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Dendritic Cell Anergy Results from Endotoxemia in Severe Malnutrition

Stephen Miles Hughes,*‡§ Beatrice Amadi,† Mwiya Mwiya,‡ Hope Nkamba,§ Andrew Tomkins,† and David Goldblatt*

Malnutrition predicts an increased risk of morbidity and mortality from infection. Defects in cell-mediated immunity, such as thymic atrophy, impaired cutaneous tuberculin responses, and reduced T cell mitogenesis in vitro, are well characterized. There has been no convincing mechanism proposed for these T cell defects. However, as T cell responses rely on signals received from APCs, this study evaluates dendritic cell (DC) function in children with severe malnutrition. Repeated sampling of peripheral blood from 81 severely malnourished children at the University Teaching Hospital, Lusaka, Zambia, demonstrated for the first time a defect in DC numbers in children with malnutrition (28 per microliter) and a recovery in cell number (48 per microliter; \( p < 0.01 \)) with standard treatment. We describe normal DC maturation in the majority of malnourished children. However, in 17% of our study patients, in association with endotoxemia we describe the novel finding of DC maturation failure (down-regulation rather than up-regulation of HLA-DR). There was a strong correlation between the strength of HLA-DR up or down-regulation and the generation of IL-10 (\( r = -0.481; p = 0.003 \)). These “anergic” DCs failed to support T cell proliferation. Defects in DC number and the immunosuppressive phenotype of DCs from severely malnourished children with endotoxemia provide a rational basis for the anergy found in severe malnutrition. *The Journal of Immunology, 2009, 183: 2818-2826.

Although most studies of the immunology of human malnutrition have focused on T cell function, over the last decade the development of appropriate T cell responses has been shown to be crucially dependent on signals from other cells. Central to this has been the role of APCs, particularly dendritic cells (DCs), in providing critical signals for directing Ag-specific T cell function (9, 10). As key members of the innate immune system, DCs are primarily responsible for innate recognition of pathogens via pattern recognition receptors, including TLRs (11) like TLR4, which binds LPS, and C-type lectins, such as the mannose receptor (12). In response to pathogen recognition, DCs initiate host responses through cytokine release and Ag presentation via MHC to T cells. Defects in DCs from individuals with HIV have been described. In adults and children, HIV infection is associated with reduced type I IFN production accompanying a dramatic reduction in plasmacytoid DCs (pDCs) at seroconversion (13) and in advanced HIV disease (14-17), and it is thought that this finding may be critical in determining innate susceptibility to opportunistic infection. We have recently demonstrated an important interplay between HIV and SM in the generation of severe immunosuppression (18) and therefore set out to explore DC function in severely malnourished children.

Materials and Methods

Study population

SM children were recruited to the study at the University Teaching Hospital, Lusaka, Zambia, between September 2003 and April 2005. Participants had either nutritional edema or severe wasting defined as weight for height (W/H) Z score of less than \(-3\) based on National Center for Health Statistics (NCHS) data (19). Recruited children met the following inclusion criteria: age between 1 and 5 years, weight \(>5\) kg, and a consenting guardian. The only exclusion criterion was a moribund clinical state.

The Research Ethics Committees of the University of Zambia, Lusaka, Zambia, and the Institute of Child Health, London, U.K., granted ethical approval for the study. At baseline, anthropometric measurements were performed and a venous blood sample was collected (for full blood count, malaria film, HIV testing, CD4 count, and study tests).
SM children were managed according to World Health Organization protocols. On admission, all children were treated with two doses of 200,000 IU of vitamin A, antibiotics (penicillin and gentamicin), antimalarials, and antithelminth medication. Frequent feeds were established with the F75 formula (75 kilocalories per 100 ml of milk). A combined mineral and vitamin mix (Nutriset) was given. Antibiotic treatment was changed for clinical deterioration. Tuberculosis infection was sought in all children with suggestive history or delayed recovery. All children with microbiological evidence of tuberculosis were treated. Upon stabilization: i.e., return of appetite and/or loss of edema, children were given more energy-dense milk feeds (F100 formula; 100 kilocalories per 100 ml of milk). As weight improved, discharge was planned and high-energy porridge feeds were introduced. A second blood sample was collected upon discharge from hospital when the child was feeding independently, had reached a W/H Z score of −1, and a parent/guardian had been educated regarding ongoing nutrition security. Study subjects were monitored regularly after discharge. On reaching a W/H Z score of 0 (i.e., full nutritional recovery), a final blood sample was collected.

All children identified as HIV seropositive during the study period were prophylactically treated with co-trimoxazole. Antiretroviral therapy (ART) was not available for any of the children in the nutrition rehabilitation unit at the outset of the study but was introduced within the district in 2005. From the time ART became available, 10 HIV-infected children were under active review, of whom eight accepted voluntary counseling and testing. Six began ART, two before reaching a W/H Z score of −1, and a parent/guardian had been educated regarding food. Guardians were counseled regarding the nature of the test and told how the test results would be linked to study data but informed that neither the study team nor the child’s clinician would know the results. The study HIV test aside, the study team encouraged all patients and parents/guardians to have a routine linked HIV test in all children following study completion rather than jeopardize recruitment or introduce bias in patient selection.

HIV testing

HIV tests were performed at study completion on the stored plasma samples of all study patients from whom blood was collected. Because of the considerable local stigma associated with HIV, it was decided to perform an anonymous linked HIV test in all children following study completion rather than jeopardize recruitment or introduce bias in patient selection. Guardians were counseled regarding the nature of the test and told how the test results would be linked to study data but informed that neither the study team nor the child’s clinician would know the results. The study HIV test aside, the study team encouraged all patients and parents/guardians to have a routine linked HIV test to facilitate optimal patient care. Posttest counseling was given with this result. Samples were tested using two different rapid test kits, Determine from Abbott Laboratories and Genie II from Bio-Rad, in keeping with Zambian Ministry of Health policy. Sero-positive children for whom a final sample was collected before the age of 1.5 years had their HIV status confirmed by commercial proviral DNA PCR (Roche Molecular Diagnostics).

Conjugated mAbs

The following mAbs were all purchased from BD Pharmingen: FITC-conjugated anti-MHC class II (L243), anti-lineage mixture Lin-1 (SK7, 3G8, SJ25C1, L27, MFp9, and NCAM16.2), anti-CD3 (UCHT1), anti-CD86 (2331 FUN-1), anti-CD83 (HB15e), and anti-isotype controls (X39, 40, 27-35), anti-CD4 (MT4); anti-CD14 (MPLP), anti-CD40 (5C3), anti-CD86 (2331 FUN-1), anti-CD123 (9F5), IFN-γ (AS.B3), and isotype controls (X40, 27-35); PerCP-conjugated anti-MHC class II (L243); and allophycocyanin-conjugated anti-CD45 (H130).

Blood DC flow cytometry

Blood was collected in EDTA. An automated blood count was performed using the KX21 hematology analyzer from Sysmex. Whole blood was mixed with Abs from BD Pharmingen in FACS buffer and incubated on ice. Lysis buffer (BD Pharmingen) was added 10 min before samples were centrifuged. One further wash cycle was performed with FACS buffer before cells were fixed in 1% paraformaldehyde, Marker expression was analyzed on a BD FACSCalibur using CellQuest Pro software (BD Biosciences). Relevant isotype control Abs were used with gating arbitrarily set below 1% positive. All Abs were used at a 1/25 dilution.

Flowcytometry of DCs

The iDCs and mature DCs (mDCs) were resuspended in their wells, stained with BD Pharmingen Abs, washed, and fixed in 1% paraformaldehyde. Marker expression was analyzed on a BD FACSCalibur using CellQuest Pro software (BD Biosciences). Relevant isotype control Abs were used with gating arbitrarily set below 1% positive. All Abs were used at a 1/50 dilution.

Cytokine ELISAs

Supernatants and plasma were frozen at −80°C. ELISA for IL-12 (BD Pharmingen), IL-10 (eBioscience) and TNF-α (eBioscience) were performed on thawed samples following the manufacturers’ instructions. From supernatants, cytokine concentrations were expressed per 400,000 cells/ml.

Plasma analytes

Plasma was frozen at −80°C, and thawed before analysis. Plasma concentration of C-reactive protein (CRP) was measured by ELISA. Albumin was measured by the Bromocresol green dye method on a Cobas Fara photometer (Roche). Endotoxin was measured using the Pyrochrome endpoint assay according to the manufacturer’s instructions (Associates of Cape Cod).

ELISPOT

Tuberculin purified protein derivative (PPD)-stimulated DCs (10,000) and autologous frozen/thawed PBMCs (200,000) were cocultured for 5 h. Cells were transferred to anti-IFN-γ (clone 1-D1-K)-coated 96-well polystyrene filter plates (Millipore). Captured cytokine was visualized after 40 h using a biotinylated detection Ab (clone 7-B6-1; Mabtech) as per the manufacturer’s instructions. Spots were counted using an ELISPOT reader from Autoimmun Diagnostika. Direct PBMC-PPD IFN-γ ELISPOT was performed using 400,000 frozen/thawed PBMCs in culture for 40 h with PPD on the coated polystyrene filter plate and visualized as described above.

Mixed leukocyte reactions

Washed mDCs were cocultured with allogeneic frozen/thawed CD14+ PBMCs (all allogeneic cells were collected from a single donor) for 6 days at a ratio of 1:10 in round-bottom, 6-well-plates at 10,000 DCs per well in RPMI 1640 supplemented with 10% human AB serum, Glutamax, penicillin, and streptomycin. Wells were divided and medium replenished after 72 h. After 6 days of coculture, cells were further stimulated for 4 h with PMA (25 ng/ml) and ionomycin (2.5 µg/ml) in combination with brefeldin A (10 µg/ml). Controls included PBMCs alone, unstimulated or stimulated with PMA/ionomycin, and further unstimulated DC-PBMCs treated with brefeldin A alone. Washed cells were fixed in 4% paraformaldehyde before permeabilization (Perm/ Wash buffer; BD Pharmingen) and staining with Ab to IFN-γ (BD Pharmingen) following the manufacturer’s instructions. Analysis was performed on a BD FACSCalibur flow cytometer with CellQuest Pro software (BD Biosciences).

Simultaneous experiments were established with PBMCs labeled with CFSE (Molecular Probes). A negative control was provided by CFSE-labeled allogeneic PBMCs without addition, and a positive control by allogeneic PBMCs cultured with 2 ng/ml PHA alone. Cell division was measured by CFSE expression in fluorescence channel 1 of the BD FACSCalibur using CellQuest Pro software (BD Biosciences).
Table I. Baseline age, anthropometry, and prevalence of edema by HIV status

<table>
<thead>
<tr>
<th>Measure</th>
<th>HIV Uninfected</th>
<th>HIV Infected</th>
<th>p Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td>42</td>
<td>39</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>1.742 (1.422–2.022)</td>
<td>1.768 (1.39–2.079)</td>
<td>NS</td>
</tr>
<tr>
<td>Male</td>
<td>22</td>
<td>23</td>
<td>NS</td>
</tr>
<tr>
<td>Weight-for-age Z score</td>
<td>-3.58 (1.01)</td>
<td>-4.62 (0.80)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Height-for-age Z score</td>
<td>-2.39 (1.15)</td>
<td>-3.24 (1.30)</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>W/HI Z score (weight after edema loss)</td>
<td>-2.94 (1.12)</td>
<td>-3.36 (0.94)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Edema</td>
<td>88%</td>
<td>51%</td>
<td>&lt;0.001</td>
</tr>
</tbody>
</table>

*Values presented are absolute numbers, percentages, medians, and interquartile ranges, means, and SD values.

Statistical analysis

All calculated anthropometric indices were determined using National Center for Health Statistics data (19). Comparisons of group means were made using t tests. All parametric or transformed normalized longitudinal data were compared with paired t tests. Nonparametric data were compared using Mann-Whitney or Wilcoxon signed ranks tests (unpaired or paired, respectively). Categorical variables were compared using Fisher’s exact test. Probability values of <0.05 were considered statistically significant. All statistical analyses were undertaken with SPSS version 11 for Mac.

Categorization

Where continuous variables have been categorized, the same strategy has been used on each occasion. Data from children at nutritional recovery (HIV-uninfected cases for circulating DC counts and myeloid DC counts, HIV-infected and uninfected cases for cultured DC IL-12 production) were analyzed to yield a value for the 2.5th centile to define the threshold below which values could be statistically considered subnormal. For cultured DC IL-12 production, the 2.5th centile cut off was established at 50pg/ml, which additionally represented the 96th centile for the production of IL-12 by these IDCs, and was thus validated as a threshold of IL-12 production beyond which mDCs should reach. The mDCs producing <50 pg/ml were therefore determined to be low producers.

Results

Study group characteristics

We recruited 81 children with SM of whom 39 were HIV infected. Table I shows the anthropometric indices by HIV status. Sixty-seven children had adequate samples collected on admission, five were too sick, and nine had insufficient samples collected. Fifty-six had quantitative DC analyses by flow cytometry, and 38 had DCs cultured from monocytes. At discharge, 49 adequate samples were collected. Samples were used to repeat tests performed on admission. Thirteen children had died and five had left hospital prematurely. Thirty-three samples were collected from children who had reached full nutritional recovery for repeat analysis. From discharge to recovery, 10 children defaulted from follow-up and three severely immunosuppressed HIV-infected children died. On admission, 39% of the children had elevated temperature, 92% had diarrhea (more than three loose or watery stools per day), and 10% had pneumonia, which was defined as cough with a respiratory rate of >40 breaths per minute. On the basis of these data and the universal elevation of inflammatory markers, we made the presumption that all of the children were infected at the time of presentation. Seven percent were proven to have tuberculosis (TB). TB was suspected in another 23%. Fifteen percent had malarial parasites seen on blood film.

Baseline bloods from this cohort of children revealed them to have low plasma IL-12 levels on admission (median, 13 pg/ml), rising significantly to discharge (79 pg/ml; p = 0.002; Wilcoxon) and to recovery (149 pg/ml; p = 0.001). Plasma IL-10 (median, 12 pg/ml) and TNF-α (median, 4 pg/ml) did not significantly alter over time. On admission, diarrhea (p = 0.03), pneumonia (p = 0.04), and fever (p = 0.07) were all associated with lower plasma IL-12 concentrations.

Quantitative DC analysis

Circulating DC numbers were quantified in peripheral blood samples taken on admission, on discharge a mean of 24 days later (Fig. 1, a and b), and on recovery a mean of 140 days after discharge. The median number of circulating DCs on admission was 28 per microliter, representing 0.45% of PBMCs. At discharge, the number of DCs had risen to 48 per microliter of peripheral blood (p < 0.01) and formed a greater percentage of PBMCs (0.75%; p < 0.01). There were no further significant changes found at the time
of recovery; the mean DC number was 45 per microliter of peripheral blood and the mean percentage of PBMCs was 0.61%.

HIV-infected children had significantly lower DC percentages ($p = 0.03$) and counts ($p = 0.005$) than HIV-uninfected children on admission (Table II). Therefore, samples from HIV-infected and -uninfected children were analyzed separately.

In HIV-uninfected children on admission, the median number of circulating DCs was 36 per microliter, representing $0.51\%$ of PBMCs. At discharge, the number of DCs had risen to 47 per microliter of peripheral blood and formed a significantly greater percentage of PBMCs ($0.76\%; p = 0.004$). In HIV-infected children on admission, the median number of circulating DCs was 22 per microliter of peripheral blood, representing $0.34\%$ of PBMCs. At discharge, the number of DCs had significantly risen to $53/\mu l$ ($p = 0.03$) and formed a greater percentage of PBMCs ($0.56\%; p = 0.27$).

At discharge, the differences between HIV-infected and -uninfected children on admission were no longer significant as a result of the mortality of the children with the lowest DC counts who were all HIV-infected; below a count of 20 DCs per microliter or 0.25% of PBMCs, virtually all had died. Children with fever, malaria, TB, pneumonia or diarrhea demonstrated no significant alteration in DC number.

Survival and DC count

We confirmed that patients with low DC counts (determined from the 2.5th centile of the results of the HIV-uninfected children in the recovery group) had accelerated mortality using a Kaplan-Meier curve ($p < 0.001$; Fig. 2). Then, using a receiver operating characteristic curve to identify the optimal DC measure, we selected an absolute DC count (area under the curve, 85%) of $<18$ cells per microliter to predict hospital mortality with 87% sensitivity and 73% specificity. We proceeded to perform multivariate survival analysis using Cox regression, confirming that this categorical DC value was one of three independent predictors of mortality. After controlling for age, sex, degree of wasting, edema, hemoglobin, inflammation (CRP, albumin, endotoxemia), HIV, and CD4 count, the independent predictors of mortality were as follows: 1) DC count of $<18$ cells per microliter (hazard ratio, 16.9; 95% confidence intervals (CI), 4.0–72); 2) diarrhea/stool frequency (hazard ratio, 1.8 per stool per day; 95% CI, 1.3–2.6); and 3) lethargy (hazard ratio, 6.4; 95% CI, 1.5–27).

DC subclasses

The very low numbers of DCs in children who died led us to specifically examine the different DC compartments in the HIV-infected and uninfected children. pDCs were significantly lower in number in HIV-infected (median, four per microliter) than uninfected children (median, 13 per microliter; $p = 0.002$; Fig. 1, c and d, and Table II) on admission. We demonstrated the same low level of pDCs in HIV-infected children at each time point (Fig. 1d). In contrast, myeloid DC numbers were found to increase significantly during nutritional rehabilitation (from admission to discharge in HIV-infected SM children: from 0.15 to 0.29% of PBMCs, $p = 0.004$, with absolute counts from 9 to 21 per microliter, $p = 0.02$; from admission to recovery in HIV-infected SM children: from 0.09 to 0.36% of PBMCs, $p = 0.04$, with absolute counts from 7 to 27 per microliter, $p = 0.03$; Table II and Fig. 1d).

The significant improvement in circulating DC numbers in the HIV-uninfected cohort was principally dependent upon the rise in myeloid DC counts from HIV-uninfected children in the recovery group. To assess factors that might specifically influence admission myeloid DC numbers, we analyzed the impact of wasting (W/H Z score), edema, malaria, CRP, albumin, and hemoglobin. The median CRP level in patients with low myeloid DC numbers (35.1) was significantly higher than that in those with normal

**Table II. DC numbers in HIV-infected and uninfected children at admission and discharge**

<table>
<thead>
<tr>
<th>Measure</th>
<th>Admission</th>
<th></th>
<th>Discharge</th>
<th></th>
<th></th>
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<th></th>
<th></th>
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<th>Significance</th>
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<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>PBMC No. ($\times 10^3/\mu l$)</td>
<td>Median</td>
<td>IQR</td>
<td>Median</td>
<td>IQR</td>
<td>Median</td>
<td>IQR</td>
<td>Median</td>
<td>IQR</td>
<td>Median</td>
<td>IQR</td>
</tr>
<tr>
<td></td>
<td>Uninfected</td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>n = 31</td>
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<td></td>
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<tr>
<td></td>
<td>Infected</td>
<td></td>
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<td></td>
<td></td>
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<tr>
<td></td>
<td>n = 25</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DC (%)</td>
<td>0.51</td>
<td>0.34–0.72</td>
<td>0.34</td>
<td>0.22–0.57</td>
<td>0.76</td>
<td>0.53–0.98</td>
<td>0.56</td>
<td>0.24–1.71</td>
<td>*</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>DC No. ((\mu l))</td>
<td>36</td>
<td>23–51</td>
<td>22</td>
<td>14–34</td>
<td>47</td>
<td>39–64</td>
<td>53</td>
<td>22–94</td>
<td>**</td>
<td>*</td>
</tr>
<tr>
<td></td>
<td>nDC/PBMC</td>
<td>0.15</td>
<td>0.06–0.20</td>
<td>0.09</td>
<td>0.05–0.18</td>
<td>0.29</td>
<td>0.18–0.40</td>
<td>0.13</td>
<td>0.10–0.52</td>
<td>NS</td>
<td>**</td>
</tr>
<tr>
<td></td>
<td>mDC/PBMC</td>
<td>0.17</td>
<td>0.07–0.25</td>
<td>0.04</td>
<td>0.01–0.08</td>
<td>0.24</td>
<td>0.15–0.34</td>
<td>0.06</td>
<td>0.04–0.20</td>
<td>**</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>pDC/PBMC</td>
<td>13</td>
<td>4–21</td>
<td>4</td>
<td>1–6</td>
<td>16</td>
<td>11–22</td>
<td>7</td>
<td>4–15</td>
<td>***</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>HLA-DR MFI</td>
<td>8.9</td>
<td>5.3–12.8</td>
<td>7.2</td>
<td>4.3–11.9</td>
<td>11.2</td>
<td>7.7–15.7</td>
<td>8.9</td>
<td>5.9–12.6</td>
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<td></td>
<td>CD86 MFI</td>
<td>25</td>
<td>13–56</td>
<td>42</td>
<td>19–86</td>
<td>29</td>
<td>14–51</td>
<td>55</td>
<td>19–85</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

*All relative DC measures are expressed as a fraction of the total PBMC count. None of the data presented in the table are parametrically distributed. Where indicated, logarithmic transformation of data was performed to permit parametric testing of significant differences (“1” in Key column). Data that could not be normalized by transformation were analyzed using the Wilcoxon signed rank test for paired data or the Mann-Whitney test for independent samples (“2” in Key column). Significance is indicated as follows: *, $p < 0.05$; **, $p < 0.01$; ****, $p < 0.001$. IQR, Interquartile range.

**FIGURE 2.** Kaplan-Meier survival curve comparing patients on admission with low DC numbers (dashed line) and patients with normal DC numbers (solid line).
myeloid DC numbers (5.7; \( p < 0.001 \)), and a strong correlation was found between myeloid DC numbers and CRP levels (\( r = -0.621; p < 0.001 \)). The odds ratios and 95% CI (in parentheses) for risk factors associated with low myeloid DC counts were as follows: albumin (<25 g/L), 5.0 (1–27); and CRP (>20 mg/L), 12.0 (2–76). We did not find any difference in age, gender, W/H Z score, hemoglobin or lymphocyte count or prevalence of fever, edema, malaria, diarrhea, pneumonia, or TB between patients with low and normal myeloid DC counts.

**Inflammation and blood DCs**

Having identified that inflammation (represented by raised CRP and lowered albumin) had a negative impact on the number of circulating myeloid DCs, we examined the range of activation phenotypes of DCs drawn from all study subjects. Up-regulation of HLA-DR and CD86 expression (as markers of DC activation) was not demonstrated in the children with biochemical evidence of inflammation. Rather, HLA-DR expression was reduced in children with high CRP levels (>40 mg/dl, \( n = 14 \)); mean HLA-DR mean fluorescence intensity (MFI), 84 compared with those with normal CRP levels (<2 mg/dl, \( n = 49 \)); mean HLA-DR MFI,132; \( p = 0.004 \).

We postulated that low DC numbers and reduced expression of DC HLA-DR might reflect immunoparesis in this group of SM children. To document “classical” immunoparesis, we examined monocytes and performed a whole blood assay to examine TNF-\( \alpha \) production in 37 of the SM children (including 26 admission samples, 19 discharge samples, and 22 recovery samples). We found decreased monocyte HLA-DR expression (median 89 vs 97%; \( p = 0.03 \)) and significantly reduced TNF-\( \alpha \) production (median 981 vs 5537; \( p = 0.002 \)) in DC cytopenic children, confirming that DC cytopenia mirrored classical immunoparesis.

We examined plasma endotoxin levels to identify a possible basis for DC immunoparesis. Lower mean numbers of blood DCs (31 vs 48 per microliter; \( p = 0.03 \)) were found in samples from which endotoxin was found (levels >0.1 endotoxin U/ml). From admission samples, endotoxin was found in 20 samples (in three patients without diarrhea and in 11 without fever). Endotoxin levels had a strong and significant negative correlation with DC HLA-DR expression (\( r = -0.505; p < 0.0001 \)), such that children with no evidence of endotoxia (levels <0.1) had a mean HLA-DR MFI of 120, in contrast to those with endotoxia (\( n = 20 \)) in whom the mean HLA-DR MFI was 63 (\( p < 0.0001 \)).

We examined other elements of the immunoparalysis syndrome and found that the plasma IL-12 concentration was significantly associated with TNF-\( \alpha \) production in the whole blood assay when controlling for age, sex, HIV status, and episode (admission, discharge, and recovery) in a linear regression (\( n = 36 \), \( r = 0.55 \); \( p = 0.001 \)). Furthermore, plasma IL-12 was significantly associated with DC HLA-DR MFI (\( n = 88 \), \( r = 0.32 \); \( p = 0.003 \)), and there was a trend toward association with the number of DC HLA-DR negative monocytes (\( n = 45 \), \( r = -0.31 \); \( p = 0.05 \)) and the number of circulating myeloid DCs (\( n = 76 \), \( r = 0.23 \); \( p = 0.05 \)).

**DC function in malnutrition**

Anticipating that low numbers of blood DCs may impair T cell function, T cell responses to recall Ag (PPD) were determined by direct PBMC-PPD IFN-\( \gamma \) ELISPOT. Fig. 3A demonstrates absent IFN-\( \gamma \)-responses at the time of admission, which tended to improve during recovery (paired \( t \) test; \( p = 0.07 \)). Uncertain whether this impaired T cell response was a result of impaired T cell function, impaired DC number, or impaired DC function, we went on to examine in more detail the function of DCs derived from monocytes drawn from SM children. Firstly, we repeated the ELISPOT assay using DCs pulsed with PPD and autologous saved cells. Fig. 3B demonstrates the same T cells’ competence to respond to PPD presented by cultured DCs, confirming that the defect demonstrated in Fig. 3A reflected the strength of a blood APC-T cell interaction rather than an absence of responsive T cells in malnutrition and/or sepsis.

We proceeded to examine in more detail the function of cultured DCs from 37 SM children on admission, assessing them in the resting immature state and after LPS activation. Samples from admission were compared with samples collected on discharge and recovery. We identified two distinct findings. Firstly, DC production of IL-12 was reduced from cells collected on admission compared with cells collected on recovery, in contrast to DC production of TNF-\( \alpha \) and IL-10, which was normal throughout (data not shown). Deficiencies were found in IL-12 production in 18 of the 37 SM children on admission. Significantly less IL-12 was produced by mDCs on admission than on recovery (median 111 vs 535 pg/ml; \( p < 0.01 \)) (Fig. 4) and significantly more children were poor producers (<100 pg/ml per 400,000 mDCs) on admission (18/37, 49%) than recovery (2/18, 11%; \( \chi^2 \) value, 7.4, \( p = 0.02 \)). In this admission group, 78% of children who subsequently died (7/9) were poor IL-12 producers in contrast to just 38% of children who survived (11/29; \( p = 0.04 \)). Poor production of IL-12 was not associated with any nutritional factor or fever, diarrhea, pneumonia, malaria, TB, HIV, or with plasma cytokine levels, but it closely correlated with the level of endotoxin in the blood from...
which the samples were drawn \((r = 0.39; p = 0.002)\). We repeated the analysis of IL-12 production after the exclusion of seven samples from which endotoxin levels exceeded 0.1 endotoxin U/ml and demonstrated the same poor production of IL-12 from admission samples (median 120) compared with recovery samples (median 535). In these nonendotoxic children, all those that died had produced low levels of IL-12 (7/7), in contrast to just 29% (7/24) of survivors \((p = 0.001)\).

The second abnormal finding in the DC phenotype was HLA-DR down-regulation, which was noted in six patients (contrasting with the expected up-regulation seen in the other 31 patients, see Fig. 5c). Three of these six patients demonstrated additional down-regulation of CD83 and/or CD86, and three demonstrated recovery of CD14 expression. On subsequent testing (at discharge and recovery), iDCs from these children responded normally to LPS stimulation (up-regulating HLA DR, CD83, and CD86). The six “down-regulators” were noted to have had significantly greater expression of HLA-DR on iDCs (median MFI, 234) than subjects with normal up-regulation (median MFI, 124; \(p = 0.01\)), suggesting earlier activation.

The six down-regulators were also shown to produce significantly more IL-10 (median, 3545 pg/ml) than “up-regulators” (median, 557 pg/ml; \(p = 0.03\); Mann-Whitney U test). The continuum of HLA-DR regulation (i.e., HLA-DR MFI (mDC)/MFI (iDC)) correlated well with mDC IL-10 production \((r = -0.481; p = 0.003)\). We further explored the relationship between iDC and mDC IL-10 production and confirmed that iDCs making quantifiable IL-10 (>10 pg/ml, \(n = 11\)) matured into mDCs making significantly more IL-10 (median, 1689 pg/ml) than iDCs that made no IL-10 (\(n = 26\), median, 486 pg/ml, \(p = 0.028\)).

Critically important in determining the activation status (HLA-DR expression) of the iDCs was the presence of endotoxin in blood, which was also highly predictive of the fall in HLA-DR MFI of cultured DCs. Children with very high endotoxin levels (LPS > 0.22 endotoxin U/ml) possessed iDCs expressing significantly more HLA-DR (median MFI, 244) than normal children (125, \(p = 0.03\)), and these same children later demonstrated a mean 1.2-fold rise (95% CI, 0.6–1.9) in HLA-DR MFI in contrast to a mean 3.4-fold rise (95% CI, 2.5–4.3) in children without endotoxemia \((p = 0.001)\). We found no significant relationships between HLA-DR expression and anthropometric indices or edema, fever, diarrhea, pneumonia, malaria, TB, or HIV or plasma cytokine levels.

DC function was further assessed by MLR to provide a standardized measure of DC support for heterologous T cell replication and T cell IFN-\(\gamma\) production. T cell proliferation, as determined by the halving of CFSE staining intensities, was strongly associated with plasma endotoxin levels \((r = 0.50, p = 0.025)\) and the expression of HLA-DR by the DCs (shown in Fig. 6). Down-regulation of HLA-DR expression predicted the generation of no more than one round of division of T cells, which was significantly fewer than the samples that demonstrated HLA-DR up-regulation \((p = 0.001\) by \(\chi^2\)). The mean percentage of IFN-\(\gamma\)-producing T

**FIGURE 4.** IL-12 production by 400,000 DCs in 1 ml of medium over 24 h. Horizontal lines represent geometric means. In samples from which no IL-12 was detected, the subject is represented at a concentration of 0.2 pg/ml. The bar dividing the chart is placed at the 2.5th centile for recovery specimens (i.e., 50 pg/ml).

**FIGURE 5.** Phenotype of cultured DCs from SM children on admission and discharge. a, Viable DCs were gated and stained with PE- and FITC-labeled mAbs. FSC, Forward scatter; SSC, side scatter. b, Two examples of HLA-DR down-regulation are shown, demonstrating the additional CD14 staining seen in three of the six patients. c, Two examples of CD4 down-regulation are shown, demonstrating the additional CD4 staining seen in three of the six patients. d, Paired results from iDCs and mDCs are linked. Down-regulators are shown in black. The same patients’ samples are shown in black again on discharge, demonstrating recovery of phenotype. d, Histograms of cell surface marker expression; the top row shows the normal pattern, the lower row shows the pattern from a down-regulator. Isotype control is shown in open histograms with gray lines, iDCs are shown in open histograms with black lines, and mDCs are shown in filled histograms.
cells rose from 2.7% on admission to 7.2% on nutritional recovery \( (p < 0.04; \text{paired} \ t \ \text{test}) \). For the entire cohort, T cell IFN-\( \gamma \) production was positively correlated with DC IL-12 production \( (r = 0.404; \ p = 0.04) \) and strongly negatively correlated with DC IL-10 production \( (r = -0.525; \ p = 0.006) \) as shown in Fig. 7.

**Discussion**

DCs are professional APCs, and by virtue of the number of TCR ligands on their surface, the quality of accessory signals they are able to provide, and their production of key cytokines such as IL-12, they influence many elements of the specific T cell response \( (10, 20, 21) \). In this study we have shown that these key cells are both deficient and qualitatively abnormal in children with SM as a result of infection, endotoxemia, and inflammation. Our study cohort had a high prevalence of severe wasting, edema, HIV, and infection and was therefore typical of severely malnourished children in the region. In keeping with previous findings \( (3) \), we identified inflammation and infection as key elements within the severe malnutrition complex. For these reasons, our study of DCs in SM children has focused not only on the scope of malnutrition but also on HIV and the inflammatory process.

We demonstrate for the first time the reduction of circulating DCs in children with severe malnutrition. As no normal ranges were available, we constructed them from HIV-uninfected children who had achieved full nutritional recovery and were interested to note that the numbers of DCs appear to be similar to the numbers generated by Teig et al. in their study of healthy German children of similar ages \( (22) \). We also confirmed that HIV-infected children had lower total DC counts, in particular the counts of pDCs \( (13, 16, 23) \), and these were lowest at the time of admission. The reason for the reduced numbers of DCs in HIV is unclear. Despite expressing CD4, DCs are rarely infected \( (24) \) but are depleted in both primary HIV infection and advancing disease. Studies in adults suggest that rising viral load correlates with falling pDC numbers \( (16) \) and that ART may variably restore DC number, although it less commonly restores function (IFN\( \gamma \) production) \( (17, 25, 26) \). Our HIV-infected study children did demonstrate a rise in total DC numbers upon treatment for SM, but there was no significant recovery of pDC numbers. The three children excluded from this analysis because of HAART commencement also failed to demonstrate recovery of pDC numbers.

The principal reason for reversible DC cytopenia in SM children was reduction in myeloid DCs. We explored the contributors to endotoxia in lowering DC number in keeping with the process of immunoparesis. Furthermore, we have demonstrated that DCs from endotoxemic SM children tend to be insensitive to endotoxin and, in contrast to responders, generate excess IL-10. Independently of this finding, in the children without endotoxia we have demonstrated reduced IL-12 production by DCs in children with SM that improves with nutritional recovery.

**FIGURE 6.** T cell division relates to DC factors: HLA-DR regulation and prior DC endotoxin exposure. A, Box plot showing the median values and ranges of the alteration in HLA-DR expression following the addition of LPS (500 ng/ml) against the number of CFSE divisions recorded in responding T cells in the MLR. In the samples in which HLA-DR expression was reduced after stimulation (down-regulation, to the left of the line of equivalence), only one round of division was seen. In all other samples in which HLA-DR was up-regulated, further rounds of division were seen. B, Scatter plot of plasma endotoxin levels against the number of CFSE divisions recorded in responding T cells in the MLR.

**FIGURE 7.** T cell IFN-\( \gamma \) production is linked to DC cytokines. The relative number of responding (IFN-\( \gamma \) producing) T cells in the MLR is inversely proportional to the mDC production of IL-10 \( (n = 26, r = -0.525; \ p = 0.006) \) \( (A) \) and positively correlated with the mDC production of IL-12 \( (n = 26, r = 0.404; \ p = 0.040) \) \( (B) \).
myeloid DC cytopenia and identified inflammation, best reflected in the CRP to be crucial. Researchers focusing on myeloid DCs and inflammation in other settings (cardiopulmonary bypass and sepsis syndromes in adults) have similarly identified reduced myeloid DCs (27) and low plasma IL-12 as causally linked in their models (28, 29). We extended our observation and were further able to show that the activation status of DCs (reflected in CD86 and HLA-DR expression) correlated strongly with endotoxin concentration in plasma. The reduction in activation status may seem contrary, but the same pattern of deactivation is recognized in monocytes in the setting of massive inflammatory insult (28, 30, 31). The explanation for this finding in SM is not clear; either LPS up-regulates HLA-DR with chemokine receptor expression and these cells leave the circulation faster than they can be replaced, as is believed to be the case for other cell types in SM (32) but for which there is limited evidence, or alternatively these cells may down-regulate their expression of HLA-DR having become anergic, as suggested by our subsequent experiments.

We describe for the first time a direct relationship between total circulating DC numbers and mortality. In HIV, low pDC counts have been thought to reflect poor prognosis (33). In systemic lupus erythematosus, disease activity has been shown to correlate negatively with total DC count (34). Based on the evidence to date, it fits that a lower DC count predicts mortality in SM. On both univariate and multivariate analyses, DC count proves to be the most powerful predictor of outcome. Using Cox regression, we identified low DC count (<18 cells per microliter), lethargy, and diarrheal frequency on admission as independent factors predicting mortality.

In addition to the quantitative abnormalities in our study cohort, we also discovered defective DC function in 54% of SM children; in 17% the down-regulation of HLA-DR expression on DCs was accompanied by amplified IL-10 production, and in 49% there was a deficiency in production of IL-12 by DCs. IL-10 is known to be immunosuppressive for DCs in experimental systems, causing down-regulation of cell surface markers and turning off cytokine production (35–37). Preservation of TNF-α production, as demonstrated in our cohort, is unusual. Down-regulation of HLA-DR on mononuclear cells has also been described previously in the context of cardiac bypass, CRP, sepsis, endotoxin, and glucocorticoids (28, 38–40), but our cohort is unique in the preservation of TNF-α production by DCs. Finally, DCs from half of our study population produced low levels of IL-12. Failure of DCs to produce IL-12 has previously been described in the contexts of immaturity (cord blood DCs fail to produce IL-12 p35; see Ref. 41) and sepsis (42), and the mechanism is thought to be related to abnormal histone methylation and consequently chromatin remodeling. The mechanism by which DCs are affected by malnutrition (and sepsis) is thus likely to be multifactorial and requires further elucidation.

The failure of up-regulation of critical DC-T cell ligands (HLA-DR and CD86) and the impaired production of IL-12 result in poor support for T cell proliferation and effector function (36). In the experiments we performed, both reduced IL-12 secretion and reduced DC ligand expression had grave consequences on the capacity of the DC to support T cell responses, as had been found in the work of others that relied upon “defective” APCs, such as CMV-infected DCs (43), or in the face of exogenous IL-10 (36).

We propose the term “anergic DCs” for the DCs we described with down-regulation of HLA-DR, elevated IL-10 secretion, and absent or low IL-12 production. These DCs did not support T cell proliferation or IFN-γ production and, combined with circulating DC depletion, may bear responsibility for the anergic response commonly seen in SM. We have found persuasive evidence for the importance of exposure to endotoxin in the development of DC anergy.

Our findings suggest a very critical role for the inflammatory process in generating anergy in severe malnutrition. We have not shown that SM itself results in a uniform pattern of DC deficiency, but rather that in the face of endotoxin exposure in the malnourished host, DCs matured poorly and support for T cell responses was blunted. As was proposed by Morgan (44), malnutrition itself may have a very limited direct effect on immune competence, whereas it is straightforward to find evidence for immune response polymorphisms determining infection risk and for consequent infection determining nutritional status. However, the evolutionary argument for the survival advantage of anergy in the face of severe malnutrition cannot be entirely disregarded. In accord with previous findings of blunted inflammatory responses (45), weakened T cell responses (4), and impaired stimulation of a one-way MLR (46), we believe that our data from this study of DCs in SM demonstrate a key place for the DC in the determination of the quality of the immune responses generated in SM. We see the ongoing successful production of immunosuppressive IL-10 as important. Whether the dynamics of IL-10 production are altered by the nutritional state of the individual remains to be determined.

Specific factors in severe malnutrition may act to augment IL-10 secretion. One such factor could be glucocorticoids. In SM, it is generally accepted that cortisol levels rise. It has never been established to what extent this rise impacts upon immunity. It is tantalizing to compare the direct effect of glucocorticoids on DCs with the changes we have demonstrated. Dexamethasone has been shown to reduce the maturation potential of iDCs and to restrict the production of IL-12 in favor of IL-10 (47).

We have shown for the first time an absolute reduction in circulating DCs in SM children that is greater in HIV-infected children than in uninfected children. We have identified a key loss of circulating myeloid DCs in SM children that recover upon nutritional therapy and resolution of inflammation. In addition to these findings we have demonstrated the independent value of the total DC count in predicting mortality. Defective DC function was described in some but by no means all SM children, either because of a down-regulation of HLA-DR expression and up-regulation of IL-10 production (the anergic DC) or because of a deficiency in IL-12 production. Defective DC function in these children was linked to impairment of T cell responses, hence providing a novel explanation for the critical impairment in CMI in SM. The presence of inflammation and particularly endotoxin appears to be critical in determining both DC numbers and function.

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Disclosures
The authors have no financial conflict of interest.

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