CD103+ Dendritic Cells Elicit CD8+ T Cell Responses to Accelerate Kidney Injury in Adriamycin Nephropathy

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ABSTRACT

CD103+ dendritic cells (DCs) in nonlymphoid organs exhibit two main functions: maintaining tolerance by induction of regulatory T cells and protecting against tissue infection through cross-presentation of foreign antigens to CD8+ T cells. However, the role of CD103+ DCs in kidney disease is unknown. In this study, we show that CD103+ DCs are one of four subpopulations of renal mononuclear phagocytes in normal kidneys. CD103+ DCs expressed DC-specific surface markers, transcription factors, and growth factor receptors and were found in the kidney cortex but not in the medulla. The number of kidney CD103+ DCs was significantly higher in mice with Adriamycin nephropathy (AN) than in normal mice, and depletion of CD103+ DCs attenuated kidney injury in AN mice. In vitro, kidney CD103+ DCs preferentially primed CD8+ T cells and did not directly induce tubular epithelial cell apoptosis. Adoptive transfer of CD8+ T cells significantly exacerbated kidney injury in AN SCID mice, whereas depletion of CD103+ DCs in these mice impaired activation and proliferation of transfused CD8+ T cells and prevented the exacerbation of kidney injury associated with this transfusion. In conclusion, kidney CD103+ DCs display a pathogenic role in murine CKD via activation of CD8+ T cells.


CDK is caused by a diverse range of insults, including infectious, toxic, inflammatory, and metabolic conditions, in which innate or adaptive immunity may protect against kidney injury.1,2 However, either persistence of these insults or dysregulation of immunity result in progressive disease, leading ultimately to end stage kidney failure.3 Dendritic cells (DCs) are central orchestrators of the immune response. They communicate with both innate and adaptive immune systems and can aggravate or attenuate kidney inflammation.4 DCs are present in the kidney and form an extensive network through tubulointerstitium to constantly sample antigens and present them to native T cells. These DCs anergize autoreactive T cells to help maintain peripheral tolerance.5,6 In kidney disease, it has been demonstrated that DCs are significantly increased in the interstitium in both experimental and human kidney diseases.7,8 However, the role of DCs in kidney disease is still not fully understood. Studies in murine models of kidney disease showed that DCs accumulating in inflamed kidneys secrete both proinflammatory and anti-inflammatory cytokines and thereby either attenuate or aggravate kidney injury. Depletion of kidney DCs enhanced kidney injury in
nephrotic nephritis and impaired recovery from ischemia-reperfusion injury, indicating a protective role of kidney DCs. However, depletion of activated kidney DCs rapidly resolved established kidney immunopathology in a mouse model of glomerular injury, suggesting a facilitatory role for kidney DCs in progression of anti–glomerular basement membrane nephritis. It is unclear why DCs are predominantly proinflammatory in some kidney diseases but anti-inflammatory in others, an incongruity that could be explained by heterogeneity of DCs within the kidney.

DCs are divided currently into two broad types: classic dendritic cells (cDCs) and plasmacytoid DCs. cDCs are subdivided further into CD4+ DCs and CD8+ DCs in lymphoid tissue and CD103+ DCs and CD103+ DCs in nonlymphoid tissue. In the kidney, cDCs commonly reside in the interstitium and express CD11c. However, CD11c is also expressed prominently on kidney macrophages, contributing to confusion about the role of DCs and macrophages. Kidney DCs are a heterogeneous population composed of several subsets. Depletion of the whole population of DCs in previous studies produced different outcomes in different diseases, suggesting that the predominant DC subsets may vary with the type and stage of disease. We previously showed that kidney CD11c+F4/80− cells, but not CD11c+F4/80+ cells, possess characteristics typical of DCs. We further found that CD11c+F4/80+ DCs can be divided into CD103+ and CD103− DCs. CD103+ DCs are of particular interest because of their important role in regulating immunity in skin, mucosa, and lungs. CD103+ DCs display distinct functional activities, including activation of CD8 T cells through antigen cross-presentation and conversion of naïve T cells into induced Foxp3 regulatory T cells. However, the role of CD103+ DCs in CKD is unknown.

The aim of this study is to define the role of CD103+ DCs and the mechanisms underlying CD103+ DC–mediated kidney injury in murine adriamycin nephropathy (AN), a model of human FSGS. We found that CD103+ DCs accelerate kidney injury, at least in part via CD8 T cell cytotoxicity.

RESULTS

Characteristics of CD103+ DCs in Normal Kidneys
To characterize the phenotype of renal mononuclear phagocytes (rMPS), we used a combination of markers including CD45, MHC-II, lineage (lin) makers (CD3, CD19, T cell receptor [TCR]−β, TCR-γδ, and CD49b), CD11c, F4/80, CD103, and CD11b. After pregating on CD45+ cells to exclude contaminating kidney epithelial cells, total rMPs, gated as MHC-II−lin− cells, could be divided into three populations based on CD11c and F4/80 expression—namely, F4/80+CD11c+ cells (rMP1), F4/80+CD11c− cells (rMP2), and F4/80−CD11c+ cells—and then F4/80−CD11c+ cells could be further subgrouped into CD103−CD11b− cells (rMP3) and CD103+CD11b+ cells (rMP4) (Figure 1A). Four subsets of rMPS were identified in normal kidneys, and the total numbers of rMP1, rMP2, rMP3, and rMP4 were 116.0±30.0×10^3, 18.9±25.6×10^3, 20.5±5.7×10^3, and 20.6±5.0×10^3 per kidney, respectively (Figure 1B). To further characterize CD103+CD11b− cells and CD103+CD11b+ cells, an extensive flow cytometry phenotypic analysis of the DC-specific markers was performed. rMP3 in normal kidneys expressed the classic DC markers CD207 and CD205, as well as a newly discovered CD103+ DC surface marker CD26, but it did not express signal regulatory protein-α (SIRPα). By contrast, rMP4 expressed high levels of SIRPα (a marker for CD11b+ DCs) and low levels of CD207, CD205, and CD26 (Figure 1C). Next, we examined transcription factors and growth factor receptors that are selectively expressed by subsets of DCs. Batf3, IRF8, and Id2, which are required for CD8α+ DC development, were selectively expressed by rMP3. IRF4, which is required for development of CD8α− DCs, was selectively expressed in CD11b− rMP4 (Figure 1D). In addition, Flt3 (the receptor for the DC growth factor, Flt3-L) was highly expressed in CD103+ rMP3, whereas the macrophage colony-stimulating factor receptor showed higher expression by rMP4 (Figure 1E). These data indicate that rMP3 and rMP4 subsets exhibit distinct phenotypes that are consistent with those of CD103+ DCs and CD11b+ DCs, respectively, in other nonlymphoid organs. We also sought to determine the physical location of rMP3 within the kidney. Immunofluorescence staining of frozen kidney sections revealed CD103+CD11c+ DCs (orange) were only distributed in the cortex of normal kidneys (Figure 1F). Most CD103+CD11c+ DCs were located in the kidney interstitium, but not in the glomeruli. Together, these experiments characterized CD103+ DCs within normal kidneys.

Kidney CD103+ DCs Are Pathogenic in Mice With AN
We next investigated the role of CD103+ DCs in diseased kidneys using the AN model. Immunofluorescence staining of kidney sections showed that the number of CD103+ cells gradually increased from week 1 to week 4 in AN mice (Figure 2, A and B). The number of CD103+CD11b− cells (rMP3), as well as the other subsets of rMPs among total kidney cells, was significantly increased in AN mice compared with those in normal mice (Figure 2C). Expression of CD103 in normal and AN kidney occurred predominantly on CD11c+ cells but was also present in subpopulations of CD4+ and CD8+ T cells while being essentially absent on F4/80+ macrophages, CD19+ B cells, and Gr1+ neutrophils (Supplemental Figure 1). Therefore, we sought to examine the in vivo function of CD103+ DCs using the CD103-saporin (CD103-SAP) antibody to deplete CD103+ DCs. Kidney CD103+ DCs were successfully depleted in AN mice treated with CD103-SAP antibody but not in AN mice treated with control IgG-SAP antibody (Figure 3A, Supplemental Figure 2A). The specificity of this CD103-SAP–depleting antibody was examined in this study. The total number of all infiltrated immune cells was significantly reduced in AN mice treated with CD103-SAP (Figure 2, A and B). However, the proportion of CD103+ DCs (rMP3), as well as CD103+CD4+ T cells and CD103+CD8+ T cells, in kidney CD45−
Figure 1. Identification of CD103+ DCs in normal kidneys. (A) Representative FACS analysis showing the gating strategy to identify CD103+ DCs in normal kidneys. After pregating on CD45+ leukocytes, the lin− MHC-II+ cells are divided into three populations based on their CD11c and F4/80 expression, including F4/80+CD11c− cells (rMP1), F4/80+CD11c+ cells (rMP2), and F4/80−CD11c+ cells. F4/80−CD11c+ cells are then further divided into two populations based on their CD103 and CD11b expression: namely, CD103+CD11b−
leukocytes was reduced in CD103-SAP–treated AN mice but not their relevant CD103− counterparts, indicating that administration of CD103-SAP antibody specifically depleted CD103+ cells (predominantly CD103+ DCs) in kidneys of AN mice (Supplemental Figure 2, C and D). Similarly, administration of CD103-SAP antibody specifically depleted CD103+ DCs in kidney draining lymph nodes (KDLNs) of AN mice, but there were no detectable changes in CD103+CD4+ T cells and CD103+CD8+ T cells (Supplemental Figure 3). Kidney function was significantly improved in AN mice treated with CD103-SAP antibody, as shown by a decrease in proteinuria and serum creatinine and an increase in creatinine clearance at day 28 (Figure 3B). Kidney injury is characterized by glomerulosclerosis, tubular atrophy, and interstitial expansion in AN. Depletion of CD103+ DCs using CD103-SAP antibody significantly attenuated all components of kidney injury in AN mice (Figure 3, C and D). There was no significant difference in kidney function and injury between untreated AN mice and AN mice treated with control IgG-SAP antibody. These results show that kidney CD103+ DCs play a pathogenic role in AN.

Kidney CD103+ DCs Do Not Directly Induce Kidney Tubular Cell Damage

To understand how CD103+ DCs are involved in the progression of kidney damage in AN mice, we performed phenotypic analysis of inflammatory mediators in rMP by flow cytometry. The subsets rMP1 and rMP2 separated from AN kidney produced much more IL-6, TNF-α, and chemokine (C-C motif) ligand 2 (CCL2) than those from normal kidney. The subsets rMP3 and rMP4 only secreted higher levels of IL-6 but not CD103− DCs (rMP3) and CD103−CD11b+ cells (rMP4). (B) Total numbers of four rMP populations per kidney are shown. Data represent the mean±SEM of at least four independent experiments using four to eight pooled kidneys. Total cell number per kidney is calculated as follows: total cells per kidney × proportion of total cells staining for CD45+ subpopulation. Data represent the mean±SEM of six mice per group. ***P<0.001 versus normal.

Figure 2. CD103+ DCs are increased in kidneys of AN mice. (A) Immunofluorescence staining for CD103 (green) in kidney sections of normal and AN (week 1, week 2, and week 4) mice. (B) Quantitative analysis of the number of CD103+ cells in kidneys of AN mice. Data represent the mean±SEM of six mice per group. (C) Quantitation of the number of four rMP subsets in kidneys of normal and AN (week 4) mice. Total cell number per kidney is calculated as follows: total cells per kidney × proportion of total cells staining for CD45+ subpopulation. Data represent the mean±SEM of six mice per group. ***P<0.001 versus normal.
Figure 3. Depletion of CD103⁺ DCs attenuates renal injury in AN mice. BALB/c mice are treated with CD103⁻ SAP or IgG-SAP antibodies on day 5 after ADR injection. Mice are euthanized on day 28. (A) Flow cytometric analysis of CD103⁺ DCs in kidneys of normal, AN+Vehicle, AN+IgG-SAP, and AN+CD103-SAP mice at day 28 after ADR injection. (B) Proteinuria, serum creatinine, and creatinine clearance are assessed in normal, AN+Vehicle, AN+IgG-SAP, and AN+CD103-SAP mice at day 28 after ADR injection. (C) Representative PAS-stained sections of renal cortices at day 28. (D) Kidney injury (glomerulosclerosis, damaged tubules, and interstitial...
molecules CD80, CD86, and B7-H1 on CD103+ DCs was significantly increased in AN mice compared with control TECs, and was further enhanced by coculture with rMP1 or rMP2, whereas there was no increased apoptosis of ADR-TECs cocultured with rMP3 or rMP4 (Figure 4, B and C). These data suggest that CD103+ DCs do not directly promote kidney tubular cell damage in AN.

Kidney CD103+ DCs Preferentially Prime CD8 T cells
DCs are professional antigen-presenting cells, which are required for presentation of internal and external antigen to T cells in vivo to protect against tissue infection or to induce tissue damage. We next investigated the T cell priming capability of rMP subsets in vitro. rMP1 and rMP2 separated from normal kidneys exhibited low T cell priming capacity in the antigen-specific CD4 and CD8 T cell proliferation assay (Figure 5). However, rMP3 and rMP4 exhibited high T cell priming capacity in the antigen-specific CD4 and CD8 T cell proliferation assay. rMP3 preferentially induced antigen-specific CD8 T cell proliferation in vitro, whereas rMP4 preferentially primed CD4 T cells (Figure 5). Moreover, the expression of costimulatory molecules CD80, CD86, and B7-H1 on CD103+ DCs was significantly increased in AN mice compared with that in normal mice, suggesting that CD103+ DCs enhanced their ability to activate T cells in vivo by upregulating cell surface receptors (Figure 6, A and B). Immunofluorescence double staining of CD103 with CD4 or CD8 revealed that numerous CD103+ DCs colocalized with CD4 T cells or CD8 T cells in kidneys of AN mice (Figure 6C), suggesting that CD103+ DCs may activate CD4 T cells or CD8 T cells in situ by cell–cell interaction in diseased kidneys.

Depletion of CD103+ DCs Abolishes the Pathogenic Effect of Transfused CD8 T Cells in SCID Mice with AN
The mechanisms underlying the pathogenic role of CD103+ DCs may involve T cell activation in diseased kidneys. Our previous studies found that depletion of CD8 T cells, but not CD4 T cells, attenuated kidney injury in AN mice, indicating that CD8 T cells contribute to kidney damage in AN.

We next investigated the interaction between CD103+ DCs and T cells in vivo by adoptive transfer and depletion experiments (Figure 7A). There was no difference in kidney functional and histologic injury between untreated AN severe combined immune deficiency (SCID) mice and AN SCID mice given CD103-SAP antibody. These findings indicate that, unlike wild-type mice with AN, depletion of CD103+ DCs did not improve kidney damage in SCID mice with AN, and our results also suggest that CD103+ DCs play a pathogenic role in AN through their interaction with other immune cells such as CD4 or CD8 T cells (Figure 7, B–D). In addition, adoptive transfer of CD8 T cells significantly worsened kidney function and exacerbated kidney histologic injury in AN SCID mice, whereas transfusion of CD4 T cells did not affect kidney histologic or functional injury. However, depletion of CD103+ DCs using CD103-SAP antibody abolished the pathogenic effect of transfused CD8 T cells in SCID mice with AN (Figure 7, B–D). These results indicate that interaction between CD103+ DCs and CD8 T cells within the kidney plays an important role in the progression of AN.

Depletion of CD103+ DCs Reduces Cytotoxicity and Proliferation of Transfused CD8 T Cells in SCID Mice with AN
To understand how CD103+ DCs interact with CD8 T cells in the AN kidney, we examined cytotoxicity and proliferation of transfused CD8 T cells in AN SCID mice. First, we measured expression of inflammatory cytokines and cytotoxic molecules. IFN-γ and TNF-α were highly expressed on transfused CD8 T cells separated from kidneys and KDLNs of AN SCID mice, whereas their expression was significantly decreased on CD8 T cells from AN SCID mice treated with CD103-SAP antibody (Figure 8A). In addition, the cytotoxic molecules perforin and granzyme B were highly expressed on transfused CD8 T cells separated from the kidneys and KDLNs of AN SCID mice, and the level of perforin and granzyme B expression by transfused CD8 T cells was reduced in CD103+ DC–depleted AN SCID mice (Figure 8B). Second, we performed a functional analysis of cytotoxicity of CD8 T cells against kidney TECs in vitro. TECs were injured by culture with ADR for 24 hours, and they were then cocultured with transfused CD8 T cells separated from kidneys and KDLNs of AN SCID mice for another 24 hours. Apoptosis of ADR-TECs was significantly increased compared with control TECs, and it was further enhanced by coculture with transfused CD8 T cells separated from kidneys and KDLNs of AN SCID mice, whereas apoptosis of TECs was reduced in ADR-TECs cocultured with transfused CD8 T cells separated from CD103+ DC–depleted AN SCID mice (Figure 8, C–F). Furthermore, the proportion of carboxyfluorescein succinimidyl ester (CSFE)–labeled transfused CD4 or CD8 T cells in AN SCID mice was assessed by flow cytometry at days 9, 14, and 28 after ADR injection (Figure 9, A and B). The percentage of CSFE-labeled CD4 or CD8 T cells among kidney CD45+ leukocytes gradually increased, whereas fluorescence intensity of
labeled T cells gradually decreased from day 2 to day 23 after transfusion in AN SCID mice, indicating that both transfused CD4 T cells and CD8 T cells proliferated in diseased kidneys. A greater number of proliferated CD8 T cells than CD4 T cells was found at days 7 and 21 after transfusion in kidneys of AN SCID mice. However, the proportion of CSFE-labeled CD8 T cells, but not CD4 T cells, was reduced at days 7 and 21 after transfusion in AN SCID mice treated with CD103-SAP antibody (Figure 9, C and D). These results suggest that CD103+ DCs play a critical role in inducing CD8 T cell activation and proliferation in AN.

### CD103+ DCs Were Activated by Kidney Damage-Associated Molecular Patterns in AN

We next investigated the potential stimuli involved in DC activation in diseased kidneys. The mRNA expression of high-mobility group box 1 (HMGB1), biglycan, and uromodulin was significantly increased from week 1 to week 4 in kidneys of AN mice compared with that in normal kidney. Levels of mRNA for all three isoforms of hyaluronan synthase were increased dramatically at week 1 in AN mice, with further upregulation through weeks 2 and 4 compared with normal mice (Figure 10A). Moreover, the expression of damage-associated molecular patterns, such as HMGB1, biglycan, and uromodulin, was increased in the kidneys of AN mice, indicating that these molecules play a critical role in activating CD103+ DCs in AN.

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**Figure 4.** Kidney CD103+ DCs do not directly induce TEC apoptosis. (A) Quantitation of cytokines and chemokines secreted by rMP subsets from normal and AN BALB/c mice after 24 hours of culture. Data represent the mean±SEM of at least three experiments with four mice each. *P<0.05; **P<0.01; ***P<0.001 versus normal. The four rMP subsets are separated from kidneys by FACS at week 4 after ADR injection. (B and C) Tubular injury in AN mice is simulated by incubating renal TECs with ADR for 24 hours. Injured renal TECs are then cocultured with four rMP subsets from AN mice for 24 hours. (B) Representative FACS analysis of apoptosis in TECs after 1-day coculture. (C) Frequency of early apoptosis (Annexin V+7AAD− cells) and late apoptosis (Annexin V+7AAD+ cells) in TECs after 1-day coculture. The values represent the mean±SEM of evaluations from each group (n=5 per group). #P<0.05 versus ADR-TEC. AAD, amino-actinomycin D; CON, control.
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In this study, we showed that CD103+ DCs are pathogenic in murine AN, via CD8 T cells. Kidney CD103+ DCs expressed DC-specific surface markers including CD207, CD205, and CD26; transcription factors Batf3, IRF8, and Id2; and growth factor receptor FLt3. Kidney CD103+ DCs were distributed only in the kidney cortex but not in the medulla. The number of kidney CD103+ DCs was significantly increased in mice with AN, and depletion of CD103+ DCs resulted in attenuation of kidney injury. Kidney CD103+ DCs preferentially primed CD8 T cells rather than CD4 T cells in vitro. Adoptive transfer of CD8 T cells significantly exacerbated kidney injury in AN SCID mice, whereas depletion of CD103+ DCs blocked activation of CD8 T cells and reduced kidney injury in AN SCID mice treated with CD8 T cells. CD8+ T cells separated from AN kidney induced tubule cell apoptosis in vitro. Without CD8+ T cells, CD103+ DCs did not cause kidney injury, suggesting that CD103+ DCs induce kidney injury through a CD8+ T cell–dependent mechanism. Regulatory T cells, γδ T cells, T cell lymphomas, tumor-infiltrating lymphocytes, and epithelial T cells have been demonstrated to express CD103 antigen in previous studies.26–33 Recently, CD103 was identified as a surface marker of CD8+ tissue-resident memory T cells in many infectious diseases.34–36 It is possible that these other immune cells could be depleted by anti–CD103-saporin. However, in this study, we found that nearly 90% of CD103 was expressed by CD11c+ DCs in normal and AN kidneys. Less than 5% of CD4, CD8 T cells, B cells, and neutrophils expressed CD103 in normal and AN kidneys (Supplemental Figure 1). Therefore, anti–CD103-saporin predominantly depleted CD103+ DCs in kidneys and KDLNs. Anti–CD103-saporin also depleted CD103-expressing CD4+ and CD8+ T cells, which could contribute to the protective effect of CD103 antibody on renal injury. However, the effects of depletion of CD103-expressing CD4+ and CD8+ T cells in AN would likely be minimal, owing to their small numbers, such that depletion by CD103 antibody did not induce a significant change in overall CD4+ and CD8+ T cell numbers (Supplemental Figures 2 and 3). Moreover, CD4+ CD103+ T cells could be regulatory T cells,26,27 whose depletion would worsen renal injury in AN. CD8+CD103+ T cells could be tissue-resident CD8 memory T cells, which would induce secondary immune responses upon encountering the same antigen. However, there is no evidence to support the importance of a secondary antigen encounter in AN.

Previous studies on DCs in kidney diseases have been focused on CD11c+ cells.14 However, depletion of CD11c+ cells in kidney diseases has shown variable and sometimes opposing effects, as a result of the elimination of both DCs and macrophages.9,10,12,37,38 With the aim of examining both kidney DCs and macrophages, we identified four subsets of mononuclear phagocytes. Nearly 60% of CD11c+ cells expressed F4/80 and displayed functions typical of macrophages. Only CD11c+F4/80+ cells showed DC phenotypes and functions; they could be divided into two subpopulations: CD103+ DCs and CD103− DCs. CD103+ DCs have been reported to be a specific population of DCs that are present in many nonlymphoid organs including the skin, lungs, kidney, and intestine.17,39 CD103+ DCs in nonlymphoid organs exhibit two main types of function: namely, maintenance of tolerance by channeling self–peptide–MHC complexes to CD4+ T cells. Kidney CD103+ DCs expressed CD11c+F4/80 and displayed functions typical of macrophages. Only CD11c+F4/80+ cells expressed CD103, whose depletion would worsen renal injury in AN. Kidney CD103+ DCs preferentially prime CD8 T cells in vitro. Four rMP subsets are isolated from kidneys of normal C57BL/6 mice by flow cytometry. (A) OVA257–264 peptide–pulsed rMP subsets are cocultured with OVA-specific CFSE-labeled CD8+ T cells isolated from OT-I mice for 4 days. (B) OVA323–339 peptide-pulsed rMP subsets are cocultured with OVA-specific CFSE-labeled CD4+ T cells isolated from OT-II mice for 4 days. Cells are harvested and stained for surface CD3. Proliferation of T cells is analyzed by flow cytometry. (A)
induction of regulatory T cells\textsuperscript{20,21} or protection against tissue infection by activation of T cells, especially preferential induction of CD8\textsuperscript{+} T cell responses by cross-presentation of foreign antigens.\textsuperscript{19,22,23} To define the role of CD103\textsuperscript{+} DCs in normal and diseased kidneys, kidney CD103\textsuperscript{+} DCs were characterized, for first time, in a model of kidney disease, AN. CD103\textsuperscript{+} DCs in the kidney showed a similar phenotype to that displayed by CD103\textsuperscript{+} DCs in the lungs, skin, and intestine, including the expression of DC markers CD207, CD205, and CD26; transcription factor Batf3, IRF8, and Id2; and growth factor receptor Flt3. Furthermore, kidney CD103\textsuperscript{+} DCs were depleted in AN mice by treatment with CD103-SAP antibody, resulting in significant improvement of kidney histology and function. Unlike in other organs, including the skin, lung, and intestine,\textsuperscript{19–23} in which CD103\textsuperscript{+} DCs have been shown to have a protective role in viral infection and tumors or to play a regulatory role by generation of regulatory T cells in mucosal immune tolerance,\textsuperscript{20,21} kidney CD103\textsuperscript{+} DCs displayed a pathogenic role in AN. The organ-specific effects of CD103\textsuperscript{+} DCs may be explained by their exposure to specific microenvironments within different organs or by disease-specific activation and modulation in various disease types. Gottschalk \textit{et al.} recently found that CD8\textsuperscript{+}CD103\textsuperscript{+} DCs capture circulating self-antigens in KDLNs and induce tolerance of cytotoxic CD8\textsuperscript{+} T cells through the coinhibitory cell surface receptor programmed death 1 ligand.\textsuperscript{40} Interestingly, CD103\textsuperscript{+} DCs separated from AN kidneys only produced low levels of inflammatory mediators and did not directly induce kidney tubule cell injury \textit{in vitro}. This raised the question of how CD103\textsuperscript{+} DCs induce kidney injury in AN.

CD8\textsuperscript{+} T cells are the main effector T cells defending against intracellular pathogens and tumors, and they have been found in interstitial and periglomerular regions in kidney disease.\textsuperscript{41–43} A critical role for CD8\textsuperscript{+} T cells in GN is supported by the attenuation of autoimmune nephrotoxic nephritis in CD8\textsuperscript{-} knockout mice or induction of glomerular injury by adoptive transfer of antigen-specific CD8\textsuperscript{+} T cells.\textsuperscript{12,44,45} Our previous study demonstrated that CD8\textsuperscript{+} T cells display a pathogenic role in AN mice.\textsuperscript{25} Tissue-resident CD103\textsuperscript{+} DCs have been demonstrated to cross-present antigens to CD8\textsuperscript{+} T cells to protect against tissue infection. In this study, we found that

![Figure 6. Activated CD103\textsuperscript{+} DCs colocalize with CD8 T cells in kidneys of AN mice. (A) Representative FACS analysis showing expression of costimulatory factors (CD40, CD80, CD86, and B7-H1) in CD103\textsuperscript{+} DCs of normal and AN mice. (B) Flow cytometric analysis of mean fluorescence intensity of costimulatory factors in CD103\textsuperscript{+} DCs of normal and AN mice. Data represent the mean±SEM of evaluations from each group (n=6 per group). *P<0.05 versus normal. (C) Immunofluorescence double staining of CD103 and CD4 or CD103 and CD8 in kidney sections of AN mice. Bar, 50 \textmu M.](image)
Depletion of CD103+ DCs reduces kidney injury in AN SCID mice treated with CD8 T cells. (A) SCID mice are treated with CD103-SAP antibodies on day 5 after ADR injection and with CD4 or CD8 T cells on day 7 after ADR injection. Mice are euthanized on day 28. (B) Proteinuria, serum creatinine, and creatinine clearance are assessed in normal, AN+Vehicle, AN+CD103-SAP, AN+CD4, AN+CD4+CD103-SAP, AN+CD8, and AN+CD8+CD103-SAP mice at day 28 after ADR injection. (C) Representative PAS-stained sections of renal cortices at day 28. (D) Kidney injury (glomerulosclerosis, damaged tubules, and interstitial volume) is assessed quantitatively. The values represent the mean±SEM of evaluations from each group (n=7 per group). **P<0.01 versus AN+Vehicle; #P<0.05 versus AN+CD8; ##P<0.01 versus AN+CD8. Original magnification, ×200.
kidney CD103+ DCs were more potent at presenting soluble antigens to CD8+ T cells than were kidney CD103− DCs. To understand the mechanisms underlying CD103+ DC mediation of kidney injury in CKD, the interaction between CD103+ DCs and CD8+ T cells was investigated. Kidney CD103+ DCs expressed high levels of costimulatory factors CD80, CD86, and B7-H1 in AN mice, suggesting that CD103+ DCs may prime CD8+ T cells by cell–cell interaction. CD8 T cells were highly activated and proliferated in the presence of CD103+ DCs and were shown to be cytotoxic to kidney cells. Depletion of CD103+ DCs abolished the pathogenic effect of trans fused CD8+ T cells in AN SCID mice, showing the co-presentation of CD103+ DCs and CD8+ T cells in causing kidney injury. This mechanism was further confirmed by the experiment in which cytotoxicity and proliferation of trans fused CD8+ T cells was reduced by depletion of CD103+ DCs in SCID mice with AN. The results are consistent with the finding in lungs infected with influenza virus, in which the development of virus-specific CD8+ T cells was severely delayed after depletion of lung CD103+ langerin+ DCs.46 Cross-talk between kidney DCs and CD8+ T cells was previously identified in autoimmune GN.12 Our study extends this observation by demonstrating that CD103+ DCs are the most important DC subset to activate CD8+ T cells in CKD.

The primary functions of CD103+ DCs are to capture, process, and present antigen to T cells and provoke immunity to pathogens. It is likely that the antigens involved in immune-mediated nephritis will be captured by kidney CD103+ DCs and presented to CD8+ T cells. CD8+ T cells primed by CD103+ DCs will be activated to become toxic and damage kidney cells.
However, the majority of CKDs have no clear immune antigens. In AN, podocytes and tubule cells are injured by ADR and other molecules (including various urinary proteins) and inflammatory cells subsequently infiltrate and proliferate within the kidney. Innate immunity is the predominant immune response in this type of sterile inflammation in which a specific antigen has not been identified.47 Kidney cell necrosis, mediated in part by ADR and noxious tubular proteins, would likely release DAMPs, triggering sterile inflammation. DAMPs include intracellular molecules such as nuclear proteins, mitochondrial components, uric acid and cellular chaperones, and extracellular matrix moieties such as hyaluronan.2,48 DAMPs are recognized by pattern-recognition receptors such as certain TLRs and nucleotide-binding oligomerization domain–like receptors that are present on DCs and macrophages. DCs play an important role in DAMP-mediated sterile inflammation.1,49,50 In this study, we found that HMGB1, biglycan, and uromodulin as well as three isoforms of hyaluronan synthase were significantly increased during the course of AN compared with normal kidneys and their respective receptors were upregulated on CD103+ DCs. We have demonstrated that HMGB1 directly activated CD103+ DCs, although other increased inflammatory factors are also involved in CD103+ DC activation in AN mice. In addition to causing tubular cell damage, the products of various urinary proteins could

![Diagram of CD103+ DC Function in Renal Injury](image)

**Figure 9.** Depletion of CD103+ DCs reduces proliferation of transfused CD8 T cells in SCID mice with AN. (A) SCID mice are treated with CD103-SAP antibodies on day 5 after ADR injection and with CFSE-labeled CD4 or CD8 T cells on day 7 after ADR injection. Mice are euthanized on days 9, 14, and 28 after ADR injection, respectively. (B) Representative FACS analysis showing the gating strategy to identify transfused CD4 or CD8 T cells in kidneys of SCID mice. After pregating on total kidney cells, the kidney leukocytes are gated as CD45+ cells, and transfused T cells are then gated as CD4\(^+\)CFSE\(^+\) or CD8\(^+\)CFSE\(^+\) cells. (C) Total kidney cells are separated from AN SCID mice at days 2, 7, and 23 after T cell transfusion. Representative FACS analysis showing transfused CD4 or CD8 T cells in kidneys of AN SCID mice. (D) Quantitation of the percentages of transfused T cells in kidneys of AN SCID mice. The values represent the mean±SEM of evaluations from each group (n=4 per group).

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CD103+ DC Function in Renal Injury

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be captured by interstitial DCs, including CD103+ DCs, to stimulate infiltrating CD8+ T cells and helper T cells. The relative importance of antigen presentation by CD103+ DCs in the overall activation of CD8+ T cells in unknown.

In conclusion, the features and role of CD103+ DCs have been defined in murine AN. Depletion of CD103+ immune cells (predominantly CD103+ DCs) protected against kidney injury, suggesting a pathologic role for CD103+ DCs in AN. The mechanisms underlying CD103+ DC mediation of kidney injury are associated with cytotoxicity of CD8+ T cells in AN. This study raises the potential of targeting pathogenic CD103+ DCs as a therapeutic approach to attenuating kidney inflammation and injury in some forms of CKD.

**CONCISE METHODS**

**Mice**

BALB/c, SCID (BALB/c background), C57BL/6, and transgenic OT-I and OT-II C57BL/6 mice were purchased from the Animal Resources Centre (Perth, Australia). Experiments were carried out in accordance with the protocols approved by the Animal Ethics Committee of Sydney West Area Health Service. Dose-finding studies defined an optimal dose of 10.4 mg/kg body weight of ADR (doxorubicin; Pharmacia & Upjohn Pty. Ltd., Australia) for BALB/c mice. ADR was injected once via the tail vein of each mouse.

**AN Murine Model and CD103-SAP Antibody Administration**

The in vivo functions of CD103 DCs in AN mice were examined by a depletion study using CD103-SAP antibody (M290). BALB/c mice were divided into four groups: normal, AN with vehicle, AN with IgG-SAP treatment, and AN with CD103-SAP treatment. For antibody treatment, mice were administered 1 mg/kg CD103-SAP or IgG-SAP antibody intraperitoneally at day 5 after ADR injection. The dose was selected according to previously published studies. Control animals received PBS only. Mice were euthanized at week 4 after ADR injection. Blood, urine, and kidneys were harvested for analysis. The proportion of rMP subsets was examined by flow cytometry. All urine and blood specimens were analyzed by the Institute of Clinical Pathology and Medical Research (Westmead Hospital), using a BM/Hitachi 747 analyzer (Tokyo, Japan).

In another study, SCID mice were divided into seven groups: normal, AN with vehicle, AN with CD103-SAP treatment, AN with...
CD4 T cell treatment, AN with CD4 T cells and CD103-SAP treatment, AN with CD8 T cells treatment, AN with CD8 T cells and CD103-SAP treatment. CD103-SAP antibody (1 mg/kg) was given intraperitoneally at day 5 after ADR injection, and CD4 or CD8 T cells (5 × 10⁶/mouse) isolated from spleen of BALB/c mice were adoptively transferred by a single tail-vein injection at day 7 after ADR injection. Mice were euthanized at weeks 4 after ADR injection. Blood, urine, and kidneys were harvested for analysis. The proportion of rMP subsets were examined by flow cytometry. CD8 T cells were separated from kidney for further analysis. All urine and blood specimens were analyzed by the Institute of Clinical Pathology and Medical Research (Westmead Hospital), using a BM/Hitachi 747 analyzer.

In parallel, to examine the proliferation of transfused CFSE-labeled CD4 or CD8 T cells, kidneys were harvested at days 2, 7, and 21 after cell transfusion. The proliferation of CFSE-labeled CD4 or CD8 T cells in the kidney was examined by flow cytometry.

Cell Suspension Preparation

Kidneys from BALB/c or SCID mice were perfused with saline before removal and digested with collagenase and DNase as previously described. Kidneys were cut into 1- to 2-mm³ pieces and placed in DMEM containing 1 mg/ml collagenase IV (Sigma-Aldrich) and 100 µg/ml DNase I (Roche) for 40 minutes at 37°C with intermittent agitation. The digested cell suspension was then passed through a 40-µm cell strainer. Mononuclear cells from kidneys were separated using 1.077 g/ml Nycodenz gradient (Axis-Shield, Oslo, Norway). Spleens and KDLNs from BALB/c mice were isolated, minced, and digested for 30 minutes at 37°C in RPMI 1640 containing 1 mg/ml collagenase D and 100 µg/ml DNase I (both from Roche). The digested cell suspension was then passed through a 40-µm cell strainer.

Flow Cytometry and Cell Sorting

For FACS analysis or sorting of kidney samples, single-cell suspensions were stained with Fc block/anti-CD16/32 (2.4G2) and antibodies to CD45.2 (104), MHC-II (M5/114), CD11c (N418), F4/80 (BM8), CD11b (M1/70), and CD103 (2E7 and M290), as well as antibodies to natural killer cell, B cell, and T cell lineages CD49b (DX5), CD19 (1D3), CD3 (145-2C11), TCR-β (H57-597), and TCR-γδ (eBioGL3), all from eBioscience. When FACS sorting was performed on the digested kidney cell suspension, cells were pregated on hematopoietic cells using anti-CD45.2 antibody. Lineages (CD49b/CD19/CD3/TCR-β/TCR-γδ) were then used to exclude natural killer cells and lymphocytes, and 4’,6-diamidino-2-phenylindole was used to exclude dead cells. F4/80-CD11c+ (rMP1), F4/80-CD11c− (rMP2), CD103+CD11b+ (rMP3), and CD103−CD11b+ (rMP4) cells were sorted using a FACSaria machine (BD Biosciences). After sorting, cells were used for phenotypic and functional assays.

Other antibodies used in this article include CD207 (eBioL31), CD205 (205yekta), SIRPa (P84), CD26 (H194-112), CD40 (1C10), CD80 (16-10A1), CD86 (GL1), B7-H1 (MH5), CD4 (GK1.5), CD8α (53-6.7), Gr-1 (RB6-8C5), Perforin (eBioOMAK-D), Granzyme B (NGZB), TLR2 (6C2), TLR4 (SA15-21), as well as corresponding isotype controls, all purchased from eBioscience or Biolegend. Cells were analyzed on an LSR II flow cytometer (BD Biosciences).

Immunofluorescence Staining of Kidney Tissue Sections

Kidney sections from normal and AN mice were stained with rat anti-mouse CD103 (M290; 1:200) antibodies and hamster anti-mouse CD11c (N418; 1:100), and then incubated with AF488 goat anti-rat IgG (1:500) and AF546 goat anti-hamster IgG (1:500). Consecutive photos from the kidney cortex to the kidney medulla were taken in each section. In another study, kidney sections from AN mice were double stained with hamster anti-mouse CD103 (2E7; 1:200) antibodies and rat anti-mouse CD4 (RM4–5; 1:50) or rat anti-mouse CD8α (53–6.7; 1:50) and then incubated with AF546 goat anti-hamster IgG (1:500) and AF488 goat anti-rat IgG (1:500). Isotype control IgGs to these rat, hamster, and goat antibodies were included in immunofluorescence staining. Tissue sections were analyzed by inverted fluorescent microscopy (Olympus BX50).

Proliferation Assay

In antigen-specific assays, isolated rMP subsets (rMP1, rMP2, rMP3, and rMP4) of normal mice were incubated with 1 µg/ml ovalbumin (OVA)257–264 peptide or OVA233–239 peptide (Mimotopes, Australia) for 2 hours at 37°C, then cocultured with OVA-specific CFSE-labeled CD8 T cells (OT-I) for 5 days or CFSE-labeled CD4+ T cells (OT-II) for 4 days. In all assays, 10⁵ T cells were cultured in round-bottom 96-well plates with 10⁴ APCs. Cell proliferation was examined with CFSE (Invitrogen) by flow cytometry.

Primary Culture of Mouse Renal TECs

Primary mouse TECs were generated following established methods adapted from Doctor et al.39 In brief, kidneys were harvested after cardiac perfusion with saline to remove blood cells. The tissue from the outer cortex was cut into pieces of approximately 1 mm³ and then digested in DMEM containing 1 mg/ml collagenase IV (Sigma-Aldrich) and 100 µg/ml DNase I (Roche) for 40 minutes at 37°C with intermittent agitation. Renal TECs were separated by centrifugation using Percoll solution and were cultured in defined K1 medium: DMEM/F12 medium supplemented with 10 ng/ml EGF, 1 ng/ml PGE₃, 5 µg/ml insulin, 5 µg/ml transferrin, 5 ng/ml sodium selenite, 5 × 10⁻¹¹ M triiodothyronine, 5 × 10⁻⁸ M hydrocortisone, 100 U/ml penicillin, 100 µg/ml streptomycin, 25 mM HEPES, and 5% FBS.

Coculture Experiments and Apoptosis Assay

Tubular cell injury in AN mice was simulated by incubation with ADR in vitro. Briefly, renal TECs (1 × 10⁵) were placed in six-well tissue culture plates in serum-free K1 medium for 24 hours and were incubated with ADR (1 µM) for 24 hours. After extensive washing with PBS, TECs (1 × 10⁵) were cocultured with FACS-sorted rMP subsets (rMP1, rMP2, rMP3, or rMP4, 1 × 10⁵ each) of AN mice for 24 hours. TECs were exposed to serum-free K1 medium alone as a control. Apoptosis of TECs was measured using FACS with Fixable Viability Dye and Annexin V (both eBioscience) staining following the manufacturer’s protocol.

In another study, CD8 T cells were isolated from kidneys or KDLNs of AN SCID mice treated with CD8 T cells or AN SCID mice treated with CD8 T cells and CD103-SAP antibody by flow cytometry. TECs (1 × 10⁵ per well) were incubated with ADR (1 µM) for 24 hours and
were then cocultured with CD8 T cells (1×10^6 per well) in the six-well plates for 24 hours. TECs were stained for Fixable Viability Dye and Annexin V and then analyzed by flow cytometry.

**Cytokine Assays**

FACS-sorted rMP subsets (rMP1, rMP2, rMP3, and rMP4) of normal and AN mice were plated at 2×10^5 cells/well in 24-well plates cultured in complete RPMI 1640 medium for 24 hours. Cytokine production was analyzed by cytometric bead array (CBA Mouse Inflammation Kits; BD Biosciences). For the CBA assay, 50 µl of supernatant was incubated with a mixture of beads coated with capture antibodies for IL-6, IL-10, CCL2, IFN-γ, TNF-α, or IL-12 p70. The addition of the Phycoerythrin-conjugated detection antibodies forms a sandwich complex. After 2 hours of incubation and one wash, samples were analyzed by flow cytometry. Standard curves were generated from analysis of titrated cytokine standards using BD CBA analysis software.

**Kidney DAMPs Preparation and Stimulation**

Kidneys from normal and AN BALB/c mice were perfused with saline before removal and were digested with LIBERASE TL as previously described. Under sterile conditions, kidneys were minced and incubated with 2 ml of Liberase TL (0.2 mg/ml; Roche) and digested for 30 minutes at 37°C with intermittent agitation. Five milliliters of PBS was added, and the digested cell suspension was then filtered through a 40-µm cell strainer. The filtrate was centrifuged (2000 rpm) to remove any cellular debris and the cell-free supernatant was filtered using 0.22-µm filters. Polymyxin B beads (20 µl, P1411; Sigma-Aldrich) were added to the supernatant and incubated at 4°C for 30 minutes to remove any contamination by endotoxin. Supernatant containing DAMPs was stored at −80°C until use.

CD103+ DCs were isolated from kidneys of normal BALB/c mice by flow cytometry. Kidney CD103+ DCs (1×10^5 per well) were incubated with or without recombinant HMGB1 (2 µg/ml) in 24-well plates for 48 hours, and then expression of surface markers on CD103+ DCs was measured by flow cytometry. In another study, CD103+ DCs (1×10^5 per well) were incubated with 1 ml of serum-free media, containing 100 µl of DAMPs from normal or AN kidney, or 100 µl of Liberase mix as the control, in the presence of anti-HMGB1 antibody or mouse IgG. The expression of surface markers on CD103+ DCs was measured by flow cytometry.

**Statistical Analyses**

Renal functional data (serum creatinine, creatinine clearance, and proteinuria) were log-transformed before analysis to stabilize the variance. Statistical tests included the unpaired, two-tailed t test using Welch’s correction for unequal variances and one-way ANOVA with Tukey’s multiple comparisons test. Statistical analyses were performed using Prism software (version 5; GraphPad). Results are expressed as the mean±SEM. P<0.05 was considered statistically significant.

**ACKNOWLEDGMENTS**

This work was supported by grants from the National Health and Medical Research Council of Australia (632665 and 1061848 to Y.W. and D.C.H.H.).

**DISCLOSURES**

None.

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