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Hemin Controls T Cell Polarization in Sickle Cell Alloimmunization

Hui Zhong,* Weili Bao,* David Friedman,†‡ and Karina Yazdanbakhsh*

Patients with sickle cell disease (SCD) often require transfusions to treat and prevent worsening anemia and other SCD complications. However, transfusions can trigger alloimmunization against transfused RBCs with serious clinical sequelae. Risk factors for alloimmunization in SCD remain poorly understood. We recently reported altered regulatory T cell (Treg) and Th responses with higher circulating Th1 (IFN-γ) cytokines in chronically transfused SCD patients with alloantibodies as compared with those without alloantibodies. Because monocytes play a critical role in polarization of T cell subsets and participate in clearance of transfused RBCs, we tested the hypothesis that in response to the RBC breakdown product hemin, monocyte control of T cell polarization will differ between alloimmunized and non-alloimmunized SCD patients. Exogenous hemin induced Treg polarization in purified T cell/monocyte cocultures from healthy volunteers through the monocyte anti-inflammatory heme-degrading enzyme heme oxygenase-1. Importantly, hemin primarily through its effect on CD16+ monocytes induced an anti-inflammatory (higher Treg/lower Th1) polarization state in the non-alloimmunized SCD group, whereas it had little effect in the alloimmunized group. Non-alloimmunized SCD CD16+ monocytes expressed higher basal levels of heme oxygenase-1. Furthermore, IL-12, which contributed to a proinflammatory polarization state (low Treg/high Th1) in SCD, was dampened in hemin-treated stimulated monocytes from non-alloimmunized SCD patients, but not in the alloimmunized group. These data suggest that unlike alloimmunized patients, non-alloimmunized SCD CD16+ monocytes in response to transfused RBC breakdown products promote an anti-inflammatory state that is less conducive to alloimmunization.

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Hemoglobin S–containing RBCs are deformed in sickle-prone conditions, resulting in chronic hemolytic anemia, shortened RBC survival in circulation, increased reticulocytosis, periodic painful vaso-occlusive crises, and end organ damage due to persistent tissue hypoxia (1). RBC transfusions remain a very important modality of treatment for patients with SCD with most patients receiving RBC transfusions in their lifetime (2). Despite its benefits, RBC transfusion results in alloimmunization in ~20–50% of patients with SCD (3). Alloantibodies can cause delayed hemolytic transfusion reactions that in SCD patients can trigger hyperhemolysis, a life-threatening poorly understood phenomena in which the transfused and the patient’s own RBCs are destroyed (3). Additionally, finding compatible units for patients with alloantibodies can be difficult, and identifying the Abs can be costly and time-consuming, often causing transfusion delays. Even with provision of Rh-D, -C, and -E Ag-matched donor RBCs, patients continue to develop Rh Abs, which may in part be due to genetic diversity of the RH locus in donors of African ancestry; many of these Abs are considered clinically significant (4). This highlights the need for better characterization of triggers of alloimmunization and identification of risk factors for alloimmunization in patients with SCD. Genetic as well as acquired patient-related factors are likely to influence the process of alloimmunization (3). We recently reported reduced peripheral regulatory T cell (Treg) and B cell suppressive function and altered Th responses with higher circulating IFN-γ, but lower IL-10 levels in alloimmunized as compared with non-alloimmunized SCD patients (5, 6). These data are consistent with a model in which a generalized immune dysregulation exists in SCD alloimmunized patients with an imbalance between the regulatory (Tregs) and effector (Th) cells, possibly as a result of an underlying inflammatory state (7), that can potentially drive pathogenic responses against transfused RBCs. Studies that address how Treg/Th differentiation and expansion are controlled may improve our understanding of how SCD alloimmunization is triggered.

The monocyte/macrophage system is responsible for extravascular clearance of transfused RBCs. Following RBC transfusion, ~10% or more of donor RBCs are cleared from the circulation within 24 h in healthy individuals (8). Levels of hemin, a breakdown product of hemoglobin, are likely to build up in monocytes/macrophages following RBC transfusions. Heme oxygenase-1 (HO-1) is normally induced in response to heme, degrading it into iron, bilirubin, and carbon monoxide, thereby reducing intracellular heme availability (9, 10). Several studies from mouse models indicate that hemin, probably through the anti-inflammatory activities of HO-1 (10), has potent immunoregulatory effects on both the innate (11) and adaptive immune response (12), regulating the secretion of alloimmunization as well as regulatory cytokines by monocytes (13, 14). In turn, monocytes can trigger and polarize Th responses (15, 16) as well as both stimulate and suppress T cell responses, depending on the monocyte subset and its activation state (16, 17). Indeed, we recently showed in a non-SCD setting...
that the CD16+ monocyte subset, which constitutes only ∼5–10% of total monocytes in healthy individuals, controls Treg/Th polarization (18), inhibiting specific Treg subsets (19) while promoting Th1 expansion via IL-12 (18). The role of HO-1 in polarization of T cell responses in human disease setting has not been investigated. Monocytes in SCD are in an activated state (20), but it remains to be determined whether they participate in modulating T cell responses in SCD alloimmunization. Because hem/HO-1 in mouse monocytes possess immunomodulatory activities (21), we hypothesized that following transfusion of RBCs, the response of human monocytes to the breakdown products of hemoglobin will play a pivotal role in polarization of T cell immune responses against transfused RBCs and ultimately in alloimmunization in human SCD.

Materials and Methods

Human samples

All studies were approved by the Institutional Review Board of the New York Blood Center. Fresh leukopaks (n = 14) containing leukocyte-enriched peripheral blood from healthy volunteer donors of the New York Blood Center were obtained without any identifiers. For SCD patients, blood was obtained solely from discarded waste bags from SCD patients undergoing erythrocythropoietic procedures. Patients were selected randomly from a cohort of heavily transfused, infectious disease–free 15- to 34-yr-olds (previously described (19)). Cells from a chronic transfusion protocol receiving leukoreduced blood matched for C, E, and K at Children’s Hospital of Philadelphia on an outpatient basis. Patients with no history of Ab production were grouped as non-alloimmunized (n = 9), and those with a history of having produced alloantibodies were grouped as alloimmunized (n = 11). The apheresis waste bags stripped of all identifiers except the alloantibody state (alloimmunized or negative) were then sent to the New York Blood Center and analyzed within 18 h of blood collection. Because patients on a chronic transfusion protocol can be transfused as regularly as every 3 wk, all data presented in this study were derived from analysis of samples obtained within a 3-wk period to ensure that each datum point represents a different subject and not a duplicate.

Cell isolation and purification

PBMCs were separated by Ficoll (GE Healthcare, Port Washington, NY) density gradient centrifugation and subjected to cell subset purification by magnetic bead purification (all from Miltenyi Biotec, Auburn, CA). CD4+ T cells and total monocytes were purified using a CD4+ T cell isolation kit and CD14 microbeads, respectively (purity >95%) following the manufacturers’ instructions. For purification of CD16+ and CD16 monocyte subsets, a CD16 monocyte isolation kit (Miltenyi Biotec) was used first to purify CD16+ monocytes by positive selection (purity >95%), and the negatively selected fraction was then incubated with CD14 microbeads to obtain the CD14+CD16– cell population (purity >95%) following the manufacturer’s instructions.

T cell stimulation assays

Purified CD4+ T cells were stained with CFSE (Invitrogen, Grand Island, NY) and mixed (1.25 × 10^5 cells/ml) with autologous purified total monocytes at a ratio of 2:1 followed by stimulation with anti-CD3 Ab (clone HIT3a, 1 µg/ml; BD Biosciences, San Jose, CA) for 7 d in U-bottom 96-well plates as previously described (19). Alternatively, purified CD14+CD16– cells and autologous CFSE-labeled CD4+ T cells (2:1 ratio) in the absence or presence of CD16+ monocytes (CD14+CD16– to CD16+ monocyte ratio of 2:1) were cultured for 7 d with anti-CD3 Ab. In the T cell stimulation experiments that were performed in the absence of monocytes, purified, CFSE-stained CD4+ T cells (1.25 × 10^5 cells/ml) were cultured for 7 d in plates precoated with anti-CD3 Ab (clone HIT3a, 1 µg/ml). For the Ab-blocking studies, anti-IL-12 p40/p70 Ab (clone 25B7; BD Biosciences, San Jose, CA) and anti–IFN-γ (clone 4S.B3; eBioscience, San Diego, CA), respectively, as previously described. Cytoplasmic IL-17 or IFN-γ expression in the CD4+ cells was also analyzed using anti–IL-17A (clone eBio64DEC17; eBioscience) and anti–IFN-γ (clone 4S.B3; eBioscience), respectively, as previously described. Percentage of FOXP3+ (IL-17A– and IFN-γ–) cells within divided (CFSE+) CD4+ T cells was used as the measurement for frequency of expanded Tregs, Th17 cells, and Th1 cells, respectively. Similarly, the frequency of expanded Helios+ or Helios+ Tregs was determined by analyzing the percentage of Helios+FOPX3+ or Helios+FOXP3hi, respectively, within CFSE+CD4+.

For HO-1 expression analysis, freshly isolated PBMCs were stained for 20 min with anti-CD3, anti-CD4, and anti-CD25 for T cell surface analysis or with anti-CD14 and anti-CD16 for monocyte subset analysis (all BD Biosciences Abs). After several washes, the cells were fixed and permeabilized (eBioscience) and then incubated with an isotype control or anti–HO-1 Ab (clone 23/HO-1; BD Biosciences) prelabeled using the Zenon labeling kit (Life Technologies, Grand Island, NY) without or with anti-FOPX3 Ab (eBioscience) for 30 min. To measure HO-1 expression following addition of hemin, PBMCs (5 × 10^5 cells/ml) were treated with different doses of hemin for 24 h and different T and monocyte subsets were analyzed using the same staining pattern as above.

For intracellular IL-12 measurements, PBMCs that had been frozen in liquid nitrogen were thawed and after several washes, divided into 200-µl aliquots (2.5 × 10^6/ml), and stimulated with IFN-γ (100 ng/ml; R&D Systems) in the absence or presence of hemin (1.25 µM) for 2 h followed by addition of LPS (0.1 mg/ml, from Escherichia coli 0111:B4; Sigma-Aldrich) for 24 h. Brefeldin A (1 µg/ml; eBioscience) was added during the last 5 h. The cells were then surface stained with PerCP conjugated anti–CD14 Ab (clone McP9; BD Biosciences) followed by treatment with a BD fixation/permeabilization solution (following the manufacturer’s instructions) and incubation with allopurinol-conjugated isotype control (IgG1) or anti–IL-12 p40/p70 (clone C8.6; BD Biosciences). The samples were then analyzed by flow cytometry.

Statistical analysis

Data are expressed as mean values ± SEM. Statistical significance of differences between groups was determined by an unpaired t test, and statistical significance of differences of paired data was determined by a paired r test. Statistical analyses were performed using PASW Statistics 18 software (IBM, Armonk, NY).

Results

Hemin can regulate CD4+ T cell polarization

To investigate the effect of hemin on Treg polarization, purified peripheral CD4+ T cells (stained with CFSE) and autologous monocytes from healthy volunteer blood donors were cultured with various concentrations of hemin and stimulated with anti-CD3 Ab for 7 d. CD4+ proliferation (CFSElo, Fig. 1A) and frequency of Tregs (FOXP3hi) in divided (CFSElo)CD4+ T cells was measured as described previously (Fig. 1B) (18, 19). Treatment with hemin resulted in a dose-dependent decrease in CD4+ T cell proliferation (Fig. 1A, 1B), consistent with previous reports (23, 24). Interestingly, however, within proliferating CD4+ T cells, the frequency of Tregs increased from 19 to 277% with increasing doses of hemin (Fig. 1C, 1D), indicating a polarizing effect of hemin on the Treg population. Hemin is the substrate, activator, and inducer of HO-1, an intracellular enzyme with complex immunoregulatory functions (9). To test whether the Treg-polarizing effect of hemin is mediated through the hemin/HO-1 axis, we used the HO-1 activity blocker ZnPPIX (9) in our CD4+ T cell monocyte coculture assay. ZnPPIX reversed hemin-mediated Treg expansion to levels similar to untreated cultures (Fig. 1E), confirming that the polarization effect of hemin is mediated through HO-1. Monocytes were key in mediating CD4+ and Treg proliferation because in their absence, hemin inhibited CD4+ proliferation only at the highest concentration (Fig. 1F), and the baseline frequency of divided Tregs was much lower in the cultures without
monocytes (1%) as compared with cultures with monocytes (9%); furthermore, addition of hemin did not promote Treg proliferation and, if anything, inhibited Treg expansion (Fig. 1G).

Analysis of proliferative Treg subsets based on expression of Helios (Fig. 2A), which was originally described as a marker to distinguish naturally occurring from peripherally induced Tregs (25), indicated that expansion of Helios+ and Helios$^{2}$ Tregs at all doses of hemin tested were comparable (Fig. 2B, 2C), suggesting that the two subsets are equally responsive to hemin. In contrast, Th1 proliferation was inhibited by hemin only at higher concentrations (Fig. 2D, 2E) whereas Th17 expansion was inhibited, albeit weakly, at lower concentrations (Fig. 2F, 2G). Collectively, these data suggest that in addition to its previously described ability to inhibit CD4$^{+}$ T cell proliferation, hemin can polarize CD4$^{+}$ T cell subsets toward Tregs and to some extent dampen Th1 and Th17 development.

**CD4$^{+}$ T cell polarization by hemin in non-alloimmunized SCD patients**

We next examined CD4$^{+}$ T cell polarization in response to hemin in a cohort of regularly transfused patients with SCD comparing alloimmunized (filled bars) and non-alloimmunized (open bars) proliferative responses. Basal Treg proliferative responses were comparable in alloimmunized and non-alloimmunized patients (Fig. 3A, comparing with no hemin concentration). Interestingly, treatment with hemin increased Treg proliferation more significantly in non-alloimmunized as compared with alloimmunized SCD patients. Indeed, at 1.25 $\mu$M hemin concentration, Treg proliferation doubled in the non-alloimmunized group (Fig. 3A, $p = 0.001$), but the increase was less pronounced in alloimmunized patients ($\sim 30\%$) (Fig. 3A, $p = 0.06$). In the absence of hemin, Helios$^{+}$/2 Treg subset proliferative responses were also comparable in alloimmunized and non-alloimmunized SCD groups (Fig. 3B). Hemin treatment increased Helios$^{+}$ Treg proliferative responses to the same extent in the two patient groups (Fig. 3B, $p = 0.04$); in contrast, Helios$^{+}$ Treg expansion was increased only in the non-alloimmunized group (Fig. 3B), suggesting that hemin has a more profound effect on the expansion of Helios$^{+}$ Tregs in non-alloimmunized as compared with alloimmunized

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**FIGURE 1.** Hemin induces CD4$^{+}$ Treg polarization. Purified, CFSE-stained CD4$^{+}$ T cells from normal healthy volunteers ($n = 14$) were cocultured with an autologous purified total monocyte fraction in the absence (Culture Medium) or presence of various concentrations of hemin (0–5 $\mu$M) and stimulated with anti-CD3 Ab for 7 d. (A) Representative histograms of the staining pattern of CFSE in total CD4$^{+}$ T cells after 7 d in cocultures showing the gating used to measure the extent of CD4$^{+}$ T cell proliferation defined as the frequency of the divided (CFSE$^{lo}$) population. (B) Dose-dependent inhibitory effect of hemin on the frequency of CD4$^{+}$ T cell proliferation (CD4$^{+}$CFSE$^{lo}$ cells) in T cell/monocyte cocultures from healthy controls. The $p$ values were analyzed by a paired t test comparing before (Medium) and after addition of hemin. (C) Representative histograms of the staining pattern of a T cell subset expressing FOXP3$^{hi}$ (Tregs) within the CD4$^{+}$CFSE$^{lo}$ population in cocultures untreated or treated with various concentrations of hemin (0–5 $\mu$M) showing the gating used to analyze the frequency of the Treg population that had undergone proliferation. (D) Dose-dependent increase in the frequency of Tregs in cocultures without or with addition of hemin (1.25 $\mu$M) and/or HO-1 inhibitor ZnPPIX (2.5 $\mu$M) at day 0. The dotted line marks the basal Treg proliferation (minus hemin or ZnPPIX). Frequency of (E) total CD4$^{+}$ and (G) Treg proliferation in cocultures treated with different doses of hemin in the absence of monocytes are shown. Unlike cocultures with monocytes, Treg proliferation is not affected by hemin, and total CD4$^{+}$ proliferation is only inhibited at the highest hemin concentration ($5\mu$M). All statistical analyses indicated by $p$ values were performed by a paired t test.

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SCD patients. We also examined Th1 (Fig. 3C) and Th17 cell (Fig 3D) proliferative responses before and after hemin treatment. In the absence of hemin, basal Th1 proliferation was comparable in the two patient groups. Following hemin treatment, Th1 proliferation was inhibited only in non-alloimmunized SCD patients ($p = 0.001$). Th17 proliferative responses were comparable in the two patients groups before hemin treatment, and neither group was affected by hemin. Collectively, these data indicate that hemin induces a more robust proliferation of Tregs, especially of the Helios$^{-}$ Treg subset, in non-alloimmunized SCD patients as compared with alloimmunized patients and that hemin inhibits Th1 polarization in non-alloimmunized SCD patients but not in the alloimmunized group.

**Hemin regulation of Treg/Th polarization by CD16$^{+}$ monocytes**

Monocytes can be divided into CD16$^{+}$ and CD16$^{-}$ monocyte subsets based on CD16 surface expression (Fig. 4A) with disparate

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**FIGURE 2.** Helios$^{+/-}$ Treg subset showing Th1 and Th17 expansion in response to hemin. (A) Representative dot plot showing Helios and FOXP3 staining pattern in divided (CFSE$^{+}$) CD4$^{+}$ T cells in T cell/monocyte cocultures on day 7. Helios$^{+}$ Tregs are defined by coexpression of Helios in a FOXP3$^{+}$ population whereas Helios$^{-}$ Tregs lack Helios expression. Frequencies of (B) Helios$^{+}$ and (C) Helios$^{-}$ Tregs that had undergone proliferation in the same cocultures from healthy control as in Fig. 1 and after hemin treatment are shown. (D) Representative histogram of IFN-γ expression in divided (CFSE$^{+}$) CD4$^{+}$ T cells on day 7 and (E) frequencies of IFN-γ$^{+}$ cells in divided CD4$^{+}$ T cells in the absence or presence of increasing concentrations of hemin in the same cocultures from healthy control as in Fig. 1 are shown. Similarly, (F) a representative histogram of IL-17 expression in divided (CFSE$^{+}$) CD4$^{+}$ T cells on day 7 and (G) frequencies of IL-17$^{+}$ cells in divided CD4$^{+}$ cells without or with hemin in cocultures from Fig. 1 are shown. The $p$ values were analyzed by a paired t test comparing before (Medium) and after addition of hemin.

**FIGURE 3.** Differences in Treg/Th polarization in response to hemin between alloimmunized and non-alloimmunized SCD patients. Purified, CFSE-stained CD4$^{+}$ T cells from regularly transfused non-alloimmunized ($n = 9$, open bars) and alloimmunized ($n = 11$, filled bars) SCD patients were cocultured with an autologous purified total monocyte fraction in the absence or presence of two different concentrations of hemin (0.625 and 1.25 μM) and stimulated with anti-CD3 Ab for 7 d. Levels of (A) total Tregs, (B) Helios$^{+}$ Treg subsets, (C) Th1, and (D) Th17 in CD4$^{+}$ T populations that had undergone proliferation were analyzed by flow cytometry. All statistical analyses comparing before and after hemin addition were performed using a paired t test; comparisons between alloimmunized and non-alloimmunized groups were performed using a Mann–Whitney U test.
biologic and functional properties (26). We have previously shown that CD16+ and CD16− monocyte subpopulations regulate polarization of distinct Treg/Th subsets in healthy controls (18, 19). To examine the role of CD16+/− monocyte subsets on T cell polarization, we performed our T cell proliferation assay system using purified CD16− monocytes cultured with autologous CD4+ cells (at 1:2 ratio) or together with a purified autologous CD16+ monocyte subset (CD16−/CD16+ monocyte ratio of 2:1) in the absence or presence of hemin. Treg, Th1, and Th17 proliferative responses were comparable in alloimmunized and non-alloimmunized SCD groups in hemin-treated or untreated cocultures T cell monocyte cocultures that lacked CD16+ monocytes (p > 0.05, Supplemental Fig. 1). In contrast, in the presence of CD16+ monocytes, basal Treg expansion, especially in Helios− Tregs, was lower in alloimmunized as compared with non-alloimmunized SCD patients (Fig. 4B). Moreover, addition of hemin resulted in doubling of total Treg and Helios+/− Treg subset proliferation in non-alloimmunized SCD patients (p < 0.05, as indicated by an asterisk above open bars), but importantly it had no effect on Treg or Th17 subset proliferation in the alloimmunized group (Fig. 4C). In the presence of CD16+ monocytes, basal Th1 proliferative responses were lower in non-alloimmunized as compared with alloimmunized patients (Fig. 4D, p = 0.04), but Th17 proliferation was comparable in the two groups (Fig. 4D). Hemin further reduced Th1 and Th17 proliferation in the non-alloimmunized group in cocultures that included CD16+ monocytes (p < 0.05, as indicated by an asterisk above open bars), but it had no effect in the alloimmunized group (Fig. 4E). Taken together, these data indicate that CD16+ monocytes from alloimm-

FIGURE 4. CD16+ monocyte control of Treg/Th proliferation before and after hemin treatment. (A) Representative dot plot analysis of PBMCs based on forward and side scatter showing the gating strategy to identify the total monocyte population. Based on the CD14 and CD16 expression patterns, CD16+ monocytes are further distinguished from the CD14+CD16− monocyte subset. Purified CFSE-stained CD4+ T cells from non-alloimmunized (n = 9, open bars) and alloimmunized (n = 11, filled bars) SCD patients were cocultured with an autologous purified CD14+CD16− monocyte subset together with autologous purified CD16+ monocytes followed by stimulation with anti-CD3 for 7 d in the presence or absence of 1.25 μM hemin. (B) Frequency of total and Helios− Tregs that had undergone proliferation in the absence of hemin are shown. (C) Fold change in proliferation of total and Helios− Treg subsets after addition of hemin was calculated (proliferation in the absence of hemin was set at 100%). The asterisks correspond to statistically significant differences in the proliferative responses before and after hemin treatment (paired t test, *p < 0.05, **p < 0.01). (D) Frequencies of IFN-γ+ and IL-17+ cells in divided CD4+ T cells from the same cocultures as in (B) are shown. (E) Fold change in Th1 and Th17 proliferation after addition of hemin was calculated (proliferation in the absence of hemin was set at 100%). The asterisks correspond to statistically significant differences in the proliferative responses before and after hemin treatment (paired t test, *p < 0.05). (F) Fold change in Treg proliferation after addition of ZnPPIX was calculated (proliferation in the absence of ZnPPIX was set at 100%). The p values indicated above the columns were calculated by a paired t test comparing before and after ZnPPIX treatment and the asterisk above the first column corresponds to the statistically significant difference, p = 0.03. All comparisons between alloimmunized and non-alloimmunized groups were performed using a Mann–Whitney U test.
munized SCD patients have a stronger ability to inhibit Treg proliferation and promote Th1 expansion as compared with CD16+ monocytes from non-alloimmunized patients. Moreover, the CD16+ subset is responsible for differential T cell polarization in response to exogenous hemin between alloimmunized versus non-alloimmunized SCD patients; that is, hemin acting through CD16+ monocytes drives Treg/Th proliferation toward an anti-inflammatory state in non-alloimmunized patients, whereas the same treatment has no effect in the alloimmunized group. To test whether differences in CD16+ monocyte-mediated Treg/Th proliferative responses was due to differential monocyte HO-1 activity in the two patient groups, ZnPPIX (2.5 μM) was added at the start of the CD4+ T cell/monocyte cocultures. In the presence of ZnPPIX, Treg expansion was not affected in cocultures without CD16+ monocytes in either of the two groups (Supplemental Fig. 1). However, in cocultures that included CD16+ monocytes, Treg expansion was inhibited by ZnPPIX in non-alloimmunized (P < 0.001, paired t test, p = 0.027, indicated in Fig. 4F as 0.03) but not in alloimmunized SCD patients (Fig. 4F), suggesting that basal CD16+ monocyte HO-1 activity with respect to Treg proliferative responses is indeed disparate in the two groups, being higher in the non-alloimmunized patients. Inhibition of HO-1 activity with ZnPPIX did not affect Th1 and Th17 proliferative responses in the presence or absence of CD16+ monocytes (data not shown), suggesting that Th1/Th17 as compared with Treg proliferative responses may be less sensitive to regulation by monocyte HO-1.

Finally, we examined the relative protein expression levels of HO-1 before and after hemin treatment. Analysis of PBMCs by flow cytometry indicated that in the absence of hemin, monocytes expressed high levels of HO-1 with highest expression in a CD16+ monocyte subset (Fig. 5A), consistent with a previous study in a non-SCD setting (21). Interestingly, we found extremely low levels of HO-1 in T cells and Tregs, although others have found HO-1 in T cells, including Tregs (Fig. 5A) (27). These data suggest that HO-1 expressed in CD16+ monocytes rather than in Tregs may be responsible for the effects of hemin observed in our study, which is consistent with studies in a transgenic mouse system showing that HO-1 expressed in Tregs had little, if any, anti-inflammatory activity, whereas when expressed in monocytes, it was highly immunosuppressive (28). Monocyte HO-1 levels were higher in SCD patients than in healthy controls (Fig. 5B), as previously reported (29–31). Importantly, non-alloimmunized SCD patients expressed significantly higher levels of HO-1 in CD16+ monocytes as compared with alloimmunized patients (Fig. 5C).

Following a 24-h treatment with hemin, HO-1 levels in T cells/Tregs remained low (<2% of cells). Monocyte HO-1 levels were upregulated, albeit only at the highest hemin concentrations used, although we did not detect any differences in upregulation of HO-1 between alloimmunized and non-alloimmunized patients (Fig. 5D). Taken together, these data indicate that basal levels of CD16+ monocyte HO-1, but not inducibility by hemin, differ between alloimmunized and non-alloimmunized SCD patients.

IL-12–mediated regulation of Treg/Th proliferation

We have previously shown that the Th1-polarizing cytokine IL-12 is involved in CD16+ monocyte regulation of Treg polarization in healthy controls (19). Specifically, CD16+ monocytes through secretion of IL-12 inhibit Treg proliferation, altering Helios+ Treg expansion. To test whether CD16+ monocyte–derived IL-12 also controls Treg/Th proliferation in SCD patients, we performed IL-12 neutralization studies in the CD4+ T cell/monocyte cocultures. Ab blocking with anti–IL-12 resulted in expansion of total Tregs,
including Helios+/− Tregs (Fig. 6A) and inhibition of Th1 expansion (Fig. 6B) in cocultures that included CD16+ monocytes, regardless of alloimmunization state, confirming its role in SCD CD16+ monocyte–mediated regulation of Treg/Th1 polarization. To test whether altered Treg/Th polarization in alloimmunized versus non-alloimmunized SCD patients may be due to differential IL-12 expression before and/or after hemin treatment, we measured IL-12 levels by ELISA in the T cell/monocyte cocultures. However, IL-12 was undetectable in the supernatants of many of the cocultures, especially those that lacked CD16+ monocytes and the ones after hemin treatment. We therefore analyzed monocyte IL-12 expression in short-term LPS-stimulated PBMCs from alloimmunized and non-alloimmunized SCD patients before and after hemin treatment. We found a greater inhibition of IL-12 in non-alloimmunized monocytes as compared with the alloimmunized group following hemin treatment (Fig. 6C), suggesting that hemin dampens IL-12 expression more effectively in non-alloimmunized patients.

**Discussion**

In the present study, we have found that hemin, a surrogate marker for transfused RBC breakdown products, can induce CD4+ T cell subset polarization mediated through monocyte HO-1, thereby establishing for the first time, to our knowledge, a role for HO-1 in T cell polarization in the human setting. Because aberrant T cell polarization, altered HO-1 expression (32–34), and monocyte activity (35–37) have been reported in various inflammatory diseases, it raises the possibility that the CD16+ monocyte HO-1/T cell polarization axis may also play a pivotal role in the etiology and prognosis of such inflammatory diseases. HO-1 activity and levels can be regulated by multiple factors (9), and therefore understanding the mechanisms of HO-1-mediated T cell polarization may not only provide insight into the etiology of diseases but also may offer potential therapy targets for such diseases (38, 39). In this regard, the present study identified differences in Treg/Th proliferation in response to hemin between alloimmunized and non-alloimmunized SCD patients. The difference was most notable under CD16+ monocyte-enriched conditions such as transfused RBC breakdown products, thus increasing the risk of further alloimmunization in this patient group. In support of this idea, it is estimated that once a patient makes an alloantibody, the risk of alloimmunization increases by 20-fold (43). Increasing evidence implicates a role for CD16+ monocytes in the control of Th polarization in autoimmune disease setting such as CD16+-mediated Th17 expansion in rheumatoid arthritis (16) and CD16+-driven Th1 polarization in immune thrombocytopenia (18).

Our data suggest that this monocyte population may also be involved in SCD alloimmunization. Clearly, longitudinal studies of SCD patients receiving RBC transfusions are needed to determine whether monocyte reactivity and Treg/Th polarization differ in patients who go on to develop alloantibodies as compared with alloimmunized SCD patients. The model predicts that because of the inability of CD16+ monocytes to switch off their proinflammatory state in response to heme, alloimmunized patients are more likely to develop a strong immune response against allogeenetic determinants on transfused RBCs, thus increasing the risk of further alloimmunization in this patient group. In support of this idea, it is estimated that once a patient makes an alloantibody, the risk of alloimmunization increases by 20-fold (43). Increasing evidence implicates a role for CD16+ monocytes in the control of Th polarization in autoimmune disease setting such as CD16+-mediated Th17 expansion in rheumatoid arthritis (16) and CD16+-driven Th1 polarization in immune thrombocytopenia (18). Our data suggest that this monocyte population may also be involved in SCD alloimmunization. Clearly, longitudinal studies of SCD patients receiving RBC transfusions are needed to determine whether monocyte reactivity and Treg/Th polarization differ in patients who go on to develop alloantibodies as compared with alloimmunized SCD patients.

**FIGURE 6.** Role of IL-12 in CD16+ monocyte control of Treg/Th polarization and response to hemin. Isotype control (−) or neutralizing anti–IL-12 p40/p70 (+) was added to the T cell/monocytes cocultures that included CD16+ monocytes from non-alloimmunized (open bars, n = 8) and alloimmunized (filled bars, n = 8) SCD patients at day 0, and frequencies of (A) total Tregs and Helios+/− Treg subsets and (B) IFN-γ+ cells in divided CD4+ T cells as measured on day 7 are shown. All statistical analyses comparing isotype versus anti–IL-12 were performed using a paired t test. (C) Frozen and then thawed PBMCs from non-alloimmunized (open bars, n = 8) and alloimmunized (filled bars, n = 7) SCD patients were stimulated with IFN-γ in the absence or presence of hemin (1.25 μM) for 2 h followed by addition of LPS for another 22 h. Frequency of IL-12–expressing monocytes in untreated and hemin treated samples are shown. The p values indicate paired t test comparison analysis of untreated versus hemin-treated samples.
those who do not. Our cohort consisted of heavily transfused SCD patients. Because the cumulative number of transfused units appears to increase alloimmunization risk (44), it remains to be determined whether differences in monocyte control of Treg/Th polarization would also be evident in less heavily transfused SCD patients or for that matter in transfused patient populations other than SCD patients.

Heme/hemin can act as the substrate for HO-1, thus activating its enzymatic activity as well as the ability to upregulate HO-1 expression, further increasing its activity (9). HO-1 is reported to be upregulated in SCD (29–31), which is consistent with our data of higher monocyte HO-1 levels in SCD patients as compared with healthy controls. In the non-SCD setting, HO-1 is considered immunosuppressive, as it was shown to inhibit T lymphocyte proliferation (9), block maturation of dendritic cells, and inhibit proinflammatory and alloageneric immune responses (45, 46). Indeed, the immunosuppressive effects of HO-1 are mediated through the anti-oxidative and anti-inflammatory activities of two of the heme breakdown products, namely carbon monoxide and biliverdin (9). Alternatively, the third byproduct, free iron, is a pro-oxidant that has to be deactivated by ferritin (9). A key finding of our study is that the heme concentrations used to elicit differences in monocyte control of T cell polarization did not induce upregulation of HO-1 expression, at least at the protein level. This suggests that heme may be acting as a substrate for HO-1 at these concentrations, inducing a more effective anti-inflammatory response in these patients due to higher CD16+ monocyte HO-1 levels as compared with the alloimmunized group.

The molecular mechanisms responsible for lower basal CD16+ monocyte HO-1 levels/activity in our alloimmunized SCD patients are unknown. One possibility is that the allosensitized group has higher baseline chronic intravascular hemolysis, a characteristic feature of SCD, which could account for lower HO-1 levels, although no differences in baseline chronic hemolysis between and non-allosensitized patients have previously been reported. Additionally, our patient cohort was undergoing exchange transfusion as part of their regular transfusion procedure to maintain their hemoglobin S levels rather than because of an acute need for transfusion, and it is therefore unlikely that the alloimmunized group had increased baseline hemolysis due to hemolytic transfusion reaction at the time of the study. Nevertheless, we were unable to examine any markers of hemolysis such as lactate dehydrogenase, bilirubin, or plasma hemoglobin levels that correlate with SCD HO-1 activity (47) because the patients were stripped of all identifiers with the exception of alloimmunization state. Genetic differences such as altered transcriptional control by microRNAs (48) or promoter polymorphisms (49, 50) may be responsible for lower CD16+ monocyte HO-1 levels, and future studies will aim to explore these possibilities.

It remains to be determined which of the key HO-1 catalytic byproducts are responsible for differences in Treg/Th polarization in alloimmunized versus non-alloimmunized SCD patients. Both carbon monoxide (51) and bilirubin (52, 53) have been shown to induce Treg expansion in mouse disease models, and their effects can be additive, indicating that both carbon monoxide and bilirubin may be responsible for promoting HO-1-mediated Treg expansion. However, the direct effects of carbon monoxide and/or bilirubin on Treg expansion in human disease settings or in culture conditions remain unexplored. Future studies focusing on the byproducts of HO-1 in SCD alloimmunization will help dissect the mechanism of HO-1-mediated Treg expansion and may identify potential therapeutic targets for prevention of alloimmunization. Interestingly, iron chelation and treatment with antioxidants can modify expression and activation of HO-1 (54, 55). This raises the possibility that transfusion-associated alloimmunization may be suppressed with peak induction of monocyte HO-1 levels through optimal use of iron chelation that can adequately lower iron levels in the monocyte/macrophage system and of antioxidants to reduce the pro-oxidant state of monocytes.

In conclusion, we have shown that the immunoregulatory function of the innate immune cells in alloimmunized SCD patients may be altered such that in response to hemin, a surrogate for transfused RBC breakdown products, these cells are no longer able to induce Treg expansion or inhibit Th1 development, anti-inflammatory responses that are induced with the same treatment in non-alloimmunized patients. Although SCD patients before and after alloimmunization were not studied, we hypothesize that in the initial step of encountering the transfused RBCs, the activation state of the monocyte/macrophage system will be key to whether T cells are polarized toward regulatory or stimulatory type cells and ultimately impact the risk of alloimmunization.

Disclosures
The authors have no financial conflicts of interest.

References