Disruption of Neutrophil Migration in a Conditional Transgenic Model: Evidence for CXCR2 Desensitization In Vivo

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We developed transgenic mice conditionally expressing the neutrophil chemoattracting chemokine KC and the β-galactosidase gene in multiple tissues. In these transgenic mice, doxycycline treatment induced a strong up-regulation in the expression of KC in several tissues, including heart, liver, kidney, skin, and skeletal muscle. Expression of KC within these tissues led to a rapid and substantial increase in the serum levels of KC (serum KC levels were higher than 200 ng/ml 24 h after treatment). Accordingly, β-galactosidase expression was also detected after injection of doxycycline and was highest in skeletal muscle, pancreas, and liver. Surprisingly, despite expression of KC in multiple tissues, no neutrophil infiltration was observed in any of the tissues examined, including skin. Doxycycline treatment of nontransgenic mice grafted with transgenic skin caused dense neutrophilic infiltration of the grafts, but not the surrounding host skin, indicating that the KC produced in transgenic tissues was biologically active. In separate experiments, neutrophil migration toward a localized source of recombinant KC was impaired in animals overexpressing KC but was normal in response to other neutrophil chemoattractants. Analysis of transgenic neutrophils revealed that high concentrations of KC in transgenic blood had no influence on L-selectin cell surface expression but caused desensitization of the receptor for KC, CXCR2. These results confirm the neutrophil chemoattractant properties of KC and provide a mechanistic explanation for the paradoxical lack of leukocyte infiltration observed in the presence of elevated concentrations of this chemokine. ***The Journal of Immunology,*** **2001, 167: 7102–7110.**

Leukocyte migration and homing is a multistep event involving leukocyte rolling, adhesion to, and migration through the endothelium (1–3). Upon exudation from the bloodstream, leukocytes enter the tissue parenchyma and respond to chemotactic gradients established by a variety of molecules. Among these chemoattractants are chemokines, small proteins that are secreted by virtually all cells in the body (4, 5).

Evidence supporting an important role for chemokines in guiding leukocyte migration and homing in vivo has been provided by analysis of a variety of genetic models (reviewed in Ref. 6). Loss-of-function experiments have shown that deletion of chemokine receptors significantly disrupts homing and inflammatory trafficking of specific leukocyte subsets (7–11). In contrast, gain-of-function transgenic studies have shown that tissue-specific expression of chemokines is associated with recruitment of specific leukocyte subsets into the expressing tissues. For instance, tissue-specific expression of the murine chemokines KC and JE (murine monocyte chemoattractant protein-1 (MCP-1)[2]), induces recruitment of neutrophils and monocytes, respectively, to the expressing tissues (12–17). The ability of JE and other chemokines to recruit leukocytes in transgenic mice seems to depend on the mode of expression of the transgenes. Widespread rather than localized overexpression of MCP-1 or IL-8 prevents or inhibits leukocyte recruitment (18, 19), but the mechanistic basis for these paradoxical findings is unclear at present (20–22).

To better understand the mechanisms regulating leukocyte recruitment in vivo, we have applied transgenesis to study the function of the murine CXC chemokine KC. KC, also known as N51, is a murine immediate early gene (23, 24) with a low or undetectable expression in most tissues (23, 25). KC expression is highly up-regulated in vitro and in vivo by a variety of factors, such as LPS, platelet-derived growth factor, and bombesin (23, 25–29). Similar to its human relatives growth-related oncogene/melanoma growth-stimulating activity (CXCL1) and IL-8 (CXCL8), KC is a potent inducer of neutrophil migration in vitro and in vivo (25). In humans, these molecules promote chemotaxis via their interaction with the receptors CXCR1 and CXCR2, but only one such receptor, CXCR2, has been identified thus far in the mouse (30–33). In previous transgenic experiments, tissue-specific expression of KC in thymus, skin, heart, lung, and brain was associated with recruitment of neutrophils into these organs (6, 12, 14, 34). The recruited neutrophils accumulated in the vicinity of cells expressing the transgene and did not appear to be activated. Interestingly, neutrophil infiltration was attenuated over time in some of these models, which was attributed to the constitutive, unphysiological expression of KC (12, 34). Thus, although genetic studies have clearly demonstrated that expression of KC is sufficient to promote neutrophil infiltration into tissues, they have also suggested that an experimental system mimicking the physiological, inducible pattern of expression of KC may be required to better understand its function.

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Received for publication August 1, 2001. Accepted for publication October 22, 2001.

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[2] Abbreviations used in this paper: MCP-1, monocyte chemoattractant protein-1; rtTA, tet-activator protein; CMC, carboxymethylcellulose; TRE, tetracycline-responsive promoter element; WBC, white blood cells; β-gal, β-galactosidase.

0022-1767/01/$02.00

Here we describe the generation of transgenic mice conditionally expressing the chemokine KC. To this end, we used the tetracycline-dependent gene expression system developed by Gossen and Bujard (35). In the transgenic model described here, expression of KC was induced by doxycycline, a tetracycline analog, in a wide variety of tissues. In agreement with previous reports, we found that localized, conditional expression of KC was associated with significant recruitment of neutrophils, but that its widespread expression inhibited neutrophil recruitment. The predominant mechanism underlying the failure of neutrophils to migrate under these conditions was the desensitization of the CXCR2 in response to high circulating levels of KC.

Materials and Methods

Transgenes

A bidirectional reporter transgene was constructed by cloning the cDNA for KC (+2 to +140) into pBI-G (Clontech, Palo Alto, CA). The activator transgene was constructed by cloning the cDNA for *rtTA* obtained from the pTet-On vector (Clontech) into the *Eco*RI site of an expression vector containing the CMV enhancer/chicken β-actin promoter and the rabbit β-globin polyadenylation signal (36). Separation of the transgenes from vector sequences after digestion with restriction enzymes was accomplished by zonal sucrose gradient centrifugation as described (37). Fractions containing the transgene were pooled, microcentrifuged through Microcon-100 filters (Amicon, MA), and washed five times with microinjection buffer (5 mM Tris-HCl (pH 7.4), 5 mM NaCl, 0.1 mM EDTA).

Generation of transgenic mice

DNA containing the transgene was resuspended in microinjection buffer to a final concentration of 1–5 ng/μl, microinjected into mouse eggs (C57BL/6J $\times$ DBA/2 F_2; Charles River Laboratories, Wilmington, MA), which were then transferred into oviducts of CD1 (Charles River Laboratories) foster mothers, according to published procedures (38). By 10 days of life, a piece of tail from the resulting animals was clipped for DNA analysis. Identification of transgenic founders was conducted by PCR analysis, as previously described (39). Identification of transgenic mice was accomplished by amplification of a segment of the *rtTA* transgene using primers (5′-CGGGTCTACCATCGAGGGCCTGCT-3′) and (5′-CCCGGGGAA TCCCCGTCCCCCAAC-3′) and amplification of a segment of the β-galactosidase (β-gal) gene (5′-ACCAGCGAATACCTGTTCCGTCAT GC-3′ and 5′-AGTAAGGCGGTCGGGATAGTTTTCTTGC-3′). As an internal control for the amplification reaction, primers for the endogenous *ZP3* gene were used (5′-CAGCTCTACATCACCTGCCA-3′; 5′-CACTGGGAAGAGACACTCAG-3′). These primers amplify a 242-bp segment of the *rtTA* transgene, a 791-bp segment of the *β-gal* gene, and a 511-bp segment of the *ZP3* gene. PCR conditions were: 95°C for 30 s; 60°C for 30 s; 72°C for 60 s for 30 cycles. The resulting transgenic mice were kept under specific pathogen-free conditions. Experiments were performed following the guidelines of the Schering-Plough Animal Care and Use Committee.

RNA analysis

RNA was extracted from tissues using RNA STAT-60, following specifications from the manufacturer (TEL-TEST, Friendswood, TX). Total RNA (20 μg) was denatured and blotted onto Biotrans membrane (ICN Biomedicals, Costa Mesa, CA). Transgene expression was assessed by hybridization to randomly primed ^{32}P-labeled *rtTA* cDNA (Stratagene, La Jolla, CA).

Quantitative PCR analysis

cDNA was generated by reverse transcription using random hexamers (Promega, Madison, WI) and oligo(dT) primers (Life Technologies, Gaithersburg, MD) from RNA. Quantitative PCR analysis was performed on an ABI 7700 sequence detection instrument (TaqMan) following manufacturer's instructions. For TaqMan analysis, 25 ng cDNA were amplified with primers at 0.9 μM final concentration, and FAM-labeled diagnostic probe at a final concentration of 0.25 μM. Primers/probe sequences for KC were as follows: (forward 5′-TGCACCCAAACCGAAGTCA-3′, reverse 5′-AGCTTCAGGGTCAAGGCAAG-3′, probe 5′-6FAM-AGCCACACT CAAGAATGGTCGCGAG-TAMRA-3′). rRNA primers/probe (PE Applied Biosystems, Foster City, CA) were used as an internal control. Quantitative PCR conditions were as follows: 50°C for 2 min; 95°C for 10 min; 40 cycles of 95°C for 15 s, 60°C for 1 min. A plasmid containing the KC gene was used as standard, ranging from 200 pg to 20 fg. Data were analyzed using Sequence Detection Systems software version 1.7.

ELISA

Sera were stored at −80°C until assayed for KC concentrations using a KC-specific ELISA kit (R&D Systems, Minneapolis, MN).

Air pouch model

The mouse air-pouch model for in vivo chemotaxis has been described in detail elsewhere (40). On experimental day 0, 5 ml sterile air was injected under the dorsal skin; the resultant space was reinjected with 3 ml sterile air on day 3. On day 5, 1 μg recombinant KC (R&D Systems) or 0.2 μg LPS (Sigma, St. Louis, MO) in 1 ml 0.5% carboxymethylcellulose (CMC; Fluka, Buchs, Switzerland) in saline was injected into the pouches. The animals were sacrificed 4 h later, and the air pouches were lavaged with 2 ml sterile PBS. The resulting cell suspensions were pelleted, resuspended in PBS, and counted under a hemocytometer. Cytospin slides were prepared, stained (Diff-Quik Stain Set; Dade Behring, Newark, DE), and differential cell counts were determined. The absolute number of neutrophils in each sample was determined by multiplying the percentage of this cell type by the total number of leukocytes per sample.

Flow cytometry

Blood was collected from the retro-orbital sinus into tubes containing EDTA (Terumo Medical, Elkton, MD) to prevent clotting. After RBC had been lysed, 10^6 cells were incubated with 5 μg/ml Fc block (BD Biosciences, San Diego, CA) and 300 μg/ml mouse IgG (Pierce, Rockford, IL). Cells were then stained with the directly conjugated primary mAbs Gr-1 (RB6−8C5) and L-selectin (CD62L; MEL-14; BD Biosciences) in PBS, 1% BSA, 0.1% sodium azide for 20 min at 4°C in the dark. To determine viability, samples were subsequently stained with 20 μl 5 μg/ml propidium iodide (Calbiochem, San Diego, CA). Events were acquired on a BD Biosciences FACScan and analyzed using the CellQuest software.

Skin grafts

A patch of skin (5 $\times$ 7 mm) was removed from the back of donor mice and kept in PBS until host animals were ready for grafting. Host Rag1$^{-/-}$ (The Jackson Laboratory, Bar Harbor, ME) were anesthetized and shaved on the back. A graft bed was prepared on the back by carefully removing a rectangular piece of skin (5 $\times$ 7 mm) without disturbing the vascular layer underneath. The donor skin was then placed onto the graft bed with the direction of hair growth opposite to that of the host. A wound dressing (Spenco Medical, Waco, TX) and a petroleum gauze (Johnson & Johnson Medical, Arlington, TX) were then placed over the graft. Finally, a Band-Aid was wrapped around the graft and thorax to prevent dislodgment of the graft. The mice were monitored during recovery, and the bandage was removed 7 to 10 days later.

Histology and β-gal histochemistry

After euthanasia, tissues were either fresh frozen with OTC medium for cryosection or fixed by immersion in 10% phosphate-buffered formalin for processing as paraffin sections. Tissues for light microscopic examination were routinely processed, sectioned at 5 μm, and stained with H&E. Fresh frozen sections (10 μm) were fixed with 2% paraformaldehyde in PBS and then used for β-gal histochemistry following a procedure described previously (41). Briefly, the slides were incubated overnight or for a specific length of time in the presence of 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Roche Molecular Biochemicals, Indianapolis, IN) and counterstained with neutral red.

Measurement of neutrophil intracellular calcium response

Neutrophils were isolated from the peripheral blood of wild-type animals or transgenic mice 24 h after injection of 500 μg doxycycline i.p. To prevent receptor recycling, all steps were performed at 4°C within 1 h after blood collection. RBC were lysed, and white blood cells (WBC) were resuspended in PBS and suspended for analysis. Cells were loaded with fluo-3-acetoxymethyl ester (Sigma) in culture medium (DMEM containing 10% serum) for 1 h at 37°C after which cells were washed three times in HBSS containing 20 mM HEPES and 0.1% BSA. Cells were then aliquoted into 96-well black-wall plates at a density of 1 $\times$ 10^6 cells/well and centrifuged briefly (300 $\times$ *g*, 2 min). All plates were precoated with poly-L-lysine. Intracellular calcium flux was measured in all 96 wells simultaneously and in real time using a Fluorescent Imaging Plate Reader (Molecular Devices, Sunnyvale, CA), and data were expressed as maximum

fluorescence vs concentration of agonist. Recombinant KC was purchased from R&D Systems.

Results

Generation of a conditional transgenic system for expression of the chemokine KC

To generate transgenic mice in which expression of the chemokine KC could be induced conditionally, we used the tetracycline-dependent gene expression system originally described by Gossen and Bujard (35). In this bigenic system the tet-activator protein (rtTA) is expressed constitutively from the "activator" transgene (Fig. 1*A*). Then, in the presence of the tetracycline analog doxycycline, the rtTA protein binds to a tetracycline-responsive promoter element (TRE) present on a "reporter" transgene and induces expression of the transgenes of choice (Fig. 1*C*). The activator transgene used here was driven by the CMV enhancer/β-actin promoter, which promotes expression of transgenes in multiple tissues (42, 43). We generated 15 founder mice carrying the activator transgene and derived 8 independent transgenic lines from them. Activator transgene expression was detected in four of these lines by analysis of skeletal muscle RNA. Further analysis of one of these lines (line 72) showed high levels of transgene expression in heart; moderate expression levels in skin, kidney, thymus, and lung; and low expression levels in spleen and liver (Fig. 1*B*). This line was selected as the activator line.

Four independent lines of transgenic mice (lines 11, 19, 31, and 39) were generated carrying the reporter transgene. This transgene contained the TRE element and two genes in opposite direction, KC and β-gal (Fig. 1*C*). Because this transgene caries only minimal promoter sequences, it should remain silent or nonexpressed in the absence of the transcriptional factor encoded by rtTa. To test whether reporter transgene expression was indeed silent in the absence of rtTA, we analyzed a large variety of tissues (bladder, bone, brain, esophagus, heart, kidney, liver, lung, muscle, pancreas, skin, spleen, stomach thymus, trachea) from all four transgenic lines for β-gal activity. With the exception the brain, where a small number of cells stained blue, β-gal activity was not detected in any other organs examined, indicating that reporter transgene expression was silent in these transgenic mice. Animals from reporter line 19 were crossed to animals from activator line 72 to generate double-transgenic animals.

Induction of transgene expression is dependent on doxycycline and occurs in multiple tissues of double-transgenic mice

To test whether doxycycline treatment induced KC expression and to determine whether this effect was dose dependent, we injected double-transgenic mice with a single i.p. dose of 20, 100, or 500 μg doxycycline. Blood was collected 24 h after injection, and KC serum levels were determined using a KC-specific ELISA. As shown in Fig. 2*A*, the levels of KC in serum increased proportionally to the amount of doxycycline injected, reaching concentrations of 224 $\pm$ 112 ng/ml in response to the highest dose of doxycycline.

Next we measured the serum levels of KC after injection of doxycycline (500 μg i.p.). Two hours after injection, the average concentration KC in the serum was 10-fold higher than baseline (from 0.1 $\pm$ 0.01 ng/ml to 1.1 $\pm$ 0.71 ng/ml). Six hours after treatment, the serum KC levels were 100-fold higher than baseline (11 $\pm$ 6.7 ng/ml). After 24 h of treatment, the serum KC levels were dramatically elevated ($\sim$2000-fold over baseline; Fig. 2*B*). After this time, the concentration of KC declined steadily reaching baseline levels 7 days after treatment. Thus, injection of a single dose of doxycycline resulted in a rapid, strong, and reversible induction of KC gene expression.

The pattern of expression of the activator transgene (Fig. 1*B*) suggested that the elevated levels of KC in circulation could be caused by doxycycline-induced expression of KC in multiple tissues. To more accurately define the source of KC in circulation, we

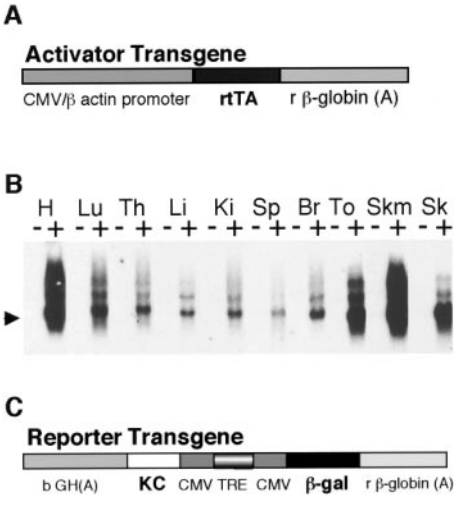

FIGURE 1. Schematic representation of the activator and reporter transgenes and analysis of activator transgene expression. *A,* Activator transgene for the expression of the tet-activator (rtTA) under control of the human CMV enhancer/chicken β-actin promoter. *B,* Northern blot analysis of the expression pattern of rtTA in selected tissues of transgenic mice. H = heart; Lu = lung; Th = thymus; Li = liver; Ki = kidney; Sp = spleen; Br = brain; To = tongue; Skm = skeletal muscle; Sk = skin. Expression of rtTA was detected in all transgenic (+) tissues tested, whereas no expression was found in nontransgenic (−) littermates. Arrow, major transcription product of the transgene. *C,* Reporter transgene for the conditional expression of β-gal and KC. CMV, CMV minimal promoter, b GH(A), bovine growth hormone polyadenylation signal; r β-globin (A), rabbit β-globin polyadenylation signal.

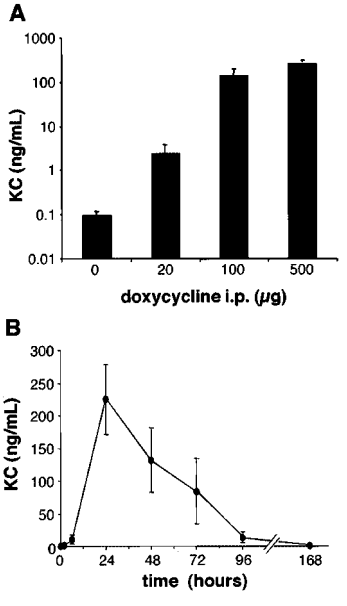

FIGURE 2. Dose- and time-dependent induction of KC expression by doxycycline. *A,* KC concentration in blood of double-transgenic animals 24 h post-doxycycline injections i.p. *B,* KC concentration in blood of double-transgenic animals that were injected with a single dose of doxycycline (500 μg i.p.). Serum was collected at the time indicated. KC concentrations in serum of at least 3 animals were determined for each dose and time point except for the 24-h time point at the 500 μg dose at which 19 animals were used. Error bars, SEM.

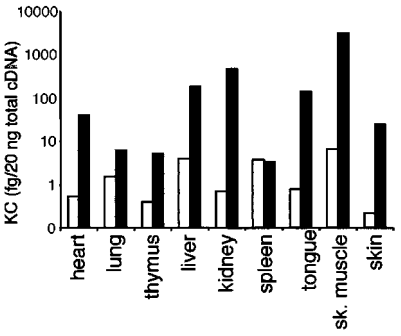

FIGURE 3. Doxycycline-induced KC expression in transgenic tissues. Quantitative RT-PCR analysis of KC in tissues from untreated transgenic mice (□) or treated mice 24 h after injection of doxycycline (500 μg i.p.; ■). Values are expressed as femtograms KC amplicon per 20 ng total cDNA. Results are representative of three experiments. sk, skeletal.

examined KC mRNA expression in various tissues by real time PCR. Tissues (thymus, kidney, spleen, lung, heart, liver, skeletal muscle, tongue, and skin) were harvested from untreated or doxycycline-treated transgenic mice (24 h after injection of doxycycline 500 μg i.p.). RNA was prepared and translated into cDNA, which was amplified using primers and probe specific for KC. As shown in Fig. 3, most tissues of untreated transgenic mice expressed very low levels of KC (<1 fg KC/20 ng total cDNA). Low levels of KC expression (1–7 fg KC/20 ng total cDNA) were detected in spleen, lung, liver, and skeletal muscle. This low basal level of KC expression in mouse tissues is in agreement with results published by Bozic et al. (25), who have used a sensitive RNase protection assay to examine expression of KC. In contrast, levels of KC in tissues of treated animals were 4- to 500-fold higher than those of untreated animals, except in spleen, where there was no induction of KC over baseline levels (Fig. 3). KC expression was highest in skeletal muscle, followed by kidney, liver, tongue, and skin.

Cellular mapping of the expression of the reporter transgene

To map the expression of the reporter transgene at the tissue and cellular level, we examined tissues from doxycycline-treated and untreated transgenic mice for evidence of β-gal activity. No β-gal activity was detected in *rtTa*-expressing tissues in the absence of doxycycline (Fig. 4*A* and data not shown). In contrast, β-gal activity was easily detected in multiple organs including skeletal muscle, pancreas, liver, kidney, stomach, small and large intestine, tongue, and heart 24 h after doxycycline treatment (500 μg i.p) (Fig. 4, *B–L*). In some organs, transgene expression was restricted to morphologically defined areas such as the cortex of the kidney (Fig. 4*E*), the exocrine pancreas (Fig. 4*C*), the epithelial lining of the stomach (Fig. 4*G*), or the epithelium of the small (Fig. 4*H*) and large intestine (Fig. 4*I*). In other tissues, such as skeletal muscle (Fig. 4*B*), tongue (Fig. 4*K*), and heart (Fig. 4*L*), a mosaic pattern of transgene expression could be clearly appreciated. In thymus (Fig. 4*F*), spleen (Fig. 4*J*), bone marrow, and brain (not shown), β-gal activity was low or absent, despite expression of the activator transgene (Fig. 1*C*). In summary, β-gal activity was detected in

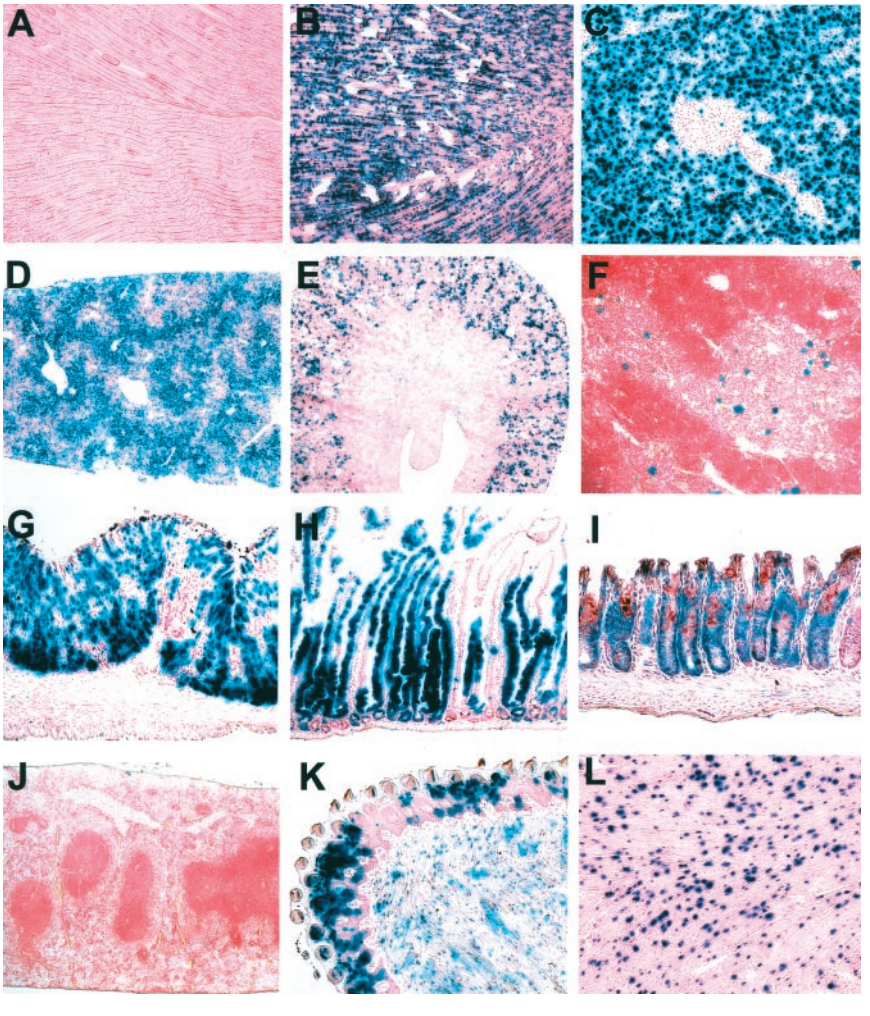

FIGURE 4. Doxycycline induces β-gal expression in double-transgenic animals. Tissues were collected from nontreated double-transgenic animals (*A*) or double-transgenic animals (*B*) 24 h following doxycycline injection (500 μg i.p.; *B–L*). Tissue sections stained for β-gal expression of skeletal muscle (*A* and *B*), pancreas (*C*), liver (*D*), kidney (*E*), thymus (*F*), stomach (*G*), small intestine (*H*), large intestine (*I*), spleen (*J*), tongue (*K*), and heart (*L*).

most of the transgenic tissues expressing the activator transgene after, but not before, doxycycline treatment.

Lack of neutrophil infiltration in tissues overexpressing KC

Expression of KC in a tissue-specific fashion induces neutrophil recruitment (12, 14, 34). To determine whether neutrophils would infiltrate tissues of animals expressing KC ubiquitously, we collected tissues from animals treated with increasing doses of doxycycline (20, 100, and 500 μg) at different time points (6–96 h postinjection) and examined them microscopically. Surprisingly, we did not detect neutrophil infiltration in the parenchyma of any of the tissues examined, regardless of the dose or duration of doxycycline treatment (Fig. 5). Neutrophils were seen only within vascular structures of skeletal muscle between 24 and 96 h after administration of the highest dose of doxycycline (Fig. 5*F*, *inset*).

KC produced by the double-positive mice in response to doxycycline is biologically active

The simplest explanation for the paradoxical lack of neutrophil infiltration observed in the previous experiment would be that the KC produced was biologically inactive. To test whether KC produced by the double-transgenic mice was biologically active, we performed skin grafting experiments. If biologically active KC were produced by the grafts, we would expect to observe a pattern of neutrophil infiltration similar to that observed in transgenic mice expressing KC in the skin (12). We grafted skin from untreated double-transgenic mice onto Rag1$^{-/-}$ mice and used as control the skin from ROSA mice. ROSA mice express β-gal constitutively in the majority of tissues (44) including skin (Fig. 6*A*). Four weeks after the procedure, when the grafts had healed, we injected the host mice with a single dose of doxycycline (500 μg i.p.); 48 h after injection of doxycycline, we collected grafted and host skin and examined β-gal expression. As expected, grafted ROSA skin stained positive for β-gal expression whereas surrounding host tissue did not stain (Fig. 6*A*). Similarly, doxycycline treatment of host mice resulted in the induction of transgene expression in grafted transgenic skin, but not in the surrounding host skin (Fig. 6*B*). To test whether neutrophils had infiltrated the grafts, we stained adjacent sections to those used for detection of β-gal activity with an Ab specific to Gr-1, a neutrophil marker. No Gr-1-positive staining was detected in the grafted skin from ROSA mice or in the adjacent host tissue (Fig. 6*C*), but a significant accumulation of Gr-1$^+$ cells was detected in tissue grafts derived from double-transgenic mice (Fig. 6*D*). Accumulation of neutrophils occurred specifically in the grafted tissue, and no infiltration was observed in the adjacent host tissue (Fig. 6, *E* and *F*). Thus, the localized induction of transgene expression in our conditional system resulted in infiltration of neutrophils, indicating that the KC produced in the transgenic tissues during doxycycline treatment is biologically active. Infiltration of neutrophils after induction of KC expression is also associated with a thickening of the epidermis in the grafted transgenic skin. Because such thickening of the epidermis is absent in the grafted control skin as well in the skin of doxycycline-treated transgenic donor mice (Fig. 5*D*), it remains to be seen whether these changes are an immediate response to the influx of neutrophils, the induction of KC expression, or a combination of these factors.

Neutrophil mobilization and L-selectin expression in induced double-transgenic mice is normal

The absence of neutrophil infiltration into tissues expressing high levels of KC could be caused by a number of factors including decreased mobilization of neutrophils from the bone marrow or marginating pool, or reduced expression of adhesion molecules important for the transmigration process, such as L-selectin. To test these hypotheses, we analyzed the number of total neutrophils and L-selectin-positive neutrophils in blood after injection of doxycycline (500 μg i.p.). As shown in Fig. 7, induction of KC expression led to a 2- to 3-fold increase in circulating neutrophils at 4 and 24 h, suggesting that the mobilization responses were preserved or even elevated ($p = 0.02$). In addition, the expression of L-selectin on the cell surface of neutrophils from doxycycline-treated transgenic animals was equivalent to that of nontreated transgenic controls, demonstrating that shedding of L-selectin did not occur in response to high levels of circulating KC.

Neutrophils of animals overexpressing KC fail to migrate to a local source of KC but respond to other chemoattractants

Next we investigated whether neutrophils could migrate toward a localized source of KC or other chemoattractants in the presence of

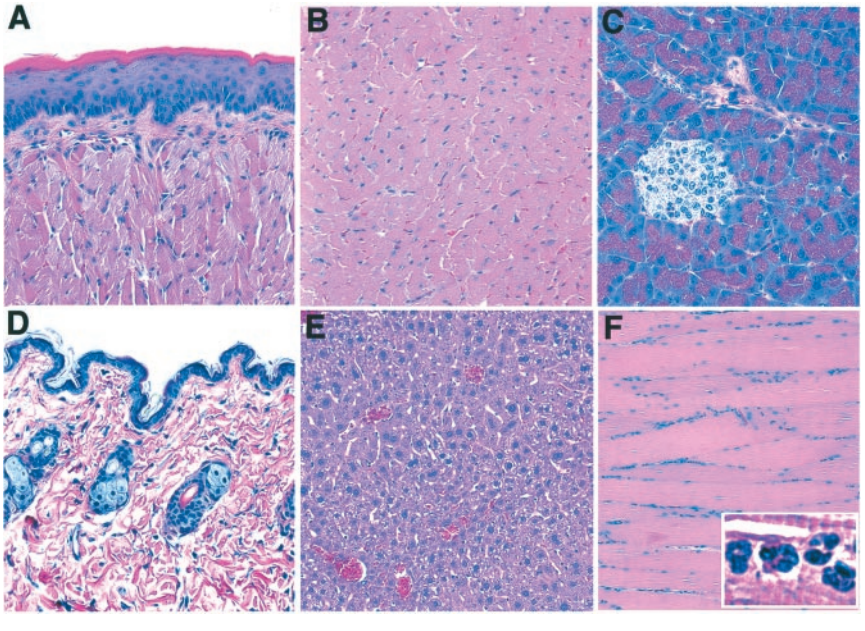

FIGURE 5. Analysis of selected tissues for neutrophil infiltration after induction of transgene expression. Tissues were collected from double-transgenic mice 24 h after injection of 500 μg doxycycline i.p., processed, and stained with H&E. *A*, Tongue; *B*, heart; *C*, pancreas; *D*, skin; *E*, liver; *F*, skeletal muscle. *Inset* in *F*, presence of polymorphonuclear cells within the vasculature of skeletal muscle (magnification, $\times$600).

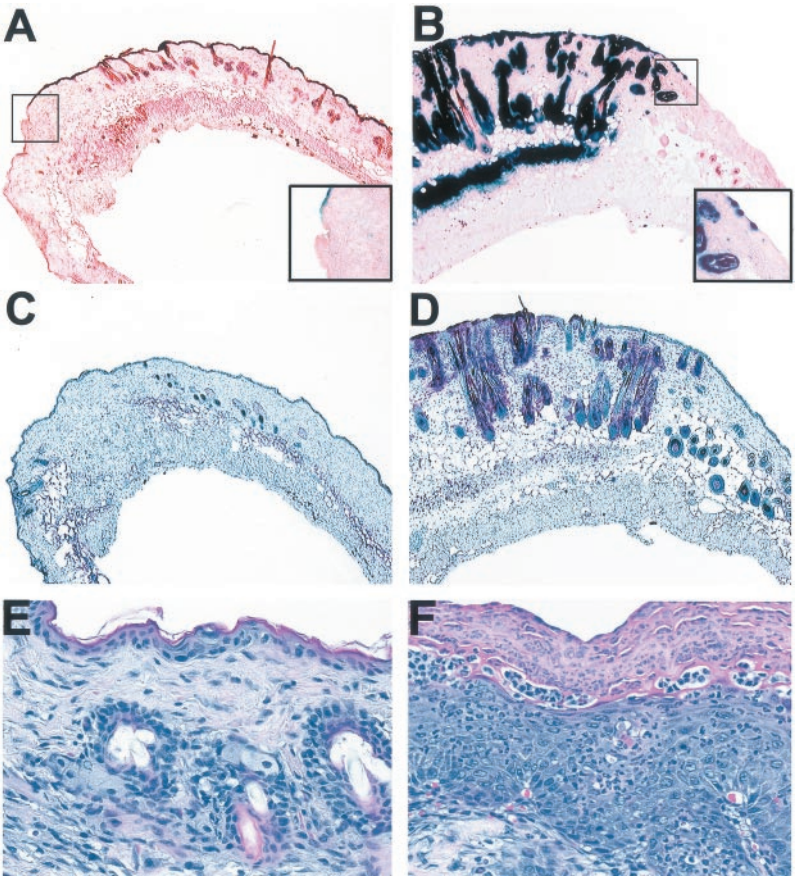

FIGURE 6. Conditional expression of KC in transgenic skin results in neutrophil infiltration. Staining for β-gal activity in ROSA skin (*A*) and in double-transgenic skin (*B*) after doxycycline treatment. GR-1+ staining of grafted skin from ROSA (*C*) or double-transgenic (*D*) mice after doxycycline treatment of Rag1$^{-/-}$ hosts (magnification, ×4). H&E staining of grafted skin from ROSA (*E*) or double-transgenic (*F*) mice after doxycycline treatment of Rag1$^{-/-}$ hosts (magnification, ×40). *Insets* in *A* and *B* show areas of grafted skin bordering host skin.

high systemic levels of KC. To this end, we generated a s.c. air pouch on the back of transgenic animals and injected it with vehicle, LPS, or KC. As shown in Fig. 8, large numbers of neutrophils infiltrated the air pouch of untreated transgenic animals in response to KC. In contrast, significantly fewer neutrophils migrated into the air pouch in response to KC, when the transgenic mice had received doxycycline treatment 24 h earlier ($p = 0.008$). The number of neutrophils recovered from the air pouch of these animals was equivalent to that obtained by injection of vehicle alone. The number of neutrophils migrating toward KC under these conditions did not change even when 10 μg recombinant KC were injected into the air pouch (data not shown). Interestingly, neutrophils of transgenic animals treated with doxycycline were still able to migrate toward other neutrophil-attracting stimuli such as those induced by LPS (Fig. 8).

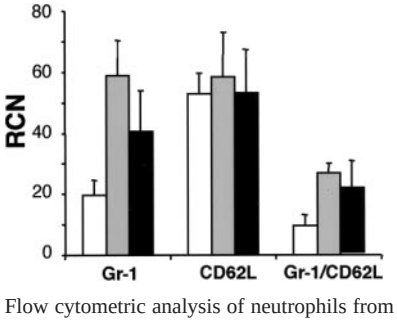

FIGURE 7. Flow cytometric analysis of neutrophils from double-transgenic mice. Blood from untreated (□) or treated double-transgenic animals was analyzed at 4 h (▨) and 24 h (■) after injection of doxycycline (500 μg i.p.) for the number of neutrophils (GR-1) and L-selectin (CD62L)-expressing cells. RCN, relative cell number; error bars, SD; $n = 3$ per treatment group.

Neutrophils from doxycycline-treated transgenic mice show reduced calcium fluxes in response to KC

The previous results indicated a selective deficit of neutrophil migration toward KC in transgenic animals overexpressing this chemokine. This defect could have resulted from down-regulation or desensitization of the receptor for KC, CXCR2. At present, we are not aware of any Ab suitable to detect surface expression of murine CXCR2. We therefore tested whether transgenic blood neutrophils could respond to KC stimulation with an intracellular calcium flux. In peripheral blood, CXCR2 is primarily expressed by neutrophils (our unpublished observation). As shown in Fig. 9, WBC from wild-type mice demonstrated a dose-dependent increase in intracellular calcium when stimulated with recombinant KC. In contrast, the KC-dependent maximum calcium response in WBC from transgenic mice was reduced by ~4-fold. Interestingly, the KC concentration inducing a half-maximal response (EC_{50}) was similar (~12 nM) for both control and transgenic WBC. These results suggest that the defect in neutrophil response in induced transgenic animals is indeed caused by receptor desensitization, down-regulation, or a combination of both.

Discussion

In this report, we describe the generation and analysis of mice conditionally expressing the chemokine KC in multiple organs. We observed that expression of KC by multiple tissues did not result in neutrophil infiltration, a result that is in contrast with previous reports that have documented neutrophil recruitment by this chemokine (12, 14, 34). The conditional properties of the transgenic system reported here allowed for a mechanistic analysis of this seemingly paradoxical response.

The bigenic conditional system described here consists of two transgenes. The first transgene (the activator transgene) encodes a

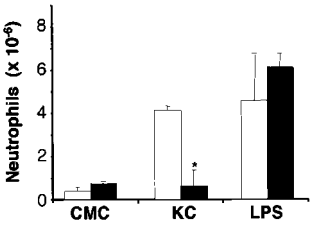

FIGURE 8. Neutrophils of transgenic mice overexpressing KC do not migrate toward a local source of KC. Vehicle CMC alone or CMC containing recombinant KC or LPS was injected into air pouches generated on the back of transgenic animals. Chemoattractants were injected into air pouches of untreated transgenic mice (□) or transgenic mice that had received doxycycline treatment (500 μg i.p.) 24 h earlier (■). The number of neutrophils in the air pouch was determined 4 h after injection of the chemoattractant. *, Significant reduction of neutrophil infiltration in air pouches in doxycycline treated vs untreated transgenic animals ($p = 0.008$). Error bars, SD; $n = 4$ per treatment group.

transcriptional factor (rtTa), which can complex with doxycycline to bind a tetracycline response DNA element present in the second transgene (reporter transgene). In agreement with previous transgenic studies using the human CMV/β-actin promoter (42, 43), high expression of the activator transgene were found in skeletal muscle, heart, tongue, and skin. Crossing animals carrying the activator transgene with animals carrying the reporter transgene yielded double-transgenic mice that did not express KC or β-gal in the absence of doxycycline. These animals, however, expressed high levels of KC and β-gal in multiple tissues after doxycycline treatment. In general, the inducibility of KC correlated with the levels of expression of the activator transgene in most tissues (skeletal muscle, tongue, skin, liver, and spleen). However, high levels of activator transgene expression were detected in the lung, and yet there was a relatively low induction of KC expression (4-fold). The converse was true for kidney, where low levels of activator transgene expression were detected, but where there was a robust KC induction (~680-fold over background). Coexpression of β-gal (encoded alongside KC in the reporter transgene) allowed precise mapping of the reporter transgene expression. Upon administration of doxycycline, β-gal expression was detected in several tissues and correlated with the expression of the activator transgene. The highest expression of β-gal was detected in exocrine pancreas and skeletal muscle. We suggest that the high expression of β-gal in pancreas was the direct result of activator transgene expression, because we and others have shown that

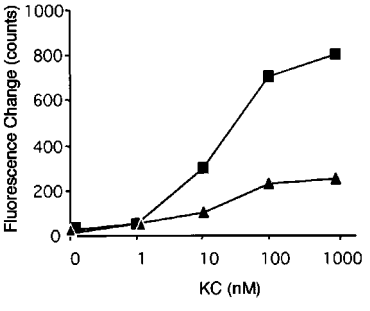

FIGURE 9. WBC from transgenic mice expressing KC after doxycycline treatment have reduced calcium flux in response to recombinant KC. WBC were isolated from peripheral blood of transgenic animals before (■) or 24 h after doxycycline treatment (500 μg i.p.; ▲). Cells were analyzed for their ability to flux calcium in response to varying concentrations of recombinant KC. The response to KC is displayed as relative fluorescence change. Data are representative of two independent experiments.

transgenes under control of the CMV/β-actin promoter are highly expressed in the exocrine pancreas (43, 45). β-Gal was not expressed in all tissues in which the activator transgene was expressed. For instance, β-gal expression was minimal or not detected in lymphoid tissues, in lung, or in the brain, tissues in which low but detectable expression of the activator transgene was demonstrated. The lack of β-gal expression in these settings could have been caused by low levels of rtTa protein or by other tissue-specific transcriptional requirements. Finally, we should point out that there was a very good concordance between β-gal and KC expression. These genes were found to be expressed at high levels in skeletal muscle, liver, heart, kidney, and skin. There was low expression of both genes in thymus and lung and no expression of either gene in spleen.

Arguably, the most interesting finding of our studies was the observation that despite high transgene expression in many tissues and increased numbers of neutrophils in circulation, no neutrophil infiltration could be detected in any of the expressing tissues. This finding suggested that neutrophil migration from the blood stream into tissue parenchyma was impaired. At least two general hypotheses could be formulated to explain this impairment in neutrophil migration: one would suggest that the transgenic mice would express a biologically inactive KC; the other would suggest a defect in neutrophil function.

To investigate whether the KC produced in the transgenic tissues was biologically active, we performed skin grafting experiments. Here we took advantage of the fact that the reporter transgene encoded both KC and β-gal, and we used β-gal expression to mark the cells expressing the transgene. Transgene expression in the grafted transgenic skin was induced by administration of doxycycline to the host animals. As expected, β-gal expression was visualized in the grafted transgenic skin, but not in the surrounding host skin. Again, as previously reported (12), expression of KC in the skin promoted neutrophil recruitment. This process was specific to the grafted skin from double-transgenic mice and was not observed in grafted control skin (derived from ROSA mice) expressing β-gal. These results indicated that the KC produced by the transgenic tissues was biologically active. Thus, besides validating the original observations regarding KC, these findings suggest that skin and other tissues from these transgenic animals can now be used to investigate the role of KC and neutrophils in transplantation biology. The ability to regulate gene expression in multiple, transplantable tissues will likely facilitate the analysis of the biological role of other chemokines.

As discussed above, the paradoxical absence of neutrophil infiltration into tissues expressing KC could also be the result of a general defect in the ability of the transgenic neutrophils to migrate. To test this hypothesis, we promoted expression of neutrophil chemoattractants by injection of LPS in a s.c. air pouch in transgenic mice with or without doxycycline treatment. LPS is by itself a potent inducer of neutrophil migration and also induces other neutrophil chemoattractants such as C5a (46). There was no difference in the number of neutrophils recruited in the pouch, suggesting that the overall capacity of neutrophils to respond to chemoattractants had been preserved, even in the presence of high systemic levels of KC. There was, however, a significant impairment in the response of neutrophils to KC after but not before doxycycline treatment. These results indicated again that the transgenic neutrophils were essentially normal before treatment and suggested that they became less capable to migrate toward a localized source of KC after doxycycline treatment. We hypothesized that the high systemic levels of KC were directly causative of the abnormal neutrophil response. A number of in vivo and in vitro studies in the literature would point in this direction. In vivo, high

levels of circulating chemokines inhibit leukocyte infiltration into tissues. High systemic levels of IL-8 attenuate proinflammatory effects of locally administered IL-8 (21, 22) or neutrophilia induced by LPS in lung (20). Furthermore, high constitutive expression of MCP-1 or IL-8 in transgenic mice does not result in leukocyte infiltration into the expressing organ or any other organ (18, 19). Moreover, tissue-specific constitutive transgenic expression of KC results over time in attenuation of neutrophil recruitment (14, 34), and monocyte recruitment induced by tissue-specific expression of MCP-1 can be abrogated in the presence of high systemic levels of MCP-1 (15). The reduced ability of leukocytes to migrate from the blood into tissue in these circumstances has been attributed to a number of factors such as shedding of L-selectin, the absence of a chemoattractant gradient, or desensitization of the cognate chemokine receptor (15, 18, 19).

Leukocyte extravasation from the blood stream into tissue is dependent on P- and possibly L- and E-selectins that mediate a low affinity adhesive rolling interaction of leukocytes on activated endothelium (47). Neutrophil emigration into tissues is dependent on shape change and shedding of L-selectin (reviewed in Ref. 48), but this effect is not dominant, because it has been demonstrated that neutrophils lacking L-selectin are still capable of infiltrating tissues (49, 50). Simonet et al. (19) found reduced L-selectin expression on the surface of blood neutrophils of mice overexpressing IL-8 and argued that this reduction could explain lack of neutrophil infiltration observed in their model. Here we found that surface expression of L-selectin on blood neutrophils remained constant even after prolonged exposure of neutrophils to high concentrations of KC. We therefore ruled out shedding of L-selectin as a factor contributing to the lack of neutrophil migration in our system.

Neutrophil migration to KC in mice is mediated through the CXCR2 receptor, a G protein-coupled seven-transmembrane receptor (33, 51). Like other G-protein-coupled seven-transmembrane receptors, the murine CXCR2 induces calcium mobilization and cell migration in response to agonist binding (52). Thus, freshly isolated transgenic WBC were tested for calcium mobilization in response to recombinant KC by calcium fluorometry. The response was greatly reduced in WBC derived from doxycycline-treated mice, indicating that the functionality of the CXCR2 receptor was affected by high concentrations of KC. Indeed, induction of transgene expression in multiple tissues resulted in a rapid increase in the levels of circulating KC reaching maximal levels of ~224 ng/ml 24 h after doxycycline injection. In vitro, similar concentrations of neutrophil chemoattracting molecules arrest neutrophil movement (53). Thus, the neutrophil migration deficit seen in our model could be due to an uncoupling of the receptor in response to KC binding, leading to desensitization or to increased internalization of the receptor. Although we have clearly demonstrated a deficit in the response of WBC to KC, we cannot rule out at this point that additional factors such as the absence of a chemotactic gradient may have prevented cell infiltration into tissues.

In summary, the development of the ubiquitous conditional system described here provided us with a tool to investigate the mechanisms underlying inhibition of neutrophil migration associated with high systemic expression of KC. Multiple tissues were shown to express KC, which resulted in high serum levels of this chemokine. Grafting of tissues conditionally expressing KC into $Rag1^{-/-}$ mice established that the KC was biologically active to promote neutrophil recruitment into the grafts, confirming previous studies (54). In the presence of high systemic levels of KC, we found an impaired neutrophil response to KC and identified desensitization of CXCR2 as a mechanism to explain the migration defect. These findings suggest that the ability of KC to promote neutrophil recruitment is dependent on how it is expressed. On the basis of these results, we hypothesize that elevated levels of KC or other CXCR2 ligands may attenuate or reduce neutrophil infiltration during pathological situations and profoundly impact host responses. We anticipate that the conditional system described here will prove to be a powerful experimental tool to test this and other hypotheses related to the biological function of chemokines.

Acknowledgments

We thank Tong-Yuan Yang, Paolo Fruscella, Linda Hamilton, Alexandra Martins, and Channa Young for technical assistance. We thank Dr. Jun-ichi Miyazaki for the plasmid containing the CMV enhancer/chicken β-actin promoter cassette.

References

1. Butcher, E. C. 1991. Leukocyte-endothelial cell recognition: three (or more) steps to specificity and diversity. *Cell 67:1033.*
2. Bevilacqua, M. P. 1993. Endothelial-leukocyte adhesion molecules. *Annu. Rev. Immunol. 11:767.*
3. Springer, T. A. 1994. Traffic signals for lymphocyte recirculation and leukocyte emigration: the multistep paradigm. *Cell 76:301.*
4. Rollins, B. J. 1997. Chemokines. *Blood 90:909.*
5. Zlotnik, A., and O. Yoshie. 2000. Chemokines: a new classification system and their role in immunity. *Immunity 12:121.*
6. Lira, S. 1999. Lessons from gene modified mice. *Forum (Genova) 9:286.*
7. Forster, R., A. E. Mattis, E. Kremmer, E. Wolf, G. Brem, and M. Lipp. 1996. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. *Cell 87:1037.*
8. Boring, L., J. Gosling, S. W. Chensue, S. L. Kunkel, R. V. Farese, Jr., H. E. Broxmeyer, and I. F. Charo. 1997. Impaired monocyte migration and reduced type 1 (Th1) cytokine responses in C-C chemokine receptor 2 knockout mice. *J. Clin. Invest. 100:2552.*
9. Gao, J. L., T. A. Wynn, Y. Chang, E. J. Lee, H. E. Broxmeyer, S. Cooper, H. L. Tiffany, H. Westphal, J. Kwon-Chung, and P. M. Murphy. 1997. Impaired host defense, hematopoiesis, granulomatous inflammation and type 1-type 2 cytokine balance in mice lacking CC chemokine receptor 1. *J. Exp. Med. 185:1959.*
10. Forster, R., A. Schubel, D. Breitfeld, E. Kremmer, I. Renner-Muller, E. Wolf, and M. Lipp. 1999. CCR7 coordinates the primary immune response by establishing functional microenvironments in secondary lymphoid organs. *Cell 99:23.*
11. Cook, D. N., D. M. Prosser, R. Forster, J. Zhang, N. A. Kuklin, S. J. Abbondanzo, X. D. Niu, S. C. Chen, D. J. Manfra, M. T. Wiekowski, et al. 2000. CCR6 mediates dendritic cell localization, lymphocyte homeostasis, and immune responses in mucosal tissue. *Immunity 12:495.*
12. Lira, S. A., P. Zalamea, J. N. Heinrich, M. E. Fuentes, D. Carrasco, A. C. Lewin, D. S. Barton, S. Durham, and R. Bravo. 1994. Expression of the chemokine N51/KC in the thymus and epidermis of transgenic mice results in marked infiltration of a single class of inflammatory cells. *J. Exp. Med. 180:2039.*
13. Fuentes, M. E., S. K. Durham, M. R. Swerdel, A. C. Lewin, D. S. Barton, J. R. Megill, R. Bravo, and S. A. Lira. 1995. Controlled recruitment of monocytes and macrophages to specific organs through transgenic expression of monocyte chemoattractant protein-1. *J. Immunol. 155:5769.*
14. Tani, M., M. E. Fuentes, J. W. Peterson, B. D. Trapp, S. K. Durham, J. K. Loy, R. Bravo, R. M. Ransohoff, and S. A. Lira. 1996. Neutrophil infiltration, glial reaction, and neurological disease in transgenic mice expressing the chemokine N51/KC in oligodendrocytes. *J. Clin. Invest. 98:529.*
15. Grewal, I. S., B. J. Rutledge, J. A. Fiorillo, L. Gu, R. P. Gladue, R. A. Flavell, and B. J. Rollins. 1997. Transgenic monocyte chemoattractant protein-1 (MCP-1) in pancreatic islets produces monocyte-rich insulitis without diabetes: abrogation by a second transgene expressing systemic MCP-1. *J. Immunol. 159:401.*
16. Gunn, M. D., N. A. Nelken, X. Liao, and L. T. Williams. 1997. Monocyte chemoattractant protein-1 is sufficient for the chemotaxis of monocytes and lymphocytes in transgenic mice but requires an additional stimulus for inflammatory activation. *J. Immunol. 158:376.*
17. Kolattukudy, P. E., T. Quach, S. Bergese, S. Breckenridge, J. Hensley, R. Altschuld, G. Gordillo, S. Klenotic, C. Orosz, and J. Parker-Thornburg. 1998. Myocarditis induced by targeted expression of the MCP-1 gene in murine cardiac muscle. *Am. J. Pathol. 152:101.*
18. Rutledge, B. J., H. Rayburn, R. Rosenberg, R. J. North, R. P. Gladue, C. L. Corless, and B. J. Rollins. 1995. High level monocyte chemoattractant protein-1 expression in transgenic mice increases their susceptibility to intracellular pathogens. *J. Immunol. 155:4838.*
19. Simonet, W. S., T. M. Hughes, H. Q. Nguyen, L. D. Trebasky, D. M. Danilenko, and E. S. Medlock. 1994. Long-term impaired neutrophil migration in mice overexpressing human interleukin-8. *J. Clin. Invest. 94:1310.*
20. Blackwell, T. S., L. H. Lancaster, T. R. Blackwell, A. Venkatakrishnan, and J. Christman. 1999. Chemotactic gradients predict neutrophilic alveolitis in endotoxin-treated rats. *Am. J. Respir. Crit. Care Med. 159:1644.*
21. Hechtman, D. H., M. I. Cybulsky, H. J. Fuchs, J. B. Baker, and M. A. Gimbrone, Jr. 1991. Intravascular IL-8: inhibitor of polymorphonuclear leukocyte accumulation at sites of acute inflammation. *J. Immunol. 147:883.*
22. Van Zee, K. J., E. Fischer, A. S. Hawes, C. A. Hebert, T. G. Terrell, J. B. Baker, S. F. Lowry, and L. L. Moldawer. 1992. Effects of intravenous IL-8 administration in nonhuman primates. *J. Immunol. 148:1746.*

23. Ryseck, R. P., H. MacDonald-Bravo, M. G. Mattei, and R. Bravo. 1989. Cloning and sequence of a secretory protein induced by growth factors in mouse fibroblasts. *Exp. Cell Res. 180:266.*
24. Oquendo, P., J. Alberta, D. Z. Wen, J. L. Graycar, R. Derynck, and C. D. Stiles. 1989. The platelet-derived growth factor-inducible KC gene encodes a secretory protein related to platelet α-granule proteins. *J. Biol. Chem. 264:4133.*
25. Bozic, C. R., L. F. Kolakowski, Jr., N. P. Gerard, C. Garcia-Rodriguez, C. von Uexkull-Guldenband, M. J. Conklyn, R. Breslow, H. J. Showell, and C. Gerard. 1995. Expression and biologic characterization of the murine chemokine KC. *J. Immunol. 154:6048.*
26. Cochran, B. H., A. C. Reffel, and C. D. Stiles. 1983. Molecular cloning of gene sequences regulated by platelet-derived growth factor. *Cell 33:939.*
27. Zullo, J. N., B. H. Cochran, A. S. Huang, and C. D. Stiles. 1985. Platelet-derived growth factor and double-stranded ribonucleic acids stimulate expression of the same genes in 3T3 cells. *Cell 43:793.*
28. Rovai, L. E., H. R. Herschman, and J. B. Smith. 1998. The murine neutrophil-chemoattractant chemokines LIX, KC, and MIP-2 have distinct induction kinetics, tissue distributions, and tissue-specific sensitivities to glucocorticoid regulation in endotoxemia. *J. Leukocyte Biol. 64:494.*
29. Kopydlowski, K. M., C. A. Salkowski, M. J. Cody, N. van Rooijen, J. Major, T. A. Hamilton, and S. N. Vogel. 1999. Regulation of macrophage chemokine expression by lipopolysaccharide in vitro and in vivo. *J. Immunol. 163:1537.*
30. Harada, A., K. Kuno, H. Nomura, N. Mukaida, S. Murakami, and K. Matsushima. 1994. Cloning of a cDNA encoding a mouse homolog of the interleukin-8 receptor. *Gene 142:297.*
31. Bozic, C. R., N. P. Gerard, C. von Uexkull-Guldenband, L. F. Kolakowski, Jr., M. J. Conklyn, R. Breslow, H. J. Showell, and C. Gerard. 1994. The murine interleukin 8 type B receptor homologue and its ligands: expression and biological characterization. *J. Biol. Chem. 269:29355.*
32. Suzuki, H., G. N. Prado, N. Wilkinson, and J. Navarro. 1994. The N terminus of interleukin-8 (IL-8) receptor confers high affinity binding to human IL-8. *J. Biol. Chem. 269:18263.*
33. Lee, J., G. Cacalano, T. Camerato, K. Toy, M. W. Moore, and W. I. Wood. 1995. Chemokine binding and activities mediated by the mouse IL-8 receptor. *J. Immunol. 155:2158.*
34. Lira, S. A., M. E. Fuentes, R. M. Strieter, and S. K. Durham. 1997. Transgenic methods to study chemokine function in lung and central nervous system. *Methods Enzymol. 287:304.*
35. Gossen, M., and H. Bujard. 1992. Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc. Natl. Acad. Sci. USA 89:5547.*
36. Niwa, H., K. Yamamura, and J. Miyazaki. 1991. Efficient selection for high-expression transfectants with a novel eukaryotic vector. *Gene 108:193.*
37. Mann, J. R., and A. P. McMahon. 1993. Factors influencing production frequency of transgenic mice. *Methods Enzymol. 225:771.*
38. Hogan, B., R. Beddington, F. Costantini, and E. Lacy. 1994. *Manipulation of the Mouse Embryo*. Cold Spring Harbor Laboratory Press, Plainview, NY.
39. Lira, S. A., R. A. Kinloch, S. Mortillo, and P. M. Wassarman. 1990. An upstream region of the mouse *ZP3* gene directs expression of firefly luciferase specifically to growing oocytes in transgenic mice. *Proc. Natl. Acad. Sci. USA 87:7215.*
40. Edwards, J. C., A. D. Sedgwick, and D. A. Willoughby. 1981. The formation of a structure with the features of synovial lining by subcutaneous injection of air: an in vivo tissue culture system. *J. Pathol. 134:147.*
41. Soares, H. D., S. C. Chen, and J. I. Morgan. 2001. Differential and prolonged expression of *Fos-lacZ* and *Jun-lacZ* in neurons, glia, and muscle following sciatic nerve damage. *Exp. Neurol. 167:1.*
42. Okabe, M., M. Ikawa, K. Kominami, T. Nakanishi, and Y. Nishimune. 1997. "Green mice" as a source of ubiquitous green cells. *FEBS Lett. 407:313.*
43. Manfra, D. J., S. C. Chen, T. Y. Yang, L. Sullivan, M. T. Wiekowski, S. Abbondanzo, G. Vassileva, P. Zalamea, D. N. Cook, and S. A. Lira. 2001. Leukocytes expressing green fluorescent protein as novel reagents for adoptive cell transfer and bone marrow transplantation studies. *Am. J. Pathol. 158:41.*
44. Friedrich, G., and P. Soriano. 1991. Promoter traps in embryonic stem cells: a genetic screen to identify and mutate developmental genes in mice. *Genes Dev. 5:1513.*
45. Zhan, Y., J. L. Brady, A. M. Johnston, and A. M. Lew. 2000. Predominant transgene expression in exocrine pancreas directed by the CMV promoter. *DNA Cell Biol. 19:639.*
46. Kalmar, J. R., and T. E. Van Dyke. 1994. Effect of bacterial products on neutrophil chemotaxis. *Methods Enzymol. 236:58.*
47. Ley, K., D. C. Bullard, M. L. Arbones, R. Bosse, D. Vestweber, T. F. Tedder, and A. L. Beaudet. 1995. Sequential contribution of L- and P-selectin to leukocyte rolling in vivo. *J. Exp. Med. 181:669.*
48. Rot, A. 1992. Endothelial cell binding of NAP-1/IL-8: role in neutrophil emigration. *Immunol. Today 13:291.*
49. Arbones, M. L., D. C. Ord, K. Ley, H. Ratech, C. Maynard-Curry, G. Otten, D. J. Capon, and T. F. Tedder. 1994. Lymphocyte homing and leukocyte rolling and migration are impaired in L-selectin-deficient mice. *Immunity 1:247.*
50. Tedder, T. F., D. A. Steeber, and P. Pizcueta. 1995. L-selectin-deficient mice have impaired leukocyte recruitment into inflammatory sites. *J. Exp. Med. 181: 2259.*
51. Cacalano, G., J. Lee, K. Kikly, A. M. Ryan, S. Pitts-Meek, B. Hultgren, W. I. Wood, and M. W. Moore. 1994. Neutrophil and B cell expansion in mice that lack the murine IL-8 receptor homolog. *Science 265:682.*
52. McColl, S. R., and I. Clark-Lewis. 1999. Inhibition of murine neutrophil recruitment in vivo by CXC chemokine receptor antagonists. *J. Immunol. 163:2829.*
53. Foxman, E. F., J. J. Campbell, and E. C. Butcher. 1997. Multistep navigation and the combinatorial control of leukocyte chemotaxis. *J. Cell Biol. 139:1349.*
54. Sawicki, J. A., R. J. Morris, B. Monks, K. Sakai, and J. Miyazaki. 1998. A composite CMV-IE enhancer/beta-actin promoter is ubiquitously expressed in mouse cutaneous epithelium. *Exp. Cell Res. 244:367.*