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Role of M2b Macrophages in the Acceleration of Bacterial Translocation and Subsequent Sepsis in Mice Exposed to Whole Body $^{137}$Cs Gamma-Irradiation

Makiko Kobayashi,* Kiwamu Nakamura,* Michael Cornforth,† and Fujio Suzuki*+

The influence of whole-body gamma-irradiation on the antibacterial host defense against Enterococcus faecalis translocation was investigated. Mice irradiated with or without 5 Gy $^{137}$Cs gamma-rays were orally infected with $10^6$ CFU/mouse E. faecalis. The pathogen was detected in the mesenteric lymph nodes (MLNs) of irradiated mice 1–4 d postinfection, whereas E. faecalis was not isolated from MLNs of normal mice. All irradiated mice died within 5 d of infection, whereas no mortality was shown in normal mice infected with the pathogen. Irradiated mice inoculated with normal mouse MLN macrophages (Mφ) were shown to be resistant against the infection, although the same mice inoculated with irradiated mouse MLNMφ (I-MLNMφ) died postinfection. I-MLNMφ were identified as IL-10$^+$IL-12$^-$CCL1$^+$LIGHT$^+$ Mφ (M2bMφ) and were shown to be inhibitory on Mφ conversion from resident Mφ to IL-10$^-$IL-12$^-$ Mφ (M1Mφ). M2bMφ were demonstrated in MLNs of mice 10–35 d after gamma-irradiation. M1Mφ were not induced by E. faecalis Ag in cultures of I-MLNMφ, whereas normal mouse MLNMφ were converted to M1Mφ in response to the Ag stimulation. After treatment with CCL1 antisense oligodeoxynucleotides, M2bMφ disappeared in MLNs of irradiated mice, and M1Mφ were generated in MLNs of these mice following E. faecalis stimulation. These results indicate that M2bMφ presented in the I-MLNMφ populations were responsible for the impaired resistance of mice irradiated with gammarays to bacterial translocation and subsequent sepsis. E. faecalis translocation and subsequent sepsis may be controlled immunologically by the intervention of M2bMφ present in MLNs. The Journal of Immunology, 2012, 189: 296–303.

A new paradigm to treat gut bacteria-associated sepsis is urgent for persons who have radiation-associated damage in the gastrointestinal system. Antibiotics are effective against these infections; however, antibiotic chemotherapies often encourage abnormal microflora and multiantibiotic-resistant enterococci generation. The objective of this study is to search a possible new strategy to control sepsis stemming from bacterial translocation in mice irradiated with whole-body gamma-rays.

It is well known that neutrophils and lymphocytes are susceptible to gamma-irradiation (1, 2), whereas monocytes/macrophages (Mφ) are relatively resistant to irradiation (3, 4). We have previously reported that the essential host defense in fighting against bacterial translocation and subsequent sepsis is mainly expressed through the function of M1Mφ (IL-10$^-$IL-12$^+$ Mφ) appearing at the bacterial translocation site (mesenteric lymph nodes [MLNs]) (5). Therefore, we tried to improve the resistance of gamma-irradiated mice to bacterial translocation and subsequent sepsis through the intervention of mouse MLNMφ. However, M1Mφ have never been generated in hosts whose M2Mφ (IL-10$^+$IL-12$^-$ Mφ) predominated, even when they are exposed to the pathogens or stimulated with selected M1Mφ inducers (5, 6). M2Mφ possess the reduced capacity to kill bacteria, and soluble factors released from M2Mφ inhibit Mφ conversion from resident Mφ to M1Mφ following stimulation with bacteria (7). Therefore, carriers of M2Mφ such as severely burned mice are greatly susceptible to bacterial translocation and subsequent sepsis (8). To date, three different subtypes of M2Mφ (M2aMφ, M2bMφ, and M2cMφ) have been described. These Mφ are discriminated from each other based on their gene expression and chemokine-producing profiles (9–13). Thus, M2Mφ that produce CCL17 and express FIZZ1 and mannose receptor genes are identified as M2aMφ; M2bMφ that produce CCL1 and express SPHK1 or LIGHT gene are classified as M2bMφ; and M2cMφ that produce CXCL13 and express FIZZ1 and mannose receptor genes are recognized as M2cMφ (10, 13). All subtypes of M2Mφ show very weak bactericidal activities, and are equally inhibitory on Mφ conversion from resident Mφ to M1Mφ (10).

In this study, Mφ in MLNs of mice irradiated with 5 Gy $^{137}$Cs gamma-rays (irradiated mouse MLNMφ (I-MLNMφ)) were identified as M2bMφ. The antibacterial defense of normal mice to Enterococcus faecalis translocation was impaired after inoculation with I-MLNMφ, M2bMφ in the I-MLNMφ populations disappeared in irradiated mice after treatment with CCL1 antisense oligodeoxynucleotide (ODN), and M1Mφ (an essential effector cell against E. faecalis translocation) appeared in these mice following the stimulation with E. faecalis. In addition, irradiated mice subjected to CCL1 gene therapy were shown to be resistant against infectious complications caused by E. faecalis oral infection. These results indicate that MLN-M2bMφ appearing in association with whole-body gamma-irradiation play a role in the impaired antibacterial resistance of the irradiated mice, suggesting CCL1 antisense gene therapy against MLN-M2bMφ is useful in controlling bacterial translocation and subsequent sepsis in gamma-irradiated hosts.

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Abbreviations used in this article: I-MLNMφ, irradiated mouse mesenteric lymph node macrophage; Mφ, macrophage; MLN, mesenteric lymph node; N-MLNMφ, normal mouse MLNMφ; ODN, oligodeoxynucleotide; PMN, polymorphonuclear neutrophil.

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Materials and Methods

Mice

Seven- to 10-wk-old BALB/c male mice and NOD/scid IL-2Rβnull mice (The Jackson Laboratory, Bar Harbor, ME) were used in these experiments. NOD/scid IL-2Rβnull mice have been defined as immunodeficient mice lacking functional T cells, B cells, and NK cells. Also, these mice have reduced dendritic functions and defective Mφ (14–17). These mice were bred with C57BL/6 Ly6G null mice (100 μg/mouse, once daily for 5 d). After multiple treatments with anti-Ly6G mAb, >90% of neutrophils were depleted from mice (5, 8, 18). The animal studies were reviewed and approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston (Institutional Animal Care and Use Committee Approval Number 0906044).

Bacteria, reagents, and media

E. faecalis (29212 strain) was purchased from the American Type Culture Collection (Manassas, VA). Before being used in experiments, E. faecalis was cultured in tryptic soy broth for 16 h at 37°C in aerobic conditions. mAbs for CCL1, CCL1, CXCL13, and recombinant CCL17 and C1L were purchased from R&D Systems (Minneapolis, MN). Recombinant CXCL13 was purchased from PeproTech (Rocky Hill, NJ). Streptavidin particles plus-DM, Cytofix/Cytperm solution, PE-conjugated anti-IL-12 mAb, FITC-conjugated anti-CCL1 mAb, anti-CD19 mAb, anti-DX5 mAb, isotype control mAbs, and IMag buffer were purchased from BD Biosciences (San Jose, CA). Biotin-conjugated anti-mouse F4/80/80 mAb was obtained from eBioscience (San Diego, CA). Heat-killed E. faecalis was prepared by heating bacteria at 65°C for 30 min. Their inactivated cell preparations were confirmed by culturing the Ag on agar plates. This Ag was stored at −80°C until needed. Single-stranded nucleic acid with anti-sense synthesis (10C/L1 (CCL1 antisense ODN 5′-GAAGCCCGAGAACATCAT-3′) was synthesized by Sigma-Prologi (Woodlands, TX). To protect antisense ODN from nucleolytic degradation in mice, CCL1 antisense ODN with phosphorothioate modification was used. As a control reagent, phosphorothioated scrambled ODN (5′-9CCATCGAGACTGCTGCTGAG-3′) was used. For cultivation of Mφ, RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) (complete medium) was used.

Whole-body [137Cs] gamma-irradiation

Mice received 3–7 Gy acute whole-body gamma-irradiation with a [137Cs] ray (0.662 MeV) irradiator (Mark I Model 30; J.L. Shepherd & Associates, University of Texas Medical Branch at Galveston (Institutional Animal approved by the Institutional Animal Care and Use Committee of the University of Texas Medical Branch at Galveston (Institutional Animal Care and Use Committee Approval Number 0906044).)

Preparation of Mφ, T cells, B cells, NK cells, and polymorphonuclear neutrophils

Mφ were prepared from MLNs of irradiated mice, as previously described (5, 6, 8). Single-cell suspensions of MLNs were obtained in PBS supplemented with 2% FBS. These cells were adjusted to 5 × 106 cells/ml in MagCellect buffer (R&D Systems). Mφ were isolated from the cell suspensions by positive selection using magnetic beads coated with anti-F4/80 mAb. Thus, the cell suspension was mixed with magnetic beads (Dynal) bearing anti-F4/80 mAb at a ratio of one cell to five beads for 30 min at 4°C. F4/80-positive cells were magnetically separated to the side of the tube, and the supernatant was eliminated. A Mφ-enriched population (>97% pure as F4/80-positive cells) was consistently obtained using this technique.

T cells, B cells, and NK cells were prepared from spleens of normal mice through the use of T cell enrichment columns (R&D Systems), anti-CD19, and anti-DX5 magnetic beads, respectively. The purity of T cells, B cells, and NK cells used in these cell experiments was shown to be >95, 95, and 91%, respectively, by flow cytometry. Polymorphonuclear neutrophils (PMN) were isolated from heparinized blood by dextran sedimentation, followed by Ficoll-Hypaque centrifugation (5, 18). RBCs were eliminated from the PMN preparations by exposure to hypotonic solution. The purity of the PMNs was shown to be 93% or more by flow cytometry performed with FITC-conjugated anti–Gr-1 mAb and Wright–Giemsas/alkaline phosphatase stainings.

Treatment of irradiated mice with CCLI antisense ODN

To deplete M2bMφ, we chose the technique to attack CCLI (CCLI antisense ODN treatment), because CCLI released from M2bMφ is essentially required for the maintenance of their M2bMφ properties (19). CCLI antisense ODN and scrambled ODN were dissolved in saline at various concentrations, respectively. Each ODN (0.2 ml) was administered s.c. to irradiated mice (mice 14 d after irradiation) twice per day for 2 d. Based on the results obtained in our preliminary studies, the route and schedule of ODN administration were determined. When irradiated mice were treated once with 6 μg/mouse CCLI antisense ODN, expression (flow cytometry) and production (ELISA) of CCLI were not demonstrated in MLN Mφ derived from these mice 1 and 2 d after treatment.

Determination of M2Mφ subsets (M2aMφ, M2bMφ, M2cMφ)

M2Mφ were considered to be M2aMφ when they produced CCLI (not CCLI and CXL13) and expressed mannose receptor (not inducible LIGHT) mRNA. M2bMφ were considered to be M2bMφ when they produced CCLI (not CCLI and CXL13) and expressed LIGHT (not inducible mannose receptor) mRNA. M2cMφ were considered to be M2cMφ when they produced CCLI (not CCLI and CCLI) and expressed mannose receptor (not inducible LIGHT) mRNA. For the chemokine production, Mφ (1 × 106 cells/ml) were cultured for 48 h without any stimulation. Culture fluids harvested were assayed for chemokines using ELISA. mRNAs for mannose receptor and LIGHT were analyzed by RT-PCR. Total RNA was extracted from Mφ (1 × 106 cells/ml) using RNA isolation, following the manufacturer’s recommendations. Within each experiment, each sample was analyzed by the amount of isolated RNA. Then, the mRNA was turned back into cDNA through reverse transcription of mRNA. PCR was performed using synthesized oligonucleotide primers from Sigma-Aldrich: mannose receptor, 5′-CCATCGAGACTGCTGCTGAG-3′ (forward) and 5′-AGCCCTGGGTTGAAGT-3′ (reverse); LIGHT, 5′-CTGCAATACGCTCTTGGAGA-3′ (forward) and 5′-GATACGTCAGCCGCTTACAG-3′ (reverse). Using a thermal cycler (GeneAmp PCR System 9600), 35 cycles of PCR were performed at 94°C for 15 s and 72°C for 20 s. The preceding products were run on 2% agarose gels containing ethidium bromide. In some experiments, Mφ preparations were immediately incubated with Cytofix/Cytoperm solution at 4°C for 20 min. After washing, the cells were incubated with FITC-conjugated anti-CCLI mAb, PE-conjugated anti–IL-12 mAb, anti–IL-10 mAb, or isotype control mAb at 4°C for 30 min. After washing, the cells were analyzed using FACSCanto flow cytometer.

Determination of M1Mφ

Mφ were considered to be M1Mφ when they produced IL-12 after stimulation with E. faecalis Ag. Thus, Mφ (1 × 106 cells/ml) were stimulated with 105 heat-killed E. faecalis. Twenty-four hours after stimulation, Mφ were harvested and IL-12+ cells were analyzed by flow cytometry. In some experiments, Transwell cultures were performed with I-MLN Mφ (5 × 106 cells/ml, upper chamber) and resident Mφ (1 × 106 cells/ml, lower chamber) that were infected with 106 bacteria. The upper chamber was cultured for 24 h in 37°C. The following formula was applied to the results: (1 – test group CFU/control group CFU) × 100 (20).

Bactericidal activity

Mφ preparations obtained (1 × 106 cells/ml) were suspended in antibiotic-free RPMI 1640 medium supplemented with 10% FBS, and 100 μl of the cell suspension (1 × 106 cells/well) was seeded into 96-well round-bottom microtiter plates. These cells were infected with 3 × 104 CFU/well E. faecalis cells. The bacteria were incubated alone in the control wells. After incubation for 3 h, samples were lysed in 0.1% Triton X-100 (Sigma-Aldrich). Serial 10-fold dilutions of these fluids were plated on tryptic soy broth agar. The number of colonies was counted after being incubated for 24 h at 37°C. For the bacitracin determination, the upper chamber was washed out with 100 μl of PBS and incubated for an additional 24 h. Culture fluids harvested were assayed for IL-12 (p35/p40 heterodimer) using ELISA.

Bacterial oral infection

In this study, mice decontaminated with a mixture of three antibiotics were challenged orally with specified numbers of E. faecalis. Sepsis stemming from orally infected enterococci was consistently demonstrated in these mice after irradiation with 3–7 Gy whole-body gamma-rays. In decontaminated mice, significant numbers of any kind of bacteria were not demonstrated. Also, mice were orally treated with an acid proton-pump inhibitor 2 d before bacterial challenge for the stabilization of oral infection. In these mice, a number of E. faecalis was required for 1 LD50. For decontamination, mice were treated for 4 d with drinking water containing 4 mg/ml penicillin, streptomycin, and bacitracin (5, 7). On the day of the final antibiotic treatment, these mice were treated orally with lansoprazole (a proton-pump inhibitor, 0.5 mg/ml) (7). Four
hours after lansoprazole treatment, mice 14 d after gamma-irradiation and irradiated mice treated with CCL1 antisense ODN or scrambled ODN were infected orally with 10^6 CFU/mouse (corresponds to <0.1 LD_{50} in normal mice) E. faecalis. For adoptive transfer experiments, MLN Mφ preparations were adjusted to 5 × 10^6 cells/ml with PBS, and 0.2 ml cell suspension was adoptively transferred i.v. to NOD/scid IL-2Rγnull mice treated with anti-Ly6G mAb. Two hours after inoculation, mice were orally infected with 1 or 3 × 10^5 CFU/mouse E. faecalis.

The severity of infectious complications caused by E. faecalis oral infection was evaluated by the following: 1) growth of bacteria in MLNs and liver, and 2) mortality rates of the test groups in comparison with the control groups. To measure the quantity of bacteria, organ specimens (MLNs and liver) were weighed and disrupted in 2 ml PBS using a Brinkman homogenizer. A serial 10-fold dilution of the homogenates was plated onto blood agar plates and incubated for 24 h at 37˚C. The colonies were counted, and the number of bacteria per gram organ was determined.

Because bacteria were not detected normally in MLNs and liver, the presence of bacteria in these organs is considered to be evidence of translocation. To determine the percentage of survival, mice will be monitored twice per day for 5 or 10 d postinfection.

**Statistical analysis**

The results obtained were statistically analyzed using an ANOVA test. Kaplan–Meier curves were constructed, and a log-rank comparison test of the groups was used to calculate p values. All calculations were performed using the program Statview 4.5 from Brain Power (Calabasas, CA). The result was considered significant if the p value was <0.05.

**Results**

**Susceptibility of gamma-irradiated mice to E. faecalis translocation**

In the first series of experiments, mice 14 d after 3, 5, or 7 Gy whole-body 137Cs gamma-irradiation (10 mice each) were observed for 20 d to determine the gamma-ray–associated mortality. As shown in Fig. 1A, 90% of the mice exposed to 7 Gy gamma-rays died. However, mortality rates were not shown in mice exposed to 3–5 Gy gamma-rays. Bacterial translocation occurs spontaneously in mice irradiated with 6–10 Gy whole-body gamma-rays (21). Therefore, mice irradiated with 5 Gy gamma-rays were mainly used in the following experiments. Normal mice and irradiated mice were infected orally with 10^6 CFU/mouse E. faecalis. MLNs and livers were obtained from these mice 1–4 d postinfection (six mice in each day). Then, the organ was homogenized respectively by a homogenizer, and the number of pathogens in each organ homogenate was determined by a standard colony-counting assay. In the results, >10^3 CFU/g organ of bacteria was detected in MLNs of irradiated mice 1 d after the infection, but the pathogen was not detected in MLNs of normal mice similarly infected with the pathogen. Also, the bacteria grew progressively in the livers of irradiated mice following infection, whereas progressive growth of pathogen was not shown in both organs of normal mice exposed to the pathogen (Fig. 1B). All of the mice exposed to 5 Gy gamma-rays died after 10^6 CFU/mouse E. faecalis oral infection, whereas 60% of the 3 Gy-irradiated mice survived after the infection (Fig. 1C). These results indicate that mice irradiated with 5 Gy gamma-rays are greatly susceptible, and mice irradiated with 3 Gy gamma-rays are moderately susceptible to E. faecalis translocation and subsequent sepsis. E. faecalis translocation and subsequent sepsis. (A) Radiation-associated mortality. After decontamination, mice (10 mice per group) exposed to 3, 5, or 7 Gy gamma-rays were observed every 12 h for 20 d to determine their mortality. (B) E. faecalis translocation. After decontamination, mice 14 d after irradiation with 5 Gy gamma-rays were orally infected with 10^6 CFU/mouse E. faecalis. One to 4 d postinfection, the growth of bacteria in MLNs and livers of these mice was determined by a colony-counting method. Data are displayed by the mean ± SEM from six mice. Data are representative of at least two independent experiments.

A role of Mφ on the host’s antibacterial resistance of gamma-irradiated mice to E. faecalis translocation

Active effector cells in host antibacterial resistance against enterococcal translocation were determined as follows: T cells, B cells, NK cells, Mφ, and PMN were isolated from normal mice and inoculated i.v. to NOD/scid IL-2Rγnull mice treated with anti-Ly6G mAb. These mice do not carry functional T cells, B cells,

<table>
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<tr>
<th>E. faecalis (CFU/Mouse)</th>
<th>Irradiated Micea (Survival/Tested)</th>
<th>Normal Micea (Survival/Tested)</th>
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<tr>
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<td>8 × 10^3</td>
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aDecontaminated mice (10 mice in each group) were exposed to 5 Gy gamma-rays, and infected orally with various doses of E. faecalis.

bND, Not done.
NK cells, Mϕ, and PMN. These mice were decontaminated and treated with a proton-pump inhibitor, and then orally infected with *E. faecalis*. In the results, the bacterial growth in MLNs was not seen in NOD/scid IL-2Rγnull mice (three mice) inoculated with Mϕ, whereas *E. faecalis* did grow in MLNs of the same mice (three mice in each group) inoculated with B cells, T cells, NK cells, or PMN (Fig. 2A). These results indicate that Mϕ are a major effector cell in host antibacterial resistance against *E. faecalis* translocation. In the next experiments, 1 × 10⁷ cells/mouse of MLNMϕ from normal mice (N-MLNMϕ) or irradiated mice (I-MLNMϕ) were adoptively transferred to NOD/scid IL-2Rγnull mice, and these mice were then orally infected with *E. faecalis*. The viability of both groups of Mϕ was shown to be 98% or more by trypan blue dye exclusion test. When all NOD/scid IL-2Rγnull mice (eight mice) inoculated with I-MLNMϕ or treated with saline (eight mice) died after *E. faecalis* infection, the same mice (eight mice) inoculated with N-MLNMϕ were shown to be resistant against the infection (Fig. 2B). These results indicate that the function of I-MLNMϕ on the anti-enterococcal resistance is different from that of N-MLNMϕ.

**Failure of M1Mϕ induction in cultures of I-MLNMϕ**

In the next series of experiments, we tried to induce M1Mϕ by stimulation with *E. faecalis* Ag (10⁵ heat-killed *E. faecalis*) in cultures of N-MLNMϕ and I-MLNMϕ. In these experiments, IL-12–expressing Mϕ were considered as M1Mϕ. In the results, in response to the stimulation with enterococcal Ag, IL-12–expressing cells appeared by 72% in the N-MLNMϕ population. However, IL-12–expressing Mϕ were not demonstrated in the I-MLNMϕ population (Fig. 3A). These results indicate that MLNMϕ of irradiated mice do not change to M1Mϕ even when they are stimulated with the Ag.

In the presence of the Ag, Mϕ conversion from resident Mϕ to M1Mϕ was examined in cultures of I-MLNMϕ. Thus, 1 × 10⁶ cells/ml N-MLNMϕ (resident Mϕ) mixed with 10⁵ heat-killed *E. faecalis* were seeded in the lower chamber of Transwell, and then these cells were cultured with 5 × 10⁵ cells/ml I-MLNMϕ (upper chamber). Twenty-four hours after cultivation, Mϕ harvested from the lower chamber were reconstituted for 24 h. Culture fluids were harvested and assayed for IL-12 by ELISA. Data are displayed as the mean ± SEM of three different experiments (three mice in each experiment). **p < 0.01 versus N-MLNMϕ.**

![FIGURE 2.](image)

**FIGURE 2.** Mϕ are major antibacterial effector cells against *E. faecalis* oral infection. (A) NOD/scid IL-2Rγnull mice treated with anti-Ly6G mAb were inoculated i.v. with 1 × 10⁶ cells/mouse B cells, T cells, NK cells, Mϕ, or PMN prepared from normal mice. Two hours after cell inoculation, these mice were infected orally with *E. faecalis* (3 × 10⁷ CFU/mouse). As a control, anti-Ly6G–treated NOD/scid IL-2Rγnull mice were infected with the pathogen in the same way. To determine the growth of bacteria, MLNs were obtained from these mice 1 d postinfection and homogenized. The numbers of bacteria in each organ homogenate were determined by a standard colony-counting assay. Data are displayed by the mean ± SEM from three mice. **p < 0.01 versus control. (B) NOD/scid IL-2Rγnull mice treated with anti-Ly6G mAb were inoculated i.v. with 1 × 10⁶ cells/mouse MLNMϕ from normal mice (N-MLNMϕ) or mice irradiated with 5 Gy gamma-rays (I-MLNMϕ). Two hours later, these mice were infected orally with *E. faecalis* (10⁷ CFU/mouse). To determine the mortality rate of these mice, they were observed every 12 h for 5 d. Data are displayed by percentage of survival (eight mice each). **p < 0.01 versus mice inoculated with N-MLNMϕ.

![FIGURE 3.](image)

**FIGURE 3.** Impaired M1Mϕ generation in mice irradiated with 5 Gy gamma-rays. (A) Enterococcal Ag-induced M1Mϕ generation. Mϕ in MLNs of normal mice (N-MLNMϕ) or irradiated mice (I-MLNMϕ) were cultured in the presence of enterococcal Ag (10⁵ heat-killed *E. faecalis*). Twenty-four hours after stimulation, numbers of IL-12+ cells in the MLNMϕ populations were prepared by flow cytometry. Data are displayed as the mean ± SEM of three different experiments (three mice in each experiment). **p < 0.01 versus N-MLNMϕ. (B) Mϕ conversion from resident MLNMϕ to M1Mϕ in Transwell cultures with I-MLNMϕ. In the presence of *E. faecalis* Ag, N-MLNMϕ (1 × 10⁶ cells/ml, lower chamber) were Transwell cultured with N-MLNMϕ or I-MLNMϕ (5 × 10⁵ cells/ml, upper chamber). Twenty-four hours after cultivation, Mϕ in the lower chamber were reconstituted for 24 h. Culture fluids were harvested and assayed for IL-12 by ELISA. Data are displayed as the mean ± SEM of three different experiments (three mice in each experiment). **p < 0.01. ***p < 0.001 versus N-MLNMϕ.
produce CCL1 and IL-10 and express LIGHT mRNA have been described as M2bMϕ (12, 13), the results shown in Fig. 4 indicate that M2bMϕ are present predominantly in the I-MLNMϕ populations.

The appearance of M2bMϕ in MLNs of mice 1, 4, 7, 10, 14, 21, 28, 35, and 42 d after 5 Gy of whole-body gamma-irradiation was examined. MLNMϕ isolated from mice various days after gamma-irradiation were analyzed for IL-10 and CCL1+ cells by flow cytometry. IL-10+CCL1+ MLNMϕ were cultured for 24 h without any stimulation. Culture fluids obtained were assayed for CCL17, CCL1, and CXCL13 by ELISA. To examine the bacteriocidal activity of Mϕ, MLNMϕ (1 × 10⁶ cells/ml) isolated from both groups of mice were infected with 3 × 10⁶ CFU/ml E. faecalis cells. After incubation for 3 h, cells were lysed and the numbers of bacteria in each cell lysate were determined by a colony-counting method. As a control, the bacteria were incubated in media. Data are displayed in (A), (B), and (D) as the mean ± SEM of three different experiments (three mice in each experiment). Data shown in (C) are representative of three independent experiments. **p < 0.01 versus N-MLNMϕ.

**FIGURE 4.** Properties of MLNMϕ from gamma-irradiated mice. Mϕ isolated from normal mice (N-MLNMϕ) and mice exposed to 5 Gy gamma-rays (I-MLNMϕ) were tested for their intracellular IL-10 (A), chemokine-producing profiles (B), LIGHT and mannose receptor mRNA expressions (C), and killing activity against E. faecalis (D). Intracellular IL-10 was analyzed by flow cytometry, and the expression of LIGHT and mannose receptor mRNAs was analyzed by RT-PCR. For the production of chemokines, 1 × 10⁶ cells/ml N-MLNMϕ or I-MLNMϕ were cultured for 24 h without any stimulation. Culture fluids obtained were assayed for CCL17, CCL1, and CXCL13 by ELISA. To examine the bacteriocidal activity of Mϕ, MLNMϕ (1 × 10⁶ cells/ml) isolated from both groups of mice were infected with 3 × 10⁶ CFU/ml E. faecalis cells. After incubation for 3 h, cells were lysed and the numbers of bacteria in each cell lysate were determined by a colony-counting method. As a control, the bacteria were incubated in media. Data are displayed in (A), (B), and (D) as the mean ± SEM of three different experiments (three mice in each experiment). Data shown in (C) are representative of three independent experiments. **p < 0.01 versus N-MLNMϕ.

**FIGURE 5.** Appearance of M2bMϕ in gamma-irradiated mice. (A) Kinetics. MLNMϕ (1 × 10⁶ cells/ml) were isolated from mice 1–42 d after 5 Gy gamma-irradiation, and IL-10+CCL1+ cells in the MLNMϕ populations were analyzed by flow cytometry. *p < 0.05, **p < 0.01 versus N-MLNMϕ. (B) Doses of gamma-rays. MLNMϕ (1 × 10⁶ cells/ml) were isolated from mice 14 d after irradiation with 3, 5, or 7 Gy gamma-rays, and IL-10+CCL1+ cells in the MLNMϕ populations were analyzed by flow cytometry. Data are displayed as the mean ± SEM of three different experiments (three mice in each experiment). *p < 0.05, **p < 0.01 versus N-MLNMϕ.

M2bMϕ depletion by CCL1 antisense ODN in mice exposed to 5 Gy gamma-rays

Because M2bMϕ were CCL1-producing cells, the effect of CCL1 antisense ODN on the properties of M2bMϕ in the I-MLNMϕ population was examined. Thus, mice, 14 d after whole-body irradiation with 5 Gy gamma-rays, were treated s.c. with various doses of CCL1 antisense ODN or scrambled ODN twice per day for 2 d. One day after the final treatment, Mϕ were isolated from MLNs of these mice and cultured for 48 h without any stimulation. Culture fluids were harvested and assayed for IL-10 as a M2Mϕ biomarker. As shown in Fig. 6A, 2.2–3.6 ng/ml IL-10 were detected in the culture fluids of MLNMϕ from irradiated mice treated with scrambled ODN. However, IL-10 was not detected in the culture fluids of MLNMϕ from irradiated mice treated with 6 µg/mouse or more of CCL1 antisense ODN. In the next experiments, MLNMϕ from irradiated mice treated with CCL1 antisense ODN or scrambled ODN were assayed for intracellular IL-10 by flow cytometry and LIGHT mRNA expression by RT-PCR. In the results, Mϕ from irradiated mice treated with CCL1 antisense ODN (6 µg/mouse) were shown to be IL-10– LIGHT+ Mϕ. Mϕ from the same mice treated with scrambled ODN were identified as IL-10+LIGHT+ Mϕ (Fig. 6B, 6C). N-MLNMϕ were shown to be IL-10– LIGHT+ Mϕ (Figs. 4C, 6B). These results indicate that MLNMϕ from irradiated mice treated with CCL1 antisense ODN are not M2bMϕ. In the next experiments, we tried to induce M1Mϕ by the bacterial Ag in irradiated mice treated with CCL1 antisense ODN. In the results, IL-12–producing cells were induced by the Ag in Transwell cultures between N-MLNMϕ and MLNMϕ from irradiated mice that were previously treated with CCL1 antisense ODN. However, IL-12–producing Mϕ were not demonstrated in cultures of MLNMϕ of irradiated mice that were treated with scrambled ODN (Fig. 7). These results indicate that M1Mϕ are inducible in MLNs of irradiated mice treated with CCL1 antisense ODN. Effect of CCL1 antisense ODN on E. faecalis translocation and subsequent sepsis in mice exposed to 5 Gy gamma-rays

In the next experiments, the susceptibility of irradiated mice treated with CCL1 antisense ODN to E. faecalis oral infection was studied. Thus, irradiated mice treated with CCL1 antisense ODN (six mice per group) were orally infected with E. faecalis, and their antibacterial resistance was tested by the growth of bacteria in MLNs and livers, and survival rates. One or 2 d postinfection, MLNs and liver were obtained from each irradiated mouse that was treated with scrambled ODN or CCL1 antisense ODN (6 µg/ mouse, s.c., twice per day). The organ in each mouse was homogenized by a homogenizer. The numbers of pathogen in each
organ homogenate were determined by a standard colony-counting assay. In the results, huge numbers of bacteria were detected in organs of irradiated mice treated with scrambled ODN. However, the pathogen did not grow significantly in MLNs and livers of irradiated mice that were treated with CCL1 antisense ODN (Fig. 8A). When all of the irradiated mice (12 mice) that were treated with CCL1 antisense ODN survived after the same infection (Fig. 8B). These results indicate that irradiated mice treated with CCL1 antisense ODN are resistant against E. faecalis translocation and subsequent sepsis.

Discussion

Neutrophils and lymphocytes are exquisitely sensitive to radiation and undergo apoptotic cell death (1, 2). In contrast, Mϕs are radioresistant cells (3, 4) and indispensable host antibacterial effector cells in irradiated hosts. It is well recognized that whole-body gamma-irradiation causes severe damage in intestinal epithelial cells, which are greatly radiosensitive (1, 22–24). Therefore, radiation-associated intestinal tissue damages are responsible for bacterial translocation and subsequent sepsis in persons exposed to radiation. In the current study, we examined the influence of whole-body gamma-irradiation on the antibacterial host defense against E. faecalis translocation. With experiments in NOD/scid IL-2Rγnull mice inoculated with various immunocompetent cells, Mϕs were shown to be a major effector cell against bacterial translocation and subsequent sepsis.

Mϕs located in the subepithelial lamina propria and MLNs are the first cells that fight against translocated E. faecalis (5). Resident Mϕs are isolated from lamina propria and MLNs of normal mice (25). Resident Mϕs are immunologically quiescent with low oxygen consumption and low levels of MHC class II gene expression. In the event of infection, resident Mϕs convert to M1Mϕ through the engagement of TLRs or IFN receptors (26, 27). M1Mϕs are actual effector cells in host antibacterial innate immune responses (28, 29). These Mϕs exhibit the following: 1) high oxygen consumption; 2) the ability to kill pathogens; 3) the ability to...
express iNOS; and 4) the ability to secrete NO, proinflammatory cytokines (IL-1, IL-6, and TNF-α). Th1 response-associated cytokines/chemokines (IFN-γ, IL-12, IL-18, CCL3, CCL5), and antimicrobial peptides (7, 30, 31). However, these M1Mφ were not demonstrated in MLNs of gamma-irradiated mice orally infected with *E. faecalis*.

We have previously demonstrated that resident Mφ do not convert to M1Mφ in circumstances in which M2Mφ predominate (32, 33). M2Mφ have been described to be implicated in the negative regulation of M1Mφ (7). Recently, three different subtypes of M2Mφ (M2aMφ, M2bMφ, and M2cMφ) were described based on their gene expression and chemokine profiles (9, 13). In this study, M6φ in MLNs of mice 10–35 d after 5 Gy gamma-irradiation were identified as M2bMφ, because these M6φ produced CCL1 and IL-10, and expressed LIGHT mRNA (Fig. 4). Therefore, we tried to deplete M2bMφ in MLNs of irradiated mice using CCL1 antisense ODN, and improve the antibacterial resistance of these mice to *E. faecalis* translocation and subsequent sepsis. In the results, M1Mφ were induced by a bacterial Ag in MLNs of gamma-irradiated mice that were previously treated with CCL1 antisense ODN. In addition, irradiated mice previously treated with CCL1 antisense ODN were shown to be resistant against oral *E. faecalis* infection. Thus, M2bMφ were successfully eliminated from irradiated mice by CCL1 antisense gene therapy, and sepsis caused by bacterial translocation was not developed in these mice orally infected with *E. faecalis*. CCL1 has been characterized as an essential chemokine for the maintenance of their M2bMφ. In the absence of CCL1, M2bMφ have been regressed to resident M6φ (19). Among possible techniques to deplete CCL1 (mAb, aptamer, small interfering RNA, antisense ODN), CCL1 antisense ODN administration was shown to be the best on the intervention of M2bMφ properties in our preliminary studies utilizing irradiated mice. Further experiments concerning the molecular basis for the functional consequences of CCL1 knockdown are needed.

In this study, M2bMφ were persistently demonstrated in MLNs of mice 10–35 d after irradiation. However, M6φ with the ability to produce IL-10, CCL1, and CXCL13 (a mixture of M2aMφ and M2cMφ) disappeared in MLNs of mice until 7 d of whole-body gamma-irradiation (34). In these experiments, I-MLNMφ were cultured for 48 h. We have performed the same cultivation in Mφ serum-free medium (Invitrogen) or RPMI 1640 medium supplemented with heat-inactivated FBS (10%). In the results, CCL1 was equally produced in two different cultures of MLNMφ. This indicates that, for the detection of M2bMφ, artificial factors acquired during cultivation procedures are minimal. Also, we have detected M2bMφ-specific biomarkers (LIGHT mRNA expression; Fig. 4C) in I-MLNMφ by RT-PCR. Because M2bMφ biomarkers were displayed by noncultured I-MLNMφ, the results indicate that M2bMφ polarization is not influenced by the 48-h culture period.

The mechanism involved in M2bMφ generation in irradiated mice remains unclear. Recently, phagocytosis-associated differentiation of resident Mφ to regulatory/M2bMφ has been demonstrated (10). In this case, Mφ-phagocytosed apoptotic inflammatory PMN expressed LIGHT/SHPK1 and produced IL-10. These results suggest a possibility that M2bMφ are generated from resident Mφ through their phagocytosis of radiation-induced damaged PMN. In our recent studies, XBP-1, a biomarker of endoplasmic reticulum stress, was shown to be expressed by MLNMφ from mice 12–24 h after 5 Gy [137Cs] gamma-ray irradiation (M. Kogiso, K. Nakamura, M.N. Cornforth, M. Kobayashi, and F. Suzuki, unpublished observations). Endoplasmic reticulum stress is known to be involved in various deleterious stress responses (35–39). Because XBP-1 Mφ are characterized as CCL2-producing cells (40) and CCL2 is known as a M2Mφ inducer (8), it is also possible that endoplasmic reticulum stress is involved in M2bMφ generation in irradiated mice. To explore these questions, more experiments are required.

In this study, we indirectly showed that M2bMφ influenced by CCL1 antisense ODN are resident M6φ, because these M6φ have been converted to M1Mφ after stimulation with bacterial Ag, and M2bMφ never convert to M1Mφ under the same stimulation. It is very difficult to directly measure how many M6φ are resident Mφ in the preparations of M2bMφ treated with CCL1 antisense ODN, because resident M6φ produce minimal amounts of cytokines and their specific surface Ag expression is not known. In addition, we have searched specific surface Ags for three subsets of M2Mφ (M2aMφ, M2bMφ, and M2cMφ) for the discrimination of each subtype of M2Mφ. However, we did not demonstrate any significant differences in the TLR expression in these M6φ populations. Further studies will be required to search their specific cell surface markers.

In conclusion, severe bacterial translocation and subsequent sepsis were developed in gamma-irradiated mice after oral infection with 10⁶ CFU/mouse *E. faecalis*. In these mice, an essential effector cell (M1Mφ) on the host antibacterial resistance was not generated in the translocation site (MLNs). The majority of Mφ in MLNs of irradiated mice were identified as M2bMφ (IL-10+IL-12- CCL1+LIGHT+ Mφ) and shown to be inhibitory on the enterococcal Ag-induced conversion of Mφ from resident Mφ to M1Mφ. After depletion of M2bMφ by treatment with CCL1 antisense ODN, M1Mφ appeared in irradiated mice, and these mice treated with CCL1 antisense ODN were shown to be resistant against *E. faecalis* translocation and subsequent sepsis. The depletion of M2bMφ may be a relevant approach in improving the host antibacterial resistance of persons who have radiation-associated damage in the gastrointestinal system.

**Disclosures**

The authors have no financial conflicts of interest.

**References**


