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Interleukin-1 Receptor Signaling Rather than That of Tumor Necrosis Factor Is Critical in Protecting the Host from the Severe Consequences of a Polymicrobe Anaerobic Infection

DANA T. GRAVES,[1]* CHIH-PING CHEN,[2] CHRISTOPHER DOUVILLE,[3] AND YANLING JIANG[3]

Department of Periodontology and Oral Biology[1] and Department of Endodontics,[3] Boston University School of Dental Medicine, Boston University, Boston, Massachusetts 02118, and Dental Department, Tri-Service General Hospital, and School of Dentistry, National Defense Medical Center, Taipei, Taiwan, Republic of China[2]

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Infection of the dental pulp leads to an osteolytic lesion that results from a polymicrobial infection consisting largely of pathogenic anaerobes. Infection causes significant morbidity and mortality mediated by bacterial factors and in some cases by the up-regulation of inflammatory cytokines. The inflammatory cytokines interleukin-1 (IL-1) and tumor necrosis factor (TNF), in particular, play a complex and central role in the responses to microbial pathogens. However, relatively little is known about the significance of these cytokines in protecting the host from focal polymicrobial anaerobic infections. To establish the relative importance of IL-1 and TNF in mediating the response to a mixed anaerobic infection, we inoculated the dental pulp of mice with six anaerobic pathogens containing functional deletions of receptors to IL-1 (IL-1R1$^{-/-}$), TNF (TNFRp55$^{-/-}$-p75$^{-/-}$), or both (TNFRp55$^{-/-}$-IL-1RI$^{-/-}$). The results indicate that IL-1 receptor signaling and TNF receptor signaling both play similarly important roles in protecting the host from local tissue damage. However, IL-1 receptor signaling is considerably more important than TNF receptor signaling in preventing the spread of infection into surrounding fascial planes, since IL-1R1$^{-/-}$ but not TNFRp55$^{-/-}$-p75$^{-/-}$ mice exhibited significantly higher morbidity and mortality. Moreover, all of the fatal infections occurred in male mice, suggesting the importance of gender differences in limiting the impact of these infections.

Convincing evidence demonstrates that infection of the dental pulp leads to an osteolytic lesion that results from a polymicrobial infection consisting largely of pathogenic anaerobes (11, 21, 25, 26). Some of the more serious sequelae are extensions of the infection into nearby fascial planes and subcutaneous tissues of the head and neck (16). This is characterized by a diffuse pattern of necrosis that may result in soft tissue abscess formation and, in some cases, sepsis. Although this is a rare complication, when it does occur it carries a high mortality rate. Moreover, the presence of an associated immunocompromising disease, such as diabetes, predisposes an individual to this complication (16).

Bacterial infection of the dental pulp is characterized by necrosis of the dental pulp followed by the formation of an osteolytic lesion (26, 29). Under normal conditions, inflammatory cytokines are absent or present in very low levels in these tissues but are induced by bacterial stimulation. The expression of these cytokines is thought to play a central role in the pathogenesis of osteolytic lesion formation (30). However, relatively little is known about the significance of this cytokine expression in lesion formation and, more generally, in protection of the host from focal polymicrobial anaerobic infections.

Interleukin-1 (IL-1) and tumor necrosis factor (TNF) are expressed by many cell types in response to inflammatory processes (7, 28). Additionally, IL-1 and TNF are able to stimulate a wide spectrum of cellular responses and often act synergistically. IL-1α and IL-1β both bind to IL-1 receptors termed type I and type II. The type I IL-1 receptor (IL-1R1) is responsible for specific signaling, while the type II receptor functions as a nonsignaling decoy receptor. Similarly, there are two TNF molecules, TNF-α and TNF-β. These molecules have a high degree of structural and sequence homology and are able to interact with two known receptors, termed TNFRp55 (also known as TNFR1) and TNFRp75 (TNFR2). These receptors have different cytoplasmic domains and, as a result, activate different signaling pathways (27). Most of the inflammatory effects have been attributed to TNFRp55, and in vivo TNFRp75 signaling acts to attenuate the inflammatory response induced by TNF (2, 22).

Experimental evidence indicates that IL-1 and TNF play a complex and central role in resistance to microbial pathogens (6). They regulate several aspects of the host response including stimulation of bactericidal activity of phagocytes, enhanced antigen presentation, induction of chemokines, and expression or activation of adhesion molecules that enhance leukocyte recruitment. However, several studies have shown that high systemic levels of IL-1 and/or TNF correlate with an unfavorable outcome in patients (8). Administration of exogenous IL-1 and/or TNF reproduces many of the pathophysiologic alterations observed in sepsis (7, 28). Hence, inhibition of IL-1 and/or TNF activity has been hypothesized to be a potential therapy for sepsis. Cytokines operate in the context of complex networks (23). Because of this, it has been difficult to precisely assess the role of a single cytokine in the process of inflammation and host resistance to infectious agents. The use of mice with targeted gene deletions has been valuable in assessing the role of a particular cytokine in physiologic homeostasis and the pathogenesis of disease states. The generation of mice with targeted functional deletions of IL-1R1 (IL-1R1$^{-/-}$), TNF type I and type II receptors (TNFRp55$^{-/-}$-p75$^{-/-}$), and both of the IL-1 and TNF type 1 receptors (IL-1R1$^{-/-}$ TNFRp55$^{-/-}$) has helped to elucidate the role of these receptors in several processes (1, 2, 6, 14, 19, 22, 24). IL-1R1$^{-/-}$ mice and

* Corresponding author. Mailing address: Department of Periodontology and Oral Biology, Boston University School of Dental Medicine, 700 Albany St. W201, Boston, MA 02118. Phone: (617) 638-8547. Fax: (617) 638-4924. E-mail: dgraves@acs.bu.edu.

TNFRp55$^{-/-}$ and/or TNFRp75$^{-/-}$ mice have been generated. These mice do not exhibit gross abnormalities and are capable of developing antibodies to exogenous antigen stimulation (14, 22). In contrast to IL-1R1$^{-/-}$ mice, TNFRp55$^{-/-}$ mice exhibit abnormal development of germinal centers in peripheral lymphoid organs and enhanced susceptibility to *Listeria monocytogenes* infection (14, 20).

To investigate the role of IL-1 and TNF in the host response to a mixed anaerobic infection that is usually well contained, we surgically exposed the dental pulp in three experimental groups and one control group and inoculated the pulp with six oral pathogens. The results indicate that IL-1R signaling and TNFR signaling both play similarly important roles in protecting the host from local tissue damage. However, IL-1R signaling is considerably more important than TNFR signaling in preventing the complication of focal infection, since IL-1R1$^{-/-}$ but not TNFRp55$^{-/-}$-p75$^{-/-}$ mice exhibited significant morbidity and mortality.

MATERIALS AND METHODS

Mice. Mice ranging in age from 10 to 14 weeks were examined, with five mice used for each datum point. These included IL-1R1$^{-/-}$, TNFRp55$^{-/-}$-p75$^{-/-}$, and IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ mice, generously provided by Jacque Peschon, Immunex Corp., Seattle, Wash. The wild-type mice with similar genetic background were C57BL/6 × 129J hybrids purchased from Jackson Laboratories (Bar Harbor, Maine). The identity of each group of mice was routinely confirmed by PCR of extracted DNA. Within a group, the number of males and females was similar for each time point. During the course of the study, 11 mice died and were replaced by additional mice, so that the total number equaled 111. The effects of infection on necrosis and osteoclast formation were determined on days 3, 7, 14, and 21. To examine the effect of infection on soft tissue abscess formation, experiments were carried out at an additional time point, day 38.

Bacterial strains and growth conditions. Surgically exposed dental pulp was inoculated with six putative oral pathogens which included one facultative anaerobic gram-positive coccus, *Streptococcus mutans* (ATCC 25175), and five anaerobic strains, consisting of two gram-positive cocci, *Streptococcus intermedius* (ATCC 27335) and *Peptostreptococcus micros* (ATCC 33270), and three gram-negative rods, *Porphyromonas gingivalis* (ATCC 33277), *Prevotella intermedius* (ATCC 25611), and *Fusobacterium nucleatum* (ATCC 49256). All bacterial strains were prepared and maintained at the Forsyth Research Center (Boston, Mass.). They were grown on anaerobic blood agar in an atmosphere of 10% CO_2, 10% H_2, and 80% N_2 at 37°C. Cultures of bacteria were grown in a commercially formulated complex broth medium, TSBY (Trypticase soy agar and brain heart infusion agar with yeast extract) or TSBY plus HK (hemin and vitamin K) (Northeast Laboratories, Winslow, Maine). On the day of surgery, bacteria were collected from the culture plates and aliquoted (10^9bacteria/ml) in viscous mixed 3% methylcellulose-enriched Trypticase soy broth (Becton Dickinson). The bacteria were preserved in an N_2 environment until use.

Lesion induction. The animals were anesthetized by intraperitoneal injection of a ketamine-xylazine solution, and the dental pulp was exposed by removing the mesial cusp of the first mandibular molars. A 100-μl volume of a viscous bacterial mixture (containing 10^8bacteria of each of the six bacterial strains described above) was placed onto the tooth surface. The mice were sacrificed on day 0, 3, 7, 14, 21, or 38 after pulp exposure and bacterial challenge by asphyxiation in a CO_2 chamber.

Specimen preparation. Following sacrifice, the mandibles were dissected and immediately immersed in 4% paraformaldehyde in phosphate-buffered saline (PBS) at 4°C for 4 h. Specimens were consecutively washed in 5, 10, and 15% glycerol in PBS for 15 min each at 4°C and decalcified for 20 days in 15% glycerol–EDTA (pH 7.1) at 4°C. Decalcification was established radiographically, and specimens were then immersed in 30% sucrose overnight at 4°C and stored at −80°C. After the specimens were embedded in HISTO PREP (Fisher Scientific, Fair Lawn, N.J.), 5-μm serial sections were prepared in the mesiodistal plane.

Image analysis and measurement. Microscopic images of the periapical tissue were captured with a high-definition RGB camera and analyzed using Image Pro Plus software (Media Cybernetics, Silver Spring, Md.). The sections showing the osteolytic lesions at their widest extent were then examined and analyzed. All images and slides were coded by one person and analyzed by another person, thus making the measurements double blind. The results were verified by a second examiner. Interexaminer and intraexaminer variation was generally less than 10%.

Pulp necrosis. Hematoxylin- and-eosin-stained cryostat sections were examined for the presence of tissue necrosis in the dental pulp. The apical one-third of the mesial root was examined for necrosis at a magnification of ×400.

TABLE 1. Pulp necrosis is more rapid in the absence of IL-1 or TNF activity[a]

Group	No. of specimens with complete necrosis on days 3 and 7/total no.[bc]	Days to complete necrosis in at least four specimens[c]
TNFp55$^{-/-}$ IL-1R1$^{-/-}$	10/10	3
IL-1R1$^{-/-}$	7/10	7
TNFp55$^{-/-}$-p75$^{-/-}$	5/10	7
Wild type	0/10	None[d]

[a] Measurements were made on days 3, 7, 14, and 21 days after bacterial inoculation. Five specimens were evaluated for each time point.

[b] By chi-square analysis, each of the experimental groups is significantly different from the controls ($P < 0.01$).

[c] The parameters were chosen because they best distinguish between the experimental and control groups while exhibiting relatively consistent results for the majority of animals in a given group at a given time point.

[d] There was complete or partial necrosis in three specimens after 21 days. The other two specimens showed no necrosis in the apical third.

Size of lesions. The size of the osteolytic lesions around the experimental mesial root end was measured using Image ProPlus software at a magnification of ×100.

TRAP analysis. Osteoclasts were identified as multinucleated, TRAP-positive cells in direct contact with bone and counted from an image projected onto a computer monitor at a magnification of ×200. The osteoclast number was then calculated as the number per millimeter of bone length.

Statistical analysis. The degree of pulp necrosis and the rates of morbidity or mortality were assessed by chi-square analysis. The number of osteoclasts and the size of osteolytic lesions were analyzed by one-way analysis of variance to find if there were differences between the groups at a given time point. The Tukey-Kramer procedure was used as a post hoc test. Significance was generally found if P was <0.05.

RESULTS

Soft tissue necrosis. In the mouse model, bacterial penetration into the root canal system causes necrosis, which eventually reaches the end of the root. When the mesial root end was examined, there was no detectable necrosis in the wild-type animals on days 3 and 7 (Table 1). Even on day 21, not all specimens showed complete necrosis of the dental pulp. In contrast, some necrosis was present in each of the experimental groups on days 3 and 7 and complete necrosis was evident in all of the experimental groups by day 14. The TNFRp55$^{-/-}$-p75$^{-/-}$ and IL-1R1$^{-/-}$ mice had similar degrees of necrosis, with four out of five mice from each group having complete necrosis of the dental pulp by day 7. In IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ mice, necrosis occurred even more rapidly, with all specimens being completely necrotic by day 3.

Osteoclastogenesis. Osteoclastogenesis can be assessed by TRAP staining of multinucleated bone-lining cells. When the developing osteolytic lesion was examined for osteoclasts, a much larger number was found in IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ mice than in wild-type mice (Fig. 1). In the wild-type mice, there was a gradual increase in osteoclast number over the entire experimental period; in contrast, a high degree of osteoclastogenesis was apparent on day 3 in each of the experimental groups (Table 2). On day 7, the number of osteoclasts remained large in the TNFRp55$^{-/-}$-p75$^{-/-}$ and IL-1R1$^{-/-}$ groups, while it increased further in the IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ mice. At later time points, the number of osteoclasts decreased in the experimental groups due in part to the lesions being extremely large.

Osteolysis. The sizes of the osteolytic lesions increased slowly throughout the experimental period in wild-type mice (Fig. 2). However, among mice lacking IL-1R and or TNFR signaling, the sizes of the osteolytic lesions increased much

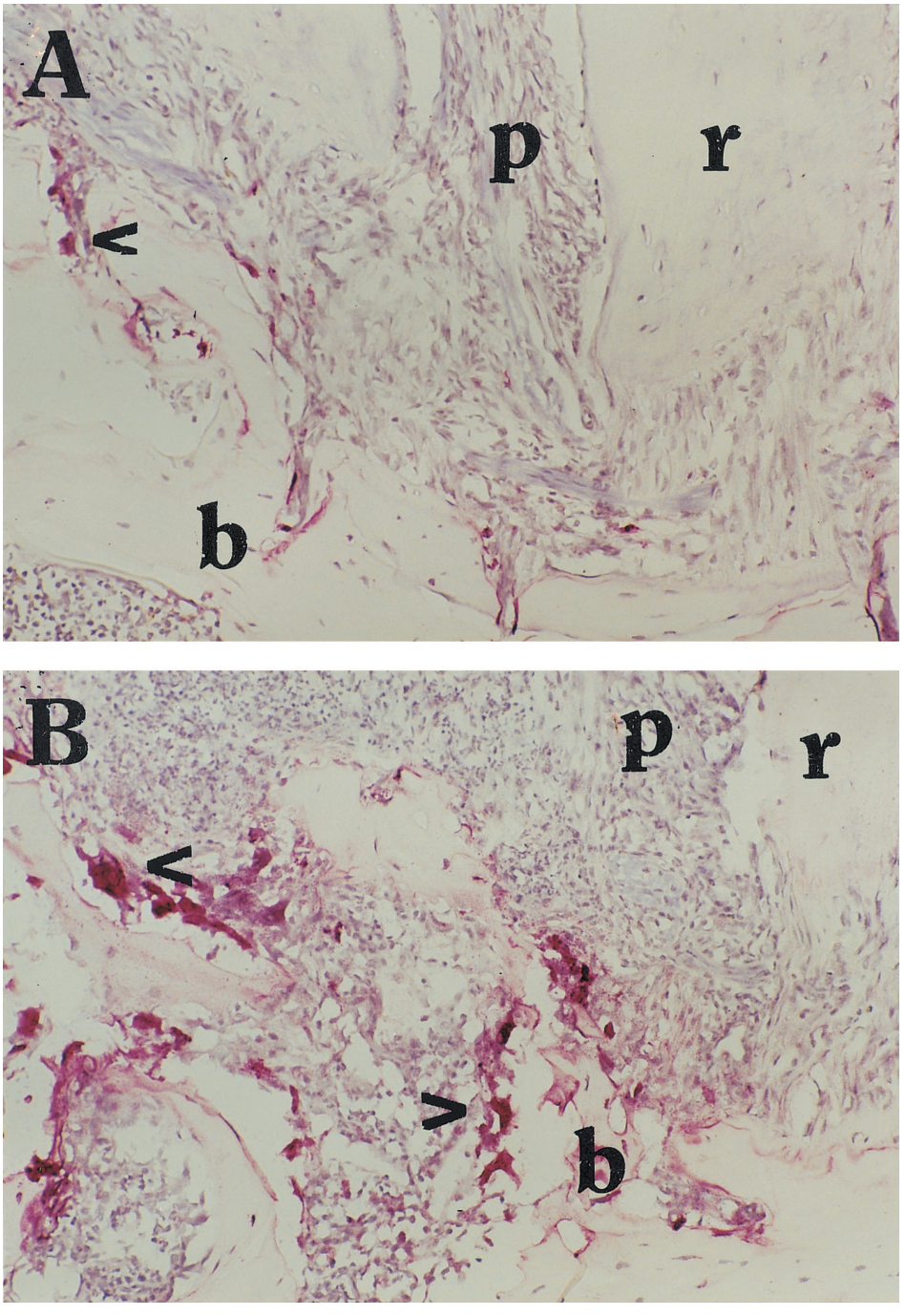

FIG. 1. Osteoclastogenesis is enhanced in mice lacking IL-1R1 and TNFR1 signaling in response to bacterial infection. Osteoclasts were identified by their characteristic appearance following TRAP staining 7 days after inoculation. (A) Wild-type mice. (B) IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ mice. r, root; p, pulp; b, bone; <, osteoclasts. Magnification, ×200.

more rapidly and to a greater extent. This was apparent as early as day 3.

Morbidity and mortality. Morbidity was assessed by the formation of a soft tissue abscess when infection spread from the intraosseous site at the tooth apex into the surrounding tissue (Fig. 3). No abscess formation was noted in the soft tissue of wild-type or TNFRp55$^{-/-}$-p75$^{-/-}$ animals. However, 17% of the IL-1R1$^{-/-}$ mice and 29% of the IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ mice developed soft tissue abscesses. In both of these groups, there was an equal distribution between males and females in the animals that developed soft tissue abscesses.

As was noted for soft tissue abscess formation, there were no deaths following surgical exposure of the dental pulp in wild-type or TNFRp55$^{-/-}$-p75$^{-/-}$ mice (Fig. 4). Out of a total of 28 IL-1R1$^{-/-}$ mice, 3 died, probably as a result of septic shock, while 8 of 33 IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ mice died. All of the deaths occurred between days 7 and 14. Surprisingly, all 11 mice that died were males, suggesting that there are important

TABLE 2. Osteoclastogenesis in IL-1R- and/or TNFR-deficient mice compared to normal mice

Time (days) after challenge	No. of osteoclasts[a] in mouse strain			
	Wild type	TNFp55$^{-/-}$-p75$^{-/-}$	IL-1R1$^{-/-}$	TNFp55$^{-/-}$ IL-1R1$^{-/-}$
3	1.04 ± 0.45	4.50 ± 0.34	5.07 ± 0.88	5.97 ± 1.07
7	2.27 ± 0.40	5.14 ± 0.63	5.22 ± 0.94	8.92 ± 1.60
14	4.56 ± 1.17	2.94 ± 0.39	3.17 ± 0.75	3.21 ± 0.39
21	4.97 ± 1.21	2.31 ± 0.27	2.95 ± 0.69	2.57 ± 0.18

[a] Osteoclastogenesis was assessed by measuring the number of osteoclasts per millimeter of bone at the root end following exposure of the dental pulp and inoculation by six oral pathogens. Osteoclasts were identified as TRAP-positive multinucleated cells lining bone at a magnification of ×200. Values represent the mean ± standard error of the mean for five specimens for each data point. By analysis of variance, the IL-1R1$^{-/-}$, TNFRp55$^{-/-}$, and IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ groups were significantly different from the wild-type group on days 3, 7, and 21 ($P < 0.01$).

sex differences in the systemic complications due to a mixed anaerobic infection in animals with compromised IL-1R or IL-1R plus TNFR signaling. When the total incidence of complications was considered, including soft tissue abscess formation and death, the various groups were clearly distinguishable (Table 3). No complications were noted in wild-type or TNFRp55$^{-/-}$-p75$^{-/-}$ animals. In contrast, 8 of 28 IL-1R1$^{-/-}$ and 16 of 33 IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ mice either had soft tissue abscess formation or died as a result of dental pulp infection. The incidence of complication in both of these groups was significantly higher than that in the control or TNFRp55$^{-/-}$ mice.

DISCUSSION

IL-1 and TNF are known to play important roles in the response to infectious agents. In studies reported here, we examined the relative contribution of IL-1R and TNFR signaling in a mixed anaerobic infection that typically causes local necrosis of the dental pulp and an osteolytic lesion at the root apex but which only rarely causes systemic complications. We assessed local tissue damage by measuring necrosis of the dental pulp, osteoclastogenesis, and the formation of a destructive osteolytic lesion. Each of these experiments indicated that there was greater local tissue destruction in the three experimental groups than in wild-type animals. It is striking that there was little difference in local tissue damage in IL-1R1$^{-/-}$ or TNFRp55$^{-/-}$-p75$^{-/-}$ animals while there was more rapid necrosis and osteolysis in IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ animals than in the two other experimental groups.

In addition to local tissue destruction, we measured morbidity, which occurred when the infection was severe enough to break through the osseous plates and involve the surrounding soft tissue. Wild-type and TNFRp55$^{-/-}$-p75$^{-/-}$ mice had no evidence of soft tissue abscess formation. In contrast, the two experimental groups which lacked IL-1R signaling, IL-1R1$^{-/-}$ and IL-1R1$^{-/-}$ TNFRp55$^{-/-}$, exhibited soft tissue involvement. This supports the notion that IL-1, in particular, is critical in restricting the spread of an anaerobic infection. It is also likely that TNFRp55 contributes to the protection of the host from anaerobic infections. This is based on the observation that nearly twice as many soft tissue abscesses were noted in the IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ mice as in the IL-1R1$^{-/-}$ mice. In vitro studies suggest that IL-1 or TNF may play an important role in stimulating the antimicrobial activity of neutrophils during infection (12, 13). Neutrophils, in particular, are critical

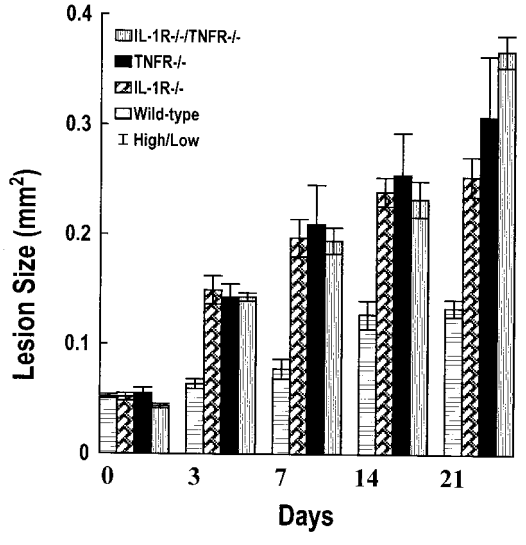

FIG. 2. Osteolytic lesions develop rapidly in mice lacking IL-1 or TNF activity. The area of each lesion at the root end was determined with a computer-assisted image analysis system at the indicated time points following exposure of the dental pulp and inoculation by bacteria. Each value represents the mean of five specimens for each time point and standard error of the mean.

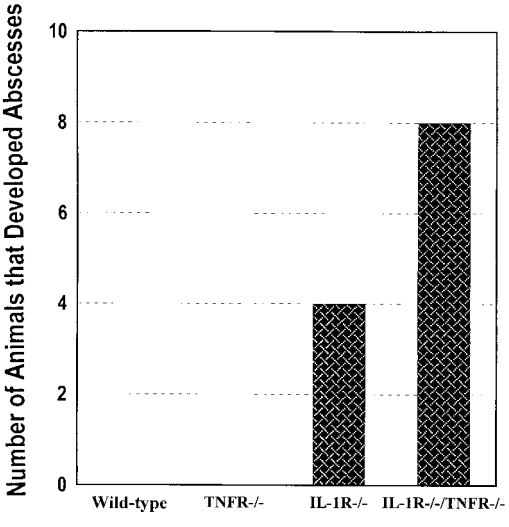

FIG. 3. Soft tissue abscess formation following infection of the dental pulp occurs in the absence of IL-1R signaling. Surgical pulp exposure followed by inoculation with six oral pathogens was carried out as described in Materials and Methods. Abscess formation was determined by gross examination. For the IL-1R1$^{-/-}$ group, 5 of 25 animals developed abscesses, 3 males and 2 females. For the IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ group, 8 of 25 animals developed abscesses, 4 males and 4 females. Animals that died (see Fig. 4) were not counted as mice that developed abscesses. By chi-square analysis, abscess formation in the IL-1R1$^{-/-}$ group was close to being significant ($P = 0.07$) and abscess formation in the IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ group was clearly significant ($P < 0.05$).

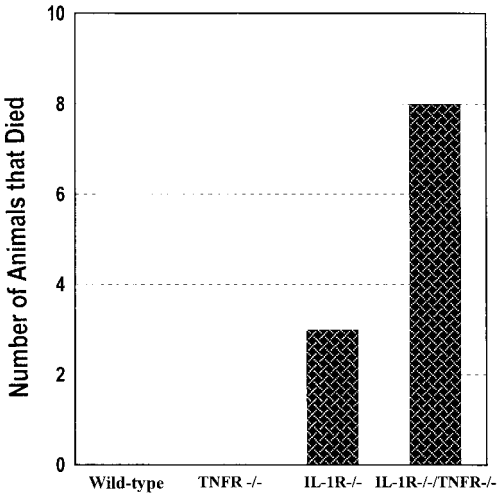

FIG. 4. Mortality following infection of the dental pulp occurs in the absence of IL-1R signaling. Surgical pulp exposure followed by inoculation with six oral pathogens was carried out as described in Materials and Methods. For the IL-1R1$^{-/-}$ group, 3 out of 28 animals died, which was not significant (chi square, $P > 0.05$). For the IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ group, 8 out of 33 animals died during the course of the experiment, which was significant (chi square, $P < 0.05$). All the animals that died were males.

in eliminating many different types of bacteria. However, the experimental results do not exclude the possibility that functional TNFRp75 in these animals renders the host more susceptible to infection in the absence of both IL-1R1 and TNFRp55 signaling.

Of the 111 mice studied, 11 died during the course of the study. All of these mice belonged to experimental groups IL-1R1$^{-/-}$ or IL-1R1$^{-/-}$ TNFRp55$^{-/-}$. In all cases, death occurred sometime between days 7 and 14 and none were noted between days 14 and 21. This suggests that in this model, it is particularly important for the host to limit the spread of infection during this period. One of the most surprising findings was that all 11 mice that died were male. The mechanisms to explain this difference are not clearly apparent. Several studies have addressed the issue of gender-based susceptibility to infection, and the results have been highly variable (3–5, 9, 10, 15, 17, 18). However, the apparent gender difference reported here is not strictly related to confining the infection to the local site, since soft tissue abscess formation occurred equally in males and females. In reviewing reports describing mortality following a severe infection, we did not find significant evidence that a given gender was an important risk factor. It is possible that IL-1 plays a more important role in the host response to infection in males and that there are interactions between cytokine networks and endocrine hormones related to survival following a mixed anaerobic infection.

TABLE 3. Incidence of soft tissue abscess formation or mortality following focal infection

Group	Mortality plus morbidity[a]
Wild type	0/25
TNFRp55$^{-/-}$-p75$^{-/-}$	0/25
IL-1R1$^{-/-}$	8/28[b]
IL-1R1$^{-/-}$ TNFRp55$^{-/-}$	16/33[b]

[a] Focal infection was induced by exposure of the dental pulp and inoculation with six oral pathogens. Morbidity was assessed as the number of animals that developed soft tissue abscess, and mortality was assessed as the number of animals that spontaneously died during the experimental period (days 3 to 21). The data are expressed as the combined number of each versus the total number of animals in each of the experimental or control groups. Mice that died were not included in the group that developed abscesses.

[b] Significantly different from the wild-type or TNFRp55$^{-/-}$-p75$^{-/-}$ groups (chi square, $P < 0.01$).

In conclusion, the results of this study demonstrate that IL-1 and TNF play an essential role in protecting the host from local damage that occurs as a result of a mixed anaerobic infection. However, when the relative importance of IL-1 or TNF is compared with respect to confining the infection to the local site, IL-1R signaling appears to play a more important role than TNFR signaling. The importance of IL-1R signaling with or without concomitant TNFRp55 signaling is highlighted by the observation that approximately 40% of IL-1R1$^{-/-}$ and IL-1R1$^{-/-}$ TNFRp55$^{-/-}$ mice either had abscess formation, which broke into the surrounding soft tissue, or died spontaneously, while none of these events occurred in wild-type control or TNFR$^{-/-}$ animals.

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