CD103⁺ Kidney Dendritic Cells Protect against Crescentic GN by Maintaining IL-10–Producing Regulatory T Cells

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ABSTRACT

Kidney dendritic cells (DCs) regulate nephritogenic T cell responses. Most kidney DCs belong to the CD11b⁺ subset and promote crescentic GN (cGN). The function of the CD103⁺ subset, which represents <5% of kidney DCs, is poorly understood. We studied the role of CD103⁺ DCs in cGN using several lines of genetically modified mice that allowed us to reduce the number of these cells. In all lines, we detected a reduction of FoxP3⁺ intrarenal regulatory T cells (Tregs), which protect against cGN. Mice lacking the transcription factor Batf3 had a more profound reduction of CD103⁺ DCs and Tregs than did the other lines used, and showed the most profound aggravation of cGN. The conditional reduction of CD103⁺ DC numbers by 50% in Langerin-DTR mice halved Treg numbers, which did not suffice to significantly aggravate cGN. Mice lacking the cytokine Flt3L had fewer CD103⁺ DCs and Tregs than Langerin-DTR mice but exhibited milder cGN than did Batf3⁻/⁻ mice presumably because proinflammatory CD11b⁺ DCs were somewhat depleted as well. Conversely, Flt3L supplementation increased the number of CD103⁺ DCs and Tregs, but also of proinflammatory CD11b⁺ DCs. On antibody-mediated removal of CD11b⁺ DCs, Flt3L supplementation ameliorated cGN. Mechanistically, CD103⁺ DCs cause cocultured T cells to differentiate into Tregs and produced the chemokine CCL20, which is known to attract Tregs into the kidney. Our findings show that CD103⁺ DCs foster intrarenal FoxP3⁺ Treg accumulation, thereby antagonizing proinflammatory CD11b⁺ DCs. Thus, increasing CD103⁺ DC numbers or functionality might be advantageous in cGN.


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In the absence of pathogen– or danger–associated molecular patterns, kidney DCs tolerate naïve T cells, whereas the presence of such patterns causes kidney DCs to mature, thereby acquiring the capacity to activate T cells. Some kidney DCs do not leave the kidney and regulate infiltrating effector Th cells. In murine models of human crescentic GN (cGN), such as nephrotoxic serum nephritis, kidney–resident DCs initially display an anti-inflammatory role, which includes the recruitment of regulatory iNKT cells. Later, when inflammation becomes chronic, DCs mature and produce chemokines and cytokines that attract and stimulated Th1 and Th17 cells, resulting in a mononuclear tubulointerstitial infiltrate that drives disease progression. From day 7 after cGN induction, FoxP3+ regulatory T cells (Tregs) play another important counter–regulatory role, and Treg depletion aggravates cGN.11–13 The suppressive mechanism used by Tregs is not exactly clear but involves the immunosuppressive cytokine IL-10.14 The recruitment of Tregs into the nephritic kidney requires the chemokine receptor CCR6 in Tregs but it is unclear which cell produces CCL20, the only known CCR6 ligand.16

More than 90% of kidney DCs belong to a DC subset characterized by expression of the integrin CD11b and the chemokine receptor CX3CR1, which shares phenotypic and functional characteristics with tissue macrophages.17–19 There is another subset of tissue DCs characterized by expression of the integrin CD103 that is not considered to overlap with macrophages.18,20–22 In the lung, liver, or intestine, CD103+ DCs constitute 20%–50%, whereas they represent a minority of <5% of DCs in the kidney.20 Their development depends on the transcription factor Batf3 and the hematopoietic growth factor Flt3L, whereas CD11b+ DCs largely depend on the NF-κB component RelB and only partially depend on Flt3L.20,22–26 In lymphatic tissues, Batf3-dependent DCs express CD8 instead of CD103.27 Batf3-dependent DCs are best characterized by expression of the integrin CD103 that is not considered to overlap with macrophages.18,20–22 In the lung, liver, or intestine, CD103+ DCs constitute 20%–50%, whereas they represent a minority of <5% of DCs in the kidney.20 Their development depends on the transcription factor Batf3 and the hematopoietic growth factor Flt3L, whereas CD11b+ DCs largely depend on the NF-κB component RelB and only partially depend on Flt3L.20,22–26 In lymphatic tissues, Batf3-dependent DCs express CD8 instead of CD103.27 Batf3-dependent DCs are best known for their ability to produce IL-12 and crosspresent antigens to CD8+ T cells.27,28–31 These T cells are important for the antiviral defense but may also cause immune-mediated diseases, such as type 1 diabetes mellitus and perhaps, FSOG.33 In the intestine, CD103+ DCs served classic DC functions in the induction of adaptive immunity after migration to draining lymph nodes.34,35 However, they induced Treg differentiation ex vivo, suggesting that they might regulate tissue homestasis.36–38 However, experimental evidence supporting this notion is lacking, and Batf3−/− mice that lack CD103+ DCs showed unaltered inflammatory bowel disease.39 The role of renal CD103+ DCs is poorly understood. Here, we addressed this question in cGN and discovered an immunoregulatory role of these DCs in the kidney.

RESULTS

CD103+ DCs Accumulate in the Kidney during cGN

We induced cGN in C57BL/6 wild–type (WT) mice and enumerated DC subsets in kidney single–cell suspensions from healthy and nephritic mice at different time points using flow cytometry. Although CD103+ DCs represented a minority among the renal immune cells under homeostatic conditions, they accumulated appreciably during cGN (Figure 1B). The numbers of CD103+ DCs did not change much in the first 4 days of cGN but more than tripled during days 4 and 10, peaking at day 7 (Figure 1C). CD11b+ DCs increased similarly (Figure 1D) as described before. These results showed that not only CD11b+ DCs but also, CD103+ DCs accumulate in kidney inflammation, suggesting that the latter might play a functional role from day 7 onward.

CD103+ DCs Are Protective in cGN

To clarify the role of CD103+ DCs in cGN, we used Batf3−/− mice, which lack these DCs but not CD11b+ DCs.26 Healthy Batf3−/− mice do not show spontaneous renal pathology.26 We first verified that CD103+ DC numbers were reduced by 99.9% in kidneys of nephritic Batf3−/− mice, whereas CD11b+ DC numbers were unchanged (Figure 2A). On day 7 after disease induction, cGN was notably aggravated in Batf3−/− mice compared with WT controls, which was evident by diminished creatinine clearance and a tendency toward higher proteinuria (Figure 2, B and C). Compared with WT mice, the histologic analysis showed significantly increased numbers of glomeruli with extracapillary proliferates, which were more severe (e.g., often circumferential in Batf3−/− versus segmental in WT mice) (Figure 2, D and E). On day 12 of cGN, kidney function was even more reduced (Figure 2, F and G). Histologic analysis revealed more severe glomerular damage (Figure 2, H and I). These results indicated that CD103+ DCs are protective in cGN.

Reduced IL-10 Production in Nephritic Batf3−/− Mice

When we analyzed parameters for intrarenal inflammation, we noted higher levels of the proinflammatory cytokine TNFα and a trend toward higher levels of IL-6 and more IFNγ–producing Th1 cells (Figure 3A). Although the anti–inflammatory cytokine TGFβ was unchanged, IL-10 was markedly reduced (Figure 3B). IL-10 plays an important regulatory role in cGN, and it can be produced by Tregs in this model.14

We determined the cellular source of IL-10 in cGN with the use of double–knockin reporter mice (Foxp3IRES-mRFP × IL-10 irex gfp–enhanced reporter), which allow sensitive simultaneous detection of IL-10 (GFp+) and FoxP3 (mRFP+). Phenotypic characterization of mRFP+ Tregs revealed expression of CCR6 (Supplemental Figure 1), consistent with its reported function to recruit Tregs into the kidney.15 Furthermore, we detected the transcription factors helios and neuropilin, suggesting thymic derivation, the IL-10 receptor, ICOSL, and CTLA-4 but not LAG3 (Supplemental Figure 1). IL-10 was detectable in intrarenal FoxP3+ Tregs, as expected, and also, CD103+ DCs were positive, but there were no IL-10+ CD11b+ DCs (Figure 3C). IL-10+ Tregs were more numerous than IL-10+ CD103+ DCs (Figure 3D). These results suggests
that CD103+ DCs maintain intrarenal IL-10 production indirectly via Tregs and to a smaller extent (also directly), by producing IL-10 themselves.

Reduction of Renal FoxP3+ Tregs but Not iNKT Cells in Nephritic Batf3−/− Mice

Tregs are potent anti-inflammatory regulators in cGN. When we enumerated FoxP3+ Tregs on day 7 of cGN, they were 65% less numerous in kidneys of Batf3−/− mice compared with WT controls (Figure 4A). By contrast, in CX3CR1GFP/GFP mice, which lack renal CD11b+ DCs but not CD103+ DCs, Treg numbers were unaltered (Figure 4B), indicating that CD103+ DCs but not CD11b+ DCs regulate intrarenal Treg numbers.

We next asked whether CD103+ DCs also regulate the other previously described renoprotective T cell subsets, iNKT cells. Intrarenal CD1d–tetramer+ TCRβ+ was not significantly reduced in Batf3−/− compared with WT mice (Figure 4D), arguing against an impaired anti-inflammatory iNKT cell response in the absence of CD103+ DCs. It has to be kept in mind that this experiment does not permit conclusions on the baseline activation status of iNKT cells or their immunosuppressive capacity and therefore, does not absolutely exclude a role of iNKT cells in Batf3−/− mice. Nevertheless, these results support a role of CD103+ kidney DCs in the maintenance of FoxP3+ Tregs but not iNKT cells.

No Influence of Batf3 Deficiency on Systemic Nephritogenic Immune Responses

The reduction of intrarenal Tregs might theoretically result from a systemic reduction of these cells in Batf3−/− mice. We, therefore, enumerated Tregs in the spleen and excluded this alternative explanation by showing unchanged numbers of FoxP3+ Tregs in nephritic WT and Batf3−/− mice (Figure 5A). Likewise, numbers of splenic IFNγ+ CD4+ Th1 cells, CD11b+ DCs, and F4/80+ macrophages were unaltered, whereas CD8+ DCs were almost absent in spleens of Batf3-deficient mice (Figure 5A).

To corroborate these findings, we asked whether Batf3−/− mice mounted a stronger nephritogenic immune response in cGN. To address this possibility, we isolated splenocytes from nephritic WT and Batf3−/− mice and restimulated them in vitro with nephrotoxic sheep serum. Cytokine analysis of supernatants showed no significant difference in the production of TNFα and IFNγ between the two groups (Figure 5B). As another parameter for the nephritogenic Th cell response, we measured serum antibody titers against sheep Ig, the nephritogenic antigen planted into the kidney. Titers of sheep IgG1, characterizing Th2 responses, and specific IgG2a, characterizing Th1 responses, were not altered between WT and Batf3−/− mice (Figure 5C). Taken together, these data argued against an intensified systemic humoral or cellular immune response in nephritic Batf3−/− mice. Thus, a local effect of renal CD103+ DCs likely affected cGN in the kidneys of these mice.
Depletion of CD103+ DCs Reduces Intrarenal Tregs

To corroborate this interpretation, we decided to deplete CD103+ DCs with the use of Langerin–diphtheria toxin receptor (DTR) mice. Langerin is expressed by renal CD103+ DCs but not by CD8+ DCs,20 the Batf3–dependent DC type in lymphatic tissues where systemic immune responses are induced.27 Therefore, Langerin-DTR mice allow us to distinguish between the roles of CD103+ kidney DCs and CD8+ splenic DCs. We induced cGN in these mice and depleted CD103+ DCs every second day by injecting diphtheria toxin (DT). As controls, WT mice were treated with the same amount of DT. On day 7 of nephritis, numbers of CD103+...
kidney DCs in Langerin-DTR mice were 50% reduced (Figure 6A), whereas the numbers of CD11b+ kidney DCs were not significantly changed (WT, 272,571 ± 63,473; Langerin-DTR, 198,501 ± 42,613; n = 4; P = 0.05). Intrarenal Treg numbers were reduced by about 50% as well (Figure 6B). Creatinine clearance seemed to be lower in mice depleted of CD103+ DCs, albeit not to a statistically significant level (Figure 6C). Crescent formation was not different (Figure 6D). The less pronounced disease aggravation in Langerin-DTR mice compared with Batf3-deficient mice is likely because of the reduction of CD103+ DCs by only 50% in the former compared with 99% in the latter. Nevertheless, these findings supported the linkage between CD103+ DCs and Tregs and argued against a role of CD8+ lymphatic tissue DCs in the regulation of intrarenal Tregs.

**Figure 3.** Reduced intrarenal anti-inflammatory cytokine production in nephritic Batf3−/− mice. (A) Concentrations of TNFα and IL-6 in supernatants of kidneys of nephritic Batf3−/− and WT mice measured by bead assay. Intracellular staining for IFNγ in CD45+ CD4+ TCRβ+ cells from kidney single-cell suspensions. (B) Quantitative RT-PCR analysis of TGFβ and IL-10 expression in kidneys from nephritic WT and Batf3−/− mice 7 days after cGN induction. mRNA levels are expressed as x-fold of healthy controls. (C) Treg numbers from kidneys of Foxp3IRES-mRFP × IL-10 ires gfp–enhanced reporter (Fir/Tiger) were stained for CD4+ and RFP+, indicating Foxp3+ cells; DCs were stained for MHC II+ CD11c+ and further subdivided into CD103+ and CD11b+ DCs. Representative contour plots depicting IL-10− and IL-10+ Treg, IL-10− and IL-10+ CD103+ kidney DCs, and IL-10− and IL-10+ CD11b+ kidney DCs. (D) Absolute numbers of renal IL-10− Foxp3+ Treg, IL-10+ CD103+ kidney DCs, and IL-10− CD11b+ kidney DCs. (E) IL-10 production measured by bead assay in supernatants of FACS–sorted CD103+ DCs, CD11b+ DCs, and CD45− nonimmune cells from WT mice 7 days after cGN induction. Data are representative of two independent experiments using (A–D) four to eleven or (E) 10 mice per group. Statistical significance was tested by (A and B) two-tailed Mann–Whitney U test or (D and E) Kruskal–Wallis test with Dunn’s multiple postcomparison test. Ctrl., control; n.d., not detectable. *P < 0.05; **P < 0.01.

Aggravated cGN Severity and Fewer Intrarenal FoxP3+ Tregs in Flt3L-Deficient Mice

Because the aggravation of cGN in Langerin-DTR mice was not pronounced enough, we wished to corroborate the protective role of CD103+ DCs by using another mouse line harboring fewer of these cells. To this end, we induced cGN in Flt3L−/− mice, in which CD8+ and CD103+ DCs are reduced in other tissues.20,41 We first determined that CD103+ DCs were reduced in kidneys of nephritic Flt3L−/− mice by >80% (Figure 7A). We also noted a significant reduction of CD11b+ DCs, albeit to a lesser extent (Figure 7B). Intrarenal Foxp3+ Treg numbers were decreased by >50% in nephritic Flt3L−/− mice compared with WT controls (Figure 7C), whereas renal iNKT cells were not significantly altered (WT, 23,260 ± 10,322; Flt3L−/−, 22,983 ± 5608; n = 3; P > 0.05), matching the observations in...
Batf3$^{-/-}$ mice (Figure 4D). Creatinine clearance was lower in Flt3L$^{-/-}$ mice than in WT controls (Figure 7D). Albuminuria and glomerular crescents, however, did not statistically differ between nephritic WT and Flt3L$^{-/-}$ mice 7 days after disease induction (Figure 7, E and F). The less severe phenotype in Flt3L$^{-/-}$ mice might be explained by the previous finding that this cytokine also fosters CD11b$^+$ DCs, albeit not as effectively as CD103$^+$ DCs.25 Flt3L Supplementation Increases Intrarenal T$_{reg}$s and Attenuates cGN When Combined with Depletion of CCR2$^+$ CD11b$^+$ DCs

Previous studies have applied Flt3L to increase protective CD103$^+$ DC numbers in the intestine and succeeded to attenuate experimental inflammatory bowel disease.42 When we used this strategy in cGN, Flt3L, indeed, increased numbers of renal CD103$^+$ DCs and T$_{reg}$s (Figure 8, A and B). However, CD11b$^+$ DCs were increased (Figure 8C), which is consistent with previous studies showing that Flt3L also exerts a minor effect on CD11b$^+$ DCs.20,23–25 This increase might counteract that of CD103$^+$ DCs, which may explain why disease severity was unaltered (Figure 8D).

To overcome this problem, we decided to remove the recruited CD11b$^+$ DCs with the use of CCR2 antibodies, which specifically targets inflammatory myeloid cells but not T cells.43 Also, under these conditions, Flt3L supplementation increased CD103$^+$ DCs and T$_{reg}$s (Figure 8, E and F), but the number of CD11b$^+$ DCs did not increase (Figure 8G). Indeed, creatinine clearance was higher and albuminuria was lower in mice supplemented with Flt3L (Figure 8, H and I). The proportion of crescentic glomeruli was not altered (Figure 8I), despite the histologic impression of improved morphology (Figure 8K). These findings showed that increasing only CD103$^+$ DCs but not CD11b$^+$ DCs increases renal T$_{reg}$s and attenuates renal function in cGN.
Figure 5. Batf3-deficiency does not affect the systemic nephritogenic immune response. (A) Quantification of splenic FoxP3+ Tregs, IFNy+ CD4+ Th1 cells, CD11b+ DCs, F4/80+ macrophages, and CD8+ DCs 7 days after NTS challenge. (B) Concentrations of TNFα and IFNγ in supernatants of kidneys of nephritic WT and Batf3−/− mice measured by bead assay. (C) Titers of mouse anti-sheep IgG and isotypes of IgG1 and IgG2a were measured in sera by ELISA. Analyses were performed 7 days after cGN induction. Results were combined from two experiments, with 4–11 mice per group. Statistical significance was tested with the two-tailed Mann–Whitney U test. ***P<0.001.

Figure 6. Conditional depletion of CD103+ DCs reduces Tregs. Numbers of (A) CD103+ DCs and (B) Tregs, (C) creatinine clearance, (D) percentage of crescentic glomeruli, and representative immunohistology in Langerin-DTR or WT mice 7 days after cGN induction that were injected with DT on days −1, 1, 3, and 5 after cGN induction. Results are representative for two experiments, with four mice per group. Statistical significance was tested with the unpaired t test. Scale bars, 50 μm. *P<0.05.
Figure 7. Flt3L−/− mice harbor less intrarenal Treg and show aggravated cGN (A) CD103+ DCs, (B) CD11b+ DCs, (C) CD4+ FoxP3+ Tregs, (D) creatinine clearance, (E) albuminuria, (F) proportion of crescentic glomeruli, and (G) representative periodic acid–Schiff–stained histologic images of glomeruli 7 days after cGN induction in WT and Flt3L−/− mice. Results are combined from three individual experiments, with 6–13 mice per group. Statistical significance was tested with the two–tailed Mann–Whitney U test. Scale bars, 50 μm. *P<0.05; **P<0.01; ***P<0.001.
CD103+ Kidney DCs Can Induce FoxP3+ Tregs and Produce the Treg–Recruiting Chemokine CCL20 Ex Vivo

To understand how CD103+ kidney DCs sustain intrarenal Tregs, we flow sorted CD103+ and as a control, CD11b+ kidney DCs and cocultured them with naive specific Th cells and antigen in vitro. As a model antigen, we used OVA, and as OVA–specific T cells, we used the well established DO11.10 transgenic T cells, which in contrast to OT-II cells, can be readily transformed into Tregs.44 We crossed DO11.10 to DEREG mice that express a GFP reporter for FoxP3 to conveniently detect Treg conversion by measuring FoxP3 induction. Furthermore, DEREG mice allow for depletion of preexisting Tregs by injecting DT. This allows for the conclusion that FoxP3+ cells have arisen from naive Th cells and have not by

Figure 8. Flt3L increases renal CD103+ DCs and Tregs and attenuates cGN when inflammatory DCs are removed. (A–D) Melanoma cells (5 × 10⁶) expressing Flt3L or OVA as a control were implanted subcutaneously into mice, and on day 9, cGN was induced. After another 7 days, (A) renal CD103+, (B) Tregs, (C) CD11b+ DCs, and (D) creatinine clearance were determined. (E–K) Same experiment as in A–D, except that inflammatory CD11b+ DCs were depleted using CCR2 antibodies. (E)Renal CD103+, (F) Tregs, (G) CD11b+ DCs, (H) creatinine clearance, (I) albuminuria, and (J) the proportion of crescentic glomeruli were determined. K shows representative histologic images stained by Gömöri Methamine silver. Results are representative of two individual experiments, with four to six mice per group in each experiment. Statistical significance was tested with the two–tailed Mann–Whitney U test. Scale bars, 50 μm. *P<0.05; **P<0.01.
expansion of preexisting T\textsubscript{regs} from the donor mice. When we analyzed these cocultures, CD103\textsuperscript{+} kidney DCs but not CD11b\textsuperscript{+} kidney DCs readily induced differentiation of sorted naive CD4\textsuperscript{+} T cells into FoxP3\textsuperscript{+} T\textsubscript{regs} (Figure 9, A and B). In contrast, CD11b\textsuperscript{+} kidney DCs induced more Th effector cells identified as CD4\textsuperscript{+} CD25\textsuperscript{+} FoxP3\textsuperscript{−} DO11.10 cells (Figure 9C).

Finally, we wondered whether CD103\textsuperscript{+} DCs might be able to recruit T\textsubscript{regs} into the kidney. Because the chemokine receptor CCR6 on T\textsubscript{regs} is essential for such recruitment,\textsuperscript{15} we stained CD11c\textsuperscript{+} MHC II\textsuperscript{+} kidney DCs from nephritic mice for the only known CCR6 ligand, CCL20.\textsuperscript{16} Indeed, among the CD103\textsuperscript{+} DCs, we noted a subset that produced considerable levels of this chemokine, whereas no CD11b\textsuperscript{+} DCs did so (Figure 9, D and E). When we isolated CD103\textsuperscript{+} DCs from nephritic kidneys, CCL20 was secreted into the supernatant by CD103\textsuperscript{+} DCs but not by CD11b\textsuperscript{+} DCs (Figure 9F). CCL20 was also produced by nonhematopoietic cells (Figure 9F). CCL20 levels in nephritic kidneys were reduced in Batf3\textsuperscript{−/−} mice compared with WT controls (Figure 9G), showing that mice that lack CD103\textsuperscript{+} DCs. Mechanistic analysis revealed a genetic tools. We found that cGN was severely aggravated in mice that lack CD103\textsuperscript{+} DCs, and some of them documented fewer T\textsubscript{regs} and more inflammation and tissue damage (for example, in the aorta), leading to exacerbated atherosclerosis.\textsuperscript{42} Furthermore, Flt3L supplementation expanded intestinal CD103\textsuperscript{+} DCs and increased T\textsubscript{regs}, thereby attenuating murine inflammatory bowel disease.\textsuperscript{42} When we applied this approach in cGN, Flt3L also increased CD103\textsuperscript{+} DCs and T\textsubscript{regs} in the kidney. Flt3L is known to increase not only CD103\textsuperscript{+} but also, CD11b\textsuperscript{+} tissue DC numbers to a lesser extent,\textsuperscript{20,23–25} and we found that this also applies to the kidney. However, CD11b\textsuperscript{+} DCs are 10 times more numerous in the kidney, so that a small relative increase raises absolute cell numbers as much as the great increase of the scarcer CD103\textsuperscript{+} DCs. This is different in intestine and lung, where CD11b\textsuperscript{+} DCs and CD103\textsuperscript{+} DCs are similarly frequent, so that Flt3L caused a comparatively stronger increase of CD103\textsuperscript{+} DCs.

The concomitant increase of both kidney DCs types might have balanced their anti- and proinflammatory effects, and cGN was unchanged. This unexpected hurdle was overcome by preventing the accumulation of proinflammatory CD11b\textsuperscript{+} DCs using CCR2-depleting antibodies, so that cGN was attenuated. It is unclear whether this approach is feasible in the human situation, but nevertheless, these findings show that increasing CD103\textsuperscript{+} DCs but not CD11b\textsuperscript{+} DCs increases intrarenal T\textsubscript{reg} numbers, supporting causality between these cell types.

We found two hypothetic mechanisms by which CD103\textsuperscript{+} renal DCs might sustain T\textsubscript{reg}. First, in coculture experiments, only these DCs but not CD11b\textsuperscript{+} kidney DCs induced FoxP3\textsuperscript{+} T\textsubscript{regs} \textit{ex vivo}. This is consistent with previous studies showing that intestinal CD103\textsuperscript{+} DCs can drive T\textsubscript{reg} differentiation after migrating to mesenteric lymph nodes when retinoid acid and TGF\textbeta are present.\textsuperscript{45,46} It remains to be seen whether these factors are also required by CD103\textsuperscript{+} kidney DCs, but it is likely that T\textsubscript{reg} induction takes place in the renal lymph nodes given that naive T cells hardly enter nonlymphoid tissues. Second, another hypothetic mechanism is the production of CCL20, the only ligand of CCR6, which is required for recruiting T\textsubscript{regs} into the inflamed brain\textsuperscript{48,49} and kidney.\textsuperscript{50} In encephalitis, CCR6 also impaired the recruitment of pathogenic Th17 cells, so that disease severity was not substantially altered,\textsuperscript{48,49} Th17 cells also play a role in cGN,\textsuperscript{51,52} but the effect of CCR6 on T\textsubscript{regs} prevailed, so that kidneys of CCR6-deficient mice contained fewer T\textsubscript{regs} and more severe cGN was noted.\textsuperscript{15} This phenotype is very similar to what we observed in nephritic Batf3\textsuperscript{−/−} mice.
Figure 9. CD103⁺ kidney DCs can induce FoxP3⁺ T<sub>reg</sub> and produce the T<sub>reg</sub>-attracting chemokine CCL20. (A–C) Kidney single-cell suspensions from C57BL/6xBALB/c mice 1 hour after ip injection of 700 µg OVA per mouse were stained for MHC II⁺ CD11c⁺ DCs and sorted according to CD103 and CD11b expression 7 days after cGN induction; 2⁻³¹⁰⁴ CD103⁺ or CD11b⁺ kidney DCs were cultured with 4⁻¹³¹⁰⁴ DO11.10 × DEREG CD4⁺ T cells purified from DT-treated mice. Shown are (A) absolute numbers of CD4⁺ FoxP3⁺ CD25⁺ KJ126⁺ T<sub>reg</sub> after 6 days and (B) their intracellular FoxP3 expression and (C) absolute numbers of CD4⁺ FoxP3⁻ CD25⁺ KJ126⁺ effector Th cells. Cytokine-induced T<sub>reg</sub> served as positive controls. Negative controls were unstimulated T cells. (D–F) CCL20 protein expression shown as (D) representative histograms and (E) MFI of flow cytometric analysis or (F) ELISA analysis of culture supernatants of
Moreover, CCL20 production was abrogated when CD103+ DCs were lacking. These findings suggest that CD103+ renal DCs regulate Treg numbers by promoting their differentiation and/or recruiting them into nephritic kidneys, but future studies are required to validate these hypotheses.

During glomerular inflammation, FoxP3+ Tregs are essential for the regulation of Th1 immune responses,11–13 and IL-10 is a major mechanism by which they do so.14 In line with diminished Treg numbers, we observed a profound reduction of IL-10 in kidneys of nephritic Batf3−/− compared with WT mice, and FoxP3+ Tregs, indeed, were a major source of this cytokine. However, also, CD103+ DCs produced some IL-10 and may, thus, potentially attenuate nephritis directly. This would explain previous studies showing a large proportion of IL-10−/− immune cells other than Tregs in the kidney.14,53 It is also consistent with the particularly strong IL-10 production by kidney DCs at late stages of cGN7 when CD103+ DCs are especially numerous. However, the superior IL-10 production by Tregs will hamper the formal experimental proof that CD103+ DC−derived IL-10 is protective.

CD103+ DCs can play a proinflammatory role under infections conditions (for example, by activating antiviral CD8+ T cell responses through crosspresentation).30,54 Our results document an anti-inflammatory function in sterile inflammation. This may help explain why the deletion of all CD11c+ DCs aggravated some models of kidney inflammation9,55 but attenuated others.3,5,7 It is possible that, in these studies, either protective CD103+ DCs or harmful CD11b+ DCs were operative.

In summary, we showed that CD103+ kidney DCs are protective in cGN and antagonize the proinflammatory functions of CX3CR1−dependent CD11b+ kidney DCs. These findings identify a major role of a minor DC subset in immune-mediated GN. Strategies to improve the functions of CD103+ DCs might be of therapeutic use in this disease and potentially, in other inflammatory diseases.

CONCISE METHODS

Mice, Reagents, and cGN Model

We bred 8- to 12-week-old C57BL/6, Batf3−/−, Flt3L−/−, Langerin-DTR, Foxp3ires-mRFP × IL-10 ires gfp–enhanced reporter, and DO11.10 × DEREG mice and kept them under specific pathogen-free conditions at the animal facility of the University Clinic Bonn. cGN was induced by intraperitoneal (ip) injection of 0.35–0.45 ml nephritis serum per mouse as previously described.3,56 CD103+ DCs were depleted in Langerin-DTR mice by ip injection of 8 ng DT per 1 g body wt.57 DO11.10 × DEREG mice were treated with 40 ng/g mouse DT ip to deplete Foxp3+ Tregs.58 Reagents were from Sigma-Aldrich (St. Louis, MO) if not specified otherwise. All animal studies have been approved by a Governmental Review Board (Landesamt für Natur, Umwelt und Verbraucherschutz, Recklinghausen, Germany); 5 × 107 B16 melanoma cells expressing either OVA or Flt3L9,60 were implanted subcutaneously. Inflammatory DCs were depleted using 20 μg of MC-21 per mouse per day, for five days. MC-21 was described as an depleting anti-CCR2 antibody.43

Histology

Light microscopy was performed on 3- to 5-μm paraffin sections of paraformaldehyde–fixed tissue stained by periodic acid–Schiff and Gömöri Methamine silver according to standard protocols. Kidney damage was histologically determined and documented by a nephropathologist blinded to the experimental groups.

Isolation of DCs from Murine Kidneys

Kidneys were digested with collagenase (Roche Diagnostics, Indianapolis, IN) and DNase-I as described previously.2 Tubular fragments were removed by filtration. CD11b+ DCs or CD103+ DCs from kidneys were sorted with a BD Aria Sorter (Becton Dickinson, San Diego, CA). Purity was usually 90%–95%.

Flow Cytometry

After treatment with Fc-blocking antibodies (Privigen; CLS Behring GmbH), cells were stained for 20 minutes at 4°C with fluorochrome–labeled mAbs against CD45 (clone 30-F11), CD11c (HL3), I-Aβ (AF6–120.1), CD11b (M1/70), CD103 (2E7), F4/80 (BM8), Ly6G (1A8), CD4 (GK1.5), TCRβ (H57–597), CD25 (pC61), and FoxP3 (FJK-16s). All antibodies were from BioLegend (San Diego, CA) if not indicated otherwise. Absolute cell numbers were determined with Calibrite APC Beads (BD Biosciences, San Jose, CA), and cells were analyzed with a BD Canto II (Becton Dickinson).

Cytokine Measurement

Cytokines in kidney supernatants were determined by Flowcytometry Bead Assay (eBioscience, San Diego, CA) according to the manufacturer’s instructions. For cell type–specific measurements, single-cell suspensions were incubated in RPMI (10% FCS; Gibco, Carlsbad, CA) with 1 μl/ml GolgiPlug (BD Biosciences) for 4 hours at 37°C. After cell surface staining, cells were fixed in 2% paraformaldehyde for 10 minutes on ice and permeabilized with PBS containing 2% BSA and 0.5% Saponin for 20 minutes at room temperature. Intracellular staining was performed in that buffer for 20 minutes at room temperature using fluorochrome-labeled mAbs against TNFα (clone MP6-XT22), IL-6 (MP5–20F3), and IFNγ (XMG1.2; BD Biosciences). CCL20 was measured by ELISA (Bio-Technne).

sorted cells from mice 7 days after cGN induction. Splenic DCs stimulated with LPS for 2 hours served as positive controls for the staining, and negative controls were stained using isotype antibodies. (G) CCL20 mRNA levels in WT and Batf3−/− mice 7 days after cGN induction. Statistical significance was tested using (A–F) Kruskal–Wallis test with Dunn’s multiple comparison test and (G) unpaired t test. contr., Control; neg., negative; pos., positive. *P<0.05.
In Vitro T<sub>reg</sub> Induction
NTS-treated mice were injected with 700 μg OVA 1 hour before DC isolation; 2×10<sup>4</sup> sorted kidney DCs were cocultured with 4×10<sup>4</sup> naïve Th cells in 200 μl RPMI medium (10% FCS; Gibco) in 96-well plates for 6 days. Naïve Th cells were obtained by sorting CD4<sup>+</sup>CD25<sup>−</sup> GFP<sup>+</sup> T cells from spleens of DT-treated DO11.10 × DEREG mice with an Aria Sorter (Becton Dickinson). Purity was usually about 95%. Because DO11.10 cells are H<sub>2</sub>-K<sup>d</sup> restricted, we DEREG mice with an Aria Sorter (Becton Dickinson). Purity was usually about 95%. Because DO11.10 cells are H<sub>2</sub>-K<sup>d</sup> restricted, we used C57BL/6 × Balb/c F1 mice as DC donors, ensuring that DO11.10 cells can recognize antigen. As a positive control for T<sub>reg</sub> generation, we coated wells with αCD3 (1 μg/ml) and added sorted T cells, αCD28 (2 μg/ml; BD Biosciences), TGFβ (5 ng/ml; Peprotech), and IL-2 (100 U/ml; Proleukin; Novartis, Basel, Switzerland).

RNA Preparation and RT-PCR
Whole-tissue RNA was extracted using the RNA NucleoSpin Kit (Macherey-Nagel) according to the manufacturer’s instructions. RNA was reverse transcribed into cDNA using the High-Capacity Reverse Transcription Kit and amplified by 40 PCR cycles using SYBR Green (Applied Biosystems, Foster City, CA). The following primer sequences were used:

IL-10 (forward): 5′-CCAGTTTTACCTGGTAGAAGTGATG-3′;
IL-10 (reverse): 5′-GTCTAGTCTGGAGTCCTGGAGTCCAGCAGACTC-3′;
TGFβ (forward): 5′-GCTCGCTTTGTACAACAGCACC-3′; and
TGFβ (reverse): 5′-GCGGTTCCACCATTAGCAGC-3′.

All samples were run in triplicate and normalized to GAPDH.

Miscellaneous Assays
For the analysis of albumin excretion and creatinine clearance, urine was collected over 18 hours using metabolic cages. Albumin concentrations in the urine were measured using a commercial mouse albumin ELISA kit according to the manufacturer’s instructions (Bethyl Laboratories, Inc., Montgomery, TX). Serum and urine creatinine concentrations were analyzed in the central laboratory of the University Hospital of Bonn. Serum levels of murine IgG1 and IgG2a specific for sheep Ig were determined by ELISA (Bethyl Laboratories, Inc.).

Statistical Analyses
Results are expressed as means±SEM. Comparisons were drawn using a two-tailed unpaired t, Kruskal–Wallis, or two–sided nonparametric Mann–Whitney U test as indicated. P values <0.05 were considered significant.

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DISCLOSURES
None.

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