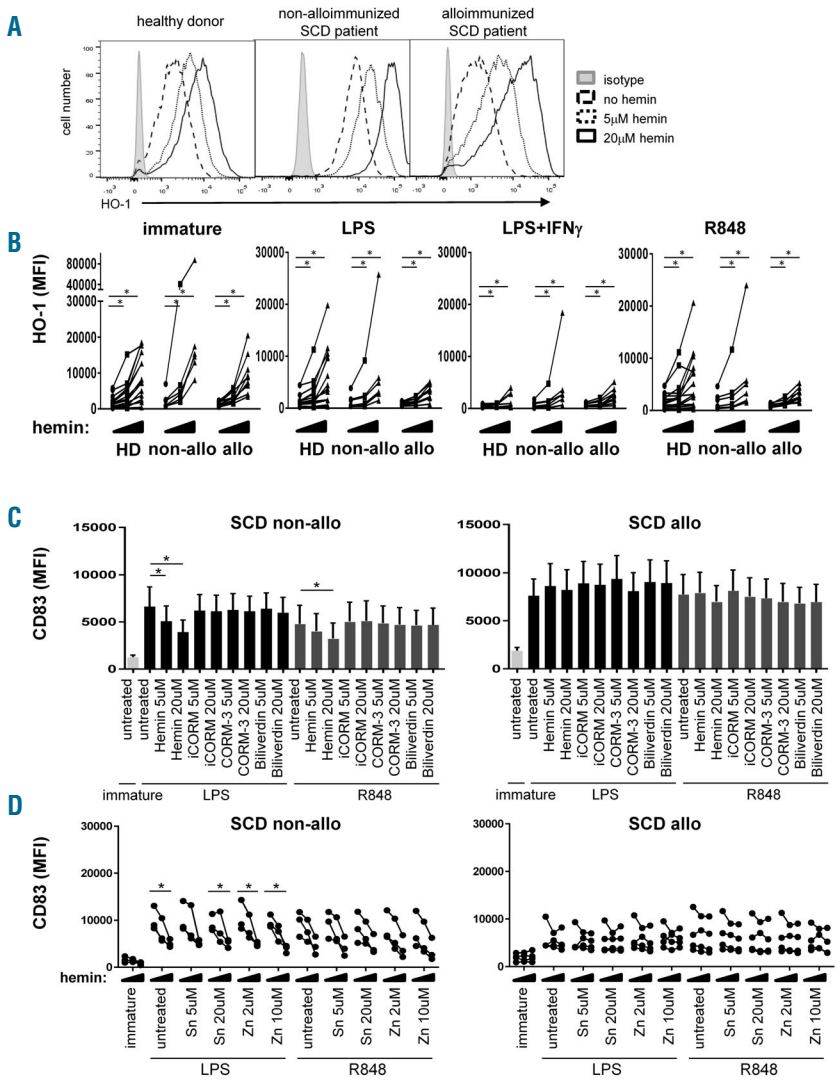

Figure 4. Expression of HO-1 by hemin-treated human monocyte derived dendritic cells (moDCs). (A and B) Immature moDCs were pre-treated or not with hemin (0, 5, or 20 μM) at day 0. When maturation was performed, 100 ng/mL lipopolysaccharide (LPS), 100 ng/mL LPS + 500 UI/mL IFNγ, or 5 μM R848 was added 2 h later. After two days, HO-1 was stained intracellularly (A and B). (A) Representative examples. (C) Immature moDCs were pre-treated or not with indicated doses of hemin products or with hemin at day 0. Maturation was performed 2 h later. After two days, CD83 levels were assessed by immunostaining. (D) HO-1 inhibitors (SnPPIX and ZnPPIX) were used to treat immature moDCs. Two hours later, hemin was added at indicated doses. After two additional hours, moDCs were matured or not. CD83 was detected at day 2. Two-tailed paired Student's *t*-tests were used to compare the expression of HO-1 or CD83 by hemin-treated moDCs (or treated with hemin products) *versus* untreated moDCs. **P*<0.05 [error bars, standard error of the mean (SEM)].

munized patients with heme treatment. Furthermore, inhibition of NF-κB signaling was identified as a mechanism by which heme could suppress DC maturation and function. Out of the NF-κB transcription factors (p50, p52, p65, RelB, c-Rel), we have narrowed down two, p50 and RelB, that are strikingly differentially activated in alloimmunized and non-alloimmunized SCD patients in the presence of heme. Differences in TLR4 expression levels may be responsible for altered p50/RelB activation, but we did not directly measure TLR4 levels on DCs derived from alloimmunized or non-alloimmunized patients, partly due to the insensitivity of commercially-available anti-TLR detection antibodies. There was no difference in inflammatory cytokines, including IL-12, between the 2 patient populations in response to LPS, suggesting that functional TLR4 levels are likely to be similar in both groups of patients. Therefore, differences between both groups could be due to differential co-receptor(s) used to detect hemin or signaling molecules "located" between TLR4 and NF-κB. We believe that uncovering this pathway represents a significant and novel finding that distinguishes DC function in alloimmunized *versus* non-alloimmunized patients, and should help the identification of downstream target genes encoding potential key molecules such as CD83, in alloimmunization. It may be that allosensitized patients have higher ongoing hemolysis and are, therefore, exposed to more cell-free heme which alters their DC responses. More accurate measurements of intravascular hemolysis including haptoglobin levels will be needed to investigate this possibility, although the reticulocyte counts, a surrogate marker for anemia, in our cohort of alloimmunized patients were not statistically different from the non-alloimmunized group (Table 1). It remains to be determined whether the p50/RelB inhibitory heme-effect is the cause or effect of alloimmunization in SCD. Future longitudinal studies can determine whether this pathway drives alloimmunization or not. As a working model, we hypothesize that the ongoing hemolysis in SCD can dampen DC maturation in non-alloimmunized patients, thereby lowering the immune response and subsequent risk of alloimmunization. However, when SCD DCs are defective in their response to heme/hemolysis, they are poised to trigger RBC-specific immune responses when transfusion occurs, resulting in

alloimmunization. Alternatively, attenuation of the NF-κB inhibitory heme-effect may be the result of alloimmunization; it may be that once patients become alloimmunized, the microenvironment changes, altering DC properties. This would be similar to the situation in other immune disorders where the microenvironment can affect immune responses and DC function.[35-37] These studies also raise the provocative possibility that alloimmunized SCD patients may display higher responses, not only against RBC antigens, but also non-specifically.[38] We found that hemin down-regulates IL-12 production, the prototypical T_H1-skewing cytokine, in moDCs from both alloimmunized and non-alloimmunized SCD patients (Figure 3). In LPS+IFNγ-treated monocytes, we previously reported greater inhibition of IL-12p40 expression by hemin in non-alloimmunized as compared to alloimmunized patients,[9] but the concentrations of hemin used in monocytes were significantly lower (<5 μM hemin) than in this study due to differences in culture conditions. It may also be that differences in molecular programing occur during differenti-

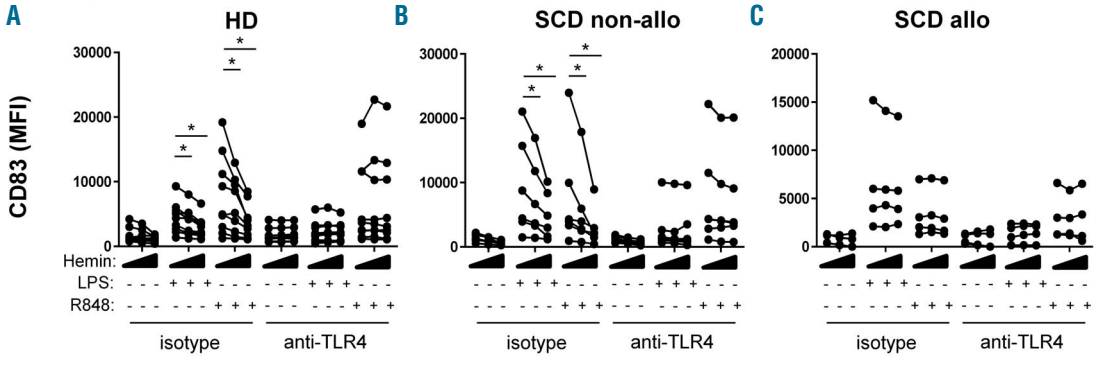

Figure 5. Hemin downregulates CD83 in a TLR4-dependent manner. Immature human monocyte derived dendritic cells (moDCs) from healthy donors (A), non-alloimmunized (B) or alloimmunized patients (C) were incubated for 1 h in the presence of either an isotype control or a blocking antibody for TLR4 (20 μg/mL). Hemin was then added for 2 h before maturation. CD83 was assessed two days later. Two-tailed paired Student's *t*-tests were used to compare the expression of CD83 by hemin-treated moDCs *versus* untreated moDCs. *P<0.05.

Table 1. Characteristics of red blood cell non-alloimmunized *versus* alloimmunized patients on chronic transfusion.

	Non-alloimmunized, n=6	Alloimmunized, n=12
Age (years), median (min, max)	18.4 (15.7, 21.9)	20.9 (16.6, 33.7)
Female	2 (33.3%)	4 (33.3%)
Transfusion indication: stroke prevention	6 (100%)	11 (91.7%)
Cumulative number of transfused RBC units, median (min, max)	552 (160, 726)	629 (266, 1659)
Iron chelation*	0 (0%)	2 (16.7%)
WBC (x10⁹/ L), median (min, max)	9.9 (5.6, 19.2)	13.15 (6.8, 22.6)
Hgb (g/dL), median (min, max)	9.1 (7.1, 11.4)	10.65 (7.6, 12.1)
Reticulocyte (x10⁹/L), median (min, max)	350 (260, 593)	414 (174, 559)
Pre-transfusion HgbS (%), median (min, max)	32.95 (21.5, 61.7)	35.2 (22.3, 54.3)
RBC alloantibody specificities		
Anti-D		10 (83.3%)
Anti-C		7 (58.3%)
Anti-E		3 (25%)
Anti-e		3 (25%)
Anti-V		2 (11.1%)
Anti-VS		1 (8.3%)
Anti-Js^a		4 (33.3%)
Anti-Kp^a		1 (8.3%)
Anti-Fy^b		1 (8.3%)
Anti-S		3 (25%)
Anti-Le^a		1 (8.3%)
Anti-CO^b		1 (8.3%)
Anti-P1		1 (8.3%)
Anti-WR^a		1 (8.3%)
RBC autoantibody		12 (100%)
Anti-HLA		11 (91.7%)

*RBC: red blood cells; WBC: white blood cell count; Hgb: hemoglobin; HgbS: sickle hemoglobin; *both patients were on Exjade therapy.*

ation of monocytes into moDCs. These data indicate that even though moDCs from alloimmunized SCD patients cannot down-regulate CD83 or inhibit T_H1 priming following heme treatment, they are still responsive to heme, thereby arguing against an analogous state of anergy or exhaustion to that described for T cells. These data also suggest that hemin-mediated inhibition of IFNγ-secreting T-cell priming by moDCs is not solely through dampening IL-12. Instead, it is likely that the inhibition of T_H1 responses is due to the downregulation of CD83. Indeed, deletion or mutation of mouse CD83 results in defective $CD4^+$ T-cell development, characterized by a dramatic reduction in both CD4 single-positive thymocytes and peripheral $CD4^+$ T cells,[39,40] as well as inhibited DC-medi-

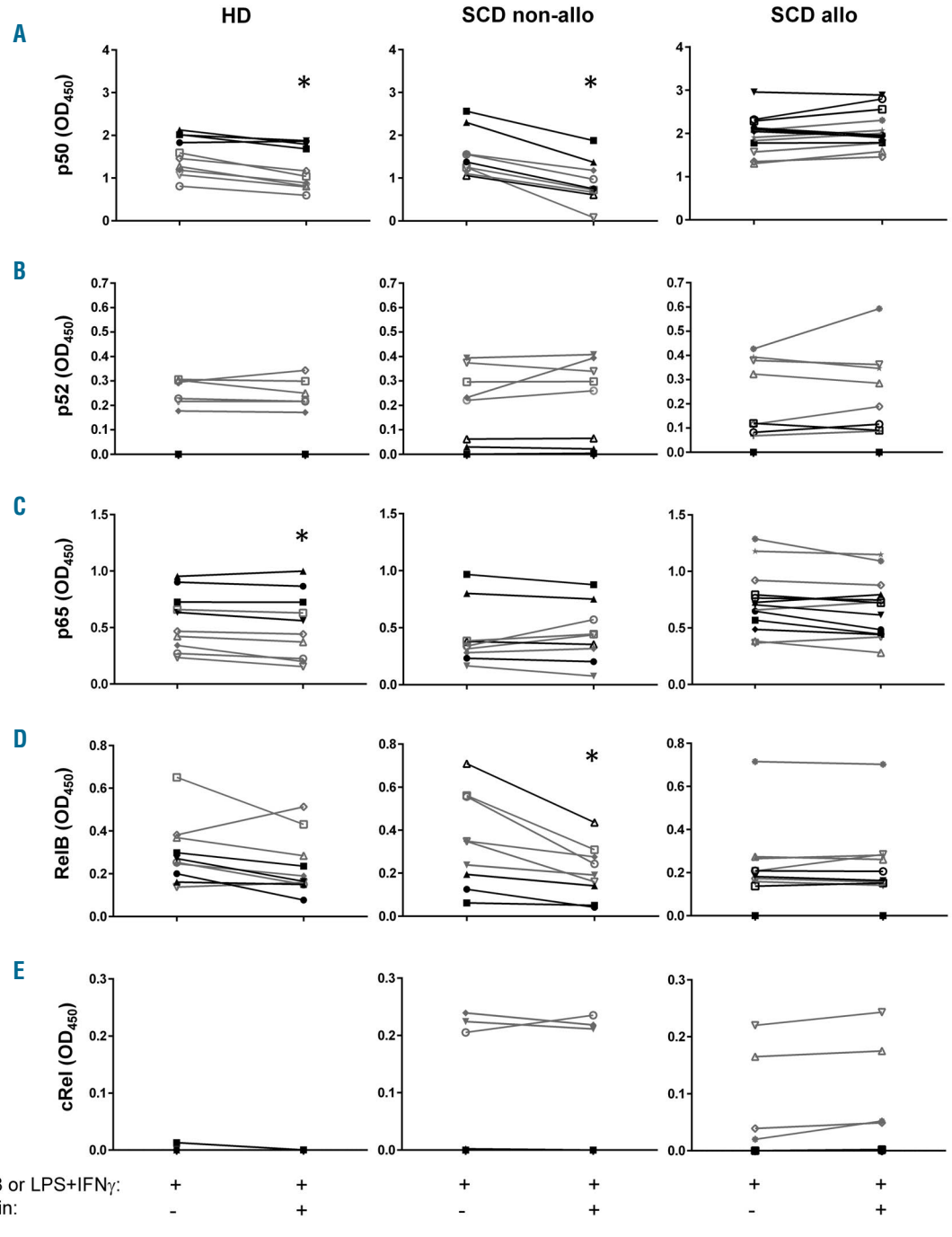

Figure 6. NF-κB inhibition by hemin. Immature human monocyte derived dendritic cells (moDCs) were matured with 5 μM R848 (gray) or 100 ng/mL lipopolysaccharide (LPS) + 500 UI/ml IFNγ (black) for 2 h in the presence or in the absence of 20 μM hemin. Nuclei were then extracted (Active Motif) and the level of transcription factors p50 (A), p52 (B), p65 (C), RelB (D) and c-Rel (E) in the nuclear fraction of moDCs was assessed (Active Motif) in moDCs derived from healthy donors (left panels), non-alloimmunized (middle panels), or alloimmunized (right panels) patients. Results are represented as OD_{450} Two-tailed paired Student's *t*-tests were used to compare moDCs stimulated alone *versus* stimulated + hemin. *P<0.05.

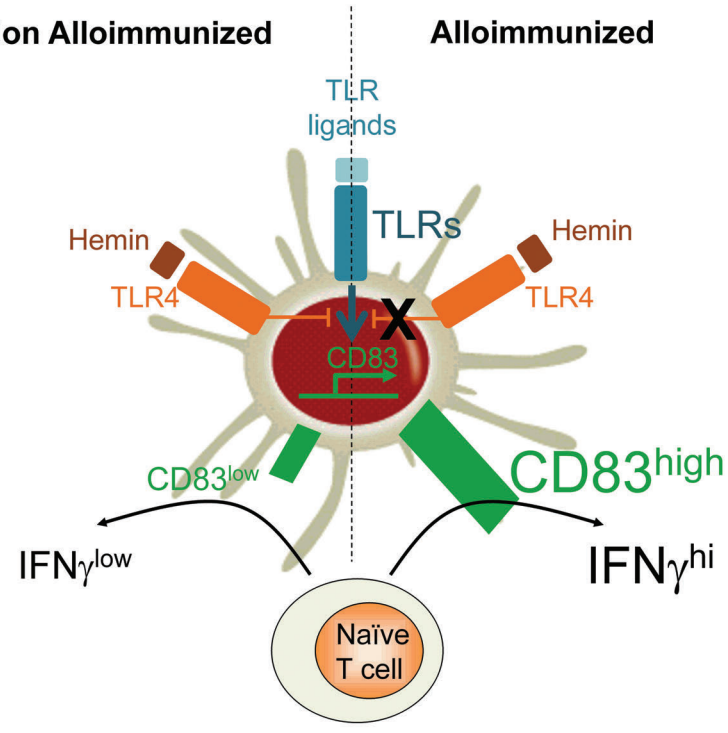

Figure 7. Proposed model. Hemin triggers TLR4 on human monocyte derived dendritic cells (moDCs), which leads to inhibition of NF-κB and downstream CD83 in non-alloimmunized sickle cell disease (SCD) patients. On the other hand, this signaling pathway is defective in alloimmunized patients, thereby preventing the inhibition of NF-κB and CD83. Hemin-exposed moDCs derived from non-alloimmunized patients ($CD83^{low}$) prime naïve T cell to produce lower levels of IFNγ than when derived from alloimmunized patients ($CD83^{high}$). This pathway could, therefore, represent a mechanism by which enhanced inflammation is induced and/or maintained in alloimmunized patients.

ated T-lymphocyte activation.[41] Moreover, circulating $CD4^+$ T cells in $CD83^{-/-}$ mice have been shown to have an altered phenotype with lower expression of TCRβ, CD5 and CD3.[39] Despite an ill-defined function for CD83, these results, together with ours, strongly suggest an instrumental role for this molecule in $CD4^+$ T-cell function. Whether CD83 affect all $CD4^+$T-cell subsets in a similar fashion still has to be investigated. Regarding IL-17A responses (T_H17-like), only low levels were observed (*Online Supplementary Figure S1C*), making it difficult to assess the effect of hemin on T_H17 responses in our system. We found IL-4 (T_H2-like) production by T cells was not affected by hemin treatment (*Online Supplementary Figure S1B*), suggesting that CD83 downregulation by hemin mainly affects T_H1, at least in our system.

Because hemin induces overexpression of HO-1, which in turn exhibits immunomodulatory function,[13,22,26] we first asked whether CD83 expression was inhibited by HO-1. In one report, inhibition of HO-1 promoted DC maturation (HLA-DR and CD86) in a p38 MAPK-CREB/ATF1-dependent manner.[42] However, these effects were comparable to LPS-treatment and the possibility of endotoxin contamination in their HO-1 inhibited DC cultures was not ruled out. On the contrary, in our system, CD83 expression is independent of HO-1. The use of either hemin products (Figure 4C) or HO-1 inhibitors (Figure 4D) did not affect CD83 downregulation in moDCs from non-alloimmunized SCD patients (or from healthy donors), supporting the conclusion that hemin does not down-regulate CD83 through HO-1. These data do not exclude the possibility that other anti-inflammatory effects of heme are mediated through HO-1.[13,22,43] For example, we found that IL-12 was inhibited by CO in DCs (*Online Supplementary Figure S4C*), invoking a role for the HO-1 pathway in IL-12 regulation. Since we did not observe a difference in inhibition of IL-12 at high concentrations of heme between alloimmunized and non-alloimmunized patients, the possibility remains that the HO-1 pathway is intact in these two groups. Provision of CO has been shown to down-regulate DC activation markers CD80 and CD86, as well as CD83, in healthy donors.[22] We did not examine the effects of CO on CD80 and CD86, but following heme treatment, CD80 or CD86 levels were not affected, and only CD83 was inhibited (in healthy donors and non-alloimmunized patients). One can also speculate that the formation of a dimer between p50 and RelB is responsible for the specificity for CD83 expression.

A key finding of our study is that heme blocks the proinflammatory signal though TLR4 signaling in DCs in healthy donors and non-alloimmunized SCD patients (Figure 7), and that this occurs through inhibition of the NF-κB activation pathway, namely p50 and RelB (Figure 6). Interestingly, most of the published literature implicates a pro-inflammatory effect of heme/TLR4 signaling.[14,18,30-32] Differences in cell types at the level of co-receptors and/or signaling molecules, involving other NF-κB subunits besides p50 and RelB[14,44] may explain the dichotomy as to why heme/heme analogs drive anti-inflammatory responses in DC, as we and others[13] have detected, but pro-inflammatory responses in these other cell types. In *in vitro* studies, heme signaling induces TNFα, albeit at low levels, and only in the absence of serum.[15,18,30] We have also observed hemin-induced very low levels of TNFα in monocytes, but only in the total absence of serum (*data not shown*). Our present study was performed in the presence of serum (5% from healthy donors), enabling us not only to detect the anti-inflammatory pathway mediated by heme, but, more importantly, the existence of a defective heme/TLR4 anti-inflammatory signaling response in alloimmunized SCD patients. LPS signaling, which also occurs through TLR4

signaling in DCs, is proinflammatory and involves upregulation of NF-κB.[14,45] One may speculate that LPS and heme have different binding sites or binding affinities for TLR4, or require additional molecules that can induce a pro- *versus* an anti-inflammatory downstream signal in DCs. In alloimmunized patients, we found that the LPS/TLR4 pro-inflammatory signal is intact, since LPS (in the absence of hemin) resulted in upregulation of CD83 and IL-12 in moDCs similar to non-alloimmunized and healthy donors, but that the anti-inflammatory heme/TLR4 pathway is defective in matured (either by TLR4 or by TLR7/8 agonists) DCs (Figure 7). Deciphering the components of this defective pathway could possibly identify targets to restore the anti-inflammatory capacity of DCs when exposed to free heme, thereby potentially reducing the risk of alloimmunization.

In summary, we have found that in non-alloimmunized SCD patients extracellular heme limits CD83 expression on DCs through a TLR4-mediated pathway, involving NF-κB, resulting in the dampening of pro-inflammatory responses (Figure 7). In contrast, in alloimmunized SCD patients, heme-driven TLR4-dependent pathway is defective, preventing NF-κB-mediated CD83 downregulation (Figure 7). Our data show that CD83 expression and T-cell priming were not inhibited by hemin in alloimmunized patients, and this could represent a mechanism by which enhanced inflammation is induced and/or maintained in this group of patients. Soluble CD83 fragments or CD83-Ig fusion protein[46] to limit inflammation may be efficacious in preventing alloimmunization. Taken together, we believe that the identification of heme-driven TLR4 signaling and NF-κB inhibition as potential brakes of SCD alloimmunization may lead to the development of diagnostic strategies to predict which patients are at risk of alloimmunization, as well as of innovative treatments to prevent alloimmunization in this patient population.

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