Uremia impairs monocyte and monocyte-derived dendritic cell function in hemodialysis patients

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Patients with chronic renal failure maintained on intermittent hemodialysis have frequent infections and a suboptimal response to vaccinations. Dendritic cells are potent antigen-presenting cells essential for the initiation and maintenance of innate and adaptive immunity. In this study we used uremic sera from hemodialysis patients to measure its impact on monocyte and monocyte-derived dendritic cell function *in vitro*. Monocytes from healthy and uremic subjects were isolated using immunomagnetic beads and differentiated into dendritic cells in the presence of either complete sera or sera from hemodialysis patients. Dendritic cells from normal patients cultured in uremic sera had decreased endocytosis and impaired maturation. These cells, however, had enhanced IL-12p70 production and increased allogeneic T-cell proliferation compared to cells of normal subjects cultured in normal sera. Monocyte derived dendritic cells of hemodialysis patients cultured in either normal or uremic sera were functionally impaired for endocytosis and maturation but had enhanced IL-12p70 production and allogeneic T-cell proliferation only when cultured with uremic sera. High concentrations of urea in normal sera inhibited all aspects of normal dendritic cell function *in vitro*. Our study suggests that hemodialysis regimes tailored to remove uremic toxins more efficiently may improve immune functions of these patients.

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Immune protection and surveillance against pathogens in humans relies on a coordinated response of innate and adaptive immune systems.[1] Chronic renal failure (CRF) patients have evidence of defects in both innate immunity (e.g. reduced polymorphonuclear leukocytes chemotaxis, phagocytosis, and bactericidal activity)[2,3] and adaptive immunity (reduced T-cell function) that contribute to generalized immunodeficiency.[4,5] Infection is a leading cause of morbidity and mortality in CRF patients (including hemodialysis (HD) and continuous ambulatory peritoneal dialysis (CAPD) patients) that may be attributed to these underlying immune defects in the innate system.[6,7] In addition, CRF patients display suboptimal responses to therapeutic vaccinations (e.g. hepatitis B) characterized by low antibody titers, and short duration of protection typical of impairment of adaptive immunity.[8,9] These deficiencies observed in CRF patients may be attributed to defects in the number and function of antigen-presenting cells, in particular dendritic cells (DC),[10] but their role in the pathogenesis of innate and adaptive immune dysfunction in CRF patients has not been defined clearly.

DC are a group of rare, heterogenous population of professional antigen-presenting cells that initiate primary immune responses, and regulate both innate and adaptive immunity.[11–13] DC are critical in stimulating naïve T-cell responses to captured foreign antigens.[14,15] Following antigen capture and processing, DC undergo maturation and migrate to secondary lymphoid tissues where they present processed antigen/peptide coupled to major histocompatibility complexes, which allow for selection and expansion of antigen-specific $CD4^+$ T-helper cells.

DC may be differentiated *in vitro* from monocyte precursors and provide a model system whereby these otherwise rare antigen-presenting cells may be generated in sufficient numbers for studies.[16] DC-based vaccination has been utilized successfully in certain clinical conditions to generate immunity to certain pathogens.[17–19] Monocytes cultured in the presence of granulocyte macrophage colony-stimulating factor and interleukin (IL)-4 for 5 days differentiate into immature monocyte-derived DC (MoDC) characterized by low expression of major histocompatibility complex class II molecules, costimulatory molecules (CD40, CD80, and CD86), and the DC maturation marker, CD83.

Bacterial lipopolysaccharide (LPS), tumor necrosis factor-α, and CD40 ligand can be used for the induction of DC maturation without cell proliferation.[20,21]

In a previous study, we established that circulating myeloid DC precursors from HD patients displayed functional impairment with reduced cell-surface costimulatory molecule expression following maturational signals.[22] We hypothesized that the presence of uremic toxins in the serum of HD patients are responsible for the observed impairment in DC functions. In this study, we used MoDC system to assess the impact of uremic sera from HD patients on monocyte and MoDC functions.

RESULTS

Monocytes

HD patients have high levels of IL-12p70. The serum concentration of bioactive IL-12p70 was significantly higher in the sera of HD patients (52.8 ± 18.1 pg/ml), CAPD patients (12.6 ± 3.8 pg/ml), and CRF patients (49.6 ± 5.5 pg/ml) when compared to healthy controls and pooled human AB serum (both below the level of detection; Mann–Whitney *U*-test $P < 0.001$).

Uremic medium inhibits normal monocyte function. To determine the effect of uremic serum on monocyte function, we studied normal monocytes cultured in uremic medium (UM). LPS-stimulated normal monocytes cultured in UM demonstrated reduced expression of costimulatory molecules (CD40, CD80, and CD86) compared to normal monocytes in complete medium (CM) (Figure 1a and Table 1). Consistent with this observation, UM-cultured monocytes displayed reduced allo-stimulatory capacity (Mann–Whitney *U*-test, $P < 0.0001$; Figure 1b) when compared to monocytes in CM. The amount of IL-12p70 present in CM and UM was below the level of detection and 13.5 ± 3.2 pg/ml, respectively (Mann–Whitney *U*-test, $P < 0.001$). Monocytes cultured in CM or UM produced no additional IL-12p70 in excess of background amount of IL-12p70 in mediums (sera of 5 HD patients used).

Uremic monocytes from HD patients are functionally abnormal. Freshly isolated uremic monocytes from HD patients exhibited reduced fluorescein isothiocyanate (FITC)-dextran uptake compared to normal monocytes (representative of three HD patients, mean fluorescent intensity (MFI) 9.2 ± 2.3 and 25.4 ± 5.2, respectively; Student's *t*-test, $P = 0.02$). Following LPS stimulation, uremic monocytes cultured in CM, but not when cultured in autologous UM, upregulated cell-surface expression of costimulatory molecules CD40, CD80, and CD86, but not to the same extent of LPS-stimulated normal monocytes cultured in CM (Figure S1 and Table 1). Consistent with this observation, uremic monocytes in CM showed greater allo-stimulatory capacity compared to uremic monocytes cultured in autologous UM (Mann–Whitney *U*-test, $P < 0.0001$; Figure S2). Uremic monocytes cultured in CM or autologous UM produced no detectable levels of IL-12p70 in excess of background levels in mediums (representative of three experiments).

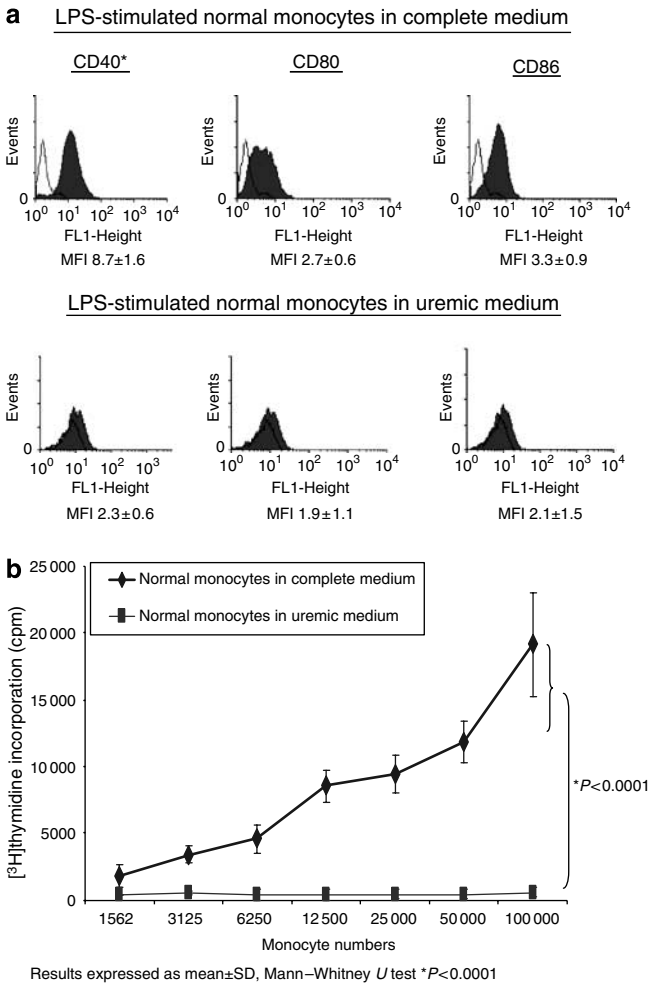

Figure 1 | Uremic medium inhibits costimulatory molecule expression and allo-stimulatory capacity of monocytes. (**a**) Cell-surface expression of costimulatory molecules on LPS-stimulated (10 ng/ml) normal monocytes of healthy blood donors cultured in either CM or UM for 24 h. Flow cytometric analysis revealed reduced cell-surface expression of costimulatory molecules (CD40, CD80, and CD86) on monocytes cultured in UM compared to cells cultured in CM (closed profiles). Open black profiles denote isotype controls. Representative histograms are shown. MFI (mean ± s.d.) below histograms representative of results of eight experiments involving the sera of eight separate HD patients (Mann–Whitney *U*-test, $^*P < 0.05$). (**b**) Allogeneic T-cell proliferation in a monocytes/T cells two-way MLR. LPS-stimulated (10 ng/ml) normal monocytes of healthy blood donors cultured in CM for 24 h induced greater allogeneic T-cell proliferation (measured as tritiated thymidine incorporated cells) compared to normal monocytes cultured in UM for 24 h (Mann–Whitney *U*-test, $P < 0.0001$). Results representative of four separate experiments.

Monocyte-derived dendritic cells

UM does not affect the differentiation of monocytes into MoDC but inhibits MoDC functions. To determine the effects of uremic serum on the differentiation of monocytes into MoDC and MoDC function, we studied normal monocytes/MoDC cultured in UM. Compared to normal immature MoDC in CM, normal immature MoDC cultured in UM exhibited reduced FITC-dextran uptake (sera of 10 separate

Table 1 | Costimulatory molecule expression of normal and uremic monocytes in complete and uremic media

Monocytes	CD40 MFI (mean ± s.d.)*	CD80 MFI (mean ± s.d.)*	CD86 MFI (mean ± s.d.)*
Normal monocytes in CM[#,†]	8.7 ± 1.6	2.7 ± 0.6	3.3 ± 0.9
Normal monocytes in UM[#]	2.3 ± 0.6	1.9 ± 1.1	2.1 ± 1.5
Uremic monocytes in CM[†]	3.3 ± 1.1	1.9 ± 0.5	2.2 ± 0.7
Uremic monocytes in autologous UM	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

CM, complete medium; MFI, mean fluorescent intensity; s.d., standard deviation; UM, uremic medium.
*$P < 0.05$ Kruskal–Wallis test.
†$P < 0.05$ Student's *t*-test (CD40 only).
#$P < 0.05$ Mann–Whitney *U*-test (CD40 only).

HD patients used, MFI 45.2 ± 6.8 and 15.5 ± 7.2, respectively; Mann–Whitney *U*-test, $P < 0.05$) and reduced cell-surface expression of CD206 (sera of 10 separate HD patients used, MFI 60.5 ± 10.2 and 8.2 ± 2.5; Mann–Whitney *U*-test, $P < 0.01$). The differentiation of normal monocytes cultured in CM or UM into MoDC was complete and unaffected by the presence of uremic serum. LPS-stimulated MoDC derived from normal monocytes cultured in UM demonstrated reduced cell-surface expression of costimulatory (CD40, CD80, and CD86) and maturation (CD83) markers (Figure 2a and Table 2).

A greater proportion of normal LPS-stimulated MoDC cultured in UM compared to CM were apoptotic and/or necrotic (sera of three separate HD patients used; Student's *t*-test, $P < 0.05$; Table 2). CD95 expression was similar in normal LPS-stimulated MoDC cultured in UM and CM (sera of 10 separate HD patients used, MFI 4.8 ± 2.2 and 3.4 ± 1.8, respectively; $P =$ NS).

Normal LPS-stimulated MoDC cultured in UM produced a greater amount of IL-12p70 (78.9 ± 24.4 pg/ml) when compared to cells cultured in CM (32.2 ± 10.2 pg/ml, sera of 10 separate HD patients used; Mann–Whitney *U*-test, $P = 0.01$). The amount of IL-12p70 present in CM and UM was below the level of detection and 9.5 ± 2.1 pg/ml, respectively (Mann–Whitney *U*-test, $P < 0.01$). Normal LPS-stimulated MoDC cultured in UM demonstrated enhanced allo-stimulatory capacity compared to cells in CM (Mann–Whitney *U*-test, $P = 0.009$; Figure 2b). In order to investigate the impact of small MW uremic toxins on MoDC functions, the effect of urea, a recognized small MW uremic toxin present at increased concentrations in the uremic sera of HD patients on normal MoDC functions was assessed.

High concentration of urea inhibits normal MoDC function. Increasing concentrations of urea were added to CM containing normal monocytes in levels equivalent to CRF patients. However, only normal monocytes cultured in CM supplemented with 40 mmol/l of urea inhibited monocyte differentiation into immature MoDC and exhibited reduced expression of costimulatory molecules and CD83 on LPS-stimulated MoDC. Monocytes cultured in CM alone or CM supplemented with lower concentrations of urea (5 and 20 mmol/l) displayed appropriate monocyte differentiation into immature MoDC and subsequent maturation following LPS stimulation (Figure 3a and Table 3). Normal immature MoDC cultured in CM containing 40 mmol/l urea demonstrated reduced FITC-dextran uptake (MFI 39.6 ± 15.2) and expression of CD206 (MFI 8.8 ± 3.2) when compared to immature MoDC cultured in CM alone (MFI 68.5 ± 10.2 and 40.5 ± 6.2, respectively, representative of six separate experiments; Mann–Whitney *U*-test, $P < 0.05$). Immature MoDC cultured in CM containing lower concentrations of urea exhibited similar endocytic capacity (FITC-dextran uptake) and CD206 expression as cells cultured in CM alone (representative of six experiments, data not shown).

A greater proportion of LPS-stimulated normal MoDC cultured in CM and 40 mmol/l urea were apoptotic and/or necrotic when compared to cells cultured in CM alone. LPS-stimulated MoDC cultured in CM containing lower concentrations of urea had similar percentage of apoptotic and/or necrotic cells as MoDC cultured in CM alone (representative of three experiments, $P =$ NS; Table 3). CD95 expression on LPS-stimulated normal MoDC was not affected by the presence of any concentrations of urea in CM (representative of six experiments, data not shown).

Increasing concentrations of urea imparted a dose-dependent inhibition in the ability of LPS-stimulated normal MoDC to produce IL-12p70 detected by enzyme-linked immunosorbent assay (ELISA). LPS-stimulated MoDC cultured in CM alone produced 35.7 ± 4.6 pg/ml of IL-12p70, whereas MoDC cultured in CM containing 5 mmol/l (28.4 ± 6.9 pg/ml), 20 mmol/l (26.1 ± 4.9 pg/ml), and 40 mmol/l (below the level of detection) of urea produced less IL-12p70 (representative of eight separate experiments; Kruskal–Wallis test, $P < 0.05$). There was no detectable IL-12p70 in CM ± urea. However, only LPS-stimulated normal MoDC cultured in CM and 40 mmol/l urea significantly inhibited allogeneic T-cell proliferation compared to MoDC cultured in the absence/lower concentrations of urea in CM (Figure 3b; Kruskal–Wallis test, $P = 0.1$).

Uremic sera of CAPD and CRF patients also affect MoDC function and survival. In order to investigate the impact of uremia and dialysis modality on MoDC functions, the effect of the sera of CAPD and CRF patients on normal MoDC functions was assessed. Compared to normal immature MoDC in CM, normal immature MoDC cultured in UM containing either sera of CAPD and CRF patients demonstrated similar capacity for FITC-dextran uptake (sera of three separate CAPD and CRF patients used, MFI 113.2 ± 15.8, 145.6 ± 89.2, and 123.6 ± 10.5, respectively; $P =$ NS). LPS-stimulated MoDC derived from normal

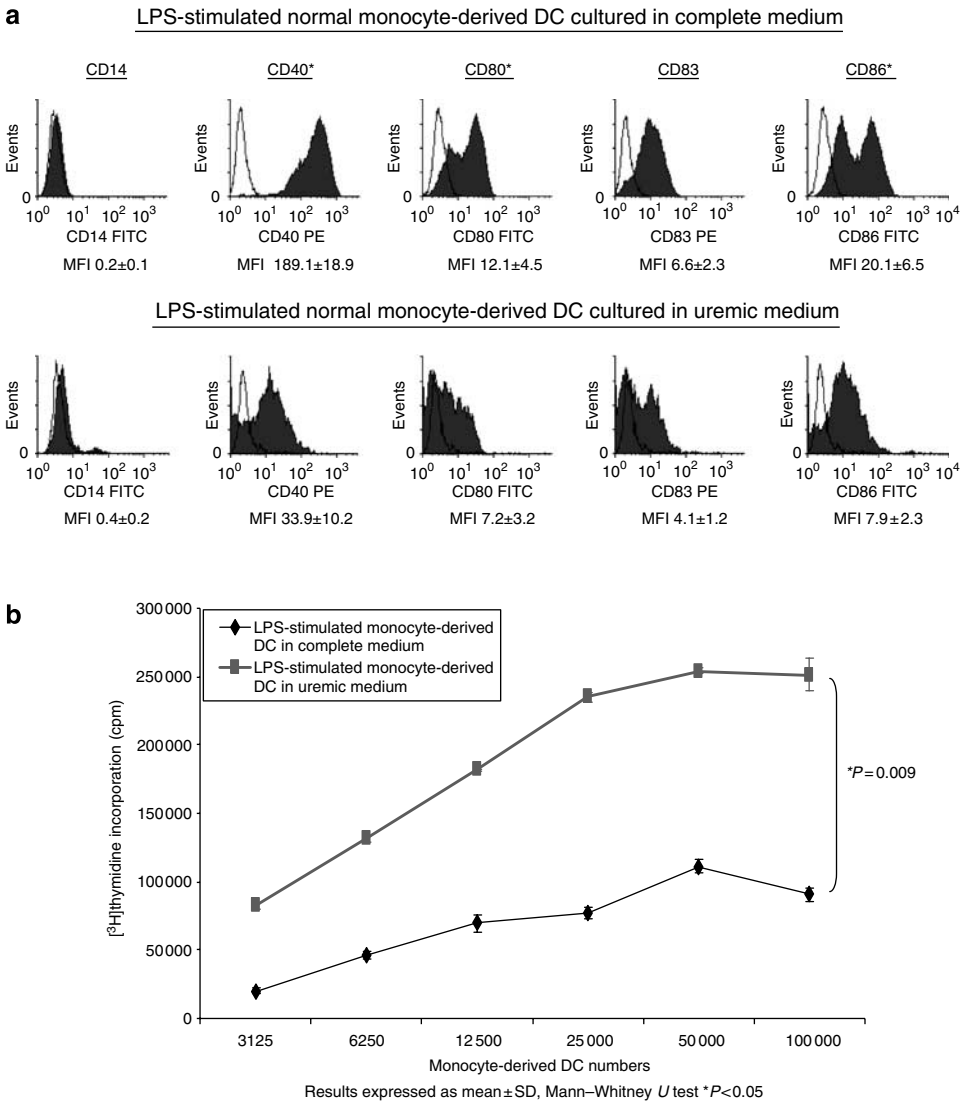

Figure 2 | Uremic medium inhibits costimulatory molecule expression on monocyte-derived DC but enhances their allo-stimulatory capacity in MLR. (**a**) Cell-surface expression of CD14, costimulatory molecules, and CD83 on normal LPS-stimulated (10 ng/ml) MoDC of healthy blood donors cultured for 48 h in either CM or UM. Flow cytometric analysis revealed reduced cell-surface expression of costimulatory molecules (CD40, CD80, and CD86) and CD83 (maturation marker) on MoDC cultured in UM compared to cells cultured in CM (closed profiles). There was negligible/absent cell-surface expression of CD14 demonstrated on both MoDC cultured in CM or UM suggesting that all monocytes had differentiated into MoDC (closed profiles). Open black profiles denote isotype controls. Representative histograms are shown. MFI (mean ± s.d.) below histograms representative of results of 10 experiments involving the sera of 10 separate HD patients (Mann–Whitney *U*-test $*P<0.05$). (**b**) Allogeneic T-cell proliferation in an MoDC/T cells two-way MLR. Normal LPS-stimulated (10 ng/ml) MoDC of healthy blood donors cultured in UM for 48 h induced a greater proliferation of allogeneic T cells compared to MoDC cultured in CM for 48 h (Mann–Whitney *U*-test, $P=0.009$). Results representative of five separate experiments.

Table 2 | Costimulatory molecule expression and viability of normal and uremic monocyte-derived dendritic cells in complete and uremic (sera of hemodialysis patients) mediums

MoDC	CD40 MFI (mean ± s.d.)*	CD80 MFI (mean ± s.d.)*	CD83 MFI (mean ± s.d.)*	CD86 MFI (mean ± s.d.)*	Apoptotic/necrotic cells (% total cells)#
Normal MoDC in CM†,‡	189.1 ± 18.9	12.1 ± 4.5	6.6 ± 2.3	20.1 ± 6.5	41.1 ± 10.2
Normal MoDC in UM†	33.9 ± 10.2	7.2 ± 3.2	4.1 ± 1.2	7.9 ± 2.3	82.4 ± 12.9
Uremic MoDC in CM‡	7.9 ± 3.2	0.4 ± 0.3	0.2 ± 0.1	0.5 ± 0.3	—
Uremic MoDC in autologous UM	1.1 ± 0.5	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.3	—

CM, complete medium; MFI, mean fluorescent intensity; MoDC, monocyte-derived dendritic cells; s.d., standard deviation; UM, uremic medium.
*$P<0.05$ Kruskal–Wallis test.
#$P<0.05$ Student's *t*-test.
†$P<0.05$ Mann–Whitney *U*-test (except CD83).
‡$P<0.05$ Mann–Whitney *U*-test.

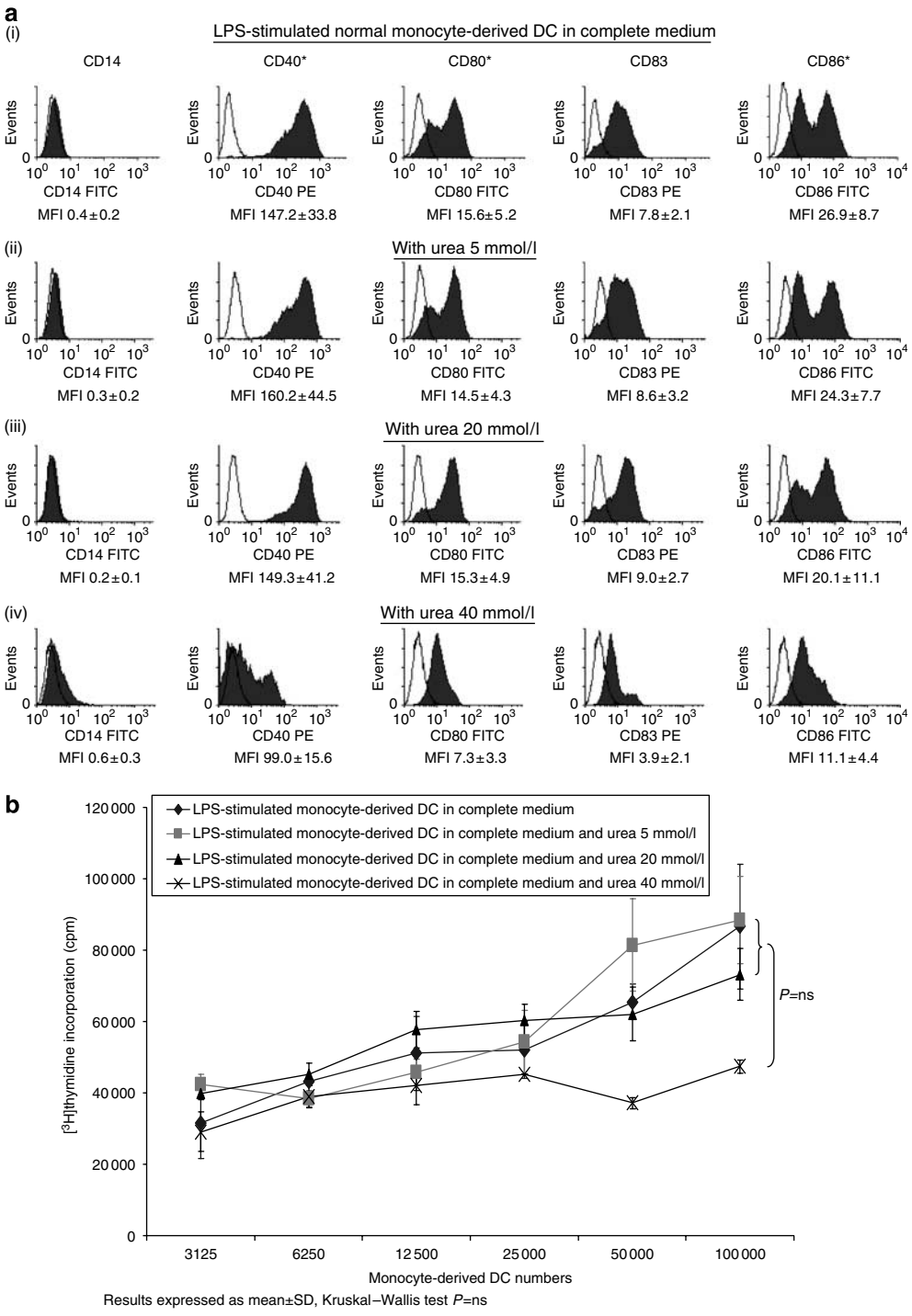

Figure 3 | The effect of urea on monocyte-derived DC costimulation molecule expression and allostimulatory capacity. (**a**) Cell-surface expression of CD14, costimulatory molecules, and CD83 on normal LPS-stimulated (10 ng/ml) MoDC of healthy blood donors cultured in CM with graded concentrations of urea for 48 h. Flow cytometric analysis revealed dose-dependent reduction in cell-surface expression of costimulatory molecules (CD40, CD80, and CD86) and CD83 (maturation marker) on mature MoDC cultured in the presence of 40 mmol/l of urea (iv) compared to cells cultured in the absence (i) or lower concentrations of urea (ii and iii). There was a slight increase in cell-surface expression of CD14 demonstrated on MoDC cultured in the presence of the highest concentration of urea (40 mmol/l) suggesting that not all monocytes in culture differentiated into DC (closed profiles). Open black profiles denote isotype controls. Representative histograms are shown. MFI (mean ± s.d.) below histograms representative of results of eight experiments involving the sera of eight separate HD patients (Mann–Whitney *U*-test comparing panels (i) and (iv) $^*P < 0.05$ for CD40, CD80, and CD86 only). (**b**) The ability of MoDC cultured in the presence of urea to stimulate allogeneic T-cell proliferation in an MLR. Normal LPS-stimulated (10 ng/ml) MoDC of healthy blood donors cultured for 48 h in CM without urea or with 5 or 20 mmol/l of urea promoted a similar amount of T-cell proliferation. MoDC cultured in the presence of 40 mmol/l induced less allogeneic T-cell proliferation compared to MoDC cultured in the absence or lower concentrations of urea (Kruskal–Wallis test, $P =$ NS). Result representative of three separate experiments.

monocytes cultured in UM (containing either sera of CAPD and CRF patients) demonstrated slightly reduced cell-surface expression of CD80 and CD86, but not CD40 and CD83. The differentiation of MoDC from monocytes was not affected by the presence of uremic serum in cell culture (Figure S3 and Table 4).

A greater proportion of normal LPS-stimulated MoDC cultured in UM compared to CM were apoptotic and/or necrotic (sera of three separate CAPD and CRF patients used, $P = $ NS; Table 4). Normal LPS-stimulated MoDC cultured in UM produced a greater amount of IL-12p70 (12.5 ± 4.7 pg/ml in UM containing sera of CAPD patients, and 155.5 ± 25.9 pg/ml in UM containing sera of CRF patients) when compared to cells cultured in CM (undetectable; Kruskal–Wallis test, $P < 0.01$). The amount of IL-12p70 present in CM and UM was both below the level of detection.

Uremic MoDC of HD patients are functionally abnormal. Uremic monocytes from HD patients cultured in either CM or autologous UM were capable of differentiating into MoDC though there was a marginal increase in the number of undifferentiated monocytes (Figure 4a). Compared to normal immature MoDC cultured in CM, uremic immature MoDC cultured in CM and autologous UM exhibited reduced FITC-dextran uptake (50.8 ± 6.7, 0.1 ± 0.1, and 0.2 ± 0.1, respectively, representative of three HD patients; Kruskal–Wallis test, $P < 0.01$) and reduced expression of CD206 (108.5 ± 15.8, 0.3 ± 0.2, and 0.3 ± 0.1, respectively, representative of three HD patients; Kruskal–Wallis test, $P < 0.0001$). Uremic LPS-stimulated MoDC cultured in CM or autologous UM demonstrated reduced expression of costimulatory molecules and CD83 compared to normal MoDC cultured in CM (Figure 4a and Table 2).

LPS-stimulated uremic MoDC cultured in autologous UM produced greater amount of IL-12p70 (34.2 ± 10.2 pg/ml) than uremic MoDC cultured in CM (below the level of detection, representative of three HD patients; Student's *t*-test, $P < 0.01$). The amount of IL-12p70 present in CM and UM was below the level of detection and 11.3 ± 5.1 pg/ml, respectively (Student's *t*-test, $P < 0.01$). As demonstrated with normal MoDC cultured in UM, uremic MoDC cultured in autologous UM exhibited greater allo-stimulatory capacity than uremic MoDC cultured in CM (Kruskal–Wallis test, $P = 0.001$; Figure 4b).

DISCUSSION

In this study, we demonstrated that uremic sera from HD patients inhibited normal monocytes and MoDC function. We established that the presence of uremic serum in cell culture prevented the upregulation of costimulatory molecule expression on monocytes (including CD40, CD80, and CD86) following LPS stimulation resulting in reduced allo-stimulatory capacity of monocytes *in vitro*. However, the presence of uremic serum did not considerably affect the differentiation of monocytes into MoDC *in vitro* but did affect the function of immature and mature normal MoDC post-differentiation. Immature MoDC generated in the presence of uremic serum displayed suboptimal capacity for endocytosis via macrophage mannose receptor pathway that may directly reflect a reduction of CD206 expression on the cell surface of these immature MoDC. Furthermore, the presence of uremic serum inhibited the maturation of MoDC demonstrated by lower expression of CD83 and costimulatory molecule expression despite LPS stimulation. However, despite the lack of phenotypic maturation displayed by

Table 3 | Costimulatory molecule expression and viability of normal monocyte-derived dendritic cells in complete and uremic (sera of peritoneal dialysis and chronic renal failure patients) mediums

MoDC	CD40 MFI (mean ± s.d.)	CD80 MFI (mean ± s.d.)	CD83 MFI (mean ± s.d.)	CD86 MFI (mean ± s.d.)*	Apoptotic/necrotic cells (% total cells)
Normal MoDC in CM	104.2 ± 54.3	7.5 ± 3.6	3.7 ± 2.8	23.1 ± 4.3	36.4 ± 12.2
Normal MoDC in UM (PD)	111.5 ± 24.6	3.2 ± 2.6	0.8 ± 0.5	11.1 ± 4.7	54.5 ± 15.2
Normal MoDC in UM (CRF)	110.2 ± 64.3	4.3 ± 2.7	1.4 ± 0.9	15.2 ± 7.2	50.1 ± 23.2

CM, complete medium; MFI, mean fluorescent intensity; MoDC, monocyte-derived dendritic cells; s.d., standard deviation; UM (CRF), uremic medium containing sera of chronic renal failure patients; UM (PD), uremic medium containing sera of peritoneal dialysis patients.
*$P < 0.05$ Kruskal–Wallis test.

Table 4 | Costimulatory molecule expression and viability of normal monocyte-derived dendritic cells in complete medium ± urea solution

MoDC	CD40 MFI (mean ± s.d.)	CD80 MFI (mean ± s.d.)	CD83 MFI (mean ± s.d.)	CD86 MFI (mean ± s.d.)	Apoptotic/necrotic cells (% total cells)
Normal MoDC in CM*	147.2 ± 33.8	15.6 ± 5.2	7.8 ± 2.1	26.9 ± 8.7	38.5 ± 10.5
Normal MoDC in CM+urea 5 mmol/l	160.2 ± 44.5	14.5 ± 4.3	8.6 ± 3.2	24.3 ± 7.7	42.9 ± 12.2
Normal MoDC in CM+urea 20 mmol/l	149.3 ± 41.2	15.3 ± 4.9	9.0 ± 2.7	20.1 ± 11.1	40.8 ± 15.5
Normal MoDC in CM+urea 40 mmol/l*	99.0 ± 15.6	7.3 ± 3.3	3.9 ± 2.1	11.1 ± 4.4	56.6 ± 20.2

CM, complete medium; MFI, mean fluorescent intensity; MoDC, monocyte-derived dendritic cells.
*$P < 0.05$ Mann–Whitney *U*-test (except CD83).

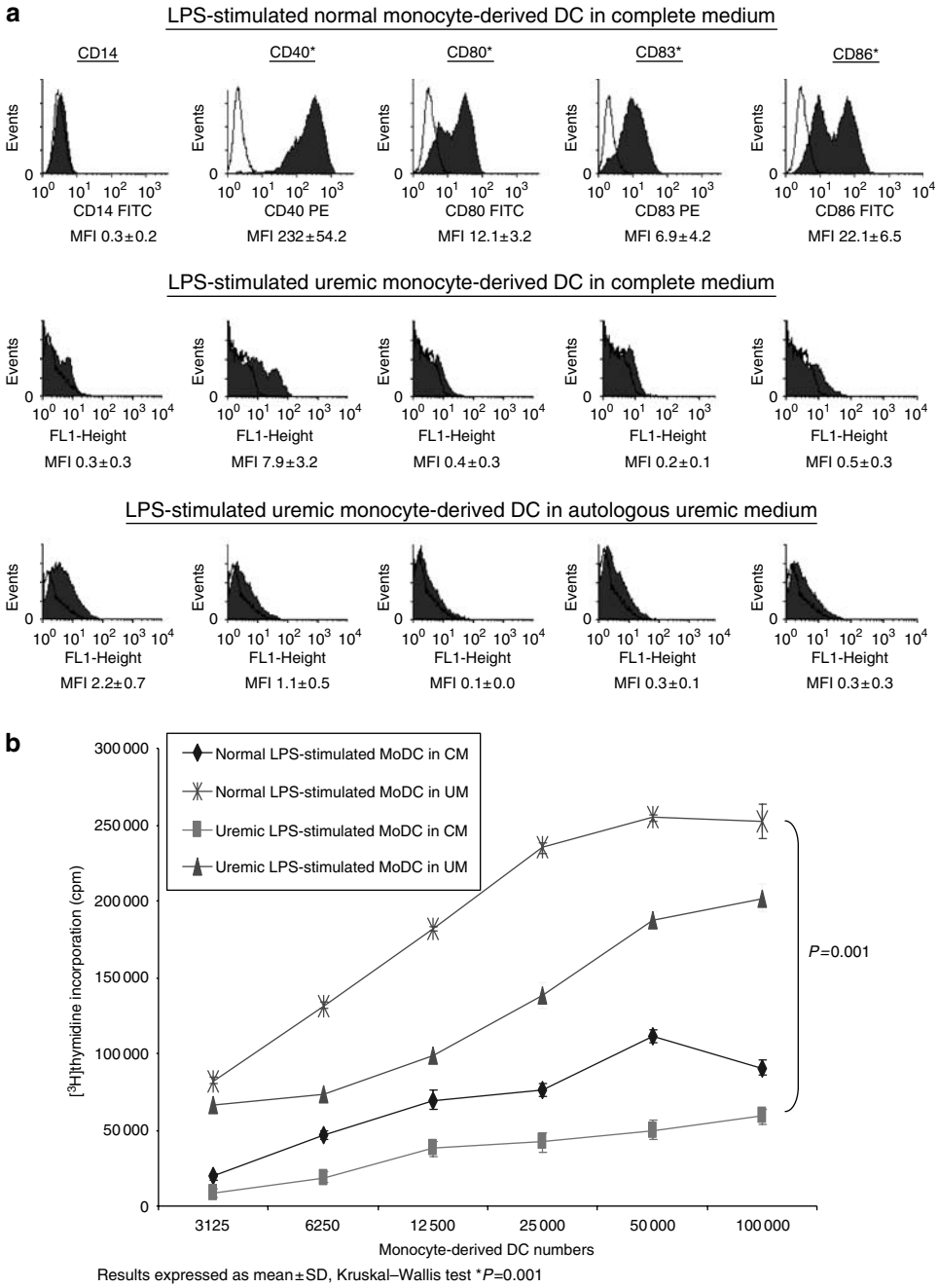

Figure 4 | The effect of uremic serum on costimulatory molecule expression and allo-stimulatory capacity of uremic monocyte-derived DC. (**a**) Cell-surface expression of CD14, costimulatory molecules, and CD83 on LPS-stimulated (10 ng/ml) uremic MoDC of HD patients cultured in CM or autologous UM for 48 h. Flow cytometric analysis revealed absent or low cell-surface expression of costimulatory molecules (CD40, CD80, and CD86) and CD83 (maturation marker) on MoDC cultured in either CM or UM. There was slight increase in CD14 expression demonstrated on uremic MoDC cultured in either CM or UM suggesting that not all monocytes in culture had differentiated into MoDC (closed profiles). Cell-surface expression of CD14, costimulatory molecules, and CD83 on normal LPS-stimulated MoDC cultured in CM are shown for comparison. Open black profiles denote isotype controls. Representative histograms are shown. MFI (mean $\pm$ s.d.) below histograms representative of results of three HD patients (Kruskal–Wallis test, $^*P<0.05$). (**b**) Allogeneic T-cell proliferation in an MoDC/T cells two-way MLR. Uremic LPS-stimulated (10 ng/ml) MoDC of HD patients cultured in UM for 48 h induced a greater proliferation of allogeneic T cells compared to uremic MoDC cultured in CM for 48 h (Kruskal–Wallis test, $P=0.001$). The ability of normal LPS-stimulated MoDC derived from healthy blood donors cultured in CM and UM to stimulate allogeneic T-cell proliferation is shown for comparison. Result representative of three separate HD patients.

LPS-stimulated MoDC cultured in UM, their allo-stimulatory capacity and ability to secrete IL-12p70 were paradoxically enhanced. A likely explanation for this unexpected finding is the presence of IL-12p70 in the uremic serum of HD patients. IL-12 is a proinflammatory cytokine that is an important regulator of Th1 response and is produced

predominantly by monocytes and DC.[23] IL-12 is particularly efficient in the induction of interferon-γ by T cells, and both IL-12 and interferon-γ may provide a positive auto-regulatory feedback to T cells and DC further promoting interferon-γ and IL-12 production, respectively.[24] Higher serum levels of IL-12 have been shown by others in the sera of chronic HD patients, and the overproduction of IL-12 has been associated with polarization of T-cell phenotype toward Th1 responses in these patients.[25,26] IL-12p70 present in uremic serum may stimulate MoDC via IL-12 receptor to enhance production of IL-12p70, which in turn augments T-cell activation and proliferation manifesting as enhanced by allo-stimulatory capacity of MoDC in the MoDC/T cell-mixed lymphocyte reaction (MLR) in our study. In addition, monocytes[27] and T cells[28] from uremic HD patients have also been demonstrated to undergo accelerated apoptosis. Consistent with these observations, our study established that uremic serum induces greater MoDC apoptosis and necrosis (and hence increased release of inflammatory mediators) that may directly result in enhanced T-cell proliferation induced by UM-cultured MoDC, further contributing to the immune dysfunction of HD patients. Unlike MoDC, LPS-stimulated monocytes cultured in either CM or UM did not produce IL-12 and may explain why MoDC but not monocytes cultured in UM exhibited enhanced allo-stimulatory capacity. The latter may perhaps reflect the lack of IL-12 receptor on monocytes[29] essential for positive auto-regulatory feedback and subsequent experiments are ongoing in our laboratory to determine the extent of IL-12 receptor expression on monocytes and MoDC (including in response to uremic serum) and whether the inhibition of IL-12 receptor on UM-cultured MoDC could inhibit their enhanced allo-stimulatory capacity and the ability to produce IL-12. Furthermore, neutralizing IL-12 activity in culture medium using a blocking monoclonal antibody may also help to distinguish which of the two factors (i.e. IL-12 or cell apoptosis) is predominantly responsible for the amplified allo-stimulatory capacity of MoDC cultured in UM.

Both low and high MW uremic toxins contained within the uremic serum or plasma of dialysis-dependent patients have been implicated in the observed dysfunction of the innate immune system including polymorphonuclear leukocytes[30,31] and mononuclear phagocytes resulting in reduced chemotaxis and oxidative activity in response to phagocytosis.[32,33] Polymorphonuclear leukocyte function has been shown to improve in CRF patients following commencement of peritoneal dialysis suggesting that these soluble uremic toxins may be removed by dialysis.[34] This study identified that high concentrations of urea (40 mmol/l), a recognized small MW uremic toxin, inhibits normal MoDC functions including reduced endocytosis and expression of costimulatory molecules following LPS stimulation comparable to the effects of uremic serum on these cells *in vitro*. Unlike UM-cultured MoDC, the presence of high concentrations of urea inhibited the ability of LPS-stimulated MoDC to produce IL-12p70 and allogeneic T-cell proliferation consistent with their reduced capacity for endocytosis or undergo maturation. However, it is likely that larger MW uremic toxins are also present within uremic serum contributing to the negative effects of uremic serum on monocytes/MoDC functions as the inhibitory effects exerted by urea on MoDC occurred only at the highest concentration of urea (40 mmol/l) in CM. Furthermore, the presence of vitamin D analogs (e.g. calcitriol) and aspirin in uremic serum is likely to contribute to the inhibitory effects of uremic serum on DC functions.

To address whether uremia or the process of HD was predominantly responsible for the inhibitory effect of uremic sera of HD patients on MoDC function, the effects of uremic sera of CRF and CAPD patients on MoDC function were analyzed *in vitro*. MoDC generated in the presence of UM containing either the sera of CAPD or CRF patients inhibited the upregulation of certain costimulatory molecules (CD80 and CD86) and exhibited enhanced apoptosis/necrosis, but not to the extent of cells cultured in UM containing sera of HD patients. These findings suggest that both uremia and the process of HD may impart a greater inhibitory effect on MoDC function and survival *in vitro* compared to uremia alone. Similar to HD patients, the sera of CAPD and CRF patients have detectable levels of IL-12p70, which may account for the enhanced production of IL-12p70 by MoDC cultured in the presence of UM but not when cultured in CM. However, the recruitment of more CAPD and CRF patients may help to confirm these findings.

A previous study has shown that monocytes isolated from HD patients exhibited low level expression of CD86 (B7.2) following LPS stimulation, leading to impaired effector activation of T cells in these patients.[35] This study confirms these conclusions and demonstrates that uremic monocytes isolated from HD patients cultured in autologous UM, and to a lesser extent when cultured in CM, have reduced ability for endocytosis, reduced expression of costimulatory molecules, and allo-stimulatory capacity following LPS stimulation. However, uremic monocytes were capable of differentiating into MoDC with appropriate cytokines but this was impaired in the presence of autologous uremic serum. Similarly, uremic MoDC generated in the presence of autologous UM or CM displayed reduced capacity for endocytosis and maturation following LPS stimulation. However, uremic MoDC cultured in autologous UM, but not when cultured in CM, exhibited enhanced production of IL-12p70 and allogeneic T-cell proliferation (similar to UM-cultured normal MoDC). This finding is likely to be dependent on the presence of IL-12 in uremic serum that may enhance the capacity of uremic MoDC to produce IL-12p70 resulting in superior T-cell activation and proliferation. The absence of any significant improvement in uremic monocytes and MoDC functions when cultured in CM compared to autologous UM suggests the likelihood of an underlying intrinsic defect of uremic monocytes and hence MoDC functions *ex vivo*, which was amplified in the presence of UM.

In this study, the observed functional impairments in monocytes and MoDC in the presence of uremic serum *in vitro* may contribute in part to the widespread immune dysfunction reported in HD patients, including a higher incidence of infections and malignancies, and displayed a suboptimal response to vaccinations. A logical next step is to determine whether improving uremic toxin clearance by utilizing a more efficient dialyzer or using a different dialysis modality (e.g. peritoneal dialysis, conventional thrice-weekly HD, and daily or nocturnal dialysis) in HD-dependent CRF patients improves DC functions. Prospective studies in selected HD patients are currently ongoing in our laboratory to address this issue. This may then enable nephrologists to determine whether specific dialysis regimen could be considered in selected HD patients with demonstrated severe immune impairment (i.e. poor or non-responders to hepatitis B vaccination) to improve their generalized immunodeficiency.

MATERIALS AND METHODS

Thirteen HD-dependent CRF patients, 13 healthy controls, 3 CAPD-dependent CRF patients and 3 CRF not maintained on dialysis were enrolled in this study. This study was approved by the Local Institutional Ethics Committee at The Queen Elizabeth Hospital and informed consent was obtained before inclusion.

All patients were prospectively recruited from outpatient clinics, in-center, and satellite dialysis units and had no clinically relevant infections in the preceding 3 months. HD patients were maintained on intermittent thrice-weekly HD using polysulfone membrane (Fresenius Medical Care AG, Bad Homburg, Germany) achieving $Kt/v > 1.5$. All patients had native or graft arterio-venous fistulas. All CAPD patients had $Kt/v > 1.6$. The mean $\pm$ s.d. calculated glomerular filtration rate (using Cockcroft Gault formula)[36] of the CRF patients not on dialysis was 24.3 ± 8.1 ml/min. Most patients were taking vitamin D supplements and/or aspirin (drugs known to suppress normal DC functions[37]).

All patients were older than healthy controls (65.8 ± 11.2 and 43.5 ± 2.4 years old; Mann–Whitney *U*-test, $P < 0.05$). Peripheral blood monocyte count of HD, CAPD, and CRF patients in this study was $0.2 \pm 0.1 \times 10^9$/l, $0.23 \pm 0.1 \times 10^9$/l, and $0.3 \pm 0.2 \times 10^9$/l (normal laboratory range $0.20–0.80 \times 10^9$/l) and was comparable to healthy control ($0.3 \pm 0.1 \times 10^9$/l; $P =$ NS). Urea concentrations in all patients were consistently less than 40 mmol/l (range 18.7–28.5 mmol/l).

Ten milliliters of peripheral venous blood were obtained from HD (pre-dialysis), CAPD, CRF patients, and healthy controls. Blood was collected in heparinised tubes (BD Biosciences, San Diego, CA, USA) and immediately centrifuged at 100 *g* for 10 min to separate serum from cells. IL-12p70 levels were determined in the sera of HD patients and healthy controls using an ELISA kit. Remaining sera collected were heat-inactivated for 30 min in a 56°C water bath to deactivate endogenous complements and were stored frozen at −70°C until use.

Reagents

FITC-, phycoerythrin-, and cychrome-conjugated mouse antihuman monoclonal antibodies used to detect cell-surface markers human leukocyte antigen-DR (clone G46-6), CD14 (clone MY4-FITC), CD40 (clone mAb89), CD80 (clone L307.4), CD83 (clone HB15A), CD86 (clone FUN1), CD95 (clone DX2), and CD206 (clone 19.2) were used. The isotype-negative controls include IgG2b isotype control (clone 27–35) and IgG2a isotype control (clone G155-178). All antibodies were obtained from BD Biosciences, except for CD14 (Beckman Coulter, Hialeah, FL, USA), and CD40 and CD83 from Immunotech (Marseille, Cedex, France). FITC-dextran, trypan blue, and bacterial LPS were purchased from Sigma-Aldrich (St Louis, MO, USA). Urea was purchased from Bio-Rad Laboratories (Hercules, CA, USA) and urea solution was prepared from the dilution of urea in distilled water. Monocytes and T cells were isolated using CD14 and CD3 microbeads, respectively (Miltenyi Biotec, Bergisch Gladbach, Germany).[38] Cell-surface expression of annexin V and propidium iodide was detected by using a commercial kit (Bender MedSystems, Vienna, Austria). Levels of IL-12p70 (BD Biosciences) were determined by an ELISA kit. Tritiated thymidine was purchased from Amersham Biosciences (Brown Deer, WI, USA). Solutions used include Ficoll-Hypaque purchased from Amersham Biosciences, phosphate-buffered saline from Sigma-Aldrich, and fluorescence-activated cell sorting lysing solution from BD Biosciences. Fetal calf serum was obtained from JRH Biosciences (Lenexa, Kansas, USA), rabbit serum from ICN Pharmaceuticals (Costa Mesa, CA, USA), and human AB serum from Australia Red Cross Service (South Australia, Australia). Staining buffer contained phosphate-buffered saline with 1% v/v fetal calf serum and 0.1% w/v sodium azide (Sigma-Aldrich). RPMI-1640 (Sigma-Aldrich) was supplemented with 10% v/v human AB or uremic serum (ABO-compatible with cultured cells), 2 mM L-glutamine (MultiCel Trace Scientific, Victoria, Australia), sodium pyruvate (ICN Pharmaceuticals), penicillin streptomycin (MultiCel Trace Scientific), and sodium bicarbonate (Amresco), and referred to as complete medium (CM) or uremic medium (UM), respectively. IL-4 and granulocyte macrophage colony-stimulating factor were purchased from Pepro Tech (Rocky Hill, NJ, USA) and Sandoz Australia (North Ryde, NSW, Australia), respectively.

Isolation of normal monocytes and MoDC from healthy blood donors

Peripheral blood mononuclear cells were isolated from buffy coat of healthy blood donors (Australian Red Cross Blood Service) by Ficoll-Hypaque density gradient separation and are referred to as normal cells henceforth. Monocytes were isolated from peripheral blood mononuclear cells using anti-CD14 microbeads using an immunomagnetic bead separation technique with purity of >98%. In brief, peripheral blood mononuclear cells were labeled with an appropriate volume of anti-CD14 microbeads and were positively selected using an automated magnetic cell separator (Automacs®; Miltenyi Biotec). Monocytes (1×10^6 cells/ml) were cultured for 24 h in medium, with LPS (10 ng/ml) added to induce maturation. To obtain MoDC, monocytes (1×10^6 cells/ml) were cultured in medium supplemented with 400 units/ml IL-4 and 800 units/ml granulocyte macrophage colony-stimulating factor and were then incubated in a humidified 37°C CO_2 incubator for 7 days to allow differentiation of monocytes into MoDC.[39] LPS (10 ng/ml) was added in the final 48 h of incubation to induce MoDC maturation. Monocytes were cultured in either CM or UM in parallel under identical conditions to allow for direct comparisons of results.

Functional analysis of normal monocytes and MoDC cultured in CM and UM

Detection of cell-surface molecules by flow cytometric analysis. LPS-stimulated normal monocytes and MoDC were resuspended in

staining buffer adjusted to a cell density of 1×10^6 cells/ml of buffer. Cells were incubated for 20 min with 10% v/v rabbit serum and were then aliquoted (1×10^5 cells) into polystyrene tubes (BD Biosciences) and stained with human leukocyte antigen-DR, CD40, CD80, CD83 (only for MoDC), CD86, CD95 (stains for Fas ligand), and CD206 (macrophage mannose receptor) monoclonal antibodies. Red cells were lysed with fluorescence-activated cell sorting lysing solution, and washed twice in staining buffer. Two-color flow cytometric analysis of 1×10^4 cells was performed by FACScan® flow cytometer with CellQuest software (BD Immunocytometry Systems, San Jose, CA, USA).

Macrophage mannose receptor-dependent endocytosis determined by FITC-dextran uptake. In brief, 1×10^6 immature MoDC were incubated in CM supplemented with 1 mg/ml FITC-dextran for 60 min in a 37°C incubator or on ice (negative control). Cells were then washed and the uptake of FITC-dextran determined by flow cytometric analysis.

Quantification of IL-12p70 by ELISA. IL-12p70 were determined in the sera (of HD patients and healthy controls), mediums, and culture supernatants of LPS-stimulated monocytes and MoDC by ELISA. In brief, 100 μl of undiluted serum and standards were aliquoted into microplates precoated with anti-human IL-12p70 monoclonal antibody. Following incubation, the wells were washed and 100 μl of diluted biotinylated anti-human IL-12p70 monoclonal antibody were added. Following incubation and washes, 200 μl of substrate solution were added. Reactions were stopped by adding stop solution (1 M phosphoric acid). Optical densities of the wells were determined using a microplate reader set to a wavelength of 450 nm. The level of detection was 7.8 pg/ml. IL-12p70 production by monocytes and MoDC in the culture supernatants is the amount of IL-12p70 detected by ELISA of culture supernatant less the background level in culture mediums (i.e. amount present in CM and UM).

Detection of apoptotic and necrotic cells. Apoptotic and necrotic MoDC were identified by surface expression of annexin V and propidium iodide, respectively, using a commercial kit. In brief, cells were washed with phosphate-buffered saline and resuspended in binding buffer (containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$) adjusted to a cell density of 2–5 $\times 10^6$ cells/ml of buffer. Positive control cells were treated 30 cm under ultraviolet light for 2 min (i.e. to render cells apoptotic) and other cells aliquoted into polystyrene tubes and stained with appropriate volumes of annexin V and/or propidium iodide. Two-color flow cytometric analysis of 1×10^4 cells was performed within 1 h.

Allogeneic T-cell stimulatory capacity determined by MLR. To investigate the allo-stimulatory capacity of monocytes and MoDC, LPS-stimulated monocytes and MoDC were co-cultured with allogeneic T cells for 5 days in a MLR. Allogeneic T cells were isolated by CD3 isolation kit by Automacs® achieving a purity of >95%. T cells were then added to reducing concentrations of monocytes/MoDC in a 96-well round bottom plates (Techno Plastic Products, Trasadingen, Switzerland) in triplicates starting at a 1:1 monocytes/MoDC:T-cells ratio. Cells were cultured in CM and incubated in a humidified 37°C incubator containing 5% CO_2 for 5 days, with 1 μCi/well of tritiated thymidine added during the last 18 h of culture. The cells were harvested on a 96-well Tomtec harvester and tritiated thymidine incorporation measured in a Microbeta Trilux liquid scintillation counter (EG&G Wallac, Turku, Finland). Results were expressed as number of tritiated thymidine incorporated cells.

Effects of urea on normal MoDC functions

To determine the effect of small MW uremic toxin on DC functions, graded concentrations of urea solution (5, 20, and 40 mmol/l) was added to CM containing normal monocytes. The functions of normal monocytes and MoDC cultured in the presence of urea were examined and compared with cells (from the same blood donor) cultured in CM in parallel.

Effects of uremic sera of CAPD and CRF patients on MoDC

To determine whether the effect of uremic sera of HD patients on MoDC function was a manifestation of the uremic state or the process of HD, normal MoDC cultured in UM containing sera of CRF not on dialysis and those maintained on CAPD were examined.

Isolation and functional analysis of uremic monocytes and MoDC from HD patients

Six of the thirteen HD patients with hemoglobin of >120 g/l consented to having an additional 20 ml of peripheral blood venesected. Blood was obtained from these patients immediately pre-HD, thus avoiding any potential effects of HD on DC. The functional characteristics of uremic monocytes ($n = 3$) and MoDC ($n = 3$) derived from HD patients were determined following their culture in either CM or autologous UM as described.

Statistical analysis

All experiments were performed at least in triplicate to ensure reproducibility. Results were expressed as mean ± s.d. Where appropriate, Mann–Whitney *U*-test and Welch-corrected Student's *t*-test (for 2 groups of data) or non-parametric Kruskal–Wallis analysis of variance test (for three or more groups of data) were used to compare results, with $P < 0.05$ deemed statistically significant and $P \geqslant 0.05$ as not statistically significant (NS). Representative histograms were shown for experiments involving flow cytometric analysis and mean ± s.d. of MFI (difference between cell-surface expression and isotype/negative control) shown. In addition, numerical tables comparing MFI of costimulatory molecules and percentage of apoptotic/necrotic cells of relevant experiments have been added for clarity (Tables 1–4). Statistics were performed using GraphPad Instat version 3.00 (GraphPad Software, San Diego, CA, USA).

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SUPPLEMENTARY MATERIAL

Figure S1. Uremic medium (sera of HD patients) inhibits the expression of costimulatory molecule on uremic monocytes.
Figure S2. Uremic medium inhibits the allo-stimulatory capacity of uremic monocytes.
Figure S3. Uremic medium (sera of CAPD and CRF patients) impairs costimulatory molecule expression on MoDC.

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