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Role of M2b Macrophages in the Acceleration of Bacterial Translocation and Subsequent Sepsis in Mice Exposed to Whole Body [\textsuperscript{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 [\textsuperscript{137}Cs] gamma-rays were orally infected with 10\textsuperscript{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 (M\textsubscript{φ}) were shown to be resistant against the infection, although the same mice inoculated with irradiated mouse MLN\textsubscript{M}φ (I-MLN\textsubscript{M}φ) died postinfection. I-MLN\textsubscript{M}φ were identified as IL-10\textsuperscript{+}IL-12\textsuperscript{−}CCL1\textsuperscript{+}LIGHT\textsuperscript{+} M\textsubscript{φ} (M2bM\textsubscript{φ}) and were shown to be inhibitory on M\textsubscript{φ} conversion from resident M\textsubscript{φ} to IL-10\textsuperscript{−}IL-12\textsuperscript{+} M\textsubscript{φ} (M1M\textsubscript{φ}). M2bM\textsubscript{φ} were demonstrated in MLNs of mice 10–35 d after gamma-irradiation. M1M\textsubscript{φ} were not induced by E. faecalis Ag in cultures of I-MLN\textsubscript{M}φ, whereas normal mouse MLN\textsubscript{M}φ were converted to M1M\textsubscript{φ} in response to the Ag stimulation. After treatment with CCL1 antisense oligodeoxynucleotides, M2bM\textsubscript{φ} disappeared in MLNs of irradiated mice, and M1M\textsubscript{φ} were generated in MLNs of these mice following E. faecalis stimulation. These results indicate that M2bM\textsubscript{φ} present in the I-MLN\textsubscript{M}φ populations were responsible for the impaired resistance of mice irradiated with gamma-rays to bacterial translocation and subsequent sepsis. E. faecalis translocation and subsequent sepsis may be controlled immunologically by the intervention of M2bM\textsubscript{φ} present in MLNs.

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 (M\textsubscript{φ}) 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 based on their gene expression and chemokine-producing profiles (9–13). Thus, M2M\textsubscript{φ} that produce CCL17 and express FIZZ1 and mannose receptor genes are identified as M2bM\textsubscript{φ} (10, 13). All subtypes of M2M\textsubscript{φ} show very weak bactericidal activities, and are equally inhibitory on M\textsubscript{φ} conversion from resident M\textsubscript{φ} to M1M\textsubscript{φ} (10).

In this study, M6b in MLNs of mice irradiated with 5 Gy [\textsuperscript{137}Cs] gamma-rays (irradiated mouse MLN\textsubscript{M}φ [I-MLN\textsubscript{M}φ]) were identified as M2bM\textsubscript{φ}. The antibacterial defense of normal mice to Enterococcus faecalis translocation was impaired after inoculation with I-MLN\textsubscript{M}φ. M2bM\textsubscript{φ} in the I-MLN\textsubscript{M}φ populations disappeared in irradiated mice after treatment with CCL1 antisense oligodeoxynucleotide (ODN), and M1M\textsubscript{φ} (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-M2bM\textsubscript{φ} 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-M2bM\textsubscript{φ} is useful in controlling bacterial translocation and subsequent sepsis in gamma-irradiated hosts.
ROLE OF M2bM IN BACTERIAL TRANSLLOCATION IN IRRADIATED MICE

Materials and Methods

Mice

Seven- to 10-wk-old BALB/c male mice and NOD/scid IL-2Rγnull mice (The Jackson Laboratory, Bar Harbor, ME) were used in these experiments. NOD/scid IL-2Rγnull 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 treated i.p. with anti-Ly6G mAb (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, CCLX, and recombinant CCL1 and CCL1 were purchased from R&D Systems (Minneapolis, MN). Recombinant CXCL13 was purchased from PeproTech (Rocky Hill, NJ). Streptavidin particles plus-DM, Cytofix/Cytoperm solution, PE-conjugated anti–IL-12 mAb, PE-conjugated anti–IL-10 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 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 properties were confirmed by culturing the Ag on agar plates. This Ag was stored at 80°C until needed. Single-stranded nucleic acid that inhibits the synthesis of CCL1 (CCL1 antisense ODN; 5'-GAAGCCCGAGAACATCAT-3') was synthesized by Sigma-Proligo. A 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'-CATCACAATGGCAGACG-3') was used. For cultivation of Mφ, RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics (100 μU/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, San Fernando, CA) at a dose rate of 1.05 Gy/min, which is reduced from the 5.08 Gy/min via lead attenuators. After gamma-irradiation, these mice were housed in the Animal BSL-2 (ABSL-2) Facility, and fed autoclaved food and water (Aseptic diet) under constant conditions. Mortality and sickness (such as decreased appetite and decreased body temperature) were not expected in mice exposed to 5 Gy or less whole-body gamma-rays.

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 MacsCellect buffer (R&D Systems). Mφ were isolated from the cell suspensions by positive selection utilizing 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–Giemsa/alkaline phosphatase stainings.

Treatment of irradiated mice with CCL1 antisense ODN

To deplete M2bMφ, we chose the technique to attack CCL1 (CCL1 antisense ODN treatment), because CCL1 released from M2bMφ is essentially required for the maintenance of their M2bMφ properties (19). CCL1 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 CCL1 antisense ODN, expression (flow cytometry) and production (ELISA) of CCL1 were not demonstrated in MLNMφ derived from these mice 1 and 2 d after treatment.

Determination of M2bMφ subsets (M2aMφ, M2bMφ, M2cMφ)

M2bMφ were considered to be M2aMφ when they produced CCL17 (not CCL17 and CXCL13) and expressed mannose receptor (not inducible LIGHT) mRNA. M2aMφ were considered to be M2bMφ when they produced CCL17 (not CCL17 and CXCL13) and expressed LIGHT (not inducible mannose receptor) mRNA. M2bMφ were considered to be M2cMφ when they produced CXCL13 (not CCL17 and CXCL13) and expressed LIGHT (not inducible mannose receptor) 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 isolator, following the manufacturer’s recommendations. Within each experiment, each sample was normalized by the amount of isolated RNA. Then, the RNA was turned back into cDNA through reverse transcription of mRNA. PCR was conducted using synthesized oligonucleotide primers from Sigma-Aldrich: mannose receptor, 5’-CCATCGGAGACTGCTGCTGAG-3’ (forward) and 5’-AGGCCCGAGAACATCAT-3’ (reverse); LIGHT, 5’-CTGCAACCGCTTGGGCT-3’ (forward) and 5’-GATACGCAGCAGCCCTCAACG-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 predicted 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-CCL1 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 FACSCanuto 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 1-MLNMφ (5 × 105 cells/ml, upper chamber) and resident Mφ (1 × 106 cells/ml, lower chamber) that were previously mixed with 105 heat-killed E. faecalis. Twenty-four hours after cultivation, the upper chamber was removed and Mφ in the lower chamber were washed with media. Then, Mφ in the chamber were cultured for an additional 24 h. Culture fluids harvested were assayed for IL-12 (p35/p40 heterodimer) using ELISA.

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 × 105 cells/well) was seeded into 96-well round-bottom microtiter plates. These cells were infected with 3 × 105 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. The following formula was applied to the results = (1 – test group CFU/control group CFU) × 100 (20).

E. faecalis 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 to be required for 1 LD50 was reduced significantly. 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 10^8 CFU/mouse (corresponds to <0.1 LD_50 in normal mice) _E. faecalis_. For adoptive transfer experiments, MLNMδ 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-2R^γnull mice treated with anti-Ly6G mAb. Two hours after inoculation, mice were orally infected with 1 or 3 × 10^7 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 Bruikman 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, >10^7 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, although normal mice are resistant against these infections. Furthermore, we tested the mortality rates of irradiated mice (5 Gy gamma-rays) orally infected with various doses of _E. faecalis_. All of the normal mice infected orally with 4 × 10^7 CFU/mouse _E. faecalis_ survived, and >2 × 10^8 CFU/mouse _E. faecalis_ was calculated as 1 LD_50 in normal mice. In contrast, 100% of irradiated mice died after the infection with 10^8 CFU/mouse _E. faecalis_, and 1 LD_50 of _E. faecalis_ in irradiated mice was shown to be 10^8 CFU/mouse (Table I).

A role of Mδ 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, Mδ, and PMN were isolated from normal mice and inoculated i.v. to NOD/scid IL-2R^γnull mice treated with anti-Ly6G mAb. These mice do not carry functional T cells, B cells,

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<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>ND&lt;sup&gt;b&lt;/sup&gt;</td>
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<sup>a</sup>Decontaminated mice (10 mice in each group) were exposed to 5 Gy gamma-rays, and infected orally with various doses of _E. faecalis_.

<sup>b</sup>ND, 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, $1 \times 10^5$ 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 N-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 (10^7 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, $1 \times 10^6$ cells/ml N-MLNMΦ (resident MΦ) mixed with $10^7$ heat-killed *E. faecalis* were seeded in the lower chamber of Transwell, and then these cells were cultured with $5 \times 10^5$ cells/ml I-MLNMΦ (upper chamber). Twenty-four hours after cultivation, MΦ harvested from the lower chamber were additionally cultured for 24 h to determine their M1MΦ properties. Resident MΦ were considered to be converted to M1MΦ, when IL-12 was detected in their culture fluids. Resident MΦ stimulated with the Ag did not change to M1MΦ when they were Transwell cultured with I-MLNMΦ, whereas IL-12–producing MΦ were induced by the Ag from the N-MLNMΦ populations (Fig. 3B). These results indicate that I-MLNMΦ are inhibitory on MΦ conversion from resident MΦ to M1MΦ.

Next, I-MLNMΦ were tested for their cytokine/chemokine-producing profiles. In the results, 91% of the I-MLNMΦ population was shown to be IL-10^3 cells (Fig. 4A). CCL1 (a biomarker for M2bMΦ) was detected in culture fluids of I-MLNMΦ, whereas IL-12 (a biomarker for M1MΦ), CCL17 (a biomarker of M2aMΦ), and CXCL13 (a biomarker of M2cMΦ) were not detected in their culture fluids. N-MLNMΦ produced a minimal amount of these cytokines (Fig. 4B). Furthermore, LIGHT mRNA (a biomarker of M2bMΦ) was expressed by I-MLNMΦ (Fig. 4C), whereas N-MLNMΦ did not express LIGHT or mannose receptor mRNA (a biomarker of M2aMΦ and M2cMΦ). I-MLNMΦ possessed weak bactericidal activities, although N-MLNMΦ killed *E. faecalis* completely (Fig. 4D). Because MΦ with the ability to
chemokines, mannose receptor mRNAs was analyzed by RT-PCR. For the production of IL-10 was analyzed by flow cytometry, and the expression of LIGHT and gamma-rays (I-MLNM) isolated from normal mice (N-MLNN) were treated s.c. with various populations was examined. Thus, mice, 14 d after whole-body irradiation, peaked 14–28 d post irradiation, and significantly increased depending on the irradiated doses of gamma-rays. This indicates that gamma-irradiation causes the appearance of M2bM from irradiated mice treated with 5 Gy gamma-rays was studied. As shown in Fig. 5B, the numbers of IL-12–producing cells were induced by the Ag in Transwell cultures from irradiated mice treated with CCL1 antisense ODN are not M2bM. In the next experiments, we tried to induce M1M by the bacterial Ag in irradiated mice treated with CCL1 antisense ODN in the culture fluids of MLNM from irradiated mice treated with 6 μg/mouse or more of CCL1 antisense ODN. In the next experiments, MLNM 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, M6 from irradiated mice treated with CCL1 antisense ODN (6 μg/mouse) were shown to be IL-10−LIGHT−M6. M6 from the same mice treated with scrambled ODN were identified as IL-10+LIGHT+M6 (Fig. 6B, 6C). N-MLNM were shown to be IL-10−LIGHT−M6 (Figs. 4C, 6B). These results indicate that MLNM from irradiated mice treated with CCL1 antisense ODN are not M2bM. In the next experiments, we tried to induce M1M 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-MLNM and MLNM from irradiated mice that were previously treated with CCL1 antisense ODN. However, IL-12–producing M6 were not demonstrated in cultures of MLNM from irradiated mice that were treated with scrambled ODN (Fig. 7). These results indicate that M1M 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 treated with scrambled ODN (5). Resident Mφ are shown to be a major effector cell against 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, Mφ were shown to be a major effector cell against bacterial translocation and subsequent sepsis.

Discussion

Neutrophils and lymphocytes are exquisitely sensitive to radiation and undergo apoptotic cell death (1, 2). In contrast, Mφ 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, Mφ were shown to be a major effector cell against bacterial translocation and subsequent sepsis. Mφ located in the subepithelial lamina propria and MLNs are the first cells that fight against translocated E. faecalis (5). Resident Mφ are isolated from lamina propria and MLNs of normal mice (25). Resident Mφ are immunologically quiescent with low oxygen consumption and low levels of MHC class II gene expression. In the event of infection, resident Mφ convert to M1Mφ through the engagement of TLRs or IFN receptors (26, 27). M1Mφ are actual effector cells in host antibacterial innate immunities (28, 29). These Mφ 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 M1ΔM were not demonstrated in MLNs of gamma-irradiated mice orally infected with E. faecalis. We have previously demonstrated that resident MΔ do not convert to M1ΔM in circumstances in which M2ΔM predominate (32, 33). M2ΔM have been described to be implicated in the negative regulation of M1ΔM (7). Recently, three different subtypes of M2ΔM (M2aΔM, M2bΔM, and M2cΔM) were described based on their gene expression and chemokine profiles (9, 13). In this study, MΔΔM in MLNs of mice 10–35 d after 5 Gy gamma-irradiation were identified as M2bΔM, because these MΔM produced CCL1 and IL-10, and expressed LIGHT mRNA (Fig. 4). Therefore, we tried to deplete M2bΔM 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, M1ΔM 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, M2bΔM 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 M2bΔM. In the absence of CCL1, M2bΔM have been regressed to resident MΔΔM (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 M2bΔM 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, M2bΔM were persistently demonstrated in MLNs of mice 10–35 d after irradiation. However, MΔΔM with the ability to produce IL-10, CCL17, and CXCL13 (a mixture of M2aΔM and M2cΔM) disappeared in MLNs of mice until 7 d of whole-body gamma-irradiation (34). In these experiments, I-MLNΔM were cultured for 48 h. We have performed the same cultivation in M collaboration (Invitrogen) or RPMI 1640 medium supplemented with heat-inactivated FBS (10%). In the results, CCL1 was equally produced in two different cultures of MLNΔM. This indicates that, for the detection of M2bΔM, artificial factors acquired during cultivation procedures are minimal. Also, we have detected M2bΔM-specific biomarkers (LIGHT mRNA expression; Fig. 4C) in I-MLNΔM by RT-PCR. Because M2bΔM biomarkers were displayed by noncultured I-MLNΔM, the results indicate that M2bΔM polarization is not influenced by the 48-h culture period.

The mechanism involved in M2bΔM generation in irradiated mice remains unclear. Recently, phagocytosis-associated differentiation of resident MΔΔM to regulatory/M2ΔM has been demonstrated (10). In this case, MΔΔM-pheragocytosed apoptotic inflammatory PMN expressed LIGHT/SHPK1 and produced IL-10. These results suggest a possibility that M2bΔM are generated from resident MΔM through their phagocytosis of radiation-induced damaged PMN. In our recent studies, XBPs-1, a biomarker of endoplasmic reticulum stress, was shown to be expressed by MLNΔM 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 XBPs-1 MΔM are characterized as CCL2-producing cells (40) and CCL2 is known as a M2ΔM inducer (8), it is also possible that endoplasmic reticulum stress is involved in M2bΔM generation in irradiated mice. To explore these questions, more experiments are required. In this study, we indirectly showed that M2bΔM influenced by CCL1 antisense ODN are resident MΔM, because these MΔM have been converted to M1ΔM after stimulation with bacterial Ag, and M2bΔM never convert to M1ΔM under the same stimulation. It is very difficult to directly measure how many MΔM are resident MΔM in the preparations of M2bΔM treated with CCL1 antisense ODN, because resident MΔM 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 M2ΔM (M2aΔM, M2bΔM, and M2cΔM) for the discrimination of each subtype of M2ΔM. However, we did not demonstrate any significant differences in the TLR expression in these MΔM 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 (M1ΔM) on the host antibacterial resistance was not generated in the translocation site (MLNs). The majority of MΔM in MLNs of irradiated mice were identified as M2bΔM (IL-10+IL-12−CCL1+LIGHT−MΔM) and shown to be inhibitory on the enterococcal Ag-induced conversion of MΔM from resident MΔM to M1ΔM. After depletion of M2bΔM by treatment with CCL1 antisense ODN, M1ΔM 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 M2bΔM 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**


apoptotic neutrophils induces an IL-12(low)IL-10(high) regulatory phenotype in macrophages. *J. Immunol.* 185: 2044–2050.


