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Summary

Sickle cell disease (SCD) is increasingly appreciated as an inflammatory condition associated with alterations in immune phenotype and function. In this cross-sectional study we performed a multiparameter analysis of 18 immune markers in 114 paediatric SCD patients divided by treatment group [those receiving hydroxycrebamide (HC, previously termed hydroxyurea), chronic transfusion (CT), or no disease-modifying therapy] and 29 age-matched African American healthy controls. We found global elevation of most immune cell counts in SCD patients receiving no disease-modifying therapy at steady state. Despite the decrease in percentage of haemoglobin S associated with CT therapy, the abnormal cellular immune phenotype persisted in patients on CT.

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Author contributions
RSN, JTH, JEH and LSK: conceived the study, analysed data, wrote the paper. IO: conceived the study, analysed data. AG: performed experiments. JR, DRA and DELP: analysed data.

Conflict of interest
The authors declare no competing financial interests.

The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

Supporting Information
Additional Supporting Information may be found in the online version of this article.
In contrast, in both univariate and multivariate analysis, treatment with HC was associated with normalization of the vast majority of leucocyte populations. This study provides additional support for HC treatment in SCD, as it appears that HC decreases the abnormally elevated immune cell counts in patients with SCD.

Keywords
sickle cell disease; immune phenotype; immunophenotype; hydroxycarbamide; chronic transfusion

Patients with sickle cell disease (SCD) are known to have altered immune systems. While immune abnormalities in SCD have historically been attributed largely to splenic dysfunction (caused by autoinfarction in early childhood leading to functional asplenia) (Barrett-Connor, 1971), growing evidence exists that the immune deviation in SCD extends beyond splenic-associated abnormalities and that SCD itself is a pro-inflammatory condition with exaggerated immune activation (Platt, 2000; Holtzclaw et al, 2004). This activation may contribute to immune-based clinical issues in SCD, including red blood cell (RBC) auto- (Castellino et al, 1999) and allo-immunization (Chou et al, 2013) and haematopoietic cell transplantation graft rejection, especially when using reduced-intensity conditioning (Iannone et al, 2003; Jacobsohn et al, 2004; Horan et al, 2005) and human leucocyte antigen-mismatched donors (Bolanos-Meade et al, 2012; Kamani et al, 2012; Kharbanda et al, 2014).

In addition to the phenomenological observations consistent with immune activation in SCD, recent work has begun to establish that immune activation contributes to the pathology of SCD. Studies have shown that patients with SCD, especially during vaso-occlusive crisis, have increased activation of neutrophils (Fadlon et al, 1998; Lard et al, 1999) and monocytes (Belcher et al, 2000; Wun et al, 2002) as well as increased cytokine levels (Duits et al, 1998; Pathare et al, 2004). There is also evidence for elevated levels and activity of invariant natural killer T cells, which have been shown to contribute to pulmonary ischaemia-reperfusion injury in murine SCD (Wallace et al, 2009) and to be elevated both at steady state and further during vaso-occlusive crisis in SCD patients (Wallace et al, 2009; Field et al, 2013).

While these recent studies have begun to link immunological abnormalities with SCD, little work has been done to investigate in detail the immunophenotype of patients with SCD at steady state, and to determine the impact of chronic transfusion (CT) or hydroxycarbamide (HC) therapy on this phenotype. CT [with a goal of reducing the proportion of haemoglobin S (HbS) to <30% via monthly RBC transfusions] is a well-established strategy for stroke prevention (Adams et al, 1998; Wang & Dwan, 2013), yet its potential impact on leucocyte counts is not well characterized. Likewise, while the ribonucleotide reductase inhibitor HC is well documented to reduce the total white blood cell (WBC) and neutrophil counts (Charache et al, 1995; Wang et al, 2011), its impact on other aspects of innate and adaptive immunity have not been fully elucidated. Therefore, to better characterize the immune phenotype in SCD patients, we conducted a large cross-sectional analysis of their immune
profiles at their baseline states of health on no therapy, CT, or HC, and compared these profiles to those of healthy controls.

**Methods**

**Study participants**

Data on paediatric patients with SCD were drawn from cross-sectional studies conducted at Children’s Healthcare of Atlanta between October 2010 and April 2013. Approval was obtained by the Emory University and Children’s Healthcare of Atlanta Institutional Review Boards. All patients enrolled had HbSS or HbSβ0 and no other condition associated with known abnormalities of the immune system. Patients were excluded if they had significant acute health problems in the 2 weeks prior to blood collection. A group of age-matched, healthy African Americans was also enrolled as controls. These individuals were recruited from contacts of patients and clinic staff. They completed a questionnaire and underwent a complete blood count (CBC) and haemoglobin electrophoresis testing to ensure that they did not have a chronic medical condition. Clinical data on patients were abstracted from the medical records. Patients were classified as being on CT therapy if they had recently received >3 consecutive planned outpatient RBC transfusions. Patients were classified as being on HC therapy if clinic notes documented that the patient was taking HC for at least 1 month. Patients were excluded from analysis if treatment classification was unclear. Patients under the age of 5 years were also excluded in an attempt to have similar age distributions in each treatment group. Each patient was evaluated using a consistent immunophenotyping strategy with a standard set of flow cytometry panels (Table SI). This analysis was performed by a single laboratory (Kean laboratory) on an LSRII flow cytometer (BD Biosciences, San Jose, CA, USA).

**Immunophenotype analysis**

Immune phenotype analysis was performed on peripheral blood drawn into a Cytochex BCT tube (Streck Inc, Omaha, NE, USA). Quantitative evaluation of total WBC, lymphocytes, monocytes and polymorphonuclear leucocytes (neutrophils) was performed based on their CD45 expression versus sideways scatter (SSC) and compared to quantitative TruCount beads (BD Biosciences, San Jose, CA, USA). Lymphocytes were defined as CD45^high/SSC^low, monocytes as CD45^mid/SSC^mid, and neutrophils as CD45^low/SSC^high. The following lymphocyte populations were then identified immunologically: total CD3^+ T cells (CD3^+/CD20^- lymphocytes), total CD20^+ B cells (CD3^-/CD20^+ lymphocytes), natural Killer (NK) cells (CD3^-/CD20^-/CD16^+), CD4^+ T cells (CD4^+/CD8^- T cells), CD8^+ T cells (CD8^+/CD4^- T cells) and CD4^+ putative T-regulatory cells (CD4^+/CD25^high/CD127^low). In addition, naïve T cells (Tn) were characterized as CD45RA^-/CCR7^-; central memory (Tcm) as CD45RA^-/CCR7^-; effector memory (Tem) as CD45RA^-/CCR7^- and terminally-differentiated effector cells (Temra) as CD45RA^-/CCR7^- for both the CD4^+ and CD8^+ T cell populations. For comparative analysis, Tcm, Tem and Temra were combined and depicted as ‘T-memory’. Naïve B cells were identified as CD27^-/IgD^+ and memory B cells as CD27^+ CD38^- IgD^- . In addition, proliferation was measured by Ki67 expression. Flow cytometry data was analysed using FloJo software (TreeStar, Ashland, OR, USA).
Statistical analysis

Statistical analyses were performed with the bioconductor module of R, SAS 9.3 (SAS Institute Inc., Cary, NC, USA) and graphics created using both R and GraphPad Prism version 6.02 (GraphPad Software, La Jolla, CA, USA, www.graphpad.com). For comparative analysis, cell count data was normalized via log transformation. Statistical differences in the cell counts for the entire SCD patient group compared to the healthy control group were assessed by a two-tailed *t*-test. Statistical differences in the cell counts between the different SCD treatment groups and controls were assessed by analysis of variance (ANOVA) with Tukey post-hoc multiple comparison analysis. Multivariable analysis with linear regression was performed to control for potential confounding variables of age (continuous variable), sex and history of a splenectomy. In addition, multivariate analysis was performed by Principle Component Analysis (PCA). PCA is a statistical method to reduce a dataset consisting of a large number of variables, many of which are highly correlated, to a smaller number of principal components (PCs, Fig S1A) (Sainani, 2014). Each PC is a vector that consists of a set of loadings, which describe the relative contribution of each variable to that PC; the largest loadings represent the most important variables distinguishing the data sets. The first 2–3 PCs typically capture the majority of variation in the data. For the PCA performed in this study, 18 immune parameters were included: WBC, neutrophils, monocytes, lymphocytes, B cells, T cells, NK cells, naïve B, memory B, CD4+, CD8+, CD4+ T-naïve, CD4+ T-memory, CD4+ T-regulatory, CD8+ T-naïve, CD8+ T-memory, CD4+ Ki67, and CD8+ Ki67. These parameters were analysed using the Bioconductor MADE4 package (Culhane et al, 2005).

Results

Patient characteristics

A total of 134 patients with SCD and 29 healthy controls were enrolled. Twenty of the 134 patients were excluded from comparative immune phenotype analysis because of not meeting study eligibility criteria (n = 3), not being able to classify treatment group (n = 9), age <5 years (n = 7), and a technical problem with the immune phenotyping (n = 1). The remaining 114 patients were divided into four clinical groups based on CT and HC therapy: (i) Patients on neither CT nor HC therapy (No Rx, n = 17). (ii) Patients receiving HC but not on CT (HC, n = 23). (iii) Patients on CT therapy but not receiving HC (CT, n = 68). (iv) In addition, a small number of patients were on both CT and HC therapy (CT + HC, n = 6). Many of the healthy African Americans were siblings of patients with SCD (8/29 were siblings of patients enrolled on this study and 14/29 were siblings of any SCD patient) and 8/25 had sickle cell trait.

Patients in the HC group had been taking HC for a median of 34·7 months (range 2·7–100·8 months) and were on a median dose of 26·3 mg/kg. Only 3/23 HC group patients had received HC for less than 6 months. CT patients had been receiving CT for a median of 59·6 months (range 5·9–144·1 months). CT + HC patients had been receiving CT for a median of 68·1 months (range 4·1–197·8 months) and HC for a median of 17·1 months (range 1·2–75·1 months), with a median HC dose of 23·2 mg/kg. The indications for CT + HC dual therapy were: HC added to CT while being weaned off CT (4/6, with the median time from last
transfusion of 39 d), HC added to CT due to an inability to suppress the percentage of
haemoglobin S [%HbS] <30% (1/6), and CT added to HC in preparation for major surgery
(1/6). Only 4/74 (5.4%) of CT patients received regular exchange transfusions with the rest
of the CT patients receiving simple transfusions. None of the patients receiving exchange
transfusions were receiving concomitant HC therapy.

Table I shows the demographic and clinical information of the patients and controls. The
groups did not significantly differ in sex distribution ($P = 0.32$) and had similar mean ages
(12.0–13.9 years, $P = 0.32$). When comparing just the four SCD patient groups, the CT
groups (CT and CT + HC) had a higher prevalence of splenectomy than the non-CT groups
(No Rx, HC) ($P = 0.05$). Also when analysing just the SCD patients, CBCs demonstrated
significant differences in haemoglobin values, reticulocyte counts and platelet counts among
the treatment groups. The %HbS was also significantly ($P < 0.0001$) different between the
SCD treatment groups with the No Rx group having the highest %HbS (83.9%) and CT
group the lowest %HbS (22.4%).

**Multiparameter flow cytometric analysis reveals widespread elevation in cell counts in
patients with SCD compared to healthy controls**

To determine if the SCD patients analysed, without regard to treatment, had an altered
immune phenotype, we first compared all SCD patients studied to the age-matched African
American controls. As shown in Fig 1, the combined SCD patients (green bars)
demonstrated significant increases in most leucocyte populations compared to controls (grey
bars), with patients demonstrating quantitatively increased cell counts for all subsets tested
with the exception of NK cells, memory B cells, and naïve CD8+ T cells (where there was
no significant difference compared to controls). Thus, SCD patients had elevated WBC
counts (13.75 ± 0.647 vs. 5.728 ± 0.334 × 10^9 cells/l, $P < 0.0001$), absolute neutrophil
counts (8.369 ± 0.526 vs. 2.986 ± 0.282 × 10^9 cells/l, $P < 0.0001$), total lymphocytes (3.786
± 0.155 vs. 2.220 ± 0.12 × 10^9 cells/l, $P < 0.0001$), monocytes (1.160 ± 0.06 vs. 0.377 ±
0.025 × 10^9 cells/l, $P < 0.0001$), total T cells (2.148 ± 0.081 vs. 1.434 ± 0.079 × 10^9 cells/l,
$P < 0.0001$) and total B cells (1.134 ± 0.078 vs. 0.429 ± 0.032 × 10^9 cells/l, $P < 0.0001$).
Total CD4+ T cells were also increased in SCD patients compared to controls (1.286 ± 0.05
vs. 0.82 ± 0.049 × 10^9 cells/l, $P < 0.0001$), and this increase in CD4+ counts was more
pronounced than the SCD-associated increase in CD8+ counts (0.607 ± 0.03 vs. 0.459 ±
0.034 × 10^9 cells/l, $P = 0.027$). As shown in Fig 1C, the increase in total CD4+ T cell counts
was driven by increases in all of the CD4+ T cell subpopulations analysed (CD4+ T-naïve,
T-memory and T-regulatory), and was accompanied by increased numbers of proliferating
(Ki67+) CD4+ T cells. In contrast, CD8+ T cells only demonstrated significant increases in
memory (not naïve) and proliferating T cells compared to healthy controls (Fig 1D). The
increase in B cells observed in the combined SCD patients was dominated by an increase in
the absolute number of naïve B cells (0.648 ± 0.05 vs. 0.294 ± 0.033 × 10^9 cells/l, $P =
0.001$) rather than memory B cells (0.026 ± 0.002 vs. 0.022 ± 0.004 × 10^9 cells/l, $P = 0.059$)
(Fig 1B). Each of the differences between the combined SCD patients and the control group
that were found to be significantly different on univariate analysis remained significant
when controlling for sex, age and history of surgical splenectomy, with the exception of
total CD8⁺ T cells, which ceased to be significantly different on multivariable analysis (Table SII).

In addition to the widespread elevation of leucocyte counts, the combined SCD patients demonstrated increased percentages of the total WBC that were neutrophils and monocytes compared to controls (Fig 2A). These increases drove a proportional decrease in the percentage of the total WBC accounted for by lymphocytes (despite the increase in the absolute lymphocyte count). Within the lymphocyte population, the combined SCD patients demonstrated an increased percentage of B cells and decreased percentage of T cells compared to controls (Fig 2B) despite their quantitative increase in T cell counts. The combined SCD patients also had an increased CD4⁺/CD8⁺ ratio compared to controls (Fig 2C).

**Elevated leucocyte counts persist despite CT therapy**

To explore the potential influence of CT and HC on the SCD immune profile, we next performed a sub-analysis involving the four SCD clinical groups described above. As shown in Fig 1, in SCD patients receiving neither CT nor HC (No Rx group, red bars), the vast majority of immune subsets tested demonstrated significantly increased cell numbers compared to the healthy controls. Similar increased cell counts were also present in the CT group (dark blue bars). In contrast, in those patients treated with HC (HC and CT + HC groups, pink and light blue bars respectively), immune subset counts were closer to those of the healthy controls. This pattern was not altered when controlling for sex, age and history of splenectomy through multivariable linear regression analysis (Table SIII). Neither of the groups treated with HC had any cell counts that were significantly lower than those observed in healthy controls.

To further investigate possible associations between HC and CT treatment (as well as surgical splenectomy, age and sex) with the elevated leucocyte counts among the SCD patients, a linear regression model was created (Table II). In agreement with the univariate analysis, this multivariable analysis also demonstrated an association of elevated cell counts for most leucocyte populations with CT treatment, but more normalized counts in association with HC treatment. Surgical splenectomy was only significantly associated with the total B cell population, with surgically splenectomized SCD patients having an estimated 1.36 times the number of B cells than non-splenectomized SCD patients when controlling for HC, CT, age and sex. Even when excluding the patients with a history of surgical splenectomy, the SCD patients continued to have a significantly increased B cell count compared to the healthy controls (mean B cell count for SCD patients with no splenectomy 1.03 × 10⁹ cells/l vs. healthy controls 0.43 × 10⁹ cells/l, \( P < 0.0001 \)).

To begin to explore the effect of iron overload on immune cell counts, the relationship between serum ferritin and immune cell counts was tested by linear regression for patients in the CT group. In this analysis, an increasing ferritin value was associated with higher immune cell counts (with this relationship being significant for most populations studied), with the exception of NK cells in which ferritin had a non-significant negative correlation with NK cell count (Table SIV, Fig S2).
Principal component analysis of the global immune profile confirms treatment-specific differences in SCD patient cohorts

We also utilized PCA, a statistical visualization strategy for complex data (see Methods), to interrogate the immune phenotype data (Fig S1). Our PCA compared (i) combined SCD patients to healthy controls (Fig S1B–C) and (ii) the four SCD treatment groups to each other and to healthy controls (Fig S1D). PCA was able to distinguish SCD patients as a group from healthy controls but demonstrated that there were also patients that overlapped with controls. This observation supported the univariate analysis shown in Fig 1, in which some SCD treatment regimens move the SCD immune signature closer to the profile of healthy controls. This idea was supported by the finding that the PC-1 for patient groups not treated with HC (No Rx and CT) was significantly different from controls, but the PC-1 for groups treated with HC (HC and CT + HC) was not.

Discussion

An elevated baseline total WBC count has long been associated with SCD (West et al, 1992) and various SCD studies (Lard et al, 1999; Wallace et al, 2009; Polanowska-Grabowska et al, 2010; Vingert et al, 2014) have reported evidence of abnormal cellular immune activation in specific cell types. Few studies, however, have investigated a more complete cellular immunological profile of patients with SCD at steady state. Wong et al (1995) described lymphocytic indices in transfused and untransfused patients with SCD compared to healthy controls, however, no patients in that study were treated with HC. Lederman et al (2014) recently reported on T cell subsets in infants treated or not with HC, however, that study did not include healthy controls or transfused patients. Our study builds on this previous work by uniquely describing immune cell counts in both paediatric SCD patients receiving HC and patients undergoing CT, and comparing these results to those in healthy African American children.

This study demonstrated that despite transfusion-mediated decreases in HbS, patients treated with CT continued to have widespread elevations in multiple immune cell counts. It is important to note that, given this study’s cross-sectional study design, the impact of CT on a particular patient’s immune profile cannot be determined. It is possible that patients who meet criteria for CT therapy have more abnormal immune profiles prior to CT therapy initiation, as suggested by previous studies that demonstrate leucocytosis is a risk factor for SCD complications (Castro et al, 1994; Platt et al, 1994; Kinney et al, 1999; Quinn et al, 2008), including stroke (Balkaran et al, 1992), the main indication for CT therapy. In addition, the potential impact of transfusion-related immunomodulation (Kaplan et al, 1984; Grady et al, 1985; Puhwa et al, 1985; Vanvakas & Blajchman, 2007; Purohit et al, 2012) and transfusional iron overload (Akbar et al, 1986; Cunningham-Rundles et al, 2000; Walker & Walker, 2000) on the immune profiles of SCD patients is not known. However, our analysis of serum ferritin, an imperfect marker of iron overload, suggests that for most cell populations iron load may contribute to increased cell counts. Finally, the vast majority of CT patients enrolled in this study were on simple transfusion therapy and thus these results may not apply to patients on exchange transfusion therapy. Despite these important
caveats, the persistence of the immunological abnormalities in the CT patients was striking, and is deserving of further longitudinal analysis.

It is notable that the one exception to the widely increased leucocyte subset counts in CT patients occurred with NK cells, in which patients treated with CT had a lower NK cell count compared to non-CT-treated patients, and for which increased ferritin was not associated with higher counts. A previous study drew a link between iron overload in transfused thalassaemia patients and decreased NK activity (Akbar et al, 1986). While our study was not designed to make definite mechanistic connections between SCD therapies and individual cell populations, the outlier-status of the impact of CT on NK cells suggests that this finding may deserve closer scrutiny.

While the causal relationship between CT or HC treatment and the immune profile cannot be determined from this cross-sectional study, the observations made in HC-treated patients are compelling. The normalization of the immunophenotype in SCD patients receiving only HC (HC group) compared to SCD patients receiving neither CT nor HC (No Rx group) is especially important given that patients treated with HC would be expected to have more severe SCD than the untreated group. Our finding that HC appears to normalize the T cell immunophenotype of SCD patients is in agreement with the results of a recent longitudinal, placebo-controlled study of HC in young children with SCD (Lederman et al, 2014).

Hydroxyurea has many proven clinical benefits for patients with SCD (Charache et al, 1995; Wang et al, 2011), however some clinicians still have reservations about recommending HC therapy and many patients refuse HC. One of the barriers to acceptance of HC therapy is a fear of side-effects including myelosuppression (Haywood et al, 2011; Lebensburger et al, 2013; Oyeku et al, 2013). Our observation that HC patients (most treated with HC for over 2 years) had a more normal immune profile rather than an abnormal reduction in immune cell counts compared to healthy controls is therefore a critical finding. This observation is consistent with the results of the Paediatric Hydroxyurea in Sickle Cell Anaemia (BABY HUG) trial, where infants with SCD treated with HC did not demonstrate an increased risk of serious infections (Wang et al, 2011; Thornburg et al, 2012) and who, at study exit, had normal protective antibody titres from routine vaccines (Lederman et al, 2014).

While few patients receiving dual therapy (CT + HC group) were available for study (n = 6) and thus it is not possible to draw strong conclusions from this group, their data are notable nonetheless as little has been published on patients receiving dual therapy. The trend towards apparent normalization of immune parameters in the CT + HC group suggests that the beneficial effect of HC on SCD-associated immune abnormalities may be robust enough to normalize the immune profile even in patients who qualify for CT. This finding leads to the provocative hypothesis that dual therapy with HC and CT may be beneficial for patients currently receiving CT. A small, retrospective study suggested that adding HC to CT may further decrease the risk of progressive cerebrovascular disease (Brousse et al, 2013). This idea that combination therapy may provide additional benefits to CT patients should be studied in a randomized trial. It is quite possible, however, that we observed a trend to more normal immune cell counts in the CT + HC group because of other cofounding variables.
(such as less severe iron overload in the CT + HC group) or simply by chance due to the small number of patients studied.

The potential immune modulatory impact of HC suggested by this study may also be relevant to the challenges in successfully transplanting patients with SCD. A retrospective report found a decreased risk of graft rejection in SCD patients who were treated with HC pre-transplant (including all patients on CT therapy) (Dedeken et al., 2014). While that report did not account for other confounding variables that changed over time that may have influenced graft rejection, it does offer some correlative support for including HC as a component of pre-transplant preparation for SCD patients. Being cognizant that the reasons for graft rejection in SCD patients are not fully understood, our findings suggest that HC may normalize the immune milieu prior to transplant and, in so doing, may potentially lower the risk of transplant rejection.

Our finding that surgical splenectomy was not significantly associated with any alterations in immune cell counts except the total B cells may be because most SCD patients are functionally asplenic. It is thus possible that the spleen does contribute to the abnormal immune phenotype in SCD patients compared to healthy controls.

While this study identified several new insights into the immune manifestations of SCD, limitations exist. First, this study was cross-sectional and thus only involved a single time-point for each of the enrolled patients. While our results are suggestive of the salutary effect of HC on the SCD immune phenotype, a prospective longitudinal study is necessary to fully evaluate both the HC and CT treatment effects. Another limitation is that the functional status of many of the analysed cells was not investigated, and changes in lymphocyte subset numbers cannot be assumed to reflect functional immune deviation. While this study included the analysis of several phenotypes that correlate with function (including markers of T cell proliferation and T and B cell memory status), antigen-specific tests of immune activation (including protective as well as alloreactive immunity) will be important to add to future studies. In addition, although this is one of the largest descriptive studies of immune phenotype in SCD, it remains limited by the relatively small sample size in the four treatment groups. Our study SCD population also overly represented patients on CT therapy, such that our ‘Combined SCD patient’ group was most influenced by this sub-group. However, the similarity of the No Rx group and the CT group suggests that many of the immune phenotype abnormalities observed in patients on CT are similar to those observed in SCD patients who are not on disease-modifying therapies. Finally, although the analysis of SCD patients in steady-state allowed us to draw conclusions about SCD itself (without the confounder of concomitant illness) on the immune profile, it is likely that patients experiencing specific SCD complications may demonstrate important changes in their immune phenotype and function. Future studies that include patients both at steady-state as well as during acute SCD complications would shed light on the impact of these complications on the immune manifestations of SCD.

In conclusion, this study has defined global elevations of immune cell counts that occur in clinically stable paediatric patients with SCD. These elevated cell counts persisted in patients treated with CT. In contrast, patients on HC demonstrated an immunophenotype...
more similar to that of healthy controls. These results provide additional support for the use of HC in patients with SCD and suggest that a large-scale longitudinal analysis of the impact of SCD treatment on immune phenotype and function is warranted.

**Supplementary Material**

Refer to Web version on PubMed Central for supplementary material.

**Acknowledgments**

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Fig 1.
Comparison of immune cell counts in sickle cell disease (SCD) patient treatment groups versus healthy controls. The SCD Combined Groups (green) includes patients with SCD in all four of the treatment subgroups. Other groups shown are: Healthy control (grey), No Rx (no chronic transfusion or hydroxycarbamide therapy, red), HC (hydroxycarbamide therapy, pink), CT (chronic transfusion, dark blue), CT + HC (chronic transfusion and hydroxycarbamide therapy, light blue). Comparisons between SCD Combined Groups and Healthy Control groups were assessed by two tailed t-tests. Comparisons between the four SCD treatment subgroups and the Healthy Control group were assessed by ANOVA; P-value above bar represents comparison to Healthy Control group, all other significant associations are shown. (A) Leucocyte populations, mean count ± standard error of the mean (SEM). (B) Lymphocyte subpopulations, mean count ± SEM. (C) CD4+ T cell subpopulations, mean count ± SEM. (D) CD8+ T cell subpopulations, mean count ± SEM. nsP > 0.05, *P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001, ****P ≤ 0.0001. WBC, white blood cell.
Fig 2.
Comparison of immune cell proportions in sickle cell disease (SCD) patient treatment groups versus healthy controls. The SCD Combined Groups (green) includes patients with SCD in all four of the treatment subgroups. Other groups shown are: Healthy control (grey), No Rx (no chronic transfusion or hydroxycarbamide therapy, red), HC (hydroxycarbamide therapy, pink), CT (chronic transfusion, dark blue), CT + HC (chronic transfusion and hydroxycarbamide therapy, light blue). Comparisons between SCD Combined Groups and Healthy Control groups were assessed by two tailed t-tests. Comparisons between the four SCD treatment subgroups and the Healthy Control group were assessed by ANOVA; P-value above bar represents comparison to Healthy Control group, all other significant associations are shown. (A) Proportion of leucocyte population with respect to total white blood cell (WBC) count, mean percentage ± standard error of the mean (SEM). (B) Proportion of lymphocyte subpopulation with respect to total lymphocyte count, mean percentage ± SEM. (C) CD4+/CD8+ mean ratio ± SEM. nsP > 0·05, *P ≤ 0·05, **P ≤ 0·01, ***P ≤ 0·001, ****P ≤ 0·0001.
### Table I

Demographic and clinical characteristics by treatment group.

<table>
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<th>No Rx, n = 17</th>
<th>HC, n = 23</th>
<th>CT, n = 68</th>
<th>CT + HC, n = 6</th>
<th>Healthy controls*, n = 29</th>
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<td>43 (63·2)</td>
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</tr>
<tr>
<td>Splenectomy, n (%)</td>
<td>1 (5·9)</td>
<td>3 (13·0)</td>
<td>22 (32·4)</td>
<td>2 (33·3)</td>
<td>0 (0)</td>
<td>&lt;0·05‡</td>
</tr>
<tr>
<td>Hb, g/l, mean (SD)</td>
<td>88 (12)</td>
<td>95 (10)</td>
<td>96 (98)</td>
<td>99 (14)</td>
<td>132 (11)</td>
<td>&lt;0·001‡</td>
</tr>
<tr>
<td>Reticulocyte count, × 10⁹/l mean (SD)</td>
<td>377 (96)</td>
<td>235 (117)</td>
<td>319 (113)</td>
<td>230 (108)</td>
<td>72 (23)</td>
<td>0·003†</td>
</tr>
<tr>
<td>%HbS†, median (IQR)</td>
<td>83·9 (12·2)</td>
<td>75·6 (14·0)</td>
<td>22·4 (19·8)</td>
<td>40·9 (13·6)</td>
<td>0 (37·7)</td>
<td>&lt;0·0001‡</td>
</tr>
<tr>
<td>Platelet count, × 10⁹/l mean (SD)</td>
<td>531 (101)</td>
<td>414 (214)</td>
<td>434 (140)</td>
<td>360 (144)</td>
<td>293 (50)</td>
<td>0·02‡</td>
</tr>
<tr>
<td>Ferritin (μg/l), median (IQR)</td>
<td>n/a</td>
<td>n/a</td>
<td>2623 (2050)</td>
<td>1188 (5275)</td>
<td>n/a</td>
<td>0·38§</td>
</tr>
<tr>
<td>Chelation therapy, n (%)</td>
<td>n/a</td>
<td>n/a</td>
<td>61 (89·7)</td>
<td>3 (50)</td>
<td>n/a</td>
<td>0·03</td>
</tr>
</tbody>
</table>

No Rx, no chronic transfusion or hydroxyurea therapy; HC, hydroxyurea therapy; CT, chronic transfusion therapy; %HbS, percentage of haemoglobin S; SD, standard deviation; IQR, interquartile range.

Differences between groups accessed by ANOVA (continuous variables) or Fisher’s exact test (categorical variables).

* Complete blood count and haemoglobin electrophoresis data were available for 25/29 controls.

† %HbS obtained at time of study laboratory draw for patients on CT and healthy controls, and at closest available date for non-CT patients.

‡ ANOVA when comparing sickle cell disease patient treatment groups (healthy controls excluded from this comparative analysis).

§ Mann–Whitney U-test comparing ferritin values for CT and CT + HC groups.
Table II
Multivariable analysis: impact of HC and CT on immune cell counts in SCD.

<table>
<thead>
<tr>
<th></th>
<th>HC ( \dagger ) + HC/no HC (95% CI)</th>
<th>CT ( \ddagger ) + CT/no CT (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total WBC</td>
<td>0.60* (0.49–0.74)</td>
<td>1.22* (1.01–1.48)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>0.49* (0.38–0.63)</td>
<td>1.40* (1.11–1.77)</td>
</tr>
<tr>
<td>Monocytes</td>
<td>0.48* (0.36–0.64)</td>
<td>1.06 (0.81–1.39)</td>
</tr>
<tr>
<td>Lymphocytes</td>
<td>0.78* (0.64–0.97)</td>
<td>1.01 (0.84–1.23)</td>
</tr>
<tr>
<td>B Cells</td>
<td>0.66* (0.49–0.90)</td>
<td>0.92 (0.69–1.22)</td>
</tr>
<tr>
<td>T Cells</td>
<td>0.89 (0.73–1.09)</td>
<td>1.16 (0.97–1.40)</td>
</tr>
<tr>
<td>NK Cells</td>
<td>0.58* (0.43–0.79)</td>
<td>0.66* (0.50–0.87)</td>
</tr>
<tr>
<td>Naïve B</td>
<td>0.65* (0.45–0.93)</td>
<td>0.80 (0.57–1.12)</td>
</tr>
<tr>
<td>Memory B</td>
<td>0.61* (0.41–0.90)</td>
<td>1.38 (0.96–1.98)</td>
</tr>
<tr>
<td>CD4</td>
<td>0.89 (0.72–1.10)</td>
<td>1.16 (0.96–1.41)</td>
</tr>
<tr>
<td>CD8</td>
<td>1.00 (0.77–1.30)</td>
<td>1.32* (1.04–1.68)</td>
</tr>
<tr>
<td>CD4 T-naive</td>
<td>1.05 (0.79–1.39)</td>
<td>1.31* (1.01–1.70)</td>
</tr>
<tr>
<td>CD4 T-memory</td>
<td>0.78* (0.64–0.95)</td>
<td>1.09 (0.90–1.31)</td>
</tr>
<tr>
<td>CD4 T-reg</td>
<td>0.73* (0.57–0.93)</td>
<td>1.33* (1.05–1.67)</td>
</tr>
<tr>
<td>CD8 T-naive</td>
<td>1.30 (0.97–1.74)</td>
<td>1.57* (1.20–2.06)</td>
</tr>
<tr>
<td>CD8 T-memory</td>
<td>0.78 (0.58–1.05)</td>
<td>1.14 (0.86–1.50)</td>
</tr>
<tr>
<td>CD4 Ki67</td>
<td>0.88 (0.64–1.22)</td>
<td>1.67* (1.23–2.26)</td>
</tr>
<tr>
<td>CD8 Ki67</td>
<td>1.00 (0.64–1.54)</td>
<td>1.74* (1.17–2.61)</td>
</tr>
</tbody>
</table>

WBC, white blood cell.

Estimated 95% confidence intervals shown for:

\( \dagger \) the ratio of indicated cell count for hydroxycarbamide (HC) treatment/no HC treatment when controlling for chronic transfusion (CT), splenectomy, age and sex for sickle cell disease (SCD) patients only.

\( \ddagger \) the ratio of indicated cell count for CT treatment/no CT when controlling for hydroxycarbamide, splenectomy, age and sex for SCD patients only.

* \( P \leq 0.05 \).