CXC Chemokine Redundancy Ensures Local Neutrophil Recruitment during Acute Inflammation

Daniel George Remick, Liza Beth Green,
David Eric Newcomb, Sunir Jain Garg,
Gerald Lee Bolgos, and Douglas Ruben Call

From the Department of Pathology, University of Michigan Medical School, Ann Arbor, Michigan

Previous publications demonstrated that elevated systemic levels of interleukin (IL)-8 decrease local neutrophil recruitment. We tested whether sustained, high plasma levels of IL-8 would prevent local inflammation after inflammatory insults. Mice carrying the transgene for human IL-8 were separated on the basis of their plasma levels of IL-8 into IL-8-positive (plasma levels >90 ng/ml) and IL-8-negative (IL-8 below detection). Presence of the IL-8 transgene did not improve survival or morbidity nor did it alter peritoneal neutrophil recruitment induced by the cecal ligation and puncture model of sepsis. In an acute lung injury model created by intratracheal injection of acid, IL-8-positive mice showed no reduction in alveolar neutrophil recruitment. There was no difference in the local recruitment of neutrophils when either thioglycollate or glycogen was injected intraperitoneally. We examined the chemotactic response to murine chemokines to test how neutrophil recruitment occurs in the setting of elevated plasma IL-8 and found that neutrophils from both IL-8-positive and -negative mice respond equally well to recombinant KC or macrophage inflammatory protein (MIP)-2. We measured KC and MIP-2 in the peritoneum after thioglycollate injection and demonstrated that IL-8-positive mice have significantly higher levels of the chemokines compared to the IL-8-negative mice. Antibody inhibition of KC and MIP-2 in the IL-8-positive mice significantly decreased peritoneal neutrophil recruitment in response to thioglycollate, clarifying their important role in the local neutrophil recruitment. Our data demonstrate that despite the presence of high plasma levels of IL-8, neutrophils may still be recruited to sites of local inflammation because of chemokine redundancy. *(Am J Pathol 2001, 159:1149–1157)*

Interleukin 8 (IL-8) belongs to a class of cytokines referred to as chemokines. These are small molecular weight peptides, usually <12,000 d that are divided into broad categories based on their protein structure. The CXC chemokines have two pairs of cysteine residues separated by an intervening amino acid whereas the CC chemokines have the cysteine residues located adjacent to one another. Other members of the chemokine family include the CX_3C and C groups.[1,2] The CXC chemokines serve to recruit neutrophils to local sites of inflammation.[3] The exact murine homolog of IL-8 has not been defined, but two candidate genes are KC and macrophage inflammatory protein 2 (MIP-2).[4]

The chemokines have several functions but one of their major attributes is the ability to induce chemotaxis of neutrophils.[5,6] With this property chemokines serve to bring these cells to sites of acute inflammation, and also to retain them once they have arrived. A portion of the mechanism responsible for this recruitment is the generation of a chemotactic/haptotactic gradient to draw the cells into the local environment.[7] Previous reports have demonstrated that high intravascular levels of IL-8 will reduce neutrophil emigration to sites of local inflammation.[8–10]

Mice carrying the human IL-8 transgene have extremely high plasma levels of this chemokine.[11] Rodent neutrophils will respond to human IL-8 by chemotaxis although a higher concentration is required.[12] We sought to test whether continuous high plasma concentrations of IL-8 would reduce neutrophil recruitment in four different animal models of acute inflammation. Given the redundancy of chemokines[1,2] we investigated whether the murine chemokines would become up-regulated to offset the high plasma IL-8 levels and allow recruitment of neutrophils to the sites of inflammation. To specifically test if local up-regulation of endogenous murine chemokines was responsible for neutrophil recruitment, we performed antibody inhibition studies.

Materials and Methods

Characterization of the Transgenic Mice

A breeding colony of mice was established from mice generously donated by Scott Simonet of Amgen, Inc. (Thousand Oaks, CA). These mice (HE8 line) carry the human IL-8 transgene and express high, systemic levels of IL-8 in the plasma.[11] Hybrid mice were obtained by crossing the IL-8 transgenic mice with BDF_1 mice (Charles Rivers, Portage, MI). At ~8 weeks of age the plasma levels of IL-8 were determined by obtaining a small sample of blood from the tail vein. IL-8 was determined using our previously described enzyme-linked immunosorbent assay (ELISA).[13] Mice were divided on the basis of their plasma levels of IL-8 into negative mice (IL-8-negative), which had undetectable levels of IL-8 (<200 pg/ml), and positive mice (IL-8-positive), which had >90,000 pg/ml of IL-8. A third group of mice was also evaluated, wild-type BDF_1. In the unmanipulated mice, a complete blood count with differential was performed. Additionally, the lungs were homogenized and the myeloperoxidase (MPO) content of the lung was determined as a measure of neutrophil sequestration, as previously described.[14] Briefly, the chest cavity was exposed and the right side of the heart perfused with 0.9% isotonic saline (normal saline) to remove blood from pulmonary circulation. The right lung was then removed and placed on ice in MPO homogenization buffer, the lung was homogenized followed by sonication. After centrifugation the supernatant was mixed with assay buffer and read in a kinetics mode on an ELISA reader.

Models of Acute Inflammation

The experiments in this article were performed in accordance with National Institutes of Health guidelines and approval from the Animal Use Committee of the University of Michigan.

Cecal Ligation and Puncture (CLP)

CLP was performed as previously described with minor modifications.[15,16] Mice were anesthetized with ketamine/xylazine (87 and 13 mg/kg of body weight, respectively) and a small midline abdominal incision was made. The cecum was isolated and punctured three times with a 16-gauge needle that resulted in 60 to 70% lethality. The needle was removed and a small amount of the stool was expelled from the punctures to ensure patency. The mice were closed using 4.0 sutures for the abdomen and stainless steel removable wound clips for the skin. After the surgery, mice were injected subcutaneously with 1 ml of prewarmed isotonic saline before being placed into their cage. Mice were treated with subcutaneous antibiotics (23 mg/kg of imipenem diluted in D5W) at 2 hours after surgery. This dose was repeated every 12 hours for a total of 3 days so that mice were receiving both antibiotics and fluids. Mice were kept in a temperature-controlled room (25°C) with light and dark cycles every 12 hours.

Collection of Data

Mice were sacrificed 24 hours after CLP, before they received their third antibiotic injection. At the time of sacrifice heparin anti-coagulated plasma was collected and stored for later cytokine analysis. A complete blood count was performed with a Coulter Counter (model Zf; Coulter Electronic, Hialeah, FL). Peritoneal washes were performed by opening the peritoneum sterilely and washing with 1 ml of 1× Hanks' balanced salt solution. This was followed with a separate wash of 15 ml with 1× Hanks' balanced salt solution. The 1-ml wash was centrifuged and supernatant was frozen for later analysis. Cells from the centrifugation were then combined with cells from the 15-ml wash. White blood cell differentials were obtained by staining with Diff-Quick (Baxter, Detroit, MI). The right lung was harvested for MPO determination. Blood was obtained from the tail vein and an automated differential was performed using a Hemavet Mascot (CDC Technologies, Oxford, CT) as previously described.[17]

Preparation of Recombinant Murine Chemokines and ELISAs

Recombinant murine KC and MIP-2 were prepared in a bacterial expression system and the recombinant protein isolated from glutathione-Sepharose columns (pGEX system; Pharmacia, Piscataway, NJ) as previously described.[18,19] The purified KC or MIP-2 protein was >95% pure as judged by denaturing sodium dodecyl sulfate-polyacrylamide gel electrophoresis. These purified proteins will induce neutrophil chemotaxis in mouse or human neutrophils and they were used to raise rabbit or goat polyclonal antibodies by immunization with Freund's complete adjuvant or Titermax (Sigma Chemical Co., St. Louis, MO). The antibodies were used to prepare ELISAs for protein quantification using our previously published methods.[13] These antibodies have specificity with no cross-reactivity in the ranges used.[18] A polyclonal antibody to KC was also raised in a goat as previously described.[19]

Cytokine Analysis

Cytokines were measured in the peritoneal fluid, bronchoalveolar lavage, and plasma. Tumor necrosis factor (TNF) was determined by cytolytic activity directed against the WEHI 164 fibrosarcoma cells as previously described.[20,21] The concentration of TNF was determined by calculation from a standard curve of recombinant human TNF (Cetus, Emmeryville, CA). IL-6 was measured by the B9 cell proliferation assay as previously described[22] and the IL-6 concentrations determined from a standard curve of human recombinant IL-6. KC and MIP-2 were quantified by an ELISA developed in our

laboratory according to our published methods.[13,18] Briefly, polyclonal antibodies were purified on protein A columns and used as capture antibodies. A portion of the same antibody was biotinylated and used for the detection antibody. Streptavidin-HRP (Jackson Immunoresearch, West Grove, PA) was used for amplification and the color was developed with TMB solution (Genzyme, Cambridge, MA). Neutrophil chemotaxis was performed using a 48-well chemotaxis chamber (Neuroprobe, Cabin John, MD) and previously described methods.[18,23] Neutrophils were prepared from the peripheral blood of either the IL-8-positive or IL-8-negative mice by dextran sedimentation and hypotonic lysis of residual red blood cells. Chemotaxis was expressed as the percentage of cells that migrated to 10^{-6} mol/L fMLP (Sigma), after background subtraction.

In Vitro *Stimulation*

Alveolar macrophages or peritoneal cells were harvested from either the IL-8-positive or IL-8-negative mice. The cells were incubated in RPMI 1640 with 1% fetal calf serum at a concentration of 1.5×10^5 cells/ml (alveolar macrophages) or 5×10^5 cells/ml (peritoneal cells). The cells were stimulated with 1 μg/ml of lipopolysaccharide and the supernatants collected after 24 hours.

Acid Lung Injury

Acid lung injury was induced by injection of 80 to 100 μl of diluted HCl, pH 1.5, following a previously described procedure.[24,25] Isotonic 0.9% saline was diluted 1:3 and the pH adjusted to 1.5 with concentrated HCl. Mice were anesthetized with ketamine/xylazine, the mice vertically suspended and the trachea exposed. Under direct visualization, the acid was instilled into the trachea using a 30-gauge needle. This was done with multiple small injections with the animal spontaneously breathing between each injection. Control mice had 0.9% isotonic saline (normal saline) injected in place of diluted acid. Immediately after surgery the neck was closed with a stainless steel surgical clip. At the time of sacrifice, heparin anti-coagulated plasma was obtained and saved for cytokine analysis. The lungs were then lavaged with 1 ml of warmed Hanks' balanced salt solution in 0.2- to 0.3-ml aliquots. This bronchoalveolar lavage fluid was centrifuged and the supernatant saved for cytokine analysis. The lungs were lavaged with a second ml of Hanks' balance salt solution and the cell pellets combined. A cell count was performed, and a differential done on a Diff-Quik-stained cytospins.

Glycogen or Thioglycollate Peritonitis

Glycogen was prepared fresh as a 2% solution (oyster shell glycogen, Sigma) in sterile isotonic saline. Thioglycollate (2.9%; Difco, Detroit, MI) was prepared and autoclaved. Either thioglycollate or glycogen was injected intraperitoneally in a 1- to 2-ml volume, as previously described.[18,19] Four hours later mice were sacrificed and
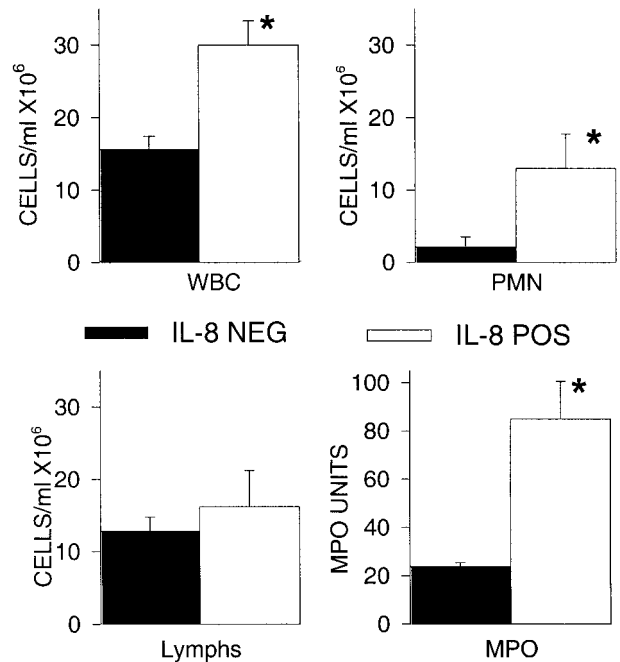

Figure 1. Pathophysiological differences between IL-8-positive and IL-8-negative mice. Mice were divided into positive and negative groups based on the plasma levels of IL-8. The IL-8-positive mice had a significantly higher WBC that was because of neutrophils. Additionally, there were increased neutrophils within the lungs as indicated by an elevated MPO. Each value is the mean ± SEM for 16 mice for the hematology parameters and 9 mice for the MPO. *, $P < 0.05$ IL-8-positive compared to IL-8-negative.

the peritoneum washed as described in the CLP model. Heparin anti-coagulated plasma was also obtained.

Antibody Neutralization Studies

For these experiments mice were injected subcutaneously with 1 ml of 50:50 mixture of goat anti-KC plus rabbit anti-MIP-2. These antibodies have previously been described and will effectively neutralize murine chemokines *in vivo*.[19] For controls, mice were injected with 1 ml of control goat serum plus control rabbit serum. Two hours later, the mice were injected with 2 ml of thioglycollate. Samples were harvested 4 hours later and the total number of peritoneal neutrophils determined as described above.

Statistical Analysis

The values for the IL-8-positive mice were compared to the IL-8-negative mice using the Student's *t*-test.

Results

Normal Parameters

We evaluated several normal parameters to differentiate the IL-8-positive mice from the IL-8-negative mice. First, the differences in plasma levels of IL-8 were significantly different, but because this was the criterion used to separate the two populations this was the expected result.
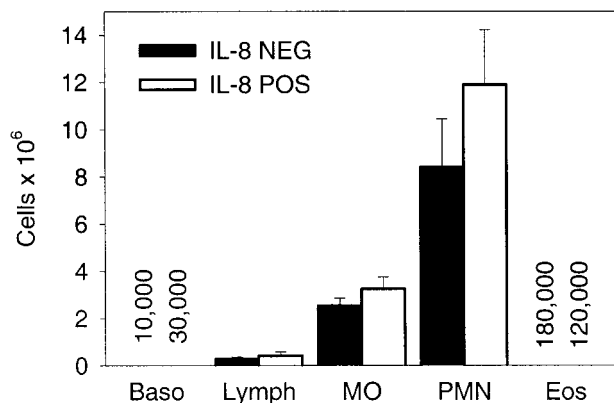
Figure 2. Peritoneal cells 24 hours after CLP. The total number of cells in the peritoneum was determined in both the IL-8-positive and IL-8-negative mice. There was no difference in the number of neutrophils in the peritoneum. Each value is the mean ± SEM of 15 IL-8-negative mice and 18 IL-8-positive mice. There were very few basophils or eosinophils present in the peritoneum, so the actual number of cells is listed with the number on the left representing the IL-8-negative mice.

Figure 3. Pulmonary cellular constituents 15 hours after acid lung injury. The total number of macrophages or neutrophils in the lung was determined in response to intratracheal injection of either acid or isotonic saline (normal saline, NS). Acid resulted in a substantial increase in the number of neutrophils recovered in the lavage fluid, with no difference between the IL-8-positive and IL-8-negative mice. Each value is the mean ± SEM for eight IL-8-positive and eight IL-8-negative mice injected with acid and four IL-8-positive and three IL-8-negative mice injected with isotonic saline.

IL-8-positive mice had a significantly higher white blood cell count because almost exclusively of the increased numbers of neutrophils (Figure 1). In a separate experiment, we examined the peripheral blood values of non-transgenic mice and IL-8-negative mice. The absolute number of peripheral blood neutrophils in the nontransgenic mice ($2.37 \pm 0.54 \times 10^6$ cells/ml) was nearly identical to the IL-8-negative mice ($2.35 \pm 0.41 \times 10^6$ cells/ml). Pulmonary MPO demonstrated substantially more neutrophils within the lung. Histological examination of the lungs showed that the neutrophils were within the blood vessels and not in the alveolar airspaces. These results confirm the previous report that the IL-8 transgenic mice have evidence of increased numbers of neutrophils both in the circulating blood and within the tissues.[11] They also indicate that mice will respond to the human IL-8 transgene because there are increased numbers of neutrophils.

Effect of IL-8 Transgene on CLP Morbidity and Mortality

Mice were subjected to CLP and followed for 7 days. There was a slightly better survival in the IL-8-positive mice, but this was not significant based on log-rank survival analysis ($P > 0.05$). In the IL-8-positive mice there were 15 out of 34 survivors, whereas in the IL-8-negative mice there were 10 out of 37 survivors. Because neutrophils represent a critical component of the innate immune response to bacterial infections we determined if there was adequate recruitment of neutrophils into the peritoneum in response to severe inflammation. Mice were subjected to CLP, treated with antibiotics, and sacrificed at 24 hours. Figure 2 shows that there was no difference between the IL-8-positive and IL-8-negative mice with regards to the total number of recruited neutrophils. There was also no difference in the number of macrophages, lymphocytes, basophils, or eosinophils recovered from the peritoneum.
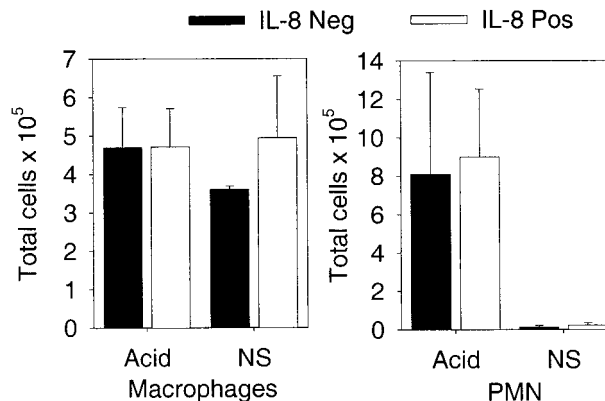
Acid Lung Injury

Aspiration of gastric contents may result in acute lung injury.[26] To mimic this scenario, we injected diluted HCl (pH 1.5) intratracheally and then determined the number of neutrophils recruited into the bronchoalveolar space. Examination of histological sections showed that neutrophils were in the alveolar space. Kinetics studies showed that in the first 8 hours there was a modest influx of neutrophils, but by 15 hours >40% of the cells in the lavage fluid were neutrophils. There were no significant differences between IL-8-positive and IL-8-negative mice ($P > 0.05$). At the 0-hour time point there were no neutrophils recovered in the bronchoalveolar lavage fluid. These findings are in contrast to the total lung MPO data that showed greater activity in the IL-8-positive mice (Figure 1). Alveolar neutrophil recruitment was examined more closely in Figure 3 where the total number of cells in the lung was quantitated. The number of neutrophils was substantially greater in response to acid injection compared to isotonic saline (normal saline), but there was no difference between IL-8-positive and IL-8-negative mice.

Peritoneal Neutrophil Recruitment to Thioglycollate or Glycogen

Intraperitoneal injection of thioglycollate has been widely used for the elicitation of neutrophils in rodents.[27] Mice were sacrificed 4 hours after intraperitoneal injection (control mice received isotonic saline) and the total number of peritoneal cells determined. Isotonic saline injection resulted in virtually no peritoneal neutrophil recruitment (Figure 4). These data parallel the lung data in which intratracheal injection of isotonic saline did not result in pulmonary neutrophil recruitment. Additionally, no detectable IL-8 was present in the peritoneal lavage fluid from either the IL-8-negative or IL-8-positive mice. In contrast, injection of either glycogen or thioglycollate resulted in neutrophil recruitment. In both models there
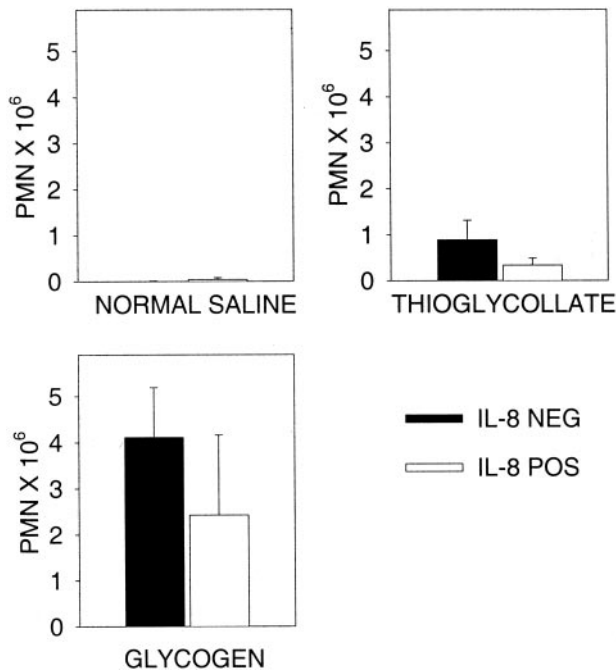
Figure 4. Peritoneal neutrophil recruitment in response to thioglycollate or glycogen. Mice were sacrificed 4 hours after injection of isotonic saline (normal saline), thioglycollate, or glycogen and peritoneal cells recovered. Both thioglycollate and glycogen recruited substantial numbers of cells to the peritoneum, with no difference between the IL-8-positive and IL-8-negative mice. Each value is the mean ± SEM for eight to nine mice for either thioglycollate or glycogen.

Figure 5. Chemokine production by alveolar and peritoneal macrophages. Cells were isolated from IL-8-negative or IL-8-positive mice and stimulated *in vitro* with lipopolysaccharide and murine chemokines were measured in the supernatant after 24 hours. There are no differences between the IL-8-positive and IL-8-negative mice. **A:** Alveolar macrophages. **B:** Peritoneal macrophages. Each value is the mean ± SEM for cells isolated from six individual mice.

were fewer neutrophils recruited in IL-8-positive mice similar to the previous report using these mice,[11] but this difference was never statistically significant ($P > 0.05$). We have previously shown that the peritoneal recruitment of neutrophils in response to 3% thioglycollate is mediated by local production of both KC and MIP-2.[19] Thioglycollate injection did not cause any increase in the local levels of IL-8, which is expected because the IL-8 is under constitutive control.

To investigate the inflammatory mediators produced after each of these inflammatory challenges, we measured TNF and IL-6 in both the plasma and at the site of inflammation. Table 1 displays the data and demonstrates that there was no difference between IL-8-negative and IL-8-positive mice. Table 1 also illustrates trends
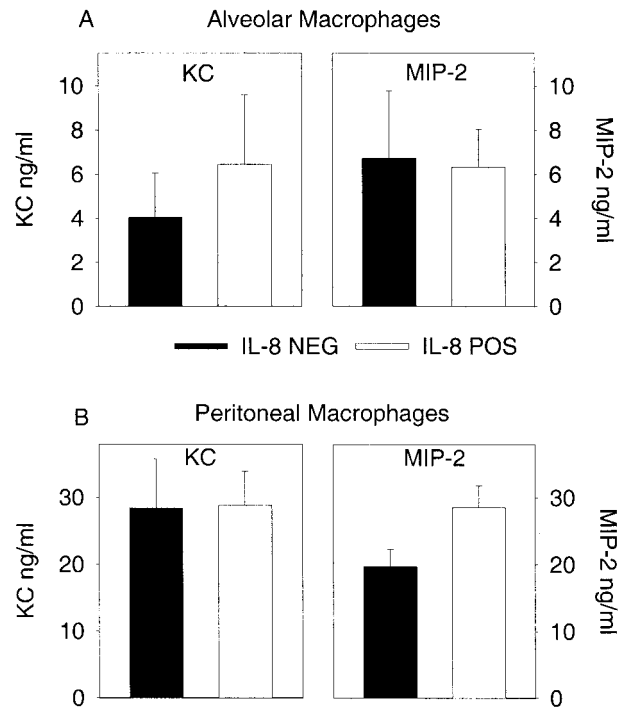
in these different models of inflammation. CLP represents the greatest inflammatory insult and has the highest levels of TNF and IL-6. It should be noted that these samples were obtained at different times after the onset of the injury and may not represent the peak values. In general, local cytokine levels were higher than systemic levels with the exception of CLP, as previously reported.[28,29]

The data indicate that neutrophils will be recruited to the local site of inflammation even in the presence of elevated plasma levels of IL-8. This suggests the possibility that additional chemotactic factors were produced in the local compartments to elicit neutrophils. We tested the ability of alveolar and peritoneal macrophages from

Table 1. Systemic and Local Cytokine Levels after Injury

	TNF pg/ml		IL-6 ng/ml	
Cytokines 24 hours after CLP*				
IL-8	Blood	Peritoneum	Blood	Peritoneum
Negative	42 ± 19	29 ± 7	284 ± 111	105 ± 44
Positive	118 ± 34	62 ± 16	260 ± 116	57 ± 25
Cytokines 15 hours after acid lung injury				
IL-8	Blood	BAL	Blood	BAL
Negative	8.7 ± 4.2	7.0 ± 5.1	0.98 ± 0.26	2.2 ± 1.3
Positive	6.5 ± 6.5	77 ± 68	0.45 ± 0.09	0.83 ± 0.3
Cytokines 4 hours after thioglycolate				
IL-8	Blood	Peritoneum	Blood	Peritoneum
Negative	BDL	BDL	0.21 ± 0.09	3.3 ± 1.6
Positive	BDL	89 ± 53	0.39 ± 0.17	11.2 ± 5.7

*Cytokines were measured in the blood, peritoneal wash, or bronchoalveolar lavage as indicated in the tables. Each value is the mean ± SEM.
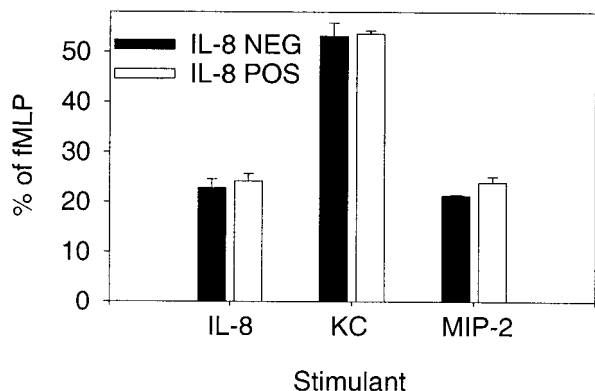BDL, below detection limit (4 pg/ml).

Figure 6. Neutrophil chemotaxis in IL-8-positive and IL-8-negative mice. Peripheral blood neutrophils were isolated and subjected to chemotaxis toward IL-8 (10^{-5} mol/L), KC (10^{-6} mol/L), or MIP-2 (10^{-6} mol/L). Neutrophils from both IL-8-positive and IL-8-negative mice exhibit similar directed migration toward the chemotactic agent. Each value is the mean $\pm$ SEM for five replicate wells and is expressed as the percentage of chemotaxis toward 10^{-6} mol/L fMLP.

Figure 7. Peritoneal chemokines after thioglycollate injection. IL-8-positive and IL-8-negative mice were injected with thioglycollate and the peritoneal contents analyzed 4 hours later. Each value is the mean $\pm$ SEM for 12 to 13 mice for the chemokine measurement. *, $P < 0.05$ IL-8-positive compared to IL-8-negative.

both IL-8-positive and IL-8-negative mice to produce the murine chemokines KC and MIP-2 after *in vitro* stimulation with 1 μg/ml of lipopolysaccharide. Figure 5 demonstrates that there was no significant difference in the inherent ability of IL-8-positive or IL-8-negative macrophages to produce the murine chemokines.

We next evaluated the neutrophils to determine whether they would still respond appropriately to a gradient of murine chemokines. Neutrophils were isolated from the peripheral blood of either IL-8-positive or IL-8-negative mice and their ability to undergo chemotaxis toward recombinant human IL-8, murine KC, or murine MIP-2 was determined using our previously described chemokine concentrations.[19] The PMN chemotactic response in both the IL-8-positive and IL-8-negative mice was the same (Figure 6). Although KC was more potent than IL-8 or MIP-2, the response of the neutrophils from the IL-8-positive and IL-8-negative mice was equivalent. These data indicate that the peripheral blood neutrophils from both groups of mice have the capacity to move toward a chemotactic stimulus. Therefore, if there were alterations in the CXCR2 chemokine receptor these changes did not result in a decrease of neutrophil function as measured by the chemotactic response.

We next addressed the mechanism of how neutrophils would respond to the inflammatory challenge, because previous work had demonstrated that high plasma levels of IL-8 would effectively reduce local neutrophil recruitment.[8–10] We studied the thioglycollate model, because the kinetics of chemokine production in this model are rapid compared to CLP[30] or acid lung injury[25] and local cytokine concentrations are easily measured. This *in vivo* model was also less complex than either the CLP or acid lung injury model. Because peritoneal neutrophil recruitment in response to thioglycollate was nearly equal in both IL-8-positive and IL-8-negative mice, we measured murine chemokines in the peritoneal fluid to determine whether enhanced local concentrations could have been generated to offset the plasma IL-8 levels. Figure 7 shows that both groups of mice have striking up-regulation of
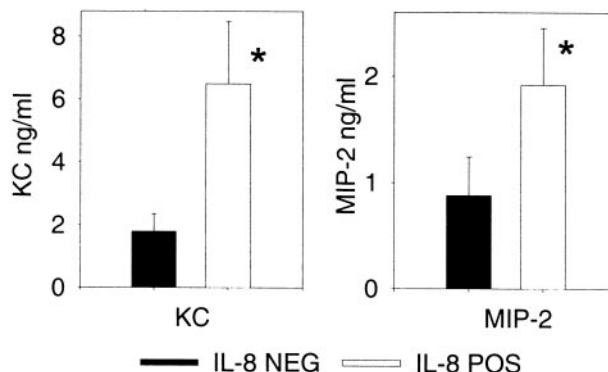
chemokines within the peritoneal cavity. The IL-8-positive mice, however, had significantly higher concentrations of both KC and MIP-2 compared to the IL-8-negative mice, showing that enhanced local production of these murine chemokines occurred. The increased levels of these chemokines may represent the biological response to overcome the IL-8 chemotactic gradient and effectively recruit neutrophils to the sites of local inflammation.

To test our hypothesis that the local up-regulation of endogenous murine chemokines was responsible for the recruitment of the neutrophils, we used antibody inhibition of the murine chemokines. For this experiment, mice were pretreated with a combination of antibodies directed against the murine chemokines KC and MIP-2[18,19,25,31] and IL-8-positive mice injected intraperitoneally with thioglycollate. These antibodies have previously been shown to significantly reduce peritoneal recruitment in response to thioglycollate injection in BALB/c mice.[19] Antibody inhibition of the murine chemokines resulted in near complete reduction in the number of recruited neutrophils (Figure 8). The number of peritoneal neutrophils was equivalent to those observed in the mice
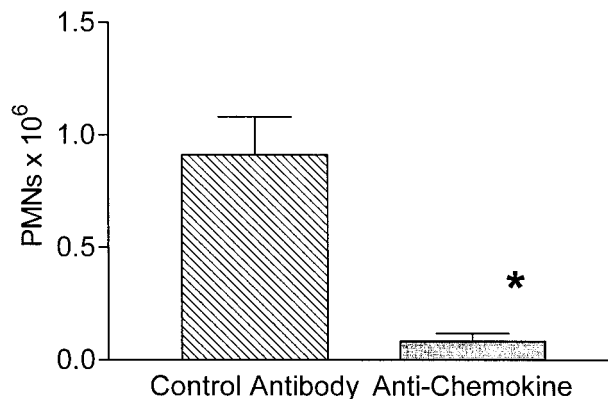

Figure 8. Anti-chemokine inhibition of peritoneal neutrophil recruitment to thioglycollate. IL-8-positive mice were pretreated with a combination of antibodies to murine KC and MIP-2, followed 2 hours later by thioglycollate injection. Antibody treatment resulted in >90% reduction in the number of recruited neutrophils. Each value is the mean $\pm$ SEM for six control antibody-treated mice and seven anti-chemokine-treated mice. *, $P < 0.005$ control antibody *versus* anti-chemokine.

injected with normal saline (thioglycollate + anti-chemokine = 42,000 total neutrophils *versus* saline = 45,000 total neutrophils). These data provide compelling evidence that the up-regulated murine chemokines were responsible for the neutrophil recruitment.

Discussion

There is extensive interest in the role of chemokines as mediators of neutrophil recruitment to sites of local inflammation.[32,33] Previous work has suggested that elevated levels of plasma chemokines, particularly IL-8, would reduce neutrophil recruitment to local sites of inflammation presumably by altering the chemokine gradient from the vascular space to the local tissue. Systemic injection of high doses of either a 77-amino acid form, or a 72-amino acid form of IL-8 reduced neutrophil recruitment.[8,9] Additionally, high-dose 77-amino acid IL-8 provided significant cardiac protection in a rabbit model of ischemia/reperfusion.[10] Previous work has demonstrated that rodent neutrophils will undergo chemotaxis to human IL-8.[12] Our investigations sought to determine whether high levels of circulating IL-8 would cause a reduction in the neutrophil recruitment in response to different inflammatory challenges. We hoped to extend previous observations concerning these mice and determine the mechanism of how neutrophil recruitment could occur even in the presence of a negative chemotactic gradient. The most severe insult occurs in the CLP model of peritonitis. After CLP, the presence of chronic high levels of IL-8 did not reduce lethality, cytokine production, or recruitment of neutrophils to the site of inflammation. Similar findings were observed in other models of acute inflammation in which the presence of high levels of IL-8 failed to reduce inflammation.

Neutrophils are the signature cell of acute inflammation, and by extension IL-8 should only be involved in acute inflammatory processes. Nevertheless, IL-8 may persist for prolonged periods in tissue culture or stimulated whole blood.[34] The persistence of IL-8 has also been documented *in vivo* in multiple studies. In chronic disease states, there are increased systemic levels of IL-8, including patients with HIV infections,[35] or β-thalassemia.[36] In longitudinal studies, peripheral blood levels of IL-8 persisted throughout several days in patients with malaria.[37] IL-8 stability has also been documented in local forms of inflammation. These data include finding IL-8 in lavage fluid for up to 7 days in patients who develop nosocomial pneumonia.[29] IL-8 has been identified within empyema fluid,[38] in patients with chronic lung diseases,[39] and has been recovered from the joint fluid of patients with arthritis.[40] High local levels of chemokines have been found in patients with lung inflammation such as acute respiratory distress syndrome[41] and asthma.[42] IL-8 has been found in the dialysate of patients on continuous peritoneal dialysis for up to 5 days,[43] and within the colonic mucosa of patients with chronic ulcerative colitis.[44] Ischemia/reperfusion injury to the heart will induce the formation of IL-8 mRNA in the myocardium and the mRNA will persist beyond 24 hours.[45] Given all of this information it is apparent that IL-8 has the potential to be long lasting and neutrophils may need to move into a local site of inflammation in the face of prolonged, elevated plasma levels of IL-8.

Our data show that neutrophil recruitment to local sites of inflammation will take place in the setting of chronically elevated systemic levels of IL-8. A possible explanation for the failure of high plasma levels of IL-8 to prevent neutrophil recruitment is desensitization of the chemokine receptors. Desensitization occurs between the CXC chemokines so that previous exposure to one chemokine renders a cell refractory to stimulation with a second chemokine.[46,47] Although this desensitization occurs *in vitro* with murine CXC chemokines,[48] the *in vivo* chemokine/receptor interaction is more dynamic. The IL-8 receptor (CXCR1) is rapidly internalized after cell surface binding and is then re-expressed on the cell surface within 10 minutes.[49] This chemokine receptor is now available for binding to the chemokine to exert its biological effect. Our data show that receptor desensitization has not occurred because neutrophils from IL-8-positive and IL-8-negative mice exhibit equal chemotaxis toward recombinant IL-8.

The failure of chronic elevated levels of IL-8 to reduce inflammation, and the specific failure to reduce neutrophil recruitment may be understood because of the complexity and redundancy of chemokines and their receptors. CXC chemokines represent important mediators for the influx of neutrophils to sites of acute inflammation.[50,51] The precise participation of the chemokines is also complicated by the presence of multiple chemokine receptors with relative specificity for the different chemokines. In general, CXC chemokines only bind the CXC chemokine receptors.[52] With the redundancy and overlap in chemokines, a complex *in vivo* focus of inflammation will involve multiple chemokines that would be available to recruit neutrophils.[53–55] The exact murine homolog of human IL-8 has not been identified,[56] but the two most probable candidates are either KC or MIP-2. Both of these chemokines will be rapidly up-regulated in a rodent model of acute lung injury.[4] Our data show that high levels of both of these chemokines are found within the peritoneum after thiolglycollate injection, and that IL-8-positive mice had significantly higher levels than the IL-8-negative mice. The peritoneal chemokine levels were higher, and the number of recruited neutrophils in multiple experiments was essentially equivalent in the IL-8-positive and IL-8-negative mice. This suggests that enhanced local production of chemokines was the appropriate biological response to generate a sufficient chemotactic gradient to induce neutrophils to migrate toward the peritoneum against the IL-8 chemotactic gradient. To provide additional support for this concept we performed antibody inhibition studies in the IL-8-positive mice. Antibody inhibition of the endogenous murine chemokines resulted in a >90% reduction in the number of peritoneal neutrophils. We believe our data support the concept that redundancy in the inflammatory response helps to ensure local neutrophil recruitment.[1,2]

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