CCR2 antagonist CCX140-B provides renal and glycemic benefits in diabetic transgenic human CCR2 knockin mice

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Sullivan T, Miao Z, Dairaghi DJ, Krasinski A, Wang Y, Zhao BN, Baumgart T, Ertl LS, Pennell A, Seitz L, Powers J, Zhao R, Ungashe S, Wei Z, Boring L, Tsou C, Charo I, Berahovich RD, Schall TJ, Jaen JC. CCR2 antagonist CCX140-B provides renal and glycemic benefits in diabetic transgenic human CCR2 knockin mice. Am J Physiol Renal Physiol 305: F1288–F1297, 2013. First published August 28, 2013; doi:10.1152/ajprenal.00316.2013.—Chemokine (C-C motif) receptor 2 (CCR2) has been implicated in the pathogenesis of type 2 diabetes and its associated comorbidities, particularly diabetic nephropathy (15). For decades, the presence of systemic markers of inflammation has been known to increase with obesity. Adipose tissue produces and secretes inflammatory cytokines, and chemokines, including TNF-α, IL-6, and CCL2, which correlate in expression levels with the degree of adiposity (13). CCL2 and inflammatory cytokines impair insulin-stimulated glucose uptake in human adipocytes and skeletal muscle cells, providing a link between inflamed adipose tissue and insulin resistance (36, 40). In fact, forced expression of CCL2 in adipose tissue, by itself, induces insulin resistance (19). In adipose tissue, macrophages are the primary source of proinflammatory mediators (53), and the number of adipose tissue macrophages increases with increasing adiposity (52). The number of macrophages in adipose tissue is dynamic, and weight loss or gain rapidly alter macrophage content in adipose tissue (6). In animal models of insulin resistance and type 2 diabetes, the CCR2-CCL2 axis is a primary control point for the entry of inflammatory macrophages into the adipose tissue of obese rodents (21, 30). In these models, both CCR2-deficient mice and CCL2-deficient mice have reduced macrophage numbers in adipose tissue and display significantly improved metabolic parameters relative to wild-type mice (9, 10, 45, 51). Moreover, CCR2 pharmacological inhibition has been shown to reduce adipose tissue inflammation and improve metabolic parameters (22, 43, 44, 54). Despite extensive efforts to identify pharmacologically suitable CCR2 drug candidates, to date only one CCR2 antagonist, orally active CCX140-B, has been shown to improve glycemic parameters (hemoglobin A1c and fasting blood glucose) in diabetic patients (16, 35).

CCR2 has also been linked to diabetic nephropathy, in both human subjects and experimental models (15). CCL2 levels increase in the glomerulus and tubulointerstitium (10, 28, 37) and correlate with macrophage recruitment (8, 25, 45). CCL2 levels in the urine also increase during disease and correlate with the levels of proteinuria and/or serum glycated albumin (3, 29, 42). Glomerular CCL2 expression is increased in patients with diabetic nephropathy (45). Diabetic mice deficient in CCL2 (9, 10, 45) or treated with CCR2 antagonists (20, 22, 32, 38) exhibit reduced proteinuria.

As indicated above, CCX140-B was recently shown to be well tolerated and effective at improving glycemic parameters in subjects with type 2 diabetes and normal renal function (16). In this report, we describe, for the first time, the effects of CCX140-B on hyperglycemia, insulin sensitivity, and renal function/pathology in two mouse models of diabetes/nephropathy. These experiments provide, in part, the rationale for the ongoing clinical evaluation of CCX140-B in two separate phase 2 clinical trials in diabetic nephropathy (www.clinicaltrials.gov, study numbers NCT01447147 and NCT01440257). Due to the specificity of CCX140-B for human CCR2, we generated mice and chemokines, including TNF-α, IL-6, and CCL2, which correlate in expression levels with the degree of adiposity (13). CCL2 and inflammatory cytokines impair insulin-stimulated glucose uptake in human adipocytes and skeletal muscle cells, providing a link between inflamed adipose tissue and insulin resistance (36, 40). In fact, forced expression of CCL2 in adipose tissue, by itself, induces insulin resistance (19). In adipose tissue, macrophages are the primary source of proinflammatory mediators (53), and the number of adipose tissue macrophages increases with increasing adiposity (52). The number of macrophages in adipose tissue is dynamic, and weight loss or gain rapidly alter macrophage content in adipose tissue (6). In animal models of insulin resistance and type 2 diabetes, the CCR2-CCL2 axis is a primary control point for the entry of inflammatory macrophages into the adipose tissue of obese rodents (21, 30). In these models, both CCR2-deficient mice and CCL2-deficient mice have reduced macrophage numbers in adipose tissue and display significantly improved metabolic parameters relative to wild-type mice (9, 10, 45, 51). Moreover, CCR2 pharmacological inhibition has been shown to reduce adipose tissue inflammation and improve metabolic parameters (22, 43, 44, 54). Despite extensive efforts to identify pharmacologically suitable CCR2 drug candidates, to date only one CCR2 antagonist, orally active CCX140-B, has been shown to improve glycemic parameters (hemoglobin A1c and fasting blood glucose) in diabetic patients (16, 35).

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THE MOVEMENT OF LEUKOCYTES throughout the body under homeostatic and inflammatory conditions is exquisitely orchestrated by the actions of chemokines and chemokine receptors (7, 48). Chemokine (C-C motif) receptor 2 (CCR2) is primarily expressed on monocytes in the circulation, although expression can also be seen on certain T cell populations. CCR2 has four known ligands, chemokine (C-C motif) ligand (CCL)2 [monocyte chemotactic protein (MCP)-1], CCL8 (MCP-2), CCL7 (MCP-3), and CCL13 (MCP-4). CCL2 is the most selective of these chemokines for CCR2 and has been the most broadly studied CCR2 ligand.

CCR2 has also been implicated in the pathogenesis of type 2 diabetes and its associated comorbidities, particularly diabetic nephropathy (15). For decades, the presence of systemic markers of inflammation has been known to increase with obesity. Adipose tissue produces and secretes inflammatory cytokines, including TNF-α, IL-6, and CCL2, which correlate in expression levels with the degree of adiposity (13). CCL2 and inflammatory cytokines impair insulin-stimulated glucose uptake in human adipocytes and skeletal muscle cells, providing a link between inflamed adipose tissue and insulin resistance (36, 40). In fact, forced expression of CCL2 in adipose tissue, by itself, induces insulin resistance (19). In adipose tissue, macrophages are the primary source of proinflammatory mediators (53), and the number of adipose tissue macrophages increases with increasing adiposity (52). The number of macrophages in adipose tissue is dynamic, and weight loss or gain rapidly alter macrophage content in adipose tissue (6). In animal models of insulin resistance and type 2 diabetes, the CCR2-CCL2 axis is a primary control point for the entry of inflammatory macrophages into the adipose tissue of obese rodents (21, 30). In these models, both CCR2-deficient mice and CCL2-deficient mice have reduced macrophage numbers in adipose tissue and display significantly improved metabolic parameters relative to wild-type mice (9, 10, 45, 51). Moreover, CCR2 pharmacological inhibition has been shown to reduce adipose tissue inflammation and improve metabolic parameters (22, 43, 44, 54). Despite extensive efforts to identify pharmacologically suitable CCR2 drug candidates, to date only one CCR2 antagonist, orally active CCX140-B, has been shown to improve glycemic parameters (hemoglobin A1c and fasting blood glucose) in diabetic patients (16, 35).

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expressing human (h)CCR2 in place of mouse CCR2 [hCCR2 knockin (KI) mice]. In hCCR2 KI mice rendered diabetic with a high-fat diet [diet-induced obesity (DIO)] or by deletion of the leptin receptor gene (db/db), CCX140-B either prevented albuminuria from worsening or reduced albuminuria outright. Improvement in renal function coincided with decreases in glomerular hypertrophy and increases in podocyte density. In DIO hCCR2 KI mice, CCX140-B also reduced adipose tissue inflammatory macrophage content, reduced fasting blood glucose and insulin levels, and improved parameters of systemic insulin sensitivity.

MATERIALS AND METHODS

In vitro assays. CCX140-B (4) for nonclinical work was generated by the Medicinal Chemistry Department at ChemoCentryx (Mountain View, CA). Human monocytes were isolated from healthy volunteer LRS chambers (Stanford Blood Center, Palo Alto, CA) using MACS separation reagents (Miltenyi Biotec, Auburn, CA). Human embryonic kidney (HEK)-293-CCR2 cells were generated by electroporation of HEK-293 cells with an expression plasmid containing the hCCR2B coding region cloned from THP-1 cells with the following PCR primers: 5'-CCAGTAGCGGCCGCTATGCTGTCCACATCTCGTTCTCG-3' and 5'-CAATCAGCGGCCGCCTCGTTTTATAAACCCAGCCGAGAC-3'. CCR2 expression was confirmed by flow cytometry (monoclonal antibody clone 48607, R&D Systems, Minneapolis, MN). Recombinant chemokines were from R&D Systems and PerkinElmer. 125I-labeled CCL2 (125I-CCL2) was from PerkinElmer. Human serum was from Bioreclamation (Hicksville, NY). Chemotaxis, Ca2+ mobilization, and radioligand binding assays were conducted as previously described (49).

Mice. C57BL/6 mice were purchased from Jackson Laboratory (Bar Harbor, ME). CCR2−/− mice were generated as previously described (5). Homozygous hCCR2 KI mice were created on a C57BL/6 background using homologous recombination in embryonic stem cells to replace the murine CCR2 coding region with the hCCR2 coding region, leaving the murine CCR2 promoter intact. The neomycin resistance gene was subsequently spliced out using Cre-recombinase. B6-Leprdb/db mice were purchased from Jackson Labs (stock no. 697). hCCR2 KI Leprdb/db mice were generated by first crossing hCCR2 KI mice with B6-Leprdb/db mice and then crossing the CCR2hu/Leprdb/db progeny with each other. Genotyping of the Lepr alleles was performed by PCR using the following primers: 5'-AGAACGGACACTCTTTGAAGTCTC-3' and 5'-CATTCAAACCATGTTTTACGTTTG-3', followed by digestion with the restriction enzyme RsaI. Leprdb/db mice have two fragments of 108 and 27 bp, Leprdb/db mice have three fragments of 27, 108, and 135 bp, and Lepr+/+ mice have one product of 135 bp. Genotyping of the CCR2 alleles was performed by flow cytometry using whole blood with antibodies specific for hCCR2 (R&D Systems, Minneapolis, MN) and mouse CCR2 (a gift of Matthias Mack, University Hospital Regensburg, Regensburg, Germany). Antibodies Ly6G-FITC (BD Biosciens.

Fig. 1. CCX140-B inhibits chemokine (C-C motif) receptor 2 (CCR2) in human primary monocytes. A and B: CCX140-B inhibited chemokine (C-C motif) ligand (CCL)2 (0.1 nM)-induced chemotaxis of human monocytes in buffer (A) and 100% human serum (B). C and D: CCX140-B inhibited CCL2 (5 nM)-induced Ca2+ mobilization in human monocytes (C) and 125I-labeled CCL2 (125I-CCL2; 50 pM) binding to human monocytes (D). E: saturation binding of [3H]CCX140-B to human primary monocytes.
to begin therapeutic intervention after for the duration of the study. Mice were generally sufficiently diabetic Research Diets) at 6 wk of age and maintained on the respective diet Brunswick, NJ) or a lean control diet (D12450B, 10 Cal% fat, Research Diets, New HOMA-IR homeostatic model assessment of insulin resistance (HOMA-IR). overnight fast (14–16 h). Insulin sensitivity was determined by the diet. Blood glucose and insulin levels were determined after an on a high-fat diet (D12492, 60 Cal% fat, Research Diets, New Systems). Another portion of blood was centrifuged to remove cells, and the plasma was analyzed by ELISA for mouse CCL2 levels (R&D sciences). A portion of blood was collected by cardiac puncture, and a portion was stained by flow cytometry with ligands and CCX140-B in Ca2⁺ mobilization assays. CCX140-B inhibited chemotaxis of human monocytes in 100% human serum toward CCL2/monocyte chemotactic protein (MCP)-1 (A), CCL8/MCP-2 (B), CCL7/MCP-3 (C), and CCL17/MCP-4 (D).

Fig. 2. CCX140-B inhibits multiple CCR2 ligands. CCX140-B inhibited chemotaxis of human monocytes in 100% human serum toward CCL2/monocyte chemotactic protein (MCP)-1 (A), CCL8/MCP-2 (B), CCL7/MCP-3 (C), and CCL17/MCP-4 (D).

HOMA-IR = glucose (in mg/dl) × insulin (in μU/ml)/405 (27). CCX140-B or vehicle control was administered subcutaneously once per day for 2 wk. Body weights were measured before the initiation of treatment and weekly thereafter. At the end of the study, blood was collected by cardiac puncture, and epididymal fat pads were excised. Blood was centrifuged to remove cells, and the plasma was analyzed by ELISA for mouse CCL2 levels (R&D Systems). The fat pads were minced and shaken for 30 min at 37°C in 25 ml buffer (DMEM containing 5% fatty acid-free BSA and 0.01% benzonase, both from Sigma-Aldrich) with 1 mg/ml collagenase type 1 (Worthington, Lake-wood, NJ). The material was filtered through a 70-μm nylon strainer and centrifuged at 400 g for 5 min. Cells were rinsed with 40 ml buffer and incubated for 2 min in 1 ml red blood cell lysing buffer (Sigma-Aldrich), after which 25 ml buffer was added and cells were collected by centrifugation. Cells were analyzed by flow cytometry with antibodies F4/80-PE-Cy5 (eBioscience, San Diego, CA) and CD11c-APC (BD Biosciences).

Table 1. Chemokine receptor selectivity profile of CCX140-B

<table>
<thead>
<tr>
<th>Receptor</th>
<th>Cell Type</th>
<th>IC₅₀, μM*</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCR1</td>
<td>THP-1</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CCR2</td>
<td>THP-1</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CCR4</td>
<td>Activated T lymphocytes</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CCR5</td>
<td>Activated T lymphocytes</td>
<td>&gt;10</td>
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<tr>
<td>CCR6</td>
<td>Activated T lymphocytes</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CCR7</td>
<td>Activated T lymphocytes</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CCR9</td>
<td>MOLT-4</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CCR12</td>
<td>Neutrophils</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CXCR1</td>
<td>Neutrophils</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CXCR2</td>
<td>Neutrophils</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CXCR3</td>
<td>Activated T lymphocytes</td>
<td>&gt;10</td>
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<tr>
<td>CXCR4</td>
<td>Activated T lymphocytes</td>
<td>&gt;10</td>
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<tr>
<td>CXCR6</td>
<td>NSO-CXCR6 transfectant</td>
<td>&gt;10</td>
</tr>
<tr>
<td>CXC7</td>
<td>MDA435-CXCR7 transfectant</td>
<td>&gt;10</td>
</tr>
<tr>
<td>C3aR</td>
<td>HEK-295-C3aR transfectant</td>
<td>&gt;10</td>
</tr>
<tr>
<td>C5aR</td>
<td>L1.2-C5aR transfectant</td>
<td>&gt;10</td>
</tr>
<tr>
<td>ChemR23</td>
<td>HEK-295-ChemR23 transfectant</td>
<td>&gt;10</td>
</tr>
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CCR, chemokine (C-C motif) receptor; CXC, chemokine (C-X-C motif) receptor; C3aR and C5aR, C3a and C5a receptor, respectively; ChemR23, chemerin receptor 23. *Potencies were determined with chemotaxis, Ca2⁺ mobilization, and radioligand binding assays.
wk. At regular intervals, mice were individually housed in metabolic cages for 18 h. Urinary albumin was measured by ELISA (Bethyl Labs, Montgomery, TX), and the urinary albumin excretion rate (UAER) was calculated as micrograms per 24 h. Urinary creatinine was measured by mass spectrometry by the Drug Metabolism and Pharmacokinetics Department at ChemoCentryx. The albumin-to-creatinine ratio (ACR) was calculated as micrograms of albumin per milligram of creatinine. At the end of the experiments, kidneys were collected, fixed in formalin, embedded in paraffin, and cut into 3-μm-thick sections. Sections were stained for podocytes by immunohistochemistry with Wilms tumor protein 1 antibody (Abcam, Cambridge, MA) by standard techniques. Glomerular cross-sectional area and podocyte number were determined in 20–30 glomeruli/mouse. Glomerulus volume and podocyte density were calculated from the immunohistochemistry data using the Weibel method (17). Mesangial expansion was measured by silver methenamine stain on 2-μm-thick paraffin sections by conventional methods.

Statistics. Data were analyzed with Prism (Graphpad Software, La Jolla, CA). Inhibition IC\textsubscript{50} values were calculated by nonlinear regression using one-site competition. Statistical significance was determined by the Mann-Whitney t-test. Values are reported as means ± SE.

RESULTS

CCX140-B inhibits CCR2 in human monocytes. Assessment of compound activity on primary cells and under physiologically relevant conditions (i.e., serum or blood) is an important and necessary part of the characterization of potential drugs (11, 39, 49). CCX140-B potently inhibited CCL2-induced chemotaxis of purified human blood monocytes with IC\textsubscript{50} values of 8 nM (Fig. 1A) in buffer and 200 nM (Fig. 1B) in the presence of 100% human serum. CCX140-B also inhibited CCL2-induced Ca\textsuperscript{2+} mobilization in monocytes with an IC\textsubscript{50} value of 3 nM (Fig. 1C). CCX140-B inhibited the binding of \textsuperscript{125}I-CCL2 to monocytes with an IC\textsubscript{50} value of 17 nM (Fig. 1D). Saturation binding experiments performed with \textsuperscript{[3H]}CCX140-B on monocytes indicated that CCX140-B has a K\textsubscript{d} value of 2.3 nM toward hCCR2 (Fig. 1E). CCX140-B also inhibited monocyte chemotaxis mediated by the other CCR2 ligands: CCL8/MCP-2, CCL7/MCP-3, and CCL13/MCP-4 (Fig. 2). Inhibitory A\textsubscript{2} values were 280 nM for CCL2, 180 nM for CCL8, 250 nM for CCL7, and 280 nM for CCL13. These data indicate that CCX140-B is a potent inhibitor of hCCR2 on primary cells under physiologically relevant conditions. CCX140-B was tested by radioligand binding, Ca\textsuperscript{2+} mobilization, and chemotaxis assays for its effects on other chemokine receptors; however, CCX140-B did not exhibit significant inhibition of any of the receptors tested (Table 1). In addition, CCX140-B was evaluated for interactions with 142 various biologically important receptors and ion channels and was found to lack activity against any of them (Supplemental Table S1 in the

Fig. 3. Leukocytes from human (h)CCR2 knockin (KI) mice respond to multiple mouse CCR2 ligands and can be inhibited by CCX140-B. Thioglycollate-elicited peritoneal leukocytes from wild-type mice (A) and hCCR2 KI mice (B) exhibited similar chemotaxis profiles to mouse (m)CCL2/MCP-1, mCCL7/MCP-3, and mCCL12/MCP-5. C and D: CCX140-B potently inhibited mCCL2/MCP-1-induced chemotaxis of leukocytes isolated from hCCR2 KI mice (C) but not from wild-type mice (D).
Supplemental Material). When tested at concentrations up to 10 μM, CCX140-B was unable to inhibit 125I-CCL2 binding or CCL2-mediated activation of mouse CCR2 (data not shown).

**CCX140-B inhibits CCR2 in hCCR2 KI mice.** Due to the fact that CCX140-B does not bind with high affinity to mouse CCR2, transgenic mice were developed that lack mouse CCR2 and express hCCR2 under the control of the mouse CCR2 promoter (hCCR2 KI mice). hCCR2 KI mice contained similar numbers of peripheral blood monocytes to wild-type mice, indicating that hCCR2 is sufficient for monocyte egress from the bone marrow (data not shown). The functionality of hCCR2 expressed in hCCR2 KI mice and the ability of CCX140-B to inhibit it were tested in vitro and in vivo. In vitro, leukocytes isolated from hCCR2 KI mice migrated toward multiple mouse CCR2 chemokines (CCL2/MCP-1/JE, CCL7/MCP-3, and CCL12/MCP-5) similarly to wild-type mouse leukocytes (Fig. 3, A and B). CCX140-B potently inhibited the chemotaxis of hCCR2 KI mouse leukocytes but not wild-type mouse leukocytes (Fig. 3, C and D). In vivo, hCCR2 KI mice were fully able to mount an inflammatory response in a CCR2-dependent sterile peritonitis model. Three days after the intraperitoneal injection of thioglycollate, hCCR2 KI mice contained large numbers of peritoneal leukocytes, similar to wild-type mice, whereas CCR2−/− control mice contained a reduced number of peritoneal leukocytes (Fig. 4A). Treatment of hCCR2 KI mice with CCX140-B caused a dose-dependent reduction in the number of peritoneal leukocytes after thioglycollate challenge (Fig. 4B): CCX140-B strongly blocked leukocyte infiltration at 30 mg/kg, partially blocked leukocyte infiltration at 10 mg/kg, and failed to block leukocyte infiltration at 3 mg/kg. The trough plasma level of CCX140 in mice treated with 30 mg/kg CCX140-B was 170 nM, which approximates the compound’s IC50 value for inhibition of the human monocyte chemotaxis toward CCL2 in 100% serum. As expected, CCX140-B had no effect on the influx of cells in wild-type control mice (data not shown). CCR2-mediated signaling in peritoneal leukocytes isolated from hCCR2 KI mice was comparable to that in leukocytes from wild-type mice, as assessed by mouse CCL2-mediated Ca2+ mobilization (Fig. 4, C and D). As expected, CCX140-B blocked this response in leukocytes from hCCR2 KI mice (Fig.

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**Fig. 4.** CCX140-B inhibits CCR2 in hCCR2 KI mice. A: hCCR2 KI mice and wild-type C57BL/6 mice were equally responsive to thioglycollate challenge, whereas CCR2−/− [knockout (KO)] mice did not accumulate peritoneal leukocytes after thioglycollate challenge. B: CCX140-B inhibited the thioglycollate response in hCCR2 KI mice in a dose-dependent manner. C: thioglycollate-elicited peritoneal leukocytes isolated from wild-type mice and hCCR2 KI mice mobilized similar amounts of intracellular Ca2+ in response to mCCL2/MCP-1. The addition of CCX140-B blocked mCCL2-mediated Ca2+ mobilization in hCCR2 KI cells (C) but not in wild-type cells (D). E and F: CCX140-B did not alter the frequency of peripheral blood monocytes (E) or the levels of plasma CCL2 (F) in thioglycollate-challenged hCCR2 KI mice. *P < 0.05 relative to vehicle-treated mice.
CCX140-B did not alter the percentage of 7/4⁺ blood monocytes in hCCR2 KI mice challenged with thioglycollate (Fig. 4D), suggesting that the reduction in peritoneal leukocyte numbers was not due to diminished egress of cells from the bone marrow. In addition, thioglycollate-challenged hCCR2 KI mice treated with CCX140-B did not contain elevated levels of plasma CCL2 (Fig. 4F).

**CCX140-B improves albuminuria in DIO hCCR2 KI mice and hCCR2 db/db mice.** hCCR2 KI mice were rendered diabetic by placing them on a high-fat diet (DIO mice) or by deleting the gene encoding the leptin receptor (db/db mice). In DIO hCCR2 KI mice, treatment with 100 mg/kg CCX140-B blocked the progressive increase in UAER (Fig. 5A) and ACR (Fig. 5B) seen in vehicle-treated animals. CCX140-B maintained lower UAER and ACR values during the entire 8-wk dosing regimen, whereas these values increased sixfold in vehicle-treated mice. In the db/db model, 8-wk-old hCCR2 KI db/db mice were treated with 100 mg/kg CCX140-B or vehicle for 6 wk. CCX140-B treatment reduced both UAER and ACR values, whereas vehicle-treated mice experienced no change or sporadic increases in these parameters (Fig. 5, C and D). To analyze the effect of CCX140-B on glomerulus size and podocyte density, kidney sections from DIO hCCR2 KI mice were stained with an antibody specific for the podocyte marker Wilms tumor protein 1. After 8 wk of treatment with CCX140-B, glomerulus size was significantly reduced and podocyte density was significantly increased compared with vehicle-treated mice (Fig. 6). Neither CCX140-B-treated nor vehicle-treated hCCR2 KI mice displayed any substantial degree of mesangial expansion (data not shown), as the DIO and db/db models reflect only some of the early changes of human diabetic nephropathy (2). Serum creatinine and blood urea nitrogen levels were mildly elevated in diabetic hCCR2 KI DIO and db/db mice, with CCX140-B generally tending to decrease both factors (data not shown).

**CCX140-B improves insulin sensitivity in DIO hCCR2 KI mice.** CCX140-B was also tested for its glycemic and anti-inflammatory effects in the DIO model. Mice were randomized to CCX140-B treatment or vehicle when fasting blood glucose levels exceeded 180 mg/dl, typically after 24–26 wk on the high-fat diet. Two weeks of 100 mg/kg CCX140-B treatment significantly reduced both fasting glucose levels (Fig. 7A) and fasting insulin levels (Fig. 7B) in these mice. As a result of the reduction in both fasting glucose and insulin, a significant improvement in insulin sensitivity was seen in CCX140-B-treated mice, as calculated by the HOMA-IR index (Fig. 7C). The improvements in glycemic control and insulin sensitivity coincided with significant reductions in the number of inflammatory (F4/80⁺/CD11c⁺) macrophages present in the epididymal fat pads of the mice (Fig. 7D). Similarly to its behavior in the thioglycollate experiments, CCX140-B improved the diabetic condition of these mice with no change in systemic CCL2 levels (Fig. 7E). Importantly, CCX140-B exerted its beneficial effects without altering body weight (Fig. 7F).

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**Fig. 5.** CCX140-B improves albuminuria in diabetic hCCR2 KI mice. Diet-induced obese (DIO) hCCR2 KI mice and hCCR2 KI db/db mice were treated with vehicle or 100 mg/kg CCX140-B for 6–8 wk. A and B: in DIO hCCR2 mice, CCX140-B initially decreased the urinary albumin excretion rate (UAER) and urinary albumin-to-creatinine ratio (ACR) and thereafter slowed the progressive increase in the these values. C and D: in hCCR2 db/db mice, CCX140-B reduced both values over the entire treatment period. *P < 0.05 and **P < 0.005 relative to vehicle-treated mice.
DISCUSSION

CCX140-B is an orally active, potent, and selective antagonist of human CCR2 and is one of a small group of chemokine receptor antagonists that have advanced into clinical development. Among these, CCX140-B is the only CCR2 antagonist for which clinical data indicative of therapeutic benefit have been published. Specifically, CCX140-B produced a reduction of fasting blood glucose and glycated hemoglobin A1C in type 2 diabetic patients (16). As shown here, using multiple assay formats, CCX140-B effectively inhibits human CCR2 with single-digit nanomolar potency. Under physiologically relevant conditions, i.e., in 100% human serum, CCX140-B inhibits CCL2-mediated chemotaxis of human monocytes with an IC50 value of 200 nM. CCX140-B inhibits the other CCR2 ligands (CCL7, CCL8, and CCL13) with similar potencies.

For many chemokine receptor antagonists taken into clinical trials, assessment of the antagonist in mouse models of disease is prevented by the fact that the antagonist does not potently bind to the mouse ortholog of the chemokine receptor. To overcome this problem, we generated hCCR2 KI mice in which the mouse CCR2 coding region was replaced with the hCCR2 coding region. Importantly, these hCCR2 KI mice displayed no alterations in peripheral blood monocyte numbers or CCL2 levels, and leukocytes isolated from hCCR2 KI mice exhibited similar responses to the murine CCR2 chemokines as did leukocytes isolated from wild-type mice. Multiple reports (5, 23, 24, 26) have shown that interfering with CCR2-CCL2 interactions can inhibit the migration of blood monocytes into multiple tissues, e.g., mice genetically deficient in CCR2 or CCL2 display significantly reduced recruitment of peripheral blood monocytes into the peritoneum after thioglycollate challenge. Our hCCR2 KI mice responded similarly to wild-type mice in the thioglycollate model, indicating that hCCR2 KI mice are able to mount a normal inflammatory response and are suitable for the assessment of CCX140-B activity in vivo. CCX140-B significantly inhibited thioglycollate-mediated monocyte recruitment into the peritoneum in these transgenic mice (to a level similar to that exhibited by CCR2+/− mice). CCR2 is known to be required for the transit of monocytes from the bone marrow to the bloodstream (46); however, CCX140-B did not interfere with this process, as indicated by the normal numbers of peripheral blood 7/4+ monocytes in compound-treated mice. This result may reflect the poor penetration of CCX140-B into the bone marrow, due to the compound’s small volume of distribution.

Recent reports on several CCR2 antagonists have created the impression that CCR2 inhibition in vivo invariably results in the elevation of systemic levels of CCL2, which could be detrimental to any CCL2-driven pathophysiological process. These findings have been reported from numerous groups with disparate molecules and have been seen in rodents, nonhuman primates, and human subjects (1, 34, 47, 50). CCX140-B does not exhibit this type of behavior in vivo, indicating that inhibition of CCR2-mediated cell trafficking can occur with no rise in systemic CCL2 levels. The reason for the difference in CCL2 elevation between the other CCR2 antagonists and CCX140-B is not known but may include lack of selectivity for CCR2 in other compounds (CCX140-B is extremely selective for CCR2 relative to all other chemokine receptors), differences between compounds in the degree of penetration of certain tissues, such as the bone marrow, and unique CCR2-binding kinetics, among others. Ongoing work in our labora-

Fig. 6. CCX140-B reverses glomerular hypertrophy and podocyte density diminution in DIO hCCR2 KI mice. DIO hCCR2 KI mice were treated with vehicle or 100 mg/kg CCX140-B for 8 wk. A and B: CCX140-B reduced glomerulus size (A) and increased the density of podocytes (B). C: representative images of podocyte nucleus staining in vehicle-treated (left) and CCX140-B-treated (right) mice. Note the smaller glomerulus size and increased number of podocyte nuclei in the kidney section from the CCX140-B-treated mouse. Original magnification: ×400. *P < 0.05 and **P < 0.005 relative to vehicle-treated mice.

A

B

C

CCX140-B EFFECTS IN DIABETIC MICE
The role of inflammation in obesity and type 2 diabetes has become increasingly clear in recent years (33). Macrophages are found in increased numbers in adipose and hepatic tissues of insulin-resistant obese humans, and this observation is faithfully reproduced in rodent models of type 2 diabetes (6, 52). CCR2 has been implicated as the major chemokine receptor responsible for the recruitment of monocytes to peripheral tissues in obese diabetic rodents. Although other CCR2 antagonists have been reported to decrease total (F4/80⁺/H11001⁺/H11001) macrophage density in adipose tissue sections (9, 44, 51), CCX140-B was also shown to decrease the number of inflammatory (F4/80⁺/CD11c⁺/H11001) macrophages in adipose tissue in DIO hCCR2 KI mice. The reduction in inflammatory macrophages correlated with improved fasting plasma glucose levels. This benefit was rapid, requiring only 2 wk of treatment. Furthermore, CCX140-B treatment reduced HOMA-IR values, indicating that CCR2 inhibition improves systemic sensitivity to insulin. The reduced HOMA-IR values correlated with reduced glucose and insulin levels in these diabetic mice. Our results provide direct evidence for a causal link between CCR2-mediated macrophage accumulation in adipose tissue and systemic effects on insulin sensitivity.

Diabetic nephropathy is one of the most common and debilitating macrovascular complications of diabetes and is a leading cause of end-stage renal disease. Key hallmarks of diabetic nephropathy include albuminuria, glomerular hypertrophy, and decreased podocyte density, among others. In diabetic nephropathy, there is increased CCL2 expression in the kidney and urine (3, 28, 29, 42) and increased numbers of macrophages in the kidney (14, 31). In diabetic leptin receptor-deficient (db/db) mice, other CCR2 antagonists have slowed or blocked the increase in albuminuria that occurs over time in these mice, but a reduction in albuminuria relative to disease severity at start of the study has not been demonstrated (22, 32, 38, 41). Here, we show that, in diabetic hCCR2 KI db/db mice, CCX140-B reduced albuminuria within 1 wk of treatment and maintained this benefit over the entire 6-wk treatment period. In DIO mice, CCX140-B also reduced albuminuria within 1 wk of the treatment start and thereafter slowed the progressive increase in albuminuria over time. Although the use of urine creatinine to normalize the amount of excreted albumin is less relevant in mice than in humans, because mice both secrete and excrete creatinine (12), CCX140-B produced an equally robust benefit in daily UAERs and ACRs. Diabetic nephropathy is...
characterized by glomerular hyperfiltration (at the single nephron level, if not systemically), which correlates histologically with glomerular hypertrophy and podocyte loss (18). Immunohistological examination of the kidneys of hCCR2-KI db/db mice indicated that CCX140-B substantially decreased glomerular hypertrophy and increased podocyte density; no other CCR2 antagonist has been reported to improve both parameters. Interestingly, the improvements in renal parameters induced by CCX140-B were not secondary to the improvements in glycemic parameters; preliminary experiments have indicated that the former occurs more quickly and/or at lower compound doses than the latter. Hence, CCX140-B may provide therapeutic renal benefits for patients with diabetic nephropathy.

In conclusion, CCX140-B is an orally bioavailable, potent, and selective CCR2 antagonist that demonstrates clear glyceremic and renal benefits in obesity-driven mouse models of type 2 diabetes and diabetic nephropathy. Recently described clinical results from a phase 2 study in type 2 diabetic patients have provided further evidence for the key role of the chemokine receptor CCR2 in the pathophysiology of type 2 diabetes. The experiments described here provide some of the rationale for the ongoing clinical study of CCX140-B in the setting of diabetic nephropathy (www.clinicaltrials.gov, study numbers NCT01447147 and NCT01440257).

REFERENCES