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

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Summary

Transgenic mice expressing the chemokine N51/KC in thymus, skin, and tongue showed a marked infiltration of a single class of inflammatory cells (neutrophils) in the sites of transgene expression. In the thymus, neutrophils were most numerous in the cortex and juxta-medullary regions, often forming aggregates or clusters. A similar, but less intense, neutrophilic infiltrate occurred in close proximity to the epidermal basal layer of the tongue and skin. No morphologic evidence of injury was observed in the thymus, skin, or tongue of these transgenic mice, indicating that N51/KC expression induces recruitment but not inflammatory activation of neutrophils. The lack of activation in the thymus resulted in a large senescent neutrophilic population that was phagocytosed by thymic macrophages and epithelial-reticular cells. These results indicate that N51/KC is a neutrophil chemoattractant in vivo and establish these transgenic mice as effective models to study the phenomena of recruitment and clearance of neutrophils, events that are critical for the initiation and resolution of the inflammatory response.

Multicellular organisms have developed sophisticated defense mechanisms to fight physical, chemical, and biological aggression. One set of such mechanisms is the inflammatory response. Central to the inflammatory process are the phenomena of recruitment, endothelial attachment, and transmigration of neutrophils and mononuclear phagocytes — inflammatory cells that represent the first line of defense against a variety of pathogens (1). Prompt recruitment of neutrophils and monocytes to the site of injury is thought to be dependent on the establishment of chemoattractant gradients and on the activation of specific sets of molecules in the endothelium and in the extracellular matrix (2–5). Molecules presumed to determine the establishment of such gradients have been recently characterized and their genes cloned. Structural similarities between these molecules have grouped them into a superfamily of proteins known as intercrines or chemokines (6, 7). All the peptides in this superfamily are cationic, secreted, small (between 70 and 100 amino acids), and contain four conserved cysteine residues that form two disulfide bonds. The first disulfide bond is located between the first and third cysteine and the second bond between the second and fourth cysteine. The position of the two NH$_2$-terminal cysteines divides the members into two families. In the first family, referred to as chemokine α or "C-X-C" branch, the cysteines are separated by one amino acid; in the second, referred to as chemokine β or "C-C" branch, the cysteines

are adjacent to one another. The best characterized member of the first family is IL-8 and of the second is monocyte chemoattracting protein 1 (MCP-1). Members of the chemokine α or C-X-C family also include: (a) platelet factor 4 (PF4); (b) human platelet basic protein, which is the precursor of the three active peptides: connective tissue–activating peptide III, β-thromboglobulin (β-TG), and neutrophil-activating peptide 2 (NAP-2); (c) human GROα/melanoma growth stimulatory activity (MGSA) and its isoforms, GROβ and GROγ; (d) rat cytokine-induced neutrophil chemoattractant (CINC); (e) chicken 9E3/CEF4; (f) human IP-10; and (g) mouse monokine induced by IFN-γ (MIG), mouse macrophage inflammatory protein 2 (MIP-2), and (h) N51/KC.

N51/KC is an immediate early gene, that encodes a secretory protein of ~8,000 daltons (8, 9). Induction of N51/KC gene expression has been documented in mitogen-stimulated fibroblasts (9, 10) and in LPS-stimulated peritoneal and lung macrophages (11, 12), endothelial (13) and vascular smooth muscle cells (14), but not in a variety of nonstimulated mouse tissues (9). The biological role of N51/KC has remained unknown to date, but it has been suggested that N51/KC might be a neutrophil chemoattractant given its structural homology with rat CINC (15). We have recently confirmed this notion and demonstrated that N51/KC is able to induce intracellular Ca^{2+} flux and that it binds specifically to neutrophils (16). To further understand the biological role of this

chemokine, we expressed it constitutively under the control of different promoters in transgenic mice. Results presented in this study prove conclusively that N51/KC is a neutrophil-specific chemoattractant in vivo.

Materials and Methods

Transgene Construction and Microinjection. The N51 cDNA (+2 to +410 bp) was cloned into the BamHI site of the plasmid lck-hGH. The lck-hGH plasmid contained the mouse lck promoter (−3,200 to +37 bp) and human GH sequences (+2 to +2,153 bp). We substituted sequences located in the 3'-untranslated segment of N51/KC, which have been postulated to contribute to its mRNA's short half-life (10–15 min) (9), for human growth hormone (hGH)[1] sequences, which provided introns and poly(A) sequences. For the construction of the K14-N51 transgene, an EcoRI fragment of the lck-N51-hGH transgene was subcloned into a plasmid containing the human K14 promoter (−2.27 kb to ATG). Transgenes were restricted from vector sequences by digestion with NotI (lck-N51) and HindIII (K14-N51). Separation of the transgenes from vector sequences was accomplished by zonal sucrose gradient centrifugation as described (17). Fractions containing the transgene were pooled, microcentrifuged through Microcon-100 filters (Amicon, Inc., Beverly, MA), and washed five times with microinjection buffer (5 mM Tris-HCL, pH 7.4, 5 mM NaCl, and 0.1 mM EDTA).

Generation of Transgenic Mice. Each transgene was resuspended to a final concentration of 1–5 ng/μl and microinjected into ([C57BL/6] × DBA/2]F$_1$; The Jackson Laboratory, Bar Harbor, ME) eggs, according to standard methodology. Microinjected eggs were transferred into oviducts of ICR (Harlan Sprague Dawley, Inc., Indianapolis, IN) foster mothers according to published procedures (18). By 10 d of life, a piece of tail from the resulting animals was clipped for DNA analysis. Identification of transgenic founders was carried out by PCR analysis, as previously described (19). Two oligonucleotide primers recognizing hGH sequences (present in both lck-N51 and K14-N51 transgenes) were used for the screening of transgene incorporation ('5-TTTGGGGTTCTGAATGTGAG-3' and 5'-AGGCACTGCCCTCTTGAAGC-3') and, as an internal control, the endogenous β-thyroid stimulating hormone (TSH) gene (5'-GTAACTCACTCATGCAAAGT-3 and 5'-TCCTCAAAG-ATGCTCATTAG-3'). These primers amplify 218- and 366-bp segments of the hGH and β-TSH genes, respectively. The resulting transgenic animals were kept under pathogen-free conditions.

RNA Analysis. RNA was extracted from tissues using RNAzol, following specifications from the manufacturer (Cinna/Biotecx, Houston, TX). RNA dot blots were performed as described (20). An 800-bp fragment of the hGH cDNA was radiolabeled with a random-priming DNA-labeling kit (Amersham Corp., Arlington Heights, IL) and was used as a probe in these experiments. The RNase protection assays were performed with an RPA II kit (Ambion Inc., Austin, TX) following the manufacturer's instructions. For each assay, 20 μg total RNA was hybridized overnight to 5 × 10^5 cpm of the probe, at 42°C. The template for the synthesis of the RNA probe was a 160-bp fragment of the N51/KC cDNA, starting 50 bases 5' of the ATG site, cloned in Bluescript SK II$^+$. The template was linearized with XhoI and transcribed with T3 RNA polymerase to generate the antisense RNA. The protected RNA hybrids were separated in a 6% polyacrylamide gel with 7

M urea and visualized by autoradiography. For in situ hybridization, a murine cDNA fragment of N51, positions 1–310 (9) was cloned into Bluescript KS +. [^{35}S]UTP-labeled sense and antisense probes were prepared from linearized template DNAs, using T3 or T7 RNA polymerases (Promega Corp., Madison, WI). Probes were degraded to an average size of 200 nucleotides by limited alkaline hydrolysis. After removal of unincorporated nucleotides using a Sephadex G-50 column (Pharmacia, Piscataway, NJ), the labeled probes were stored at −80°C in a solution containing 10 mM dithiothreitol and 1 μg/ml yeast RNA. In situ hybridization was performed as previously described (21).

Protein Analysis. Immunoprecipitations and Western blots were done essentially as described (22). Biosynthetic labeling with [^{35}S]methionine was done as follows: 10^6 thymic cells from lck-N51 transgenic and control mice were isolated and washed twice in DMEM lacking methionine, resuspended in 300 μl DMEM containing 1 mg/liter methionine and 2.5% dialyzed FCS plus 1 mCi [^{35}S]methionine/ml, and incubated overnight (16 h) at 37°C and 10% CO$_2$. The cells were centrifuged and the immunoprecipitates were subjected to SDS-PAGE, stained with Coomassie blue, destained, treated with Entensify (NEN-DuPont, Boston, MA), and exposed to Kodak X-Omat AR film at −70°C for 7 d.

Microscopy and Histochemistry. 15 lck-N51 transgenic mice and 15 age-matched control mice were used in this study. Animals (*n* = 5/group/time point) were killed at 15, 50, and 100 d of age. Immediately after euthanasia, the thymus was removed and immersion fixed in McDowell-Trump solution (23). Tissues for light microscopy were processed by routine methods, sectioned at 6 μm, and stained with hematoxylin and eosin (H&E). Quantification of neutrophils in the thymus was performed on H&E paraffin–embedded material. Neutrophils, characterized as a cell with a diameter of 10–12 μm with a multilobulated nucleus and granular cytoplasm, were counted per high power field (at a magnification of 400). A minimum of 20 fields of thymic cortex and 20 fields of medulla were evaluated per tissue section. Statistical differences in the mean number of neutrophils in the thymus of transgenic mice, as compared with age-matched controls, were determined by the analysis of variance (ANOVA) test with statistical significance at $p \leqslant 0.05$. Naphtol AS-D chloracetate esterase histochemical staining for neutrophils was done essentially as described by Leder (24). Selected tissues for evaluation by transmission electron microscopy (TEM) were post-fixed in 1% osmium tetroxide, dehydrated in a graded series of methanol baths, and embedded in epoxy resin. 1 μm-thick sections were cut, stained with toluidine blue, and examined by light microscopy to delineate areas to be evaluated by TEM. Ultrathin sections were placed on copper mesh grids, stained with uranyl acetate and lead citrate, and examined with a Zeiss EM-10C transmission electron microscope.

Flow Cytometry. Flow cytometry was carried out using an Epics Profile II flow cytometer and cell sorter (Coulter Corp., Hialeah, FL). Thymic cells (10^6 cells/reaction) were incubated with FITC- or PE-labeled antibodies for 30 min on ice, spun, washed once with 3% FCS in PBS, and resuspended in 1 ml of the same solution. With the exception of F4/80 (Serotec Ltd., Kidlington, Oxford, UK) all the antibodies used for fluorescence-activated cell sorter analysis were purchased from PharMingen (San Diego, CA). An average of 20,000 cells was recorded in each case.

Results

Generation of Transgenic Animals Expressing the Chemokine N51/KC in the Thymus. Because the expression of this specific

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[1] *Abbreviation used in this paper:* hGH, human growth hormone.

A

B
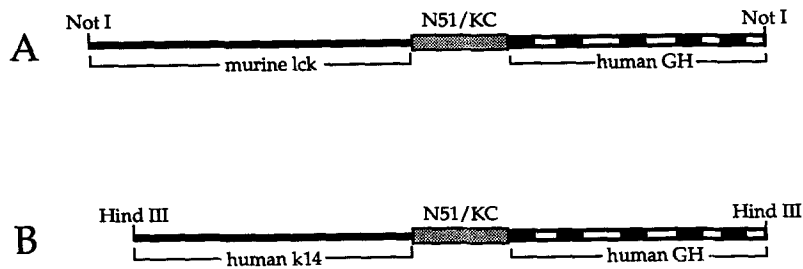
Figure 1. Diagram of the transgenes microinjected into mouse eggs. (A) The lck-N51 transgene. The murine lck promoter drives the expression of the N51/KC cDNA. (B) The K14-N51 transgene. The human keratin promoter drives the N51/KC cDNA. In both transgenes, hGH sequences provide introns, exons, and polyadenylation sequences.

chemokine had not been studied via transgenesis, the potential problem of toxicity and lethality tailored our choice of promoter elements. The thymus was selected as the site of transgene expression because N51/KC is not expressed in this organ (9) and because lesions to the thymus are not incompatible with survival. To target the expression of the transgene to the thymus, we used the proximal lck promoter fused to the cDNA encoding N51/KC, plus hGH sequences. This transgene is referred to here as the lck-N51 transgene (Fig. 1). Fertilized (C57BL/6J × DBA/2J)F$_1$ mouse eggs, were injected with the lck-N51 transgene and transferred to foster mothers. PCR analysis of their offspring resulted in the identification of 11 lck-N51 founder mice. The resulting transgenic mice were healthy, fertile, and did not have any obvious macroscopic phenotype. To obtain a large number of animals for expression analysis, we mated nine of the founders to wild-type mice and established eight transgenic lines.

Transgene mRNA was detected at varying levels in the thymus of all lck-N51 lines tested (Fig. 2). Occasionally, very low levels of transgene expression could be detected in the

spleen, but not in 13 other organs (data not shown); a finding that is in agreement with the reported pattern of expression of the lck promoter in transgenic mice (25). Transgene mRNA was detected at the highest levels in the thymus of lines 48 and 60. In situ hybridization analysis of the thymus of animals in these lines revealed prominent transgene expression in the cortex and lower levels in the medulla, suggestive of an increased expression of the transgene in immature T cells (Fig. 2). Western blot analysis revealed the presence of N51/KC immunoreactive material in the thymus of transgenic animals (Fig. 3). Additional studies involving [^{35}S]methionine labeling of thymocytes and subsequent immunoprecipitation, showed that N51/KC could be readily detected in the media of thymocytes derived from transgenic but not control animals (Fig. 3), and that such material was not detected in the cellular pellets (data not shown), suggesting rapid secretion of the N51/KC protein to the extracellular medium.

Flow Cytometry Analysis Detects an Increased Subset of Granulocytes in the Thymus. Flow cytometry analysis of thymic cells from at least three different lck-N51 lines indicated the
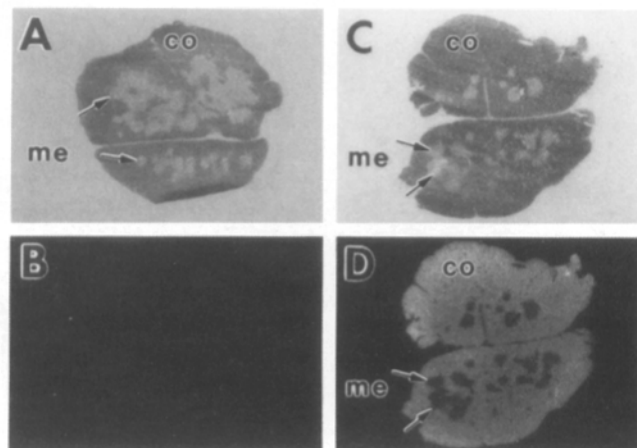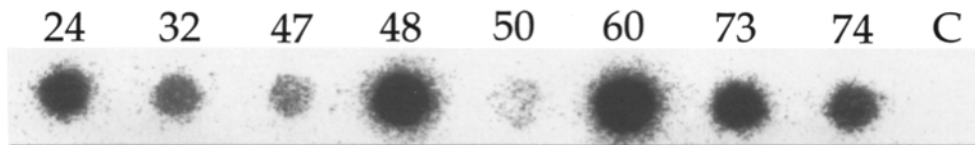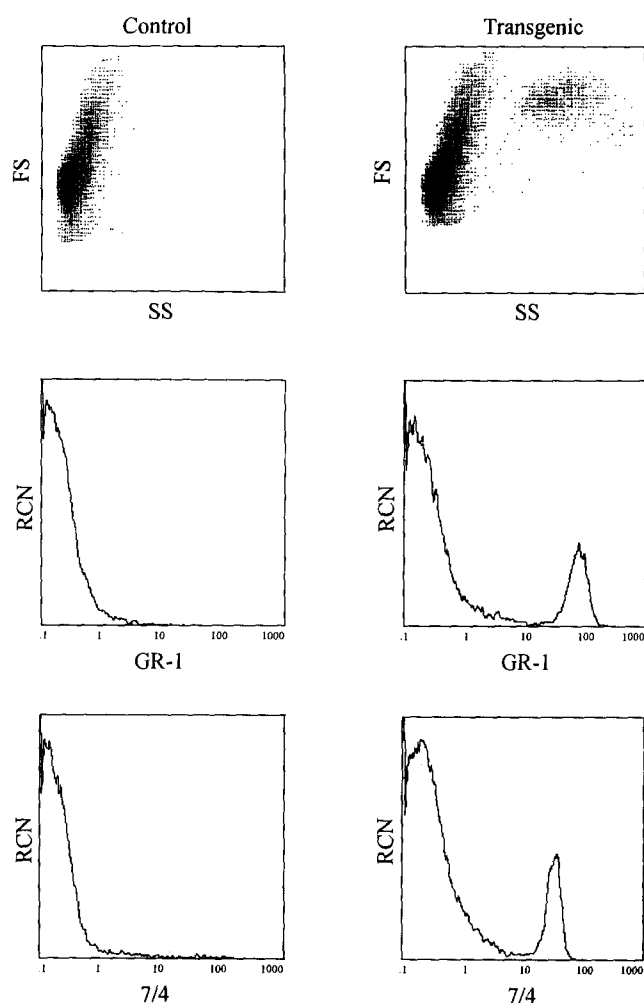

Figure 2. lck-N51 transgene mRNA expression in the thymus. (*Top*) Dot-blot hybridization of thymus total RNA (10 μg) from distinct transgenic lines. (*Bottom*) In situ hybridization analysis of N51/KC in thymus sections of control (*A* and *B*) and of a lck-N51 transgenic mouse of line 48 (*C* and *D*). The sections were hybridized with N51/KC riboprobes and photographed under bright-field (*A* and *C*) and dark-field (*B* and *D*) illumination. (*co*) Cortex; (*me*) medulla.
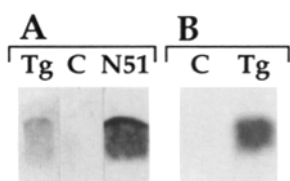
Figure 3. Expression of N51 protein in the transgenic mice. (A) Thymus cells isolated from a lck-N51 transgenic (Tg) (line 60) and a control (C) mouse were lyzed and immunoprecipitated with anti-N51 antibody. The immunocomplexes, along with 100 ng of purified baculovirus expressed N51 (N51), were analyzed by SDS-PAGE and immunoblotting using anti-N51 antibody. (B) Thymus cells isolated from a control and a lck-N51 transgenic mouse (line 48) were biosynthetically labeled overnight with [^{35}S]methionine. The conditioned media were immunoprecipitated with anti-N51 antibody and the immunocomplexes were analyzed by SDS-PAGE and fluorography.
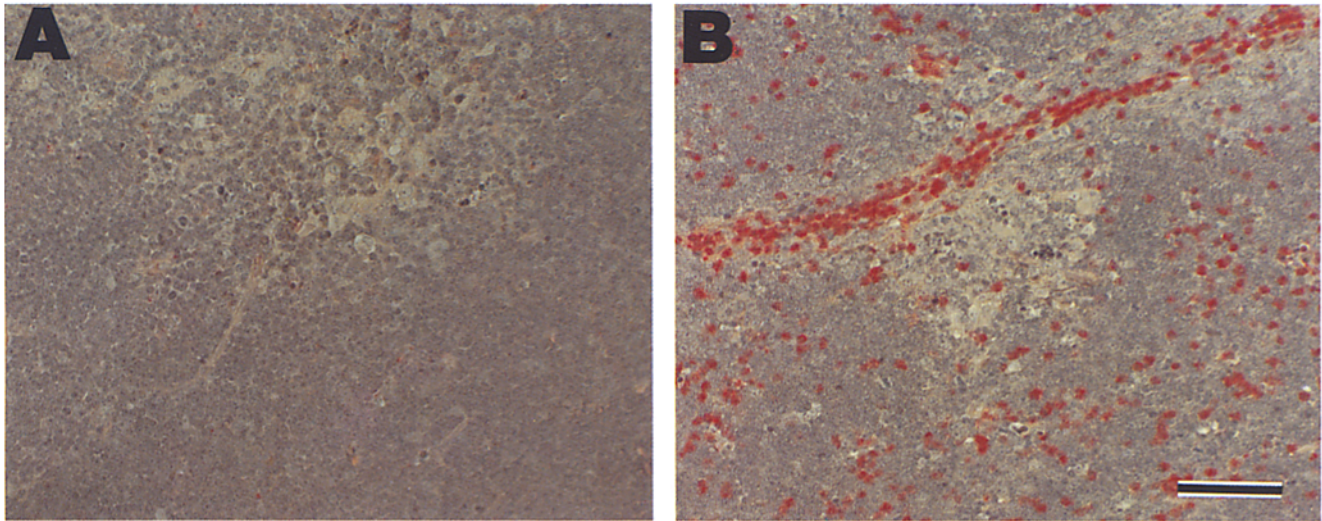
presence of a distinct cell population in the thymus. This population was composed of cells not significantly larger than thymocytes but of much higher granularity (Fig. 4). Over 95% of such cells were stained with antibodies directed against the granulocytic markers GR-1 and 7/4. The number of positive GR-1 cells in the thymus of transgenic mice averaged 7–10-fold higher than that of control animals (data not shown). Since the GR-1 antibody identifies an epitope present on all granulocytes, we sought to characterize this population in detail, using the antibody 7/4 that recognizes an antigen present solely on neutrophils (26). In addition, we examined whether other cell types could be increased in the thymus of these transgenic mice. We did not detect an increase in the number of F4/80 (monocyte/macrophage lineage) or NK 1.1 (NK cells) positive cells, nor could we detect changes in the CD4/CD8 T cell populations (data not shown). These results suggest that the infiltration is predominantly unicellular (neutrophils) and that at least one parameter of T cell differentiation is not significantly affected by the expression of the transgene or by the presence of the neutrophils in the thymus.

Characterization of the Cellular Infiltrate in the Thymus. The thymus was the only organ that contained a prominent cellular infiltrate during extensive histopathological analysis of the lck-N51 animals. A rare neutrophil was observed in the thymic cortex or medulla of control animals, whereas a marked neutrophilic infiltrate was present in the thymic cortex of all four lck-N51 transgenic lines examined. The neutrophilic infiltrate was not accompanied by morphological features of injury in the surrounding tissue as determined by light and electron microscopy, and the severity of the infiltrate was proportional to the levels of transgene expression. To assess whether the presence of neutrophils was associated with an increased vascular permeability we used Evan's blue dye. No significant differences could be detected among thymuses from control and transgenic mice from lines 48 and 60 (*n* = 3, data not shown), indicating that the expression of the chemokine N51/KC did not induce a significant increase in vascular leakage. To further characterize the neutrophilic infiltration, we performed semiquantitative analysis of neutrophil numbers in thymic sections of animals in line 48. In the thymus of these mice, aggregates or clusters of 10 or more neutrophils, often with a perivascular distribution, were frequently observed (Fig. 5). Statistically significant (*p* ≤0.05) increases in neutrophil counts, as compared with age-matched controls, occurred in the thymic cortex of lck-N51 transgenic animals at all ages analyzed (Fig. 6). The neutrophilic infiltrate of the thymus was greatest in the 15-d-old group. The number of neutrophils per unit area decreased over time, and was approximately equivalent in the 50- and 100-d-old animals. Despite the marked infiltration of the transgenic thymus with neutrophils, we did not detect significant changes in the absolute or relative numbers of circulating granulocytes, lymphocytes, red cells, or platelets (data not shown).

In addition to a significantly increased number of neutrophils, the thymus of 50- and 100-d-old transgenic animals contained cells with bright eosinophilic, spindloid, in-

Figure 4. Flow cytometry analysis of transgenic and control thymic cells. (*Top*) Forward (*FS*) and side (*SS*) scatter analysis. Note the presence of a distinct class of cells in the thymus of transgenic animals (lck-N51 line 48). (*Middle*) Thymic cell suspensions analyzed for the expression of a pan-granulocytic marker, Gr-1. (*Bottom*) Thymic cell suspensions analyzed for the expression of a neutrophil differentiation antigen recognized by the mAb 7/4. (*RCN*) Relative cell number. These results are representative of more than five separate experiments.

Figure 5. Light photomicrographs of the thymus of 15-d-old mice. No chloroacetate esterase (CAE)-positive cells, indicative of neutrophils, are present in the control thymus (*A*), whereas numerous CAE-positive cells are present in the lck-N51 (line 48) transgenic mouse (*B*). (*A* and *B*) Bars: 100 μm.

tracytoplasmic inclusions. Ultrastructural analysis indicated that such cells were thymic macrophages and epithelial-reticular cells (Fig. 7). The number of macrophages in the transgenic thymus, however, remained unchanged; we were not able to detect an increase in the number of tissue macrophages by flow cytometry or by immunohistochemistry using the F4/80 antibody (data not shown), indicating that monocytes were not being recruited to the thymus as a consequence of transgene expression. Both cell types often contained numerous crystalloid inclusions that greatly distended the cytoplasm, and in some instances, perforated the cell membrane. These inclusions, which were similar in both cell types, were contained in lysosomal structures, and were characterized as electron dense, rod-shaped, linear crystalloid structures measuring up to 7.5 μm in length, with straight sides and blunt, tapering ends (Fig. 7). Several crystalloid inclusions lying parallel to one another were occasionally seen. Other rod- or irregular-shaped crystalloid inclusions were enmeshed with granular electron-dense material and lipid, characteristic of lipofuscin or residual bodies.

In addition to intracytoplasmic crystalloid inclusions, macrophages and epithelial-reticular cells also contained phagocytized cellular and nuclear debris, with characteristics of degenerative or necrotic neutrophils in varying stages of apoptosis. Degenerative neutrophils, characterized by a fragmented, condensed electron-dense nucleus and cytoplasm containing primary granules, were frequently observed. The degenerative neutrophils were contained within, or partially enveloped by, macrophage or epithelial-reticular cell pseudopodia (Fig. 7).

Expression of N51/KC in the Epidermis also Results in a Neutrophilic Infiltrate. Since the expression of N51/KC in the thymus resulted in a neutrophilic infiltrate that did not induce injury, we decided to probe its chemotatic nature by expressing it in other tissues. The reasons for this were two-

fold: first, to ask whether the neutrophil chemoattractant properties of N51/KC were independent of the tissue substrate; and, second, to investigate whether the nature of the infiltrate would remain the same. We selected the epidermis as our next target tissue because it has a reduced granulocytic and lymphoid background and because it provides a convenient experimental access. To target the expression of N51/KC to the skin and other stratified epithelia, we used a 2.3-kb segment of the human keratin 14 promoter fused to the N51/KC cDNA plus hGH sequences (referred to here as the K14-N51 transgene). This promoter targets the expression of transgenes to most stratified squamous epithelia, including epidermis, tongue, and esophagus (27). We generated seven founders bearing the K14-N51 transgene. To examine K14-N51 transgene expression, we carried out RNase protection experiments in founders and their offspring. Skin and tongue RNA from animals in lines 35 and 38, but not that of the other founders or control mice, completely protected the ^{32}P-labeled riboprobe (Fig. 8). Thus, in this set of transgenic experiments, the penetrance was reduced (two expressing lines out of seven) when compared with that obtained with the lck-N51 transgene (eight expressing lines out of eight) and the levels of expression were severalfold lower when compared with that of lck-N51 animals. Notwithstanding the low levels of transgene expression, a higher number of neutrophils was observed in the tongue and tail skin of the expressing lines (35 and 38) than in age-matched controls. Similar to what was observed with the lck-N51 transgene, the infiltrate in tongue and skin was neutrophilic and not associated with any morphologic features of injury. The neutrophilic infiltrate in the tongue was adjacent to and within the squamous mucosa, whereas the neutrophilic infiltrate in the skin was observed in close proximity to the outer root sheath cells of the hair follicles and the basal layer of the epidermis (Fig. 9). The presence of neutrophils in these areas most likely results

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Cortex

Medulla
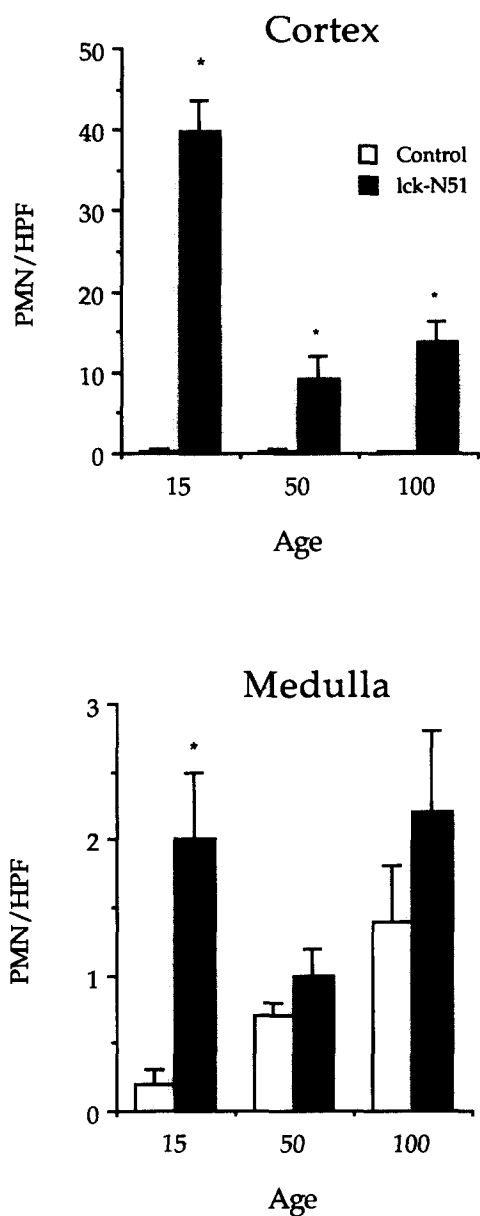
Figure 6. Quantification of neutrophils in the thymus of control and lck-N51 transgenic mice. Columns represent mean number of polymorphonuclear neutrophils (*PMN*) per high powered field (*PMN/HPF*). (*) Statistically increased over age-matched controls (ANOVA, $p \leqslant 0.05$). Error bars = ± SEM (n = five animals).

from local transgene expression, since these cells represent known sites for K14 gene expression (27, 28). The chronic expression of N51 in these compartments to date (>50 d of age) has not affected the growth of the skin and adnexa, nor resulted in healing abnormalities or tumors.

Discussion

Results from this study indicate that transgenic mice chronically expressing the N51/KC chemokine have a significant neutrophilic infiltrate in the sites of transgene expression. In

these tissues, the neutrophils are distributed in close proximity to cells expressing the transgene and often around vessels. The perivascular distribution and subsequent distribution throughout the parenchyma of these tissues, imply that the neutrophils migrate from the vascular lumen into the adjacent parenchyma. These results conclusively demonstrate that the N51/KC chemokine functions as a neutrophil chemoattractant in vivo. Whereas it is possible that some of the chemotactic role of N51/KC may be mediated indirectly, via generalized activation of the endothelium, the striking pattern of neutrophil infiltration in the vicinity of cells expressing the N51/KC chemokine in both transgenic models argues in favor of a direct chemoattractant role for this molecule. Reinforcing this notion is the recent experimental evidence that N51/KC binds and induces intracellular calcium changes in human neutrophils (16).

The presence of neutrophils in the parenchyma indicates that the N51/KC chemokine is capable of activating a number of processes that allow for neutrophilic infiltration, including transendothelial and basement membrane migration and navigation throughout the intercellular space. These phenomena are thought to be active in nature, and have not been conveniently modeled because of lack of a suitable animal system. We began to analyze the process of neutrophil infiltration in the thymus by quantitating the number of neutrophils at different ages. In the thymus, the greatest degree of neutrophil infiltration was present in the younger animals (<15 d of age). Although still significantly greater than age-matched controls, the number of neutrophils in the thymus decreased at later time points, i.e., at 50 and 100 d of age. This reduction in neutrophil numbers, however, cannot be ascribed to reduced transgene expression because there is no significant decrease in mRNA expression at those time points (data not shown). The specific mechanism responsible for this reduction remains to be elucidated but may reside in a reduction in active recruitment followed by senescence and death of the resident neutrophil population. The average half-life of neutrophils in the blood is 6 h after their release from the bone marrow (29). After neutrophils migrate into tissues, they do not return to circulation, but remain in the tissue spaces for ~1–2 d and undergo apoptosis if left unstimulated (29, 30). The precise mechanisms involved in disposal of senescent neutrophils are not well understood, but it has been known since Metchnikoff (31) that they can be phagocytosed by macrophages. In the present study, the decrease in thymic neutrophils in older mice was associated with the presence of bright, eosinophilic cells with cytoplasmic inclusions that we identified as macrophages and epithelial-reticular cells. Such phenomenon was observed in the thymuses of transgenic mice from three distinct pedigrees, which rules out the possibility that it might have been due to a position effect. Although one cannot exclude the possibility that the appearance of cytoplasmic inclusions was a consequence of a direct action of N51/KC on those cells, the presence of cytoplasmic inclusions on macrophages engaged in active phagocytosis of degenerating neutrophils strongly suggests that such inclusions may represent a phagocytic byproduct. Crystalloid inclusions
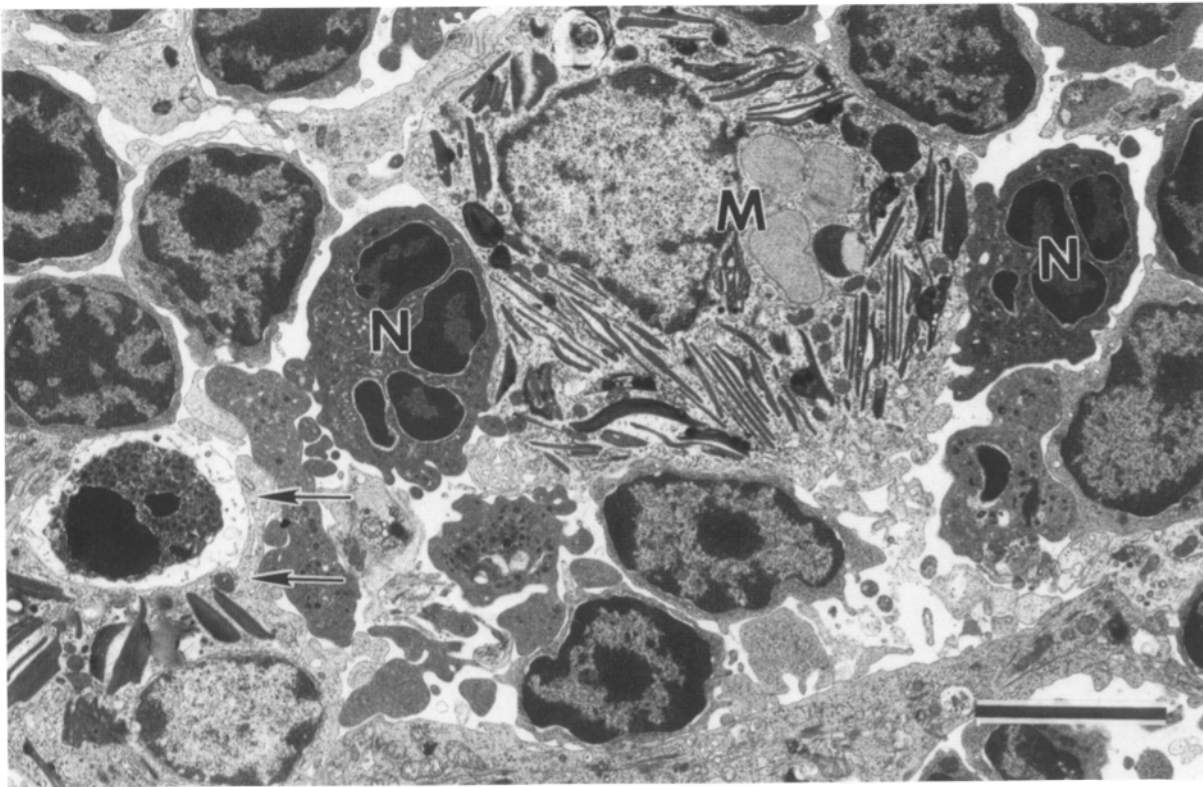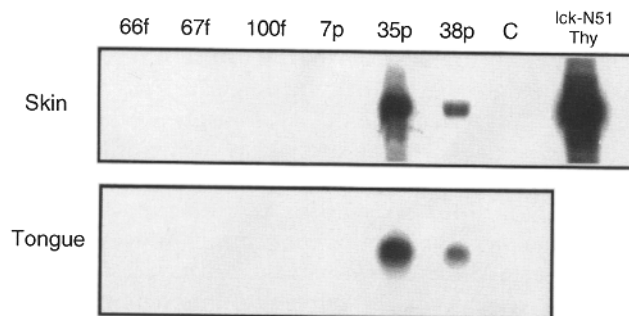
Figure 7. Transmission electron micrograph of a thymus of a 200-d-old lck-N51 transgenic mouse. Several neutrophils (*N*) are in close proximity to thymic macrophages (*M*) that contain numerous crystalloid inclusions. Macrophage pseudopodia (*arrows*) surround an apoptotic neutrophil. Section stained with lead citrate and uranyl acetate. Bar, 5 μm.

in macrophages, with characteristics similar to those described in the present study, have also been reported in the peritoneal macrophages of adult mice administered buffy-coat white cells (32), in the bone marrow of mice with myeloid leukemia, and in bone marrow, spleen, lung, illeum, and Kuppfer cells of liver but not thymus of aged mice (33). It has been postulated that such inclusions represent phagocytic byproducts of granulocytes (33). By electron microscopy, degenerating granulocytes have also been identified in the cytoplasm of the peritoneal macrophages (32), glomerular macrophages (34),

Figure 8. K14-N51 transgene expression in tongue and skin of transgenic and control mice. Total RNA (10 μg) was used for these RNase protection experiments. As positive control (*lck-N51 Thy*), we used 2 μg of thymus RNA from a lck-N51 animal (line 48). (*f*) Founder; (*p*) progeny.

and macrophages from acutely inflamed joints (35). Thus, the recognition of senescent but otherwise intact neutrophils by phagocytic cells, including thymic macrophages and epithelial-reticular cells, may represent an injury-limiting neutrophil disposal mechanism, important in the normal resolution of inflammation.

Previous studies using transgenic mice have reported that expression of cytokines can result in an overt inflammatory response or in severe hematopoietic abnormalities. In some cases, the granulocytic infiltration seems to be the product of a strong proliferative stimulus for a given cell lineage, as observed in transgenic mice overexpressing the IL-5 gene, which develop splenomegaly and the full pathway of eosinophil differentiation with massive eosinophilia (36, 37). In other cases, the inflammatory component seems to result from recruitment of several different cells to the site and activation of synthesis and release of a variety of inflammatory mediators. Allison et al. (38) have demonstrated that mice expressing the IL-2 gene in the pancreas develop pancreatitis affecting predominantly the exocrine pancreas with progression to diabetes and subsequent death. The inflammatory reaction was characterized by massive numbers of macrophages, some lymphocytes, and eosinophils. In animals surviving to weaning age, the pancreatitis resolved into a peri-islet infiltrate comprising mainly small lymphocytes and some macrophages. Similarly, Katsuki et al. (39) demonstrated a perivascular lym-
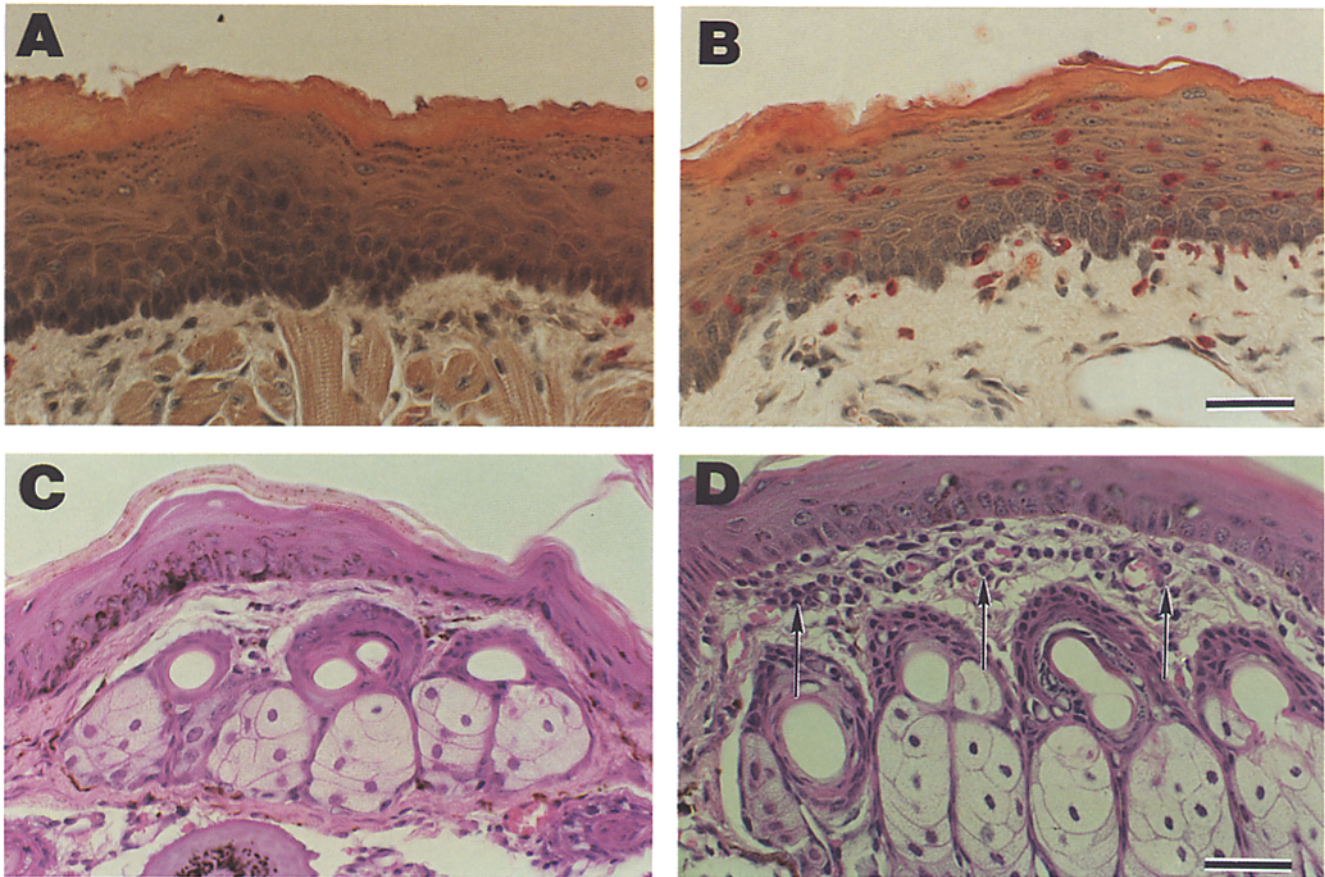
Figure 9. Light photomicrographs of the tongue of a 52-d-old control (*A*) and K14-N51 transgenic (*B*) mouse. A rare CAE-positive cell is present in control tongue, whereas numerous CAE-positive cells with morphologic characteristics of neutrophils, are observed in the squamous epithelium and lamina propria of the tongue of the transgenic animal. Light photomicrograph of the skin from the tail of 22-d-old control (*C*) and K14-N51 transgenic (*D*) mouse. The epidermis and superficial dermis of transgenic animals contain several neutrophils (*arrows*) (H & E). Bars: (*A–D*) 50 μm.

phocyte accumulation in the cerebellar meninges which was followed by increased cell infiltration of neutrophils and monocytes in the cerebellum in transgenic mice with an intact human genomic IL-2 gene or a mouse metallothionein I promoter–human IL-2 chimeric gene. A multicellular inflammatory reaction is also observed in mice expressing TNF genes in their joints (40), skin (41), or pancreas (42–44) and in mice expressing IL-10 in the pancreas (45). Yet, in other studies, the expression of cytokines results in an inflammatory process in areas not associated with the transgene expression. Transgenic mice overexpressing IL-4 in the thymus, develop an inflammatory occular lesion (blepharitis) with characteristic histopathological features seen in allergic reactions (mononuclear cells and a striking number of eosinophils). In the thymus, IL-4 transgene expression perturbs T cell maturation, reducing the number of double positives and peripheral T cells, but does not result in recruitment of inflammatory cells (46, 47). In contrast to these well characterized transgenic models, the cellular infiltrate resulting from the overexpression of N51/KC is monocellular, restricted to the site of transgene expression, and is benign in nature. The lack of a full-blown inflammatory reaction to the chronic expression of N51/KC suggests that under physiological con-

ditions, N51/KC functions primarily as a chemoattractant molecule, and that the inflammatory activation of neutrophils (i.e., degranulation) requires other inflammatory mediators. The rapid activation of N51/KC in fibroblasts, macrophages, and the endothelium might therefore, constitute one of the first levels of response towards lesion, ensuring the arrival of a particular class of cells to the site of injury.

The most relevant feature of the N51/KC transgenic models described here is the genetically programmed recruitment of a single class of inflammatory cells to a particular tissue. These models, therefore, will be particularly useful to study the physiological pathways required for the establishment of the inflammatory response. Given the striking specificity of N51/KC towards neutrophils, these models will be useful to study the mechanisms involved in diapedesis, providing much needed information on the interactions of neutrophils with the endothelial wall, and serve as valuable animal models in which to evaluate compounds that affect such interactions. Other long-standing questions on inflammation, such as the nature of the chemotactic gradient, may also be addressed by these models. The distinction between fluid- or solid-phase chemotactic gradients has not been possible up to now, because the chemotactic assays currently available rely on the establish-

ment of fluid-phase gradients. Since C-C and C-X-C chemokine family members bind negatively charged proteoglycans (4) it is possible that they could bind to chemically related substances (glycosaminoglycans) in the vessels or in the tissue parenchyma to form a solid- rather than a fluid-phase gradient. Evidence in favor of the formation of solid-phase gradients has been recently obtained for chemokines such as IL-8 (48) and MIP-1β (49). Therefore, these transgenic mice might offer the opportunity to further discriminate between these gradient models. Finally, the N51/KC transgenic models may also contribute to our understanding of the fate of the infiltrating neutrophils, an issue that is central to the resolution of the inflammatory response.

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