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ORIGINAL ARTICLE

Blood dendritic cell levels associated with impaired IL-12 production and T-cell deficiency in patients with kidney disease: implications for post-transplant viral infections

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Summary

Reduced pretransplant blood myeloid dendritic cell (mDC) levels are associated with post-transplant BK viremia and cytomegalovirus (CMV) disease after kidney transplantation. To elucidate potential mechanisms by which mDC levels might influence these outcomes, we studied the association of mDC levels with mDC IL-12 production and T-cell level/function. Peripheral blood (PB) was studied in three groups: (i) end stage renal disease patients on hemodialysis (HD; $n = 81$); (ii) chronic kidney disease stage IV-V patients presenting for kidney transplant evaluation or the day of transplantation (Eval/Tx; $n = 323$); and (iii) healthy controls (HC; $n = 22$). Along with a statistically significant reduction in mDC levels, reduced $CD8^+$ T-cell levels were also demonstrated in the kidney disease groups compared with HC. Reduced PB mDC and monocyte-derived DC (MoDC) IL-12 production was observed after *in vitro* LPS stimulation in the HD versus HC groups. Finally, ELISpot assays demonstrated less robust $CD3^+$ INF-γ responses by MoDCs pulsed with CMV pp65 peptide from HD patients compared with HC. PB mDC level deficiency in patients with kidney disease is associated with deficient IL-12 production and T-cell level/function, which may explain the known correlation of $CD8^+$ T-cell lymphopenia with deficient post-transplant antiviral responses.

Introduction

Kidney transplantation is the treatment of choice for patients with end stage renal disease (ESRD) and acceptable operative risk, conferring a clear survival benefit over similar wait-listed individuals [1]. Acute rejection has decreased dramatically over the past decades in large part due to more potent immunosuppressive agents [2]. Although graft and patient survival rates continue to improve, post-transplant viral infection remains a major complication of the procedure [3–5].

Dendritic cells (DCs)are a rare and important heterogeneous population of professional antigen-presenting cells that serve as a critical link between innate and adaptive immune responses. DCs differentiate from several types of precursors and then undergo maturation by a variety of exogenous stimuli, including microbial products. DC maturation/activation is followed by a number of functional and phenotypic changes that ultimately leads to T-cell activation. Two distinct lineages of blood DCs (BDCs) exist in humans [6,7]: myeloid DCs (mDCs) and plasmacytoid DCs (pDCs). Myeloid DCs originate from myeloid precursors, produce large amounts of IL-12, and induce strong T-helper 1 and cytotoxic T lymphocyte (CTL) responses. Plasmacytoid DCs derive from lymphoid precursors, display a more plasma cell-like morphology,

and are the main producers of the antiviral cytokine, interferon-α (IFN-α).

Advances in rare event flow cytometric analysis allow quantification of BDC levels and have proved useful for immunologic monitoring in various disease states and in organ transplantation [8–13]. Several groups, including our own, have demonstrated significantly lower BDC levels in patients with kidney disease compared with healthy controls [12,14,15]. These BDC levels are further reduced following transplantation, in part from the surgical procedure itself, but also because of exogenous immunosuppression [11,12,16]. With increasing time post-transplant, as immunosuppression is reduced, BDC levels return to pretransplant levels, yet are still reduced compared with those in healthy controls [12].

Given the importance of DCs to immunity and the variability of levels observed in the kidney disease population, we have chosen to explore the association of BDC levels with post-transplant viral infections. In two previous reports, we demonstrated an association of *pretransplant* mDC level deficiency with *post-transplant* BK viremia [17] and CMV disease [18]. In an effort to evaluate how pretransplant BDC deficiency might influence these outcomes mechanistically, we studied the correlation of BDC deficiency with T-cell level and function in patients with kidney disease.

Patients and methods

Patients

All study subjects provided informed consent in accordance with a protocol approved by the Johns Hopkins University (JHU) Institutional Review Board (Protocol # NA 00033825). Peripheral blood was studied in 3 groups: (i) patients with ESRD at hemodialysis units affiliated with JHU (HD; $n = 81$); (ii) patients with chronic kidney disease (CKD) stage IV-V presenting for kidney transplant evaluation or on the day of arrival for kidney transplantation at The Johns Hopkins Hospital (Eval/Tx; $n = 323$); and (iii) healthy controls (HC; $n = 22$).

Analysis of peripheral blood dendritic and other immune cells

BDCs were analyzed by four-color flow cytometry, as previously reported by our group [12,17,18] and others [11,15], with limited modifications. The four-color Dendritic Value Bundle Kit was obtained from BD Biosciences (San Jose, CA, USA), which includes fluorescein isothiocyanate (FITC)-conjugated antilineage 1 (Lin1) cocktail antibodies, anti-HLA-DR-PerCP, anti-CD11c-APC, anti-CD123-PE, and isotype control mouse IgG2a-APC and IgG1-PE antibodies. The Lin1 cocktail contains monoclonal antibodies (mAbs) against CD3 (T cells), CD16 and CD56 (natural killer cells), CD19 and CD20 (B cells), and CD14 (monocytes/macrophages). To minimize selective cell loss during the preparation procedure, cells were first stained with mAbs, followed by erythrocyte lysis. Briefly, blood cells were incubated at room temperature for 20 min with PE-, PerCP-, FITC-, and APC-conjugated mAbs, after initial incubation with Fc block. Erythrocytes were then lysed with fluorescence-activated cell sorting lysing solution (BD Biosciences). After washing with phosphate-buffered saline, the stained cells were analyzed with a FACSCalibur™ four-color flow cytometer (BD Biosciences).

Data were analyzed with CellQuest software (BD Biosciences). Total BDCs were defined as cells positive for PerCP-conjugated anti-HLA-DR and negative for FITC-conjugated anti-Lin1 ($HLA\text{-}DR^{+}Lin1^{-}$), with mDCs additionally positive for PE-conjugated anti-CD11c and pDCs positive for APC-conjugated anti-CD123 mAbs. Other blood cells measured with mAb from BD Biosciences included T cells (CD3/CD8/CD45/CD4 mix) and monocytes (CD14; MϕP9). Total WBC concentration was measured with an automated cell counter (Millipore, Darmstadt, Germany). The absolute concentrations of all cell types were calculated from the WBC count multiplied by the percentage of each subtype, as determined by fluorescence-activated cell sorting (FACS), and expressed as cells/ml peripheral blood.

It should be noted that the Dendritic Value Bundle Kit (BD Biosciences), which was the first commercially available kit for DC detection, only allows separation of DCs into two major types, myeloid and plasmacytoid. However, additional DC subtypes are now known, with newer methods available to detect and isolate these cells [19]. However, as the current report builds on our previous publications [12,17,18], we elected to continue to use the older technique.

Ex vivo PBMC stimulation

Peripheral blood mononuclear cells (PBMCs; 1×10^6/well) were cultured in 96-well plates and stimulated with LPS (1 μg/ml) for 24 h. Intracellular staining for IL-12p40 (Clone 20C2; BD Biosciences) and IL-12p70 (Clone HP40; eBioscience (San Diego, CA, USA)), gated on mDCs ($Lin1^{-}HLADR^{+}CD11c^{+}$), was measured by FACS.

In vitro monocyte-derived DC generation and stimulation

$CD14^{+}$ monocytes were isolated from PBMCs by positive selection (Miltenyi Biotec (San Diego, CA, USA)) according to the manufacturer's protocol. Monocyte-derived dendritic cells (MoDCs) were induced *in vitro* by culture of these cells in human recombinant IL-4 (20 ng/ml) and GM-CSF (100 ng/ml) for 6 days. These cells expressed a $CD1a^{+}CD14^{-}$ phenotype by flow cytometry, indicating differentiation into MoDCs, which were then stimulated

(2×10^5/well) with LPS (1 μg/ml) for 24 h. Supernatants were collected and IL-12 measured by multiplex assay (Millipore).

CMV serostatus

CMV serostatus was determined using a commercial kit, VIR-ELISA anti-IgG (VIRO-IMMUN Labor-Diagnostika GmbH, Oberusel, Germany), according to the manufacturer's instructions.

ELISpot assay

A multiscreen, 96-well filtration plate (Millipore) coated with antihuman IFN-γ antibody (Human IFN-γ ELISpot kit; Mabtech (Cincinnati, OH, USA)) was used. $CD3^+$ T cells from patients or healthy donors were isolated (CD3 Microbeads; Miltenyi). $CD3^+$ T cells (1×10^6 in 100 μL volume) were added to each well alone, or co-cultured with autologous MoDCs (1×10^5/well) with or without CMV peptide (PepTivator CMV pp65, Miltenyi), with medium alone as a negative control, or with PHA (Sigma–Aldrich (St. Louis, MO, USA)) as a positive control. All ELISpot assays were carried out in triplicate. After 24 h incubation at 37 °C/5% CO2, cells were removed by washing the plates four times with PBS containing 5% Tween 20 and twice with PBS. Fifty microliters of biotinylated anti-IFN-γ antibody was added (1:1000 dilution; Mabtech) and incubated for 3 h at room temperature. The ELISpot plate was washed an additional six times with PBS/Tween 20 and incubated for 2 h with streptavidin-ALP substrate (Mabtech) followed by the addition of an alkaline phosphatase conjugate substrate (Mabtech). The resulting spots were counted semi-automatically with Bioreader 4000 (Pro-X, BIOSYS GmbH, Werner Freber Germany). Results were expressed as number of cells secreting IFN-γ per well.

Statistical analysis

Peripheral blood immune cell levels, cytokine concentrations, and ELISpots were analyzed by one-way ANOVA or *t*-test for normally distributed data and the nonparametric Kruskal–Wallis or Mann–Whitney *U*-test for non-normally distributed variables. Correlation of mDC levels with $CD8^+$ T-cell levels was performed using a linear regression model after log transformation. $P < 0.05$ was considered as significant.

Results

Reduction of T cell and monocyte levels in patients with kidney disease

In our previous reports, we demonstrated a significant reduction of BDC levels in patients with kidney disease compared with healthy controls [12,17]. To determine whether other immune cell types are similarly depressed with kidney disease, we measured levels of T cells and monocytes in ESRD patients recruited from a hemodialysis unit (HD) or in CKD stage IV-V patients presenting for kidney transplant evaluation or kidney transplantation (Eval/Tx) and compared them with levels in healthy controls (HC). Consistent with our previous report, BDC levels, including both mDC and pDC subsets, were significantly lower in patients with kidney disease compared with healthy controls (Fig. 1a and b). Interestingly, there was a clear trend toward lower levels in the subjects from the HD units versus those presenting for evaluation or transplantation. A similar pattern was observed for $CD4^+$ and $CD8^+$ T-cell levels, with the lowest levels observed in the HD patients (Fig. 1c and d). Similar results were observed with monocytes (Fig. 1e). Despite depressed levels of the various PBMC subsets in patients with kidney disease compared with HC, total WBC levels were equivalent between groups (Fig. 1f).

Our previous work demonstrated an association between pretransplant mDC deficiency and post-transplant reactivation of BKV and CMV [17,18]. Given the importance of DCs to maintenance of the lymphocyte pool and the critical role $CD8^+$ T cells play in antiviral immunity, we determined the correlation of mDC and $CD8^+$ T-cell levels in the Eval/Tx and HD groups. As shown in Fig. 2a, after log transformation of $CD8^+$ T-cell levels, results indicated that a unit increase in mDC was associated with a 0.16 log $CD8^+$ T-cell value increase. This correlation, while statistically significant, is relatively low. Thus, the $CD8^+$ T-cell frequency is only partially explained by the mDC level, with other variables likely that are unable to be described in the current analysis.

Myeloid dendritic cell IL-12 production impaired in ESRD patients

To explore potential BDC functional abnormalities that may accompany the *in vivo* numerical deficiency observed in kidney disease patients, we measured peripheral blood mDC IL-12 production after activation *ex vivo* in patients from the HD group and compared it to that in controls. As shown in Fig. 3, the percentage of (a) IL-12p40 and (b) IL-12p70 producing mDCs (of total mDCs) was significantly lower after LPS stimulation of PBMCs from patients in the HD compared with HC group. To explore further the relationship between peripheral blood mDC level and IL-12 production, we compared the percentage of IL-12 producing mDCs after LPS stimulation *ex vivo* in the peripheral blood of subjects in the HC group stratified by mDC quartile. Results demonstrated a significantly lower percentage of IL-12p40 and IL-12p70 producing mDCs (of total

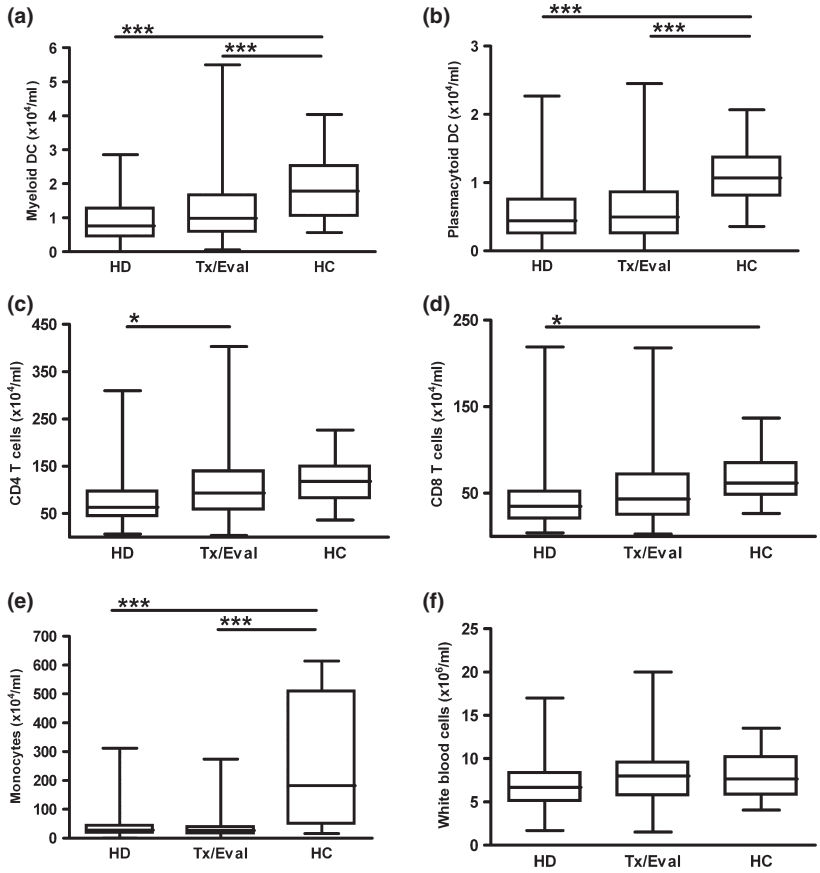

Figure 1 Comparison of mean blood cell levels between groups. Peripheral blood cell levels were analyzed by flow cytometry. Significant differences in levels were observed between groups for individual cell subtypes, including (a) myeloid DCs, (b) plasmacytoid DCs, (c) $CD4^+$ T cells, (d) $CD8^+$ T cells, and (e) monocytes, with levels lowest in ESRD patients from hemodialysis units (HD; $n = 81$) compared to patients with kidney disease presenting for transplant evaluation or on the day of transplantation surgery (Eval/Tx; $n = 323$) and healthy controls (HC; $n = 22$). (f) No significant differences between groups were observed for total WBC levels. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

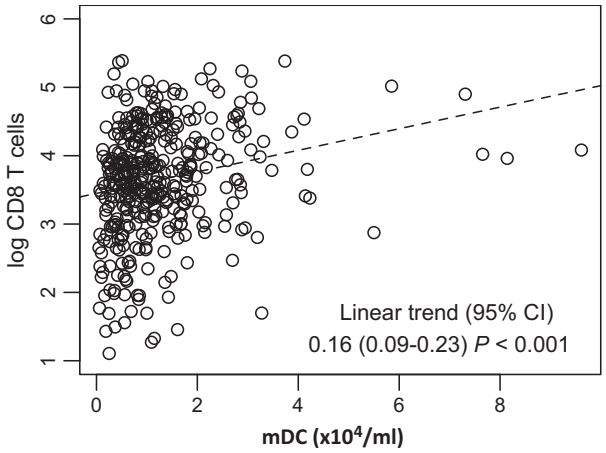

Figure 2 Correlation of myeloid DC and $CD8^+$ T-cell levels. $CD8^+$ T-cell levels in the Tx/Eval ($n = 323$) and HD ($n = 81$) groups were log-transformed. A unit increase in myeloid DC (mDC) was associated with a 0.16 log $CD8^+$ T-cell value increase (95% CI 0.09–0.23; $P < 0.001$).

mDCs) in the lowest quartile compared with highest quartile (Fig. 3c and d). These experiments suggest that reduction in peripheral blood mDC levels is associated with a lower percentage of IL-12 producing mDCs in the total DC pool, regardless of the study population.

As mDCs derive from several different progenitors, including monocytes, we also measured IL-12 production from MoDCs generated from the peripheral blood of HD and HC subjects after LPS stimulation in culture. This approach allowed comparison of cytokine production from equal numbers of DCs in culture, despite being derived from patient populations with significantly different peripheral blood monocyte and DC levels. Results demonstrated significantly lower IL-12p40 concentration in response to LPS stimulation in MoDCs derived from HD compared with HC subjects (Fig. 3e), with a nonsignificant trend observed with IL-12p70 concentrations (Fig. 3f). Thus, ESRD status is not only associated with reduced IL-12 production from existing mDCs in the peripheral

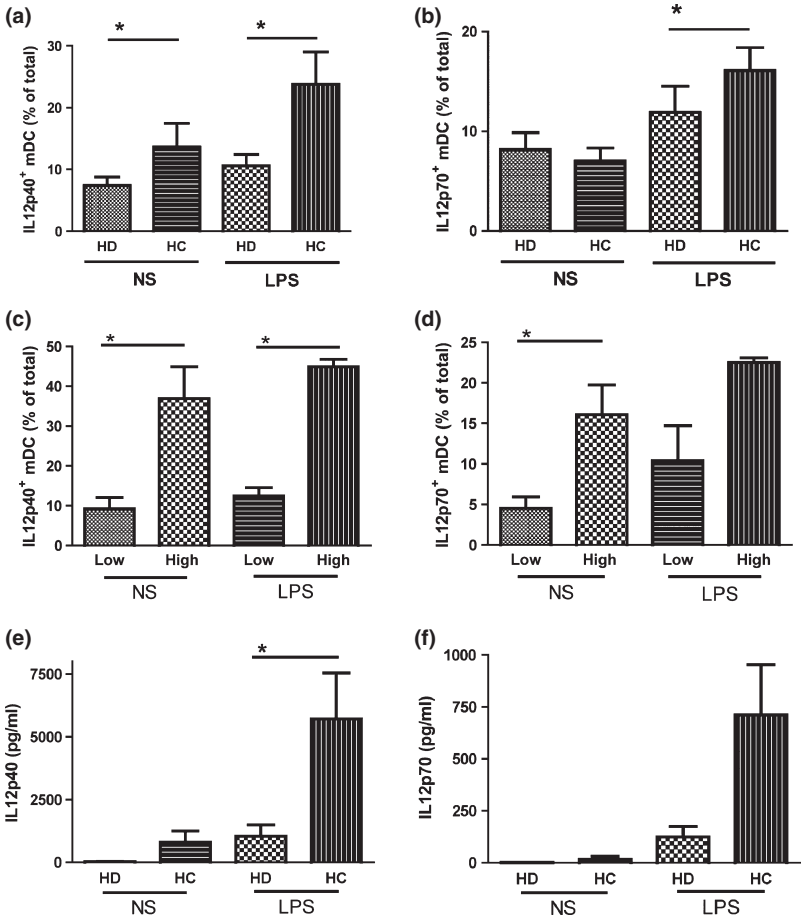

Figure 3 Myeloid DC IL-12 production in ESRD patients. Upon stimulation of PBMCs with LPS, the percentage of (a) IL-12p40 and (b) IL-12p70 producing mDCs was significantly lower in ESRD patients on hemodialysis (HD; $n = 18$) compared with healthy controls (HC; $n = 10$). The percentage of (c) IL-12p40 and (d) IL-12p70 producing peripheral blood mDCs was lower at baseline and after LPS stimulation in HC subjects with peripheral blood mDC levels in the lowest quartile (Low; $n = 4$) compared with highest (High; $n = 4$) quartile. Finally, LPS stimulation of equal numbers of monocyte-derived DCs resulted in lower IL-12p40 (e) and IL-12p70 (f) concentrations in the HD ($n = 17$) versus HC ($n = 16$) group. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$.

blood but also from mDCs-derived *ex vivo* from monocytes.

Altered MoDC-induced T-cell IFN-γ production

To explore potential abnormalities of T-cell activation resulting from putative DC functional defects associated with ESRD, we used the ELISpot assay to compare IFN-γ production by peripheral blood T cells between subjects in the HD and HC groups. In the first set of experiments, MoDCs were added in culture to purified $CD3^+$ T cells. Results demonstrated a statistically significant increase in IFN-γ spots in the HC group when MoDCs were added to culture (Fig. 4a). In contrast, cultures in the HD group demonstrated more variability in baseline IFN-γ spots that did not significantly increase with addition of MoDCs. A similar pattern was observed when IFN-γ responses to CMV antigen were measured (Fig. 4b). In the HC group, addition of MoDCs and CMV antigen to culture resulted in a significant increase in IFN-γ spots over addition of CMV antigen or T cells alone. However, in HD subjects, higher variability in baseline IFN-γ spots was again observed that did not demonstrate significant increase after addition of viral antigen or MoDCs. CMV IgG testing revealed all patients in the HD group to be seropositive, whereas only 2 of 6 subjects in the HC group were seropositive.

Discussion

Given the importance of effective DC–T cell interactions to antiviral immunity, experiments in the current report were designed to determine whether BDC deficiency is associated with abnormalities in T-cell level and function. Along with healthy controls, we studied two separate

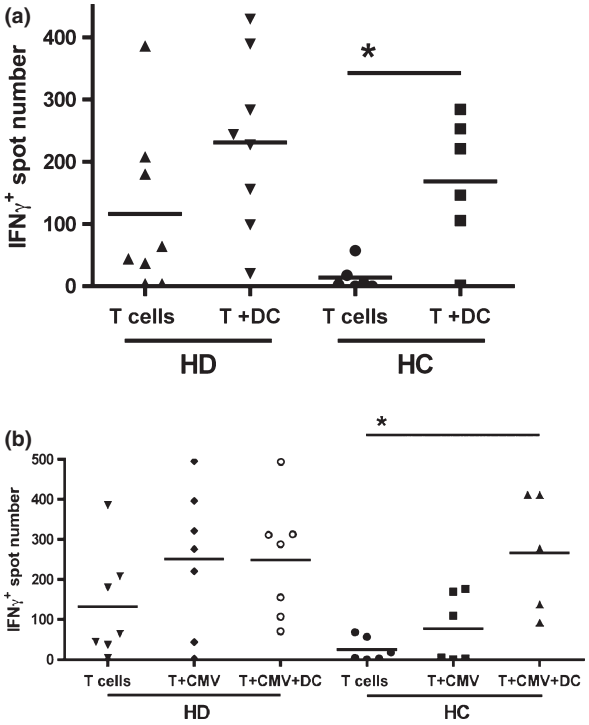

Figure 4 MoDC stimulation of T-cell responses. (a) Significantly greater augmentation of IFNγ-producing $CD3^+$ T cells over baseline was observed in the healthy control (HC; $n = 6$) versus hemodialysis (HD; $n = 7$) group during co-culture with autologous monocyte-derived DCs (MoDCs). (b) Likewise, addition of MoDC and CMV peptide to $CD3^+$ T-cell culture resulted in significantly greater augmentation of IFNγ -producing cells over baseline only in the HC group. $^*P < 0.05$ and $^{**}P < 0.01$.

groups of patients with kidney disease, including ESRD patients from a local HD unit (HD) and CKD IV-V patients presenting either for their transplant evaluation or on the day of kidney transplantation (Tx/Eval), which included some patients with failing or failed allografts on immunosuppression. We found that BDC levels were lowest in patients from the HD unit, which is not entirely unexpected, given that many patients presenting for evaluation or transplantation had not yet required dialysis, while all of those patients from the HD unit were end stage. Previous studies have shown a correlation of mDC deficiency with reduction in glomerular filtration rate [15]. We do not believe that inclusion of patients with failing allografts on immunosuppression in the Tx/Eval group explains the higher DC levels in the Tx/Eval versus HD groups. Heavy immunosuppression immediately post-transplant is associated with a reduction (not increase) in average DC levels compared with pretransplant wait-listed individuals, and although these average DC levels increase with reduction in immunosuppression beginning around 3 months post-transplant, they do not rise higher than pretransplant levels [12]. It is possible that the lower BDC levels in the HD unit patients reflect the more debilitated state of these patients, the majority of whom had multiple medical co-morbidities and would never be considered for transplantation. However, in our previous studies, we have been unable to demonstrate an association of serum albumin, a marker for nutritional status, with either BDC level or post-transplant viral infection [17,18]. Likewise, in our combined work, we have never been able to demonstrate an association of time on HD with either of these outcomes. Thus, it appears that there are differences in blood DC levels between predominately wait-listed and nonwait-listed individuals, although the precise mechanism for why a reduction in BDC levels is observed with kidney failure still remains unclear. Future large multivariate analyses, beyond the scope of this report, may shed some light on this interesting finding.

IL-12 is a cytokine critical for mDC activation of antiviral cytotoxic T-cell responses. In our previous work, we demonstrated significantly lower levels of IL-12 after stimulation of PBMCs in culture with LPS pretransplant in those patients who subsequently developed post-transplant BK viremia [17]. These results suggested that a functional DC deficiency accompanied the reduction in level and supported our hypothesis that pretransplant DC deficiency promotes the development of BK viremia. However, although LPS activates toll-like receptor 4 present on the surface of DCs to produce IL-12, it was not clear from these studies whether the IL-12 production in culture was specifically from DCs, as other cell types in the peripheral blood can also produce this cytokine. Using intracellular staining by FACS to focus specifically on mDC production of this cytokine, we were able to demonstrate a lower frequency of IL-12 positive mDCs (of total mDCs) after LPS stimulation of PBMCs from the HD patients compared with healthy controls, supporting our hypothesis that a functional DC deficiency accompanies the deficiency in level we have observed in kidney disease patients.

As further confirmation of the correlation between BDC level and IL-12 production, we demonstrated a lower frequency of IL-12-positive mDCs in healthy controls who had total mDC levels in the lowest quartile compared with those in the highest quartile. Interestingly, the deficiency in mDC IL-12 production, at least in kidney disease patients, extends beyond circulating mDCs to DCs derived *in vitro* from monocytes, although admittedly, some mDCs measured in the peripheral blood may have derived from monocyte precursors. Thus, functional mDC deficiency, at least as defined by IL-12 production, is correlated with mDC level in the blood of both healthy individuals and those with kidney disease. However, kidney disease patients are much more likely to have lower mDC levels and therefore deficient mDC IL-12 production.

Virus-specific T-cell responses are critical to antiviral immunity post-transplant, including those for BKV and CMV, with deficient responses clearly associated with both of these infections [20–30]. In our study, we examined the relationship between mDC deficiency and T-cell level/function. For the first time, we have demonstrated that a reduction in peripheral blood T-cell levels exists in patients with kidney disease that correlates with the previously observed reduction in mDC levels. Furthermore, T cells from HD patients failed to exhibit the normal pattern of IFN-γ production observed in healthy controls in response to addition of MoDCs alone or with CMV antigen. Although only two of the individuals in the HC group were CMV seropositive, we demonstrated a statistically significant increase in IFN-γ spots over baseline despite seronegativity, similar to a recent report by Bestard *et al.* Our choice to study pretransplant T-cell responses is also supported by Bestard *et al.* [31], who demonstrated that low *pretransplant* CMV-specific T-cell responses predicted the development of *post-transplant* CMV infection, regardless of IgG serostatus, induction therapy, and prophylactic regimen. These findings suggest that immune susceptibility to viral infection exists prior to transplantation, which is consistent with our previous reports demonstrating the association of pretransplant BDC levels with post-transplant viral infection [17,18]. Admittedly, our experiments do not address directly whether the observed deficient T-cell responses are a direct result of the documented mDC functional deficiencies or, alternatively, are pre-existing and independent of the DC functional status. We have merely shown a correlation of depressed mDC level with deficient IL-12 production and that MoDCs from these individuals fail to stimulate T cells properly. Future studies could use HLA-matched MoDCs from healthy controls with mDC levels in the highest quartile to determine whether they augment T-cell IFN-γ responses in kidney disease patients compared with autologous MoDCs, which would argue for DC deficiency as the primary underlying immunologic defect in kidney disease.

In summary, we have demonstrated for the first time a correlation between peripheral blood mDC level and function in kidney disease patients that may explain the deficient T-cell responses known to predispose individuals in this population to viral infections post-transplant [32]. Antiviral medications will no doubt continue to play an important therapeutic role during periods of inadequate immunity [33]. However, if mDC deficiency is indeed found to play a major role in T-cell dysfunction, future therapies for management of viral infections post-transplant may also involve the *in vivo* administration of cytokines or other growth factors to boost endogenous mDC level and function.

Authorship

PC: Participated in writing of paper, performance of research, and data analysis. QS, FFN, NA and ESK: Participated in performance of research. YH: Participated in design and performance of research. MGA and ST: Participated in performance of research and writing of paper. DLS: Participated in performance of research, writing of paper, and data analysis. KAM and KLW: Participated in research design, writing of paper, performance of research, and data analysis.

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