Ferrous Ferric Chloride Downregulates the Inflammatory Response to *Rhodococcus aurantiacus* Infection in Mice

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The healthy drink Pairogen is mainly composed of ferrous ferric chloride water that reportedly changes the status of intracellular water from oxidative to antioxidative. Here, we investigated whether Pairogen affects host immune function in a murine model of granulomatous inflammation in response to *Rhodococcus aurantiacus* (*R. aurantiacus*) infection. Longitudinal ingestion of Pairogen markedly improved the survival of infected mice in a concentration-dependent manner. Compared to mice received water, mice that ingested 10-fold-diluted Pairogen displayed rapid bacterial elimination, decreased production of tumor necrosis factor (TNF)-α and interleukin (IL)-6, and high levels of IL-10 in organs during the initial phase of infection. Moreover, histological studies showed significant reduction in the number and size of granulomas as well as amelioration of oxidative stress in the livers of mice ingested 10-fold-diluted Pairogen at 14 d post-infection. These characteristics were further pronounced in first-generation (F1) mice that also ingested 10-fold-diluted Pairogen. Following stimulation with heat-killed *R. aurantiacus*, the production of TNF-α, IL-6, and IL-10 by macrophages from F1 mice was similar to that detected in vivo, while their gene expression levels in these cells were significantly lower than the levels in macrophages from mice received water. Heat-killed *R. aurantiacus* also induced the expression of heme oxygenase-1 mRNA in the cells from F1 mice. Taken together, these results indicate that Pairogen contributes to the negative regulation of the immuno-inflammatory response to *R. aurantiacus* infection in mice by modulating the cellular redox state. Longitudinal ingestion of Pairogen further enhances the defense function in mouse progeny.

**Key words** Pairogen; ferrous ferric chloride; immuno-inflammatory response; *Rhodococcus aurantiacus*; redox state; mouse

The healthy drink Pairogen (Akatsuka Co., Mie, Japan) consists of 90% ferrous ferric chloride (FFC) water and 10% purified ingredients, including rice vinegar, Japanese apricot vinegar, apple vinegar, persimmon vinegar, vitamin B2, vitamin B3, vitamin C, glucose, fructose, citric acid, and malic acid.1,2 FFC is a special aqueous iron composed of a complex of ferrous chloride and ferric chloride and is known to participate in intracellular redox reactions.3,4 FFC water is obtained by immersing FFC ceramic beads (Akatsuka Co.) in water. Pairogen and FFC water are reported to influence multiple biological functions in living organisms. For example, Hasegawa et al. demonstrated that FFC water stimulates plant growth, particularly that of roots.5 The addition of either FFC water or Pairogen to a medium showed an equal effect on inducing the proliferation and differentiation of epidermal cells derived from both humans and mice.1,4,6 Moreover, treating mice with skin lotion containing FFC stimulates the proliferation and differentiation of skin cells as well as hair growth.7 However, little is known about the effects of FFC and Pairogen on modulating immune defense functions in animals or humans.

In response to microbial pathogen invasion, the host immune system is quickly activated via pattern-recognition receptors, leading to the innate immune response in which the release of various inflammatory mediators and the recruitment of inflammatory cells constitute the host’s primary line of defense during the initial stages of infection.8,9 Pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and interleukin (IL)-1β, as critical inflammatory mediators, initiate an inflammatory cascade to eliminate pathogens and promote tissue repair, whereas the generation of anti-inflammatory cytokines is essential for limiting an uncontrolled hyperinflammatory response.10,11 The balance between pro-inflammatory and anti-inflammatory cytokines in the innate immune response not only plays key roles in homeostasis maintenance and host survival, but also influences the severity and duration of the adaptive immune response.12,13 Increasing evidence indicates that several inflammation-related processes, including regulation of pro-inflammatory and anti-inflammatory cytokine production, depend on cellular redox signaling. Augmented oxidative stress such as overproduction of reactive oxygen species results in increased gene expression and secretion of pro-inflammatory cytokines and initiates tissue damage.14–16 In contrast, an antioxidant response involving generation of antioxidant enzymes and the addition of antioxidant agents negatively regulates the inflammatory response.17,18 Therefore, maintenance of redox homeostasis is critical for host immune function. *Rhodococcus aurantiacus* (*R. aurantiacus*) is an intracellular psychrophilic Gram-positive bacterium closely related to members of the genera *Corynebacterium*, *Mycobacterium*, and *Nocardia*. Infection with *R. aurantiacus* induces a non-necrotic and epithelioid granulomatous inflammation in mice, which resembles human sarcoidosis.19 Our previous studies have demonstrated that this inflammatory response is mainly regulated by the balance between TNF-α, IL-6, and IL-10 production during the initial phase of infection.20

In the present study, we assessed the role of Pairogen in regulating the immuno-inflammatory response to *R. aurantiacus* infection in mice. Our results indicate that longitudinal...
ingestion of Pairogen suppressed an excessive inflammatory response in mice and that the immune defense function was further enhanced in their progeny when they were also treated with Pairogen.

**MATERIALS AND METHODS**

**Mice**  Pairogen was supplied by Akatsuka Co. and diluted 1000-, 100-, and 10-fold with sterile water. Female and male C57BL/6 mice purchased from SLC Inc. (Shizuoka, Japan) were used as the parental generation (P mice). The animal protocol was approved by the Institutional Animal Care and Use Committee of Hokkaido University. After 7 d of acclimatization, 4-week-old female P mice received 1000-, 100-, or 10-fold-diluted Pairogen and male P mice were given a drink of 10-fold-diluted Pairogen. Breeding of male and female P mice received 10-fold-diluted Pairogen after weaning. Eight-week-old female P and F1 mice were used for in vivo infection studies, and male F1 mice were used for in vitro experiments. P and F1 mice that received sterile water were used as controls. All the mice were housed in a temperature-controlled room with a 12-h light/dark diurnal cycle and had free access to food and drink throughout the experiment.

**Infection of Mice with *R. aurantiacus***  Female P and F1 mice that received 1000-, 100-, or 10-fold-diluted Pairogen or sterile water, were inoculated via a lateral tail vein with 1×10^8 colony forming units (CFU) of viable *R. aurantiacus* (strain 80005) suspended in 0.2 mL of phosphate-buffered saline (PBS). 0.2 mL of PBS was injected as a control for *R. aurantiacus*. The survival of the mice was monitored daily up to 14 d post-infection, and survival curves were plotted.

**Determination of the Bacterial Counts in Organs**  Tissues were harvested from infected mice at the indicated times post-infection and homogenized in RPMI 1640 medium (Sigma, MO, U.S.A.) (0.1 g/10 mL); 100 µL of the organ homogenates and their serial 10-fold dilutions were plated onto nutrient agar plates (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan). Viable colonies were counted at 48 h after culture. Using this method, we could detect >10^7 bacteria/g of organ.

**Preparation of Organ Extracts**  The murine spleens and livers were aseptically removed at the indicated times post-infection and suspended in RPMI 1640 medium containing 1% (w/v) CHAPS (Wako Pure Chemical Industries, Ltd., Kyoto, Japan). Ten percent (w/v) homogenates were prepared with a Dounce grinder. The homogenates were clarified by centrifugation at 2000×g for 30 min, and the supernatants were stored at −80°C for cytokine assays.

**Peritoneal Macrophages Culture**  The peritoneal macrophages were harvested from uninfected 8-week-old F1 male mice treated with 10-fold-diluted Pairogen or sterile water and suspended in 0.83% ammonium chloride solution containing 10% (v/v) Tris buffer (pH 7.65) to lyse erythrocytes. The cells were resuspended in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin, and then dispersed into 24-well plates at 5×10^5 cells/well for cytokine assays and 35-mm dishes at 2×10^5 cells/dish for RNA extraction. After culture for 2 h at 37°C, floating cells were removed, and the attached macrophages were further cultured for 12 h and then stimulated with heat-killed *R. aurantiacus* (multiplicity of infection (MOI) of 2).

**Measurement of Cytokine Production**  Enzyme-linked immunosorbent assay (ELISA) was used to determine cytokine concentrations in the organ extracts and culture supernatants. The TNF-α level was determined as described previously. A purified hamster anti-TNF-α monoclonal antibody (mAb) and a purified rabbit anti-TNF-α polyclonal antibody (Endogen, MA, U.S.A.) were used as capture and detection antibodies, respectively. A standard curve was constructed for each experiment by serially diluting recombinant TNF-α (R&D systems, Minneapolis, MN, U.S.A.). IL-6 concentration was also measured by ELISA. Plates were coated with purified rat anti-IL-6 mAb (BD Bioscience, Inc., CA, U.S.A.) and incubated with culture supernatants or organ extracts. IL-6 was detected with a biotinylated rat anti-IL-6 mAb (BD Bioscience, Inc.). All ELISAs were run with recombinant IL-6 (BD Bioscience, Inc.). IL-10 concentrations were measured using the DuoSet ELISA development kit (R&D systems) according to the manufacturer’s protocol. The sensitivities of the ELISAs were 50 pg/mL for TNF-α and 20 pg/mL for IL-6 and IL-10.

**Quantitative Real-Time Polymerase Chain Reaction (PCR) analysis**  Total RNA was isolated from peritoneal macrophages using the Trizol reagent (Invitrogen, CA, U.S.A.) and reverse transcribed to first-strand cDNA using the Moloney murine leukemia virus reverse transcriptase (TOYOBO Co., Osaka, Japan). The cDNA was utilized for quantitative real-time PCR with the Power 2× SYBR Green PCR master mix and monitored on an ABI Prism 7000 sequence detection system (both from Applied Biosystems, CA, U.S.A.). The PCR conditions were 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. The primer sequences of the

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**Table 1. Primers Used for Quantitative Real-Time PCR**

<table>
<thead>
<tr>
<th>Gene name</th>
<th>Direction</th>
<th>Primer sequence</th>
<th>Amplicon size (bp)</th>
<th>Accession No.</th>
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<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>5'-GGGTGATCCGTCCTCCCAAAAGG-3'</td>
<td>95</td>
<td>NM_013693</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>5'-TTGAGAAGATGTCATCGTGGTGGAGG-3'</td>
<td>79</td>
<td>NM_031168</td>
</tr>
<tr>
<td>IL-6</td>
<td>Forward</td>
<td>5'-AAAGAATGATGATGATGCTACCAACTG-3'</td>
<td>73</td>
<td>NM_010548</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-GTACTCCAGAGACAGAGGAAGAAT-3'</td>
<td>97</td>
<td>NM_008689</td>
</tr>
<tr>
<td>IL-10</td>
<td>Forward</td>
<td>5'-GGGACAACTACTCTGTAACCGACCTC-3'</td>
<td>86</td>
<td>NM_010442</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5'-TTCCGATAAGGCTTGTCGACACCC-3'</td>
<td></td>
<td></td>
</tr>
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</table>
selected genes are shown in Table 1. Relative gene expression vs. untreated control was normalized by using the expression of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) housekeeping gene.

**Histological Analysis** Animals were sacrificed, and the removed liver samples were fixed in 10% neutral formalin and embedded in paraffin blocks. Tissue sections of 2 μm were stained with hematoxylin and eosin (H&E) using standard techniques. In each experiment, sections were made from three different areas of each organ. The size of the granuloma was calculated by the diameter of each granuloma measured with an ocular micrometer. The mean size of a granuloma and the mean number of granulomas per field were measured from four random optical fields within each section. Granuloma area was determined from the size of granuloma multiplied by the number of granulomas. Paraffin sections were also used for immunohistochemistry of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of oxidative DNA damage. The sections were stained with mouse anti-mouse 8-OHdG (Japan Institute for the Control of Aging, Shizuoka, Japan), followed by application of the immunoperoxidase technique using the STAT-Q rapid IHC system (Innovex Biosciences, CA, U.S.A.). As a negative control, slides were incubated with normal mouse immunoglobulin G (IgG) (Santa Cruz Biotechnology Inc., CA, U.S.A.). Stained cells were quantified using the Image J software, and the data are expressed as the number of cells per field.

**Statistical Analysis** All the data are expressed as mean ± standard deviation (S.D.). Significant differences between the values in the mouse groups were calculated using Student’s t tests or one-way analysis of variance (ANOVA) with Tukey’s post-hoc tests. The p-values of <0.05 were considered statistically significant.

**RESULTS**

**Effect of Pairogen on the Survival of Infected Mice**

Mice in each group were inoculated with 1×10⁸ CFU of viable *R. aurantiacus*, and survival was monitored for 14 d. The P and F1 mice that received water showed a 70% survival rate at 14 d post-infection (Fig. 1A). Although no significant difference was observed in the survival rate for the P mice received water and those treated with either 1000- or 100-fold-diluted Pairogen, P mice treated with 10-fold-diluted Pairogen had an 85% survival rate at 14 d post-infection (Fig. 1A). Higher survival rates were also observed in the F1 mice treated with 1000- or 100-fold-diluted Pairogen relative to those received water (80, 90%, respectively); moreover, all the F1 mice treated with 10-fold-diluted Pairogen survived over 14 d after infection (Fig. 1B). PBS injection had no effect on the survival of mice receiving either Pairogen or water (data not shown).

**Effect of Pairogen on Bacterial Elimination in Infected Mice** After challenge with *R. aurantiacus*, in vivo kinetics of bacterial growth and elimination in P and F1 mice treated with 10-fold-diluted Pairogen were compared with those in P and F1 mice received water. Bacterial proliferation was undetectable in each group of infected mice (Fig. 2). The P and F1 mice that received water displayed a decrease in bacterial burden in their livers and spleens, which fell to an undetectable level at 11 d post-infection (Fig. 2). In contrast, the bacterial counts rapidly declined and were no longer detectable at 9 d post-infection in the organs of P mice treated with 10-fold-diluted Pairogen, and at 7 d post-infection in the spleens and at 8 d post-infection in the livers of F1 mice treated with 10-fold-diluted Pairogen (Fig. 2).

**Effect of Pairogen on Cytokine Production in Infected Mice** Because the balance between pro-inflammatory and anti-inflammatory cytokine production in the early phase of *R. aurantiacus* infection plays key roles in mouse survival and the development of granulomatous inflammation,²⁰,²¹ we investigated the effect of Pairogen on cytokine secretion. There was no cytokine secretion detected in the organs of P and F1 mice that ingested Pairogen alone (Figs. 3A–C). Infection with *R. aurantiacus* induced a high level of TNF-α in the spleens of P and F1 mice that received water on days 1–7.
post-infection and in their livers on days 1–5 post-infection; in contrast, P and F1 mice treated with 10-fold-diluted Pairogen showed a significant decrease in TNF-α production in organs in the early phase of infection, especially F1 mice (Fig. 3A). The mice received water also exhibited peak levels of IL-6 in spleens at 3d post-infection and in livers at 1d post-infection; thereafter, IL-6 production gradually diminished through 7d (Fig. 3B). A marked reduction in IL-6 release was noted on days 1–7 post-infection in the organs of P mice treated with 10-fold-diluted Pairogen, and this reduction was even more pronounced in F1 mice treated with 10-fold-diluted Pairogen (Fig. 3B). The IL-10 level was also measured in the organs of the mice from each group, and size of granulomas in the livers of P and F1 mice, particularly in the latter (28.7, 18.3%, respectively) (Fig. 5A). The

Effect of Pairogen on Granuloma Formation and Redox State in Livers After R. aurantiacus was injected, hepatic histological changes were compared between the mice received water and those treated with 10-fold-diluted Pairogen at 14d, which is a sufficient time for granuloma formation. A large number of non-necrotic and epithelioid granulomas were observed in the livers of P and F1 mice received water (mean area of granulomas per field, 36.8%), and treatment with 10-fold-diluted Pairogen significantly decreased the number and size of granulomas in the livers of P and F1 mice, particularly in the latter (28.7, 18.3%, respectively) (Fig. 5A). The
Fig. 3. Kinetics of Cytokine Production in Mouse Organs

After infection with $1 \times 10^8$ CFU of *R. aurantiacus* or uninfection, the production of TNF-α (A), IL-6 (B), and IL-10 (C) were measured on days 1–7 in the spleens and livers of P and F1 mice treated with water or 10-fold-diluted Pairogen. The results represent the mean±S.D. values from 2 independent experiments in which 3 mice per group were used at each time point. $^p<0.05$, $^{*p}<0.01$, $^{**p}<0.001$ vs. P mice treated with *R. aurantiacus* and water; $^v<p<0.05$, $^{*v}<0.01$, $^{**v}<0.001$ vs. P mice treated with *R. aurantiacus* and 10-fold-diluted Pairogen.
Fig. 4. Expression of Cytokines, NF-κB, and HO-1 by Mouse Macrophages

Under condition of stimulation with heat-killed *R. aurantiacus* (MOI 2), the production of TNF-α, IL-6, and IL-10 (A), and their gene expression (B) as well as the gene expression of NF-κB and HO-1 (C) were measured in the peritoneal macrophages from F1 mice treated with water and those treated with 10-fold-diluted Pairogen. The results represent the mean±S.D. values from 3 independent experiments. \(^p<0.05, *p<0.01, †p<0.001\) vs. cells from F1 mice treated with water.
Immuno histochemical analysis results for 8-OHdG are shown in Fig. 5B. At 14 d post-infection, a significant population of 8-OHdG-positive cells was observed throughout the lobules of mice received water (73/field). In contrast, there was a marked decrease in the number of these cells in the lobules of P and F1 mice that were treated with 10-fold-diluted Pairogen (59, 40/field, respectively). PBS injection and Pairogen ingestion did not induce detectable histological changes in the livers of mice (Figs. 5A, B).

DISCUSSION

To our knowledge, the present study is the first to indicate that the healthy drink Pairogen contributes to the modulation of host inflammatory and immune responses to bacterial infection. Compared with mice ingested water, P and F1 mice that were longitudinally ingested serial dilutions of Pairogen showed enhanced resistance to R. aurantiacus infection. Moreover, all F1 mice treated with 10-fold-diluted Pairogen survived infection. However, administration of 10-fold-diluted Pairogen just before or just after bacterial infection failed to augment mouse resistance and survival (data not shown). FFC water, which is the main component of Pairogen, reportedly regulates cellular functions by changing the state of intracellular water from oxidative to antioxidative.3,4 It has also been reported that a well-controlled homeostatic redox balance is the most critical event for host survival during host-microbe interaction in Drosophila.25 Considering our observations and these previous reports, we conclude that the improved natural resistance of mice to R. aurantiacus resulted from the well-regulated cellular redox state due to long-term ingestion of Pairogen.

It is well known that an imbalanced cytokine response to invading pathogens results in aggravated immune responses, septic shock, and even death.14,26 Our previous studies have also shown that the excessive production of TNF-α and IL-6 during the initial phase of R. aurantiacus infection is responsible for mouse death, although the clearance of bacteria
depends on the presence of TNF-α, but not of IL-6. In the present study, after challenge with *R. aurantiacus*, P and F1 mice treated with 10-fold-diluted Pairogen exhibited significant decreases in TNF-α and IL-6 secretion and bacterial burden in organs compared with those received water, particularly the F1 mice. Similarly, macrophages from F1 mice treated with 10-fold-diluted Pairogen expressed reduced levels of TNF-α and IL-6 as well as reduced mRNA expression under stimulatory condition. Therefore, it is evident that longitudinal administration of Pairogen suppressed the excessive expression of TNF-α and IL-6 when mice were infected with *R. aurantiacus*, which subsequently led to acceleration of bacterial clearance and amelioration of survival. NF-κB is a redox-sensitive transcription factor, and its expression and activity are inhibited by antioxidants such as N-acetylcysteine and butyrate in lipopolysaccharide (LPS)-induced inflammation. In the current study, the macrophages from F1 mice treated with 10-fold-diluted Pairogen also showed a decreased level of NF-κB gene expression triggered by heat-killed *R. aurantiacus*. This result suggests that Pairogen contributes to the negative regulation of NF-κB gene expression in macrophages, which results in a decrease in the gene expression levels of TNF-α and IL-6, because the expression of these genes is reported to be regulated by NF-κB. In the macrophages from F1 mice treated with 10-fold-diluted Pairogen, the elevation in IL-10 mRNA expression induced by heat-killed *R. aurantiacus* was mildly but significantly attenuated compared with that in the cells from F1 mice received water. Although the molecular mechanism by which IL-10 gene expression is regulated is not well understood, a number of studies have...
reported the inhibitory effects of antioxidants on IL-10 expression and secretion. Of interest, in contrast to a reduction in the mRNA expression level of IL-10, a continuous augmentation of its secretion was found in the macrophages from F1 mice treated with 10-fold-diluted Pairogen following stimulation. Consistent with the in vitro results, P and F1 mice treated with 10-fold-diluted Pairogen also showed a higher level of IL-10 in the initial phase of infection, relative to the mice received water, especially the F1 mice. Our previous studies have demonstrated that TNF-α, IL-6, and IL-10 are negatively regulated by each other in the inflammatory response to R. aurantiacus infection and that the balance among the three cytokines determines the development of granulomatous inflammation. Hence, we consider the increased release of IL-10 in mice treated with 10-fold-diluted Pairogen and their macrophages to be due to reduction in TNF-α and IL-6 production. Although several antioxidants have been reported to interfere early in inflammatory responses by blocking or modifying the signal transduction of cytokines, administration of 10-fold-diluted Pairogen just before or just after R. aurantiacus infection did not show any effects on cytokine expression and the development of inflammation in mice (data not shown).

Following R. aurantiacus infection, there was significant amelioration of oxidative stress in the livers of P and F1 mice treated with 10-fold-diluted Pairogen compared with that in mice received water, which was accompanied by a marked decrease in hepatic granuloma formation. These findings imply that longitudinal ingestion of Pairogen attenuates R. aurantiacus-induced granulomatous inflammation by regulating the redox state in tissues and cells. Numerous studies have indicated that HO-1 possesses anti-inflammatory and anti-oxidative properties and that its induction is an adaptive defense mechanism to protect cells and tissues against injury in many diseases. In our in vitro experiments, stimulation with heat-killed R. aurantiacus resulted in a higher level of HO-1 gene expression in the macrophages from F1 mice treated with 10-fold-diluted Pairogen compared with that in the cells from F1 mice received water, suggesting that the negative regulation of R. aurantiacus-induced inflammation by Pairogen is partially dependent on HO-1 production. Evidence suggests that the immune response is a redox-regulated process and that the redox state of resting immune cells determines the type and extent of immune response to pathogen stimulation. The importance of redox homeostasis is further supported by the present study in which the coordination of the cellular redox state by Pairogen contributes to depression of excessive inflammatory and immune responses to R. aurantiacus infection in mice.

On the basis of these data, we conclude that longitudinal administration of Pairogen prior to infection augments their resistance to bacterial infection and suppresses excessive cytokine expression by modulating the cellular redox state in R. aurantiacus-infected mice, which consequently result in accelerated bacterial clearance, enhanced survival, and suppression of granulomatous inflammation. Moreover, the effects of Pairogen can be enhanced in mouse progeny that also ingest Pairogen, suggesting that the well-controlled redox state of immune cells by Pairogen in parental generation effectively affects the immunity and disease resistance of progeny. Our findings provide the basis for the application of Pairogen as a potential redox regulator in combating infectious disease and improving health.

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