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11. NK, the only test participant to show some improvement across the REM deprivation interval (on his second deprivation night), had spent more than 5 hours of the interval in the awake state and more than 25% of his total sleep time in stage 1 sleep (see Figs. 2 and 3). Thus, the improvement may have resulted from the waking state consolidation process.
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Neutrophil and B Cell Expansion in Mice that Lack the Murine IL-8 Receptor Homolog

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Interleukin-8 (IL-8) is a proinflammatory cytokine that specifically attracts and activates human neutrophils. A murine gene with a high degree of homology to the two known human IL-8 receptors was cloned and then deleted from the mouse genome by homologous recombination in embryonic stem (ES) cells. These mice, although outwardly healthy, had lymphadenopathy, resulting from an increase in B cells, and splenomegaly, resulting from an increase in metamyelocytes, band, and mature neutrophils. Thus, this receptor may participate in the expansion and development of neutrophils and B cells. This receptor was the major mediator of neutrophil migration to sites of inflammation and may provide a potential therapeutic target in inflammatory disease.

IL-8 is a member of a family of proinflammatory cytokines that are related by a C-X-C motif, where X is any amino acid between two cysteines. IL-8 is a major factor in acute inflammation, being responsible for the activation of neutrophils and their chemotaxis to the site of acute injury (*1, 2*). Neutrophils destroy bacteria by phagocytosis and the release of superoxides and peroxides, providing the first line of defense in fighting infection; the response is rapid and is neither acquired nor antigen specific (*3*). Many cells produce IL-8 in vitro, and it has been implicated in neutrophil migration and, to a lesser extent, T-cell migration, to sites of IL-8 injection (*4*). Neither mouse nor rat IL-8 has been identified (*5*), but antibodies (Ab) to human IL-8 inhibit lung inflammation in rats (*6*), which suggests the presence of a similar molecule in rodents.

Two high-affinity human IL-8 receptors have been cloned and characterized (*7–9*). These receptors share 77% amino acid sequence identity and are members of the superfamily of seven transmembrane domain receptors that are coupled to GTP-binding proteins. We have cloned a murine homolog of the human IL-8 receptor by screening a mouse genomic library at reduced stringency with complementary DNA (cDNA) probes from both human IL-8 receptors (*7, 8*). DNA sequencing shows that the mouse receptor is encoded by a single exon (as are the two human receptors) containing a 350–amino acid open reading frame with 68% and 71% amino acid identity with human IL-8 receptors A and B (*10*). Using several different restriction enzymes and genomic DNA blots hybridized under low-stringency conditions, we found a single cross-hybridizing band (*10*), suggesting that unlike the human genome, the murine genome contains a single gene for the putative IL-8 receptor. We refer to this gene as the murine IL-8 receptor homolog (mIL-8Rh).

To determine the function of this receptor in inflammation, we used homologous recombination in ES cells to generate a mouse strain lacking this gene. We constructed a gene-targeting vector by deleting the single exon containing the open reading frame of the mIL-8Rh and replacing it with the neomycin resistance gene (*neo*). This ensures the complete elimination of the gene after homologous recombination (Fig. 1A). Of 814 individual ES clones screened by genomic blot hybridization, 7

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were identified as having undergone homologous recombination; 3 of these were used to generate germline mice. Two mouse lines were selected for interbreeding to produce mice homozygous for the deletion of the mIL-8Rh (Fig. 1B). We confirmed the deletion of the entire mIL-8Rh gene by using the complete exon as a probe (*10*).

Outwardly, the homozygous receptor–deleted mice were indistinguishable from their wild-type and heterozygous litter mates in size and health, but showed morphologic changes at necropsy. In all mIL-8Rh(−/−) mice examined, the spleens ranged from two to four times normal size and cervical lymph nodes were enlarged 3- to 10-fold. Most other lymph nodes were enlarged, although the degree of enlargement varied among animals (*10*); inguinal and popliteal lymph nodes, however, were grossly normal. Comparative histopathology (*10*) of mIL-8Rh(+/+) and (−/−) mice showed that the splenomegaly resulted from expansion of the splenic white pulp by proliferation of myeloid elements (metamyelocytes, bands, and neutrophils) and megakaryocytes. Longitudinal sections of the femur and tibia revealed grossly white marrow in mIL-8Rh(−/−) mice compared to the normal red appearance in mIL-8Rh (+/+) mice. Histologically, there was a great increase in bone marrow cellularity composed of the normal myeloid maturation series. The erythroid series was unaffected. Approximately 25% of the mIL-8Rh(−/−) animals analyzed had multiple foci of granulopoiesis in the periportal region of the liver (*10*). There was no indication of parenchymal infiltration, inflammation, or hepatic damage. The presence of metamyelocytes, bands, and neutrophils in normal ratios suggests that extramedullary myelopoiesis was occurring in the liver,

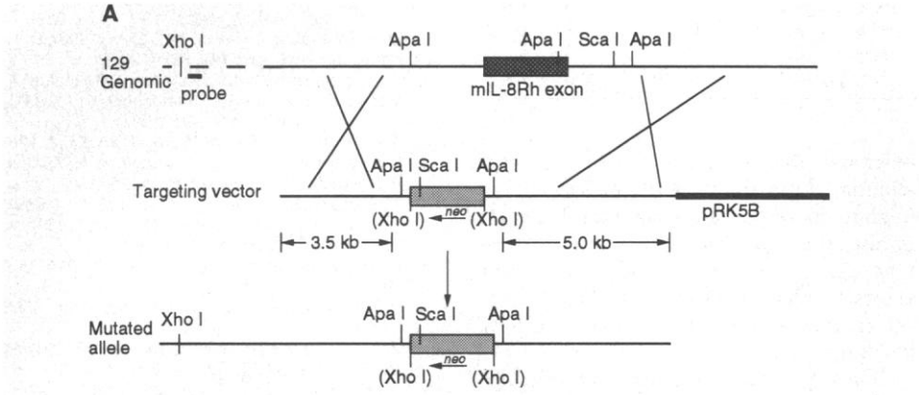

Fig. 1. Targeting of the mIL-8Rh gene. (**A**) Restriction map of the wild-type mIL-8Rh gene (top). A neomycin resistance gene under control of the Pgk promoter (*15*) was inserted into the Apa I–deleted region with synthetic DNA linkers that allowed ligation to the Apa I ends and created new Xho I sites on each side of *neo* (middle). An oligo probe was designed from sequences not on the targeting vector. After homologous recombination, the mutated allele could be detected by a Xho I or Sca I digest (bottom). (**B**) Genomic DNA was isolated from tail snips of the offspring of interbreeding heterozygous mice, digested with Sca I, and analyzed by Southern blot hybridization with the oligo probe indicated in (A). Genotypes of mice are indicated as wild-type (+/+), heterozygous (+/−), and homozygous receptor–deleted (−/−).

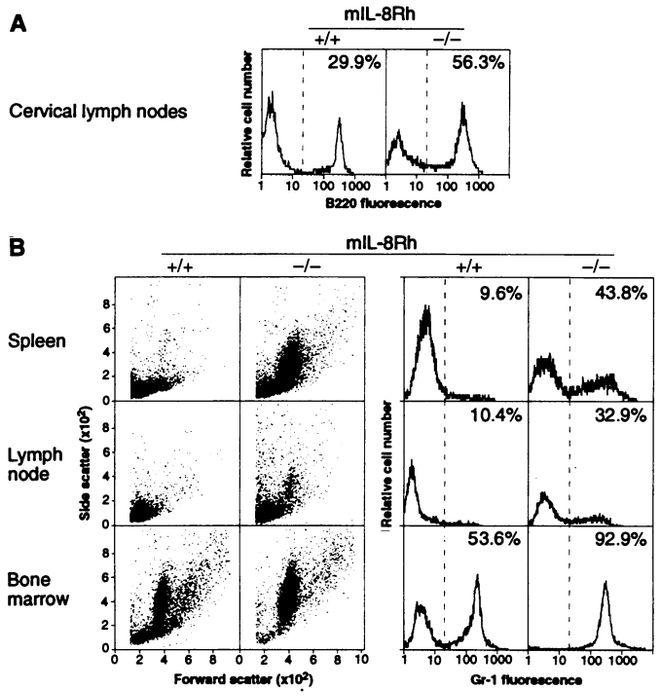

Fig. 2. Neutrophil and B cell expansion in mIL-8Rh(−/−) mice. (**A**) Representative analysis of B220-positive cells from cervical lymph nodes. Cell counts of lymph nodes multiplied by the percentage of B220-positive cells indicate 2.4×10^6 total cells with 0.7×10^6 B cells in mIL-8Rh(+/+) mice, and 19×10^6 total cells with 10.6×10^6 B cells in mIL-8Rh(−/−) mice. (**B**) Single-cell suspensions from bone marrow, spleen, and lymph node of mIL-8Rh(+/+) and (−/−) mice were analyzed with fluorescein isothiocyanate–conjugated antibody to Gr-1 (clone RB6-8C5), a cell surface marker for neutrophils.

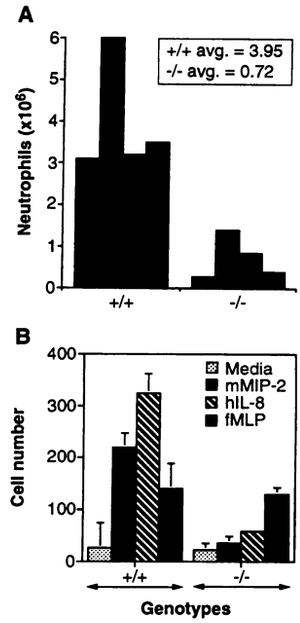

Fig. 3. Thioglycollate-induced migration of neutrophils. (**A**) The mIL-8Rh(+/+) and (−/−) mice were injected intraperitoneally with 3 ml of thioglycollate. After 3 hours, peritoneal exudate cells were harvested by peritoneal lavage. Total cell numbers were determined with a hemacytometer. Percentage of neutrophils was determined by differential cell counts from cytospin slide preparations. The number of neutrophils was calculated by multiplying percentage of neutrophils by total cell counts. Bars represent individual mice. (**B**) In vitro migration of purified bone marrow neutrophils was measured in a microchemotaxis chamber experiment with a multiwell Boyden chamber (*16*). After a 40-min incubation at 37°C, the number of neutrophils that migrated in response to the indicated chemokine (50 nM) or media alone was determined in three chambers per group.

lymph node, and spleen. Despite the relative myeloid hyperplasia of the marrow, the mIL-8Rh(−/−) mice were not anemic and had normal amounts of red blood cells and hemoglobin, a normal hematocrit value, and did not display an increase in nucleated red blood cells (*10*).

In lymph nodes, the medullary cords were expanded by abundant foci of myelopoiesis, Russell bodies, and plasma cells, and they compressed the adjacent medullary sinuses. This apparent increase in B cells was confirmed by flow cytometric analysis with an Ab to the B cell marker B220 (Fig. 2A), and was nearly 10-fold. The number of circulating neutrophils increased approximately 12-fold in the mIL-8Rh(−/−) mice (*10*), and the increased cellularity and alteration of granulocyte to lymphocyte populations was evident when flow cytometric analysis of spleen, lymph node, and bone marrow was performed (Fig. 2B). The thymus and all other organs appeared normal. T cell populations were also normal. We have not observed more marked changes in the above phenotypes with advancing age up to 20 weeks. Heterozygous [mIL-8Rh(+/−)] mice did not display any overt phenotype.

The mIL-8Rh(−/−) and mIL-8Rh (+/+) mice were tested for their neutrophil migration response after injection of thioglycollate (Fig. 3A). The number of neutrophils from the mIL-8Rh(−/−) mice that migrated to the peritoneum was one-fifth that of mIL-8Rh(+/+) mice, indicating that the acute migration ability of neutrophils was compromised. These data are even more compelling when one considers that the total number of neutrophils in the mIL-8Rh(−/−) animals was greatly increased, providing these animals a much greater potential pool of neutrophils than wild-type animals. The locomotor function of the neutrophils was not impaired, because in vitro migration studies indicated that mIL-8Rh(−/−) neutrophils could migrate in response to fMLP but not in response to any of the ligands (mMIP-2 and hIL-8) identified for this receptor (Fig. 3B) (*10*). To determine whether aspects of neutrophil function other than migration were affected, we tested purified bone marrow neutrophils for their ability to kill bacteria (Table 1). Both mIL-8Rh(−/−) and mIL-8Rh(+/+) neutrophils were effective at intracellular and extracellular killing of bacteria, showing that mIL-8Rh–independent functions of neutrophils were unaffected.

Although we anticipated effects on the inflammatory and migratory response of neutrophils in the mIL-8Rh(−/−) mice, the extramedullary myelopoiesis was unexpected and excessive in light of the marked concurrent production by the bone marrow and the circulating neutrophil counts in the absence of an inflammatory focus. We demonstrated that the loss of this receptor eliminated the ability of the neutrophils to migrate in response to thioglycollate administration and the appropriate chemokine ligands in vitro. These data support efforts to block neutrophil-mediated damage in inflammation by removing IL-8 or blocking its receptor.

Table 1. Bacterial killing by neutrophils. Bone marrow neutrophils from mIL-8Rh (+/+) and (−/−) mice were tested for their ability to kill *Staphylococcus aureus* (*17*). The initial inoculum was 70 × 10^6 bacteria and 2 × 10^6 neutrophils. All values are ×10^6.

mIL-8Rh	Total live	Phagocytosed bacteria	Number of intracellular bacteria	
			Live	Killed
+/+	1.9	68.3	0.31	68.0
+/+	2.7	67.5	0.21	67.3
−/−	2.9	67.2	0.18	67.0
−/−	2.4	67.8	0.21	67.6

The expansion of the neutrophil lineage could indicate that this receptor is involved in the negative regulation of neutrophil production. Alternatively, the immune system could detect the mIL-8Rh(−/−) neutrophils as nonresponsive and continue to stimulate neutrophil production in order to replace normal neutrophil function. A third possibility is that this receptor is expressed on other cell types and may be involved in the regulation of other cytokines. A disruption in the function of a particular cytokine could have profound effects on a number of different cell types and immunological functions. The immune system could be compensating for the loss of properly functioning neutrophils by boosting the humoral arm of the immune response. In humans, IL-8 negatively affects IL-4 expression in tonsillar and circulating B cells (*11*). IL-6 drives B cells to become plasma cells, and we detected increased IL-6 in mIL-8Rh(−/−) sera (average of 4.8 ng/ml as compared to wild-type amounts that are below the 0.1 ng/ml limit of detection), whereas IL-4 expression remained in the normal range (*10*). The increased IL-6 expression could explain the observed plasma cells in lymph nodes of mIL-8Rh(−/−) mice, because transgenic mice overexpressing IL-6 also presented lymphadenopathy resulting from plasmacytosis (*12*). With regard to the granulopoiesis, the MIP-1α chemokine can inhibit hematopoietic stem cell proliferation (*13*). A C-X-C–related molecule was cloned from an expression cDNA library made from a bone marrow stromal cell line (*14*). It has not been determined whether this molecule, stromal cell–derived factor–1, is involved in the regulation of bone marrow hematopoiesis, but its discovery in a bone marrow stromal cell line suggests this possibility. These studies, together with our results, raise the prospect that chemokines may play a much more complex role than solely affecting leukocyte trafficking, and that they may also be involved in the regulation of hematopoiesis and myelopoiesis.

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