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ORIGINAL ARTICLE

Renal epithelial cell-derived monocyte colony stimulating factor as a local informant of renal injury and means of monocyte activation

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Summary

Monocyte accumulation in renal allografts is associated with allograft dysfunction. As monocyte influx occurs acutely following reperfusion, we investigated the effect of ischemia-reperfusion injury (IRI) on monocyte colony stimulating factor (m-CSF), a key cytokine in monocyte recruitment. We hypothesized that renal tubule epithelial cells (RTECs) could produce m-CSF in response to IRI, which could in turn promote monocyte activation. Real time PCR was used to measure levels of intragraft m-CSF transcripts in patients during IRI and clinical rejection. Also, m-CSF production by RTECs following IRI simulation *in vitro* was measured using ELISA. Monocyte expression of CD40 and CD80 was then analyzed using flow cytometry following co-culture with supernatants of RTECs after IRI. Monocyte expression of CD40, CD80 and HLA-DR was then examined following treatment with rh-m-CSF (10, 36, and 100 ng/ml), as was monocyte size and granularity. We found that intragraft m-CSF transcription was significantly increased postreperfusion ($P = 0.002$) and during clinical rejection ($P = 0.002$). We also found that RTECs produced m-CSF in response to IRI *in vitro* ($P = 0.036$). Monocytes co-cultured with the supernatants of postischemic RTECs became activated as evidenced by increased expression of CD40 and CD80. Also, monocytes treated with recombinant m-CSF assumed an activated phenotype exhibiting increased size, granularity and expression of CD40, CD80, CD86, and HLA-DR, and demonstrating enhanced phagocytic activity. Taken together, we suggest that renal tubular cell derived m-CSF is a stimulus for monocyte activation and may be an important target for control of IRI-associated immune activation.

Introduction

Monocytes are among the first responders to arrive in the renal allograft following graft reperfusion, constituting up to 60% of infiltrating cells [1–3]. They are known to assume an activated phenotype *in vivo* and stimulate adaptive immune responses through their production of degradative products and phagocytic activity, increasing the likelihood of antigen capture for presentation, and expression of co-stimulatory molecules, fostering a pro-immune result of antigen presentation [4,5]. Monocyte infiltration during acute rejection has been shown to have a negative correlation with allograft survival [6–8], and more recently shown to relate closely to the degree of dysfunction associated with an acute rejection event [9]. Moreover, in the setting of T-cell depletion, monocytes have been shown to actively infiltrate renal tubules, producing monocytic tubulitis and facilitating substantial

renal dysfunction despite remarkably sparse lymphocytic infiltration [10]. This later observation has suggested that renal tubules elicit a chemo-attractant-fostering monocyte diapedesis across the tubular basement membrane.

One cytokine that has gained attention for its role in monocyte recruitment and activation is monocyte colony stimulating factor (m-CSF) [11]. Renal tubule cells have recently been described as a source of m-CSF during immune-mediated native kidney disease [12]. Intrarenal m-CSF expression has been shown to be associated with local macrophage proliferation in animal models of kidney inflammation and in human glomerulonephritis [12,13]. Importantly, we have shown that m-CSF is a prominent part of the postreperfusion transcriptome in human renal transplantation under nondepletional induction conditions [14]. We have therefore sought to determine the role of m-CSF in promoting human allograft rejection with a focus on its role in monocyte activation. Herein, we show that renal tubule-derived m-CSF is produced as a result of ischemia-reperfusion injury (IRI) and directly fosters monocyte differentiation and activation.

Methods

Transcriptional analysis for m-CSF in human renal allograft biopsies

All subjects involved in this study were, after receipt of informed consent, enrolled in a National Institutes of Health Institutional Review Board-approved clinical protocol authorizing tissue procurement for research. Consecutive human renal allograft biopsies were procured intraoperatively using an 18-gauge needle core device within 30 and 60 min of allograft reperfusion, or at the time of percutaneous diagnostic biopsy, and snap-frozen in liquid nitrogen within 1 min of procurement. Biopsies were then homogenized in Trizol reagent (Life Technologies, Grand Island, NY, USA) and converted to cDNA. Transcriptional analysis was performed using real time polymerase chain reaction (PCR) as previously described [15]. Briefly, each cDNA sample from individual samples (100 ng) was used as a template for RT-PCR assays containing forward and reverse primers (900 nM each) and 6-carboxyfluorescein (6-FAM)-labeled probes (250 nM) for four replicates of m-CSF (Applied Biosystems, Foster City, CA, USA). In addition, forward and reverse primers for 18s ribosomal RNA (100nM each), VIC^{TM} dye (a fluorescein derivative)-labeled (200 nM), were used as an internal control. Reaction mixtures were subjected to the following amplification scheme: one cycle at 50 °C for 2 min and one cycle at 99 °C for 10 min, followed by 35 cycles at 99 °C for 15 s and 60 °C for 1 min. RT-PCR data were analyzed using Sequence Detection System version 1.7.1 software included with the ABI PRISM 7700 Sequence Detector (Applied Biosystems). Final quantification was derived using the comparative threshold cycle (C_t) method as previously described [15]. All experimental samples are expressed as *n*-fold difference relative to the calibrator (normal kidney obtained from normal living donors prior to donor nephrectomy).

Renal tubule cells, monocytes and ischemic culture conditions

Cryopreserved primary human renal proximal tubule epithelial cells (RTECs) were obtained from Clonetics, Inc (East Rutherford, NJ, USA). Cells were seeded at 20 000 cells/ml, sub-cultured, and maintained in T75-cm^2 flasks in 15 ml of growth medium (Clonetics Inc.), and incubated at 37 °C, 5% CO_2. Growth media was changed every 1–2 days and cells were split when confluence reached 80–90%, which occurred at approximately days 5–7 of culture. Cells were then transferred to T25-cm^2 flasks until confluence for experimental studies. All experiments were performed during the fourth to sixth passage.

Renal tubule epithelial cells were made ischemic using a hypoxia chamber. The chamber facilitated cell flasks to be placed in a vacuum-sealed disk in which nitrogen gas was used to replace room air in the chamber via an inflow tract. Oxygen was thus displaced with nitrogen within the flask and room air was inhibited from entering the chamber by the outflow of nitrogen gas. After approximately 10 min of nitrogen treatment, both the inflow and outflow tracts were closed and the entire hypoxia chamber was transferred to 4 °C for 24 h. Cold ischemia was induced by placing the culture flasks on ice. Following ischemia and/or cold treatment, RTECs were either analyzed immediately or rewarmed at 37 °C and 5% CO_2 for 24 h. Changes in cell morphology following cold ischemia and/or rewarming were noted. Renal tubule cell supernatants were collected after each test period. These supernatants were then analyzed for m-CSF production using an m-CSF ELISA (Quantikine, Inc, Minneapolis, MN, USA). Aliquots of the same supernatants were also incubated with elutriated monocytes.

Human elutriated monocytes from healthy volunteer donors were obtained from the National Institutes of Health Blood Bank following informed consent. Within each experimental condition, monocytes were from a single donor. Prior to all experiments, monocytes were rested overnight at 4 °C in RPMI 1640 supplemented with 5% fetal calf serum.

Cell co-cultures and flow cytometry

Renal proximal tubule cells were subjected to cold ischemia and rewarming as described above. Rested elutriated

monocytes (5×10^6/ml) were co-cultured with either RTECs recently fed with fresh media or with the supernatants of RTECs after cold ischemia and rewarming for 24 h.

The monoclonal antibodies and their fluorescein isothiocyanate (FITC) or phycoerythrin conjugates used for flow cytometry included mouse isotype control antibodies IgG1, IgG2a, and IgG1 (Becton Dickinson, San Jose, CA, USA); and human-specific mouse antibodies directed against CD14, CD16, CD40, CD80, and HLA-DR (Becton Dickinson). Monocytes were collected from the supernatants of co-cultures, washed once with 4 °C FACS buffer (PBS supplemented with 5% FBS and 0.05% sodium azide), and stained with labeled monoclonal antibodies at 4 °C for 30 min. After a final wash with cold FACS buffer, cells were analyzed by FACScan (Becton Dickinson) gated on $CD14^+$ cells. Forward and side scatter gates were used to evaluate both large and small populations; in addition, the original flow gates were established to eliminate artificial doublets.

Monocytes ($5–10 \times 10^6$ cells/ml) were incubated with 0, 10, 36, or 100 ng/ml of recombinant human m-CSF (R&D Systems, Minneapolis, MN, USA) for a period of 24, 48, or 96 h. Following incubation with m-CSF, cells were washed and stained for surface markers and analyzed as described above. To test for changes in phagocytic ability following treatment with m-CSF, monocytes were incubated with 1 mg/ml FITC-dextran for 30 min following 24, 48, and 96 h of treatment with recombinant m-CSF and analyzed by flow cytometry for an increase in FITC-dextran incorporation. Based on our flow gates (data not shown), our forward and side-scatters are gated in large and small populations and there was no change between them. Our original flow gates are established to eliminate artificial doublets.

Statistics

Significance was determined using a Mann–Whitney Rank Summary test and a Student's *t*-test. Significance was determined as $P < 0.05$, with a two-tailed analysis of data.

Results

m-CSF transcript levels are elevated *in vivo* upon human renal allograft reperfusion and during human allograft rejection

To assess the potential role of m-CSF in clinical renal transplantation, we examined intragraft levels of m-CSF transcripts by RT-PCR in biopsies acquired within 30 min to 1 h of allograft reperfusion in 29 patients. The study population included patients treated with a depletional induction regimen (alemtuzumab; $n = 13$) or a nondepletional induction regimen (daclizumab, $n = 16$). All patients received methylprednisolone, 500 mg, intraoperatively, prior to reperfusion. Results were compared with preprocurement donor kidney biopsies. The levels of m-CSF transcripts were significantly elevated in postreperfusion samples ($P = 0.002$) indicating that m-CSF transcription is part of the human response to renal allograft reperfusion (Fig. 1). The increase in transcript levels was observed in both alemtuzumab-treated patients ($P = 0.004$) and nondepleted individuals ($P = 0.039$).

To determine whether m-CSF transcripts were also similarly elevated during allograft rejection, we studied renal allograft biopsies taken from patients ($n = 43$) undergoing acute cellular rejection. m-CSF was significantly increased during allograft rejection ($P = 0.002$) (Fig. 1). This was evident both in patients treated with nondepletional induction therapy ($n = 24$, $P = 0.017$), and patients experiencing rejection following alemtuzumab induction ($n = 19$, $P = 0.077$). The latter group failed to reach statistical significance because of a single outlier with markedly elevated m-CSF nearly 20-fold higher than that of the rest of this group, disrupting the variance of the sample set. Notably m-CSF transcripts in both nondepletional and depletional biopsies in patients with stable function were not significantly different from native donor biopsies ($P = 0.554$). Living donor kidney biopsies prior to transplantation are the most optimal representation of normal kidneys. Samples from these donors were pooled to avoid artifact related to individual conditions. This methodology has been validated by numerous other studies as an appropriate calibrator [8]. Evaluation in this way allows for the investigation with regard to how transplanted kidneys differ from normal kidneys; and, by pooling donors, demographic variability is minimized.

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Renal tubule epithelial cells produce m-CSF upon ischemic stress and deliver activating signals to monocytes

Having established that m-CSF transcripts are induced during clinical allograft reperfusion and rejection, we sought to determine whether RTECs might be a relevant

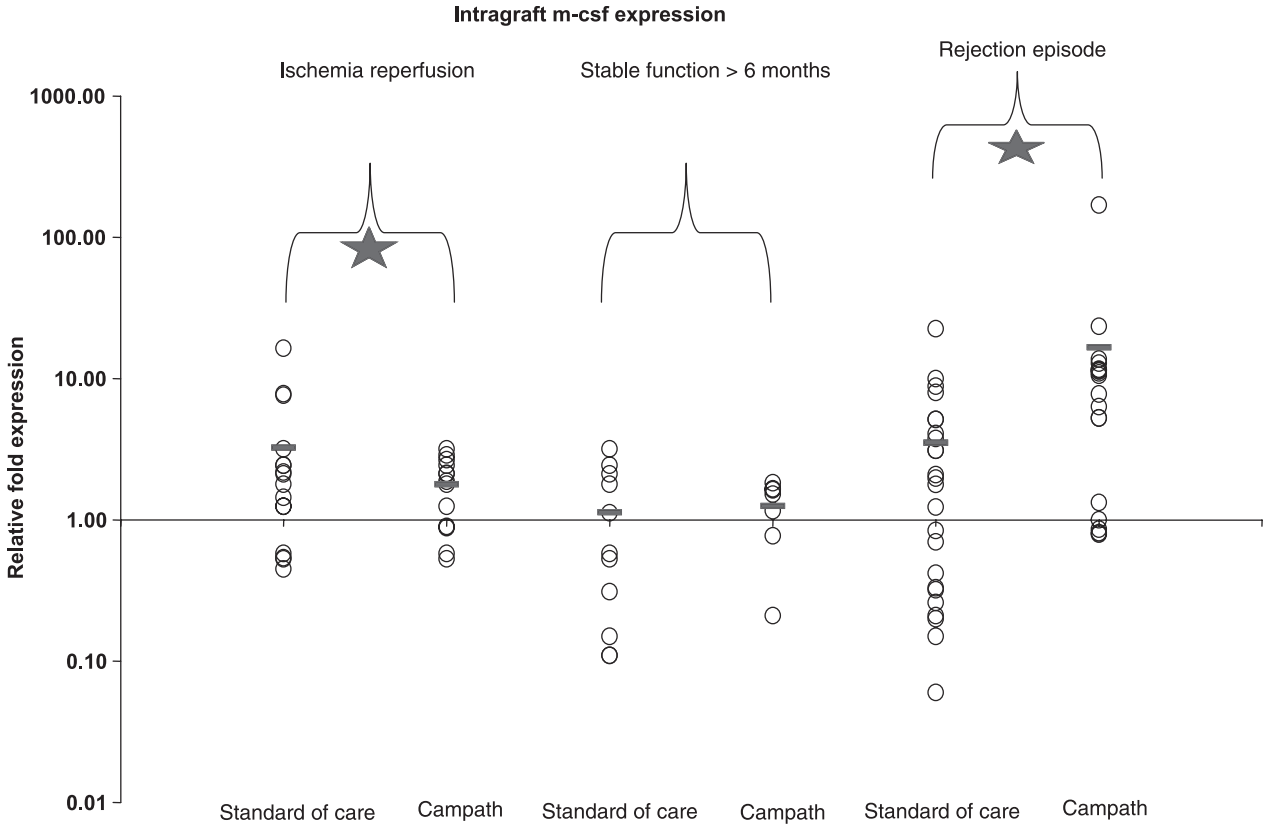

Figure 1 Measurement of intragraft m-CSF mRNA transcripts during ischemia reperfusion, stable function, and during clinical rejection in both nondepletional and depletional (Campath-1h) human protocols. Following ischemia reperfusion, m-CSF mRNA was significantly elevated ($P = 0.002$) in both treatment groups. Elevated m-CSF levels were also observed during periods of rejection ($P = 0.002$). Notably, there was no difference observed in the stable function group ($P = 0.554$).

source of this cytokine in the transplant setting. We therefore studied primary RTECs under various conditions *in vitro* to evaluate their response to cold ischemia and rewarming to mimic the stress commonly associated with organ transplantation. ELISA analysis of RTEC supernatants demonstrated that m-CSF protein was produced by these cells and that their production was significantly increased following cold ischemia and rewarming compared with unmanipulated cell cultures or cultures subjected to cold ischemia alone ($P = 0.036$) (Fig. 2). Our data suggest that there is a small basal level of m-CSF produced by unstimulated RTECs.

To determine whether human RTEC-derived proteins had direct stimulatory effects on monocytes, purified monocytes were treated with RTEC supernatants from unmanipulated cultures, and cultures subjected to cold ischemia and rewarming. Interestingly, monocytes treated with postischemic supernatants showed a significant increase in surface expression of the critical co-stimulatory molecules CD40 and CD80 (Fig. 3) indicating that RTECs can generate a direct activating signal to infiltrating monocytes independent of other cell sources. Supernatants from each experimental condition represent a total of 24 h in that test period. For example, super-

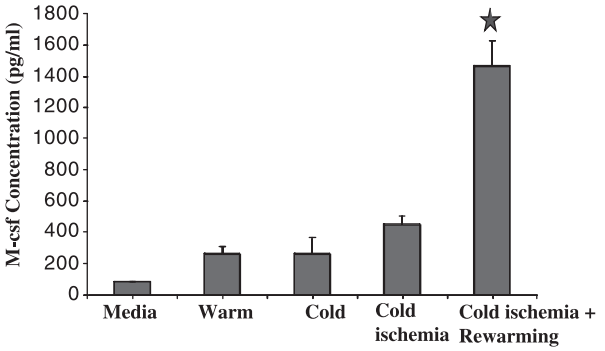

Figure 2 Renal tubule cells produce increase levels of m-CSF in response to ischemia and rewarming. Renal tubule cells were subjected to cold ischemia and/or warm and cold media. Subsequently, m-CSF was measured using ELISA. m-CSF was significantly elevated following cold ischemia and rewarming ($P = 0.036$).

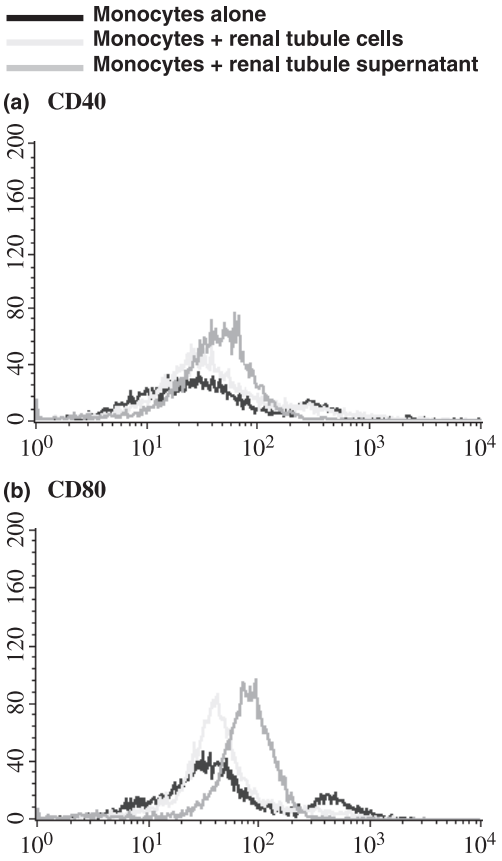

Figure 3 Human monocytes treated with the supernatants of renal tubule cells following ischemia-reperfusion injury express increased CD40 and CD80. Renal tubule cells were subjected to ischemia-rewarming injury. Monocytes were then co-cultured with either renal tubule cells + supernatant or the supernatant alone. Monocytes increased their surface expression of the co-stimulation markers (a) CD40 and (b) CD80 following treatment with renal tubule cell supernatant.

natants from both cold and cold ischemia samples were collected after 24 h. Both the warm and cold ischemia + rewarming represent a total treatment of 24 h as well. Based on our time course experiments, the difference could not be explained by time alone, but rather is unique to the experiment itself. It is further notable that RTECs were not killed or damaged; viability was not affected as assessed by trypan blue staining. Renal tubule cells became more granular in morphology and enlarged in response to ischemia and rewarming.

m-CSF induces monocyte activation *in vitro*

To determine whether m-CSF acting alone could provide sufficient stimulatory signals to foster monocyte activation, we investigated the effects of purified recombinant

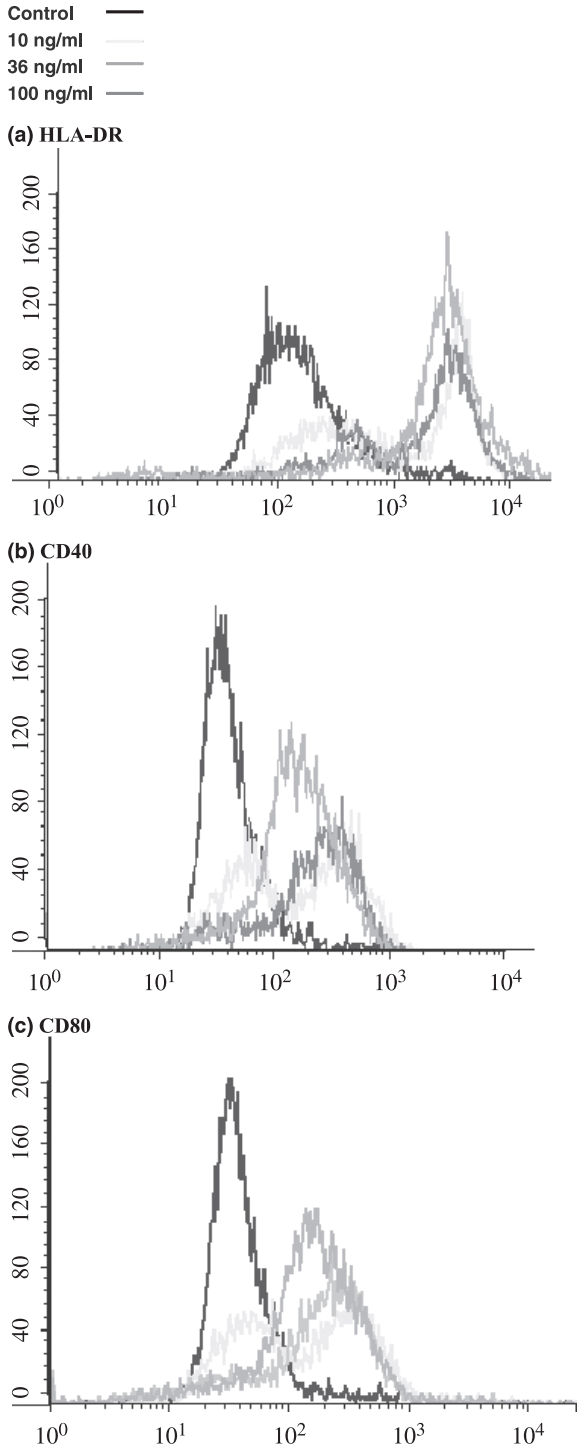

Figure 4 Human monocytes treated with recombinant human m-CSF increase surface marker expression of HLA-DR, CD40, and CD80. Human elutriated monocytes were treated with 10, 36, and 100 ng/ml of rh-m-CSF and then analyzed for surface markers HLA-DR (a), CD40 (b), and CD80 (c). Following treatment with both 36 and 100 ng/ml m-CSF, monocytes increased their surface marker expression of HLA-DR, CD40, and CD80.

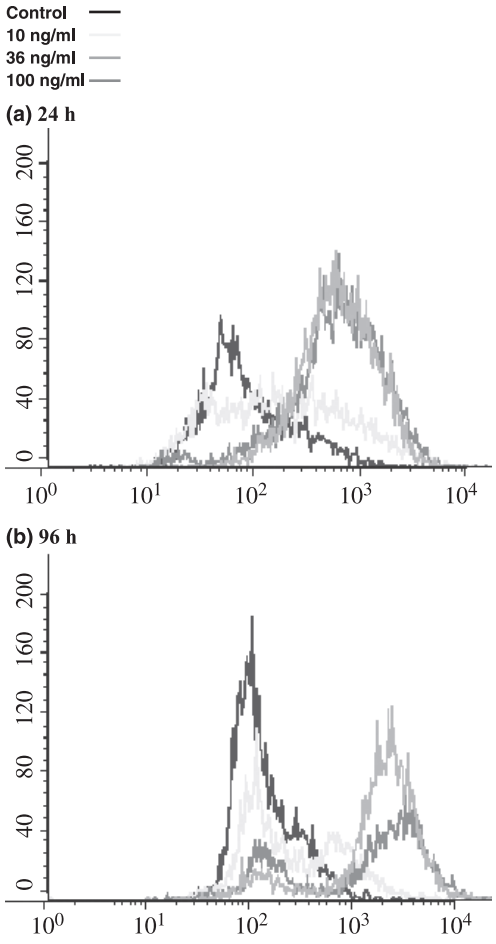

Figure 5 m-CSF increases monocyte surface marker expression of FITC-dextran. Monocytes were treated with 10, 36, and 100 ng/ml m-CSF for 24 h (a) or 96 h (b) and then analyzed for surface marker FITC-dextran to assess monocyte engulfment. After treatment with m-CSF at both 24 and 96 h, monocyte FITC-dextran surface marker expression was increased in a dose-dependent manner.

m-CSF on human monocytes *in vitro* looking both at induction of surface molecules relevant to antigen presentation, and the process of antigen uptake. When treated with recombinant human m-CSF, human monocytes demonstrated a dose-dependent increase in MHC class II molecule expression (HLA-DR) in concert with the critical co-stimulatory molecules CD40 and CD80 (Fig. 4). Expression increased for all markers through 36 ng/ml and plateaued thereafter.

Similarly, treatment with m-CSF led to a marked increase in monocyte phagocytic activity. Monocyte FITC-dextran uptake increased significantly following treatment with 36 and 100 ng/ml of recombinant m-CSF administration, with a significant effect within 24 h that persisted beyond 96 h of treatment (Fig. 5). In addition, m-CSF treatment led to general monocyte activation as indicated by dose- and time-dependent increases in monocyte size and granularity (Fig. 6). Forward- and side-scatter gates allowed for the evaluation of both large and small populations and there were no changes between them. In addition, it is notable that the original flow gates utilized in this study were established to eliminate artificial doublets.

Discussion

There is increasing awareness that monocytes are important participants in renal allograft rejection. They are among the earliest cells infiltrating a reperfused allograft; their presence increases during rejection and increases proportionally with advancing organ dysfunction [16–22]. These observations have become more apparent in the context of lymphocyte depletion, and several studies have demonstrated that monocytes have important roles in donor antigen capture and presentation [23,24]. In this study, we have investigated the role of m-CSF, a cytokine with known influence on monocyte function and increasingly recognized as a factor in native renal disease. The findings reported herein are particularly notable with regard to alloimmune response initiation.

As monocytes are almost immediately responsive to a reperfused allograft, the mechanisms linking IRI to monocyte recruitment require definition. In this study, we have shown that m-CSF is part of the immediate postreperfusion milieu in human renal allografts and that RTECs are a potential primary source of its production. That renal tubule cells are induced to produce m-CSF is in keeping with the development of monocyte tubulitis even in severely T-cell-depleted individuals, and suggests a mechanism by which monocytes are initially drawn across the endothelium into an allograft. More importantly, the direct activating effects of m-CSF of monocytes provide a mechanism for enhanced antigen uptake and presentation. In doing so, they suggest that m-CSF inhibition could be helpful in limiting alloimmune response initiation.

Our study indicates that m-CSF can activate monocyte antigen uptake and suggests that it can increase antigen presentation by enhancing co-stimulation. In this regard, m-CSF may be regarded as a primer of monocyte activation. Xu recently described monocytes as a necessary intermediary between allograft vascular endothelial cells and T cells for proper antigen presentation and subsequent co-stimulation [25]. Taken together, we have shown that ischemia reperfusion stimulates the production of renal tubule derived m-CSF, which can then activate monocyte phagocytosis and co-stimulation. These

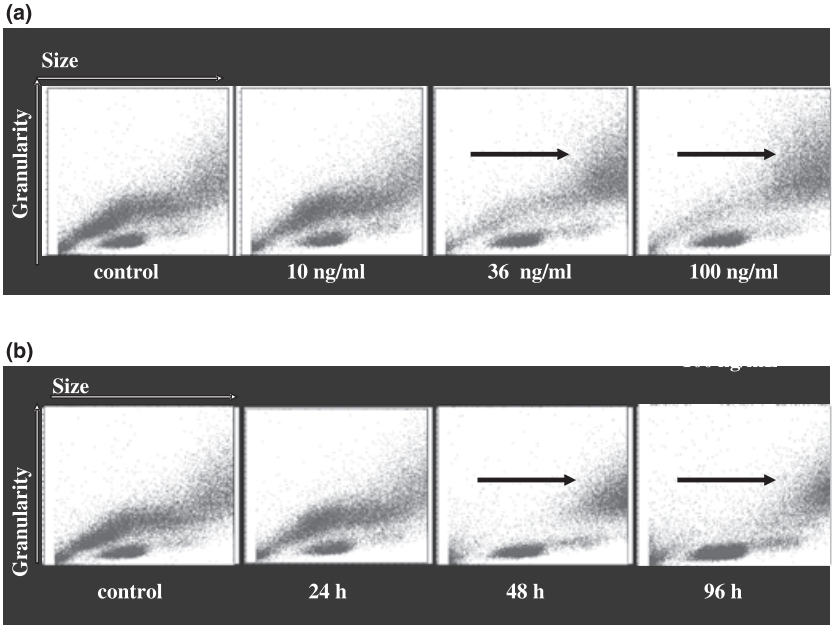

Figure 6 m-CSF increases monocyte size and granularity in dose- and time-dependent manner. (a) Following monocyte treatment with 10, 36, and 100 ng/ml m-CSF for 24 h, monocyte size and granularity appear to increase with increasing doses. (b) Monocyte size and granularity appeared to increase in a time-dependent manner at 24, 48, and 96 h following treatment with 36 ng/ml of m-CSF.

primed monocytes are the catalyst for future rejection episodes upon cross-talk with T cells.

To hone in on the effects of m-CSF on monocyte activation, we treated human monocytes with a range of doses of recombinant human m-CSF based on an optimal dose of 36 ng/ml. We similarly saw an increase in CD40 and CD80 as well as in HLA-DR and FITC-dextran as measured by flow cytometry in response to m-CSF administration. It is necessary to point out that although we did rest the elutriated monocytes overnight, it is impossible to say with certainty that these monocytes were entirely in a resting state prior to treatment with m-CSF. We can, therefore, only make generalized comments on the presence of an increased shift of these specific cell differentiation markers as compared with the control group. With this in mind, we did observe an increase in CD40, CD80, and HLA-DR in all doses of m-CSF tested. There does appear to be a dose-response when comparing the10 ng/ml group to the 36 ng/ml-treated cohort, but no additional increase in effect was observed upon comparing the 36 ng/ml treatment to the 100 ng/ml group. These results show that m-CSF does appear to activate monocytes and enhance their ability towards co-stimulation. We extrapolate that these data may implicate m-CSF in assisting monocyte cross-talk with other immune cells. Finally, in response to treatment with m-CSF, monocytes increase their surface marker expression of FITC-dextran in a time-dependent manner, showing a greater effect after 96 h of m-CSF treatment. This suggests that m-CSF also augments monocyte phagocytotic abilities.

Taken together, these data suggest a role for renal tubule cell derived m-CSF in the activation of monocytes. Targeting m-CSF specifically with the use of an anti-m-CSF antibody is a logical next step in further evaluation of the role of m-CSF in renal allograft rejection. Similarly, blockade of the m-CSF receptor, c-fms, holds promise and has been shown to reduce macrophage accumulation in renal inflammation and attenuate proliferation in a rat model of acute allograft rejection [19–22]. Further, use of the *op/op* mouse, which spontaneously lacks m-CSF, to study renal transplantation could also assess the role of renal derived m-CSF on allograft infiltrates and subsequent rejection. Our preliminary results using this animal model suggest an allograft survival advantage when m-CSF is not involved (unpublished observations, KAS, ADK) but these animals are exceptionally fragile and definitive studies using them in transplant studies have been difficult to perform. Further studies are necessary to better understand the role of renal tubule-cell-derived m-CSF in allograft dysfunction and rejection. Clearly, though, however important m-CSF may be in promoting renal allograft rejection, it is certainly the interplay of many factors that ultimately contribute to graft dysfunction. In addition, m-CSF is may be increased in pro-inflammatory states outside the realm of transplantation. The significance of our observations lies in the recognition of monocytes' contribution to renal allograft dysfunction and the importance of renal tubule-cell-derived m-CSF in the activation of monocytes, thereby suggesting that the allograft itself may participate in its own demise.

Authorship

KAS: designed research study, performed research study, wrote paper, collected data, and analyzed data. RLK: collected data and contributed important reagents. SCH: designed research study. SME: analyzed data. ADK: designed research study, analyzed data, and wrote paper.

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