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Role of Polymorphonuclear Neutrophils on Infectious Complications Stemming from *Enterococcus faecalis* Oral Infection in Thermally Injured Mice[1]

Yasuhiro Tsuda,* Kenji Shigematsu,* Makiko Kobayashi,*† David N. Herndon,† and Fujio Suzuki[2]*†

Thermally injured mice are susceptible to *Enterococcus faecalis* translocation. In this study, the role of polymorphonuclear neutrophils (PMN) on the development of sepsis stemming from *E. faecalis* translocation was studied in SCID-beige (SCIDbg) mice depleted of PMN (SCIDbgN mice) or macrophages (Mϕ) and PMN (SCIDbgMN mice). Sepsis was not developed in SCIDbgN mice orally infected with *E. faecalis*, while the orally infected pathogen spread systemically in the same mice inoculated with PMN from thermally injured mice (TI-PMN). SCIDbgMN mice were shown to be greatly susceptible to sepsis caused by *E. faecalis* translocation, while orally infected *E. faecalis* did not spread into sepsis in the same mice that were previously inoculated with Mϕ from unburned SCIDbg mice (resident Mϕ). In contrast, orally infected *E. faecalis* spread systemically in SCIDbgMN mice inoculated with resident Mϕ and TI-PMN, while all SCIDbgMN mice inoculated in combination with resident Mϕ and PMN from unburned SCIDbg mice survived after the infection. After cultivation with TI-PMN in a dual-chamber transwell, resident Mϕ converted to alternatively activated Mϕ, which are inhibitory on the generation of classically activated Mϕ (typical effector cells in host antibacterial innate immunities). TI-PMN were characterized as immunosuppressive PMN (PMN-II) with abilities to produce cc-chemokine ligand-2 and IL-10. These results indicate that PMN-II appearing in response to burn injury impair host antibacterial resistance against sepsis stemming from *E. faecalis* translocation through the conversion of resident Mϕ to alternatively activated Mϕ. *The Journal of Immunology,* 2008, 180: 4133–4138.

Infection is a leading cause of morbidity and mortality in thermally injured patients (1, 2). These patients are particularly susceptible to infection with bacterial pathogens, and local infections easily escalate into infectious complications (3–5). The translocation of bacteria beyond the intestinal lumen is one of the mechanisms that promotes infectious complications (6, 7). In fact, bacterial translocation has been demonstrated in 16 to 40% of patients with multiple organ dysfunction syndrome and intestinal ischemia (8). The manifestation of multiple organ dysfunction syndrome and intestinal ischemia is frequently demonstrated in thermally injured patients (9, 10). Bacterial translocation is postulated to occur in several clinical conditions, such as bacterial overgrowth in small bowel, damage to the gut barrier, and states of systemic immunosuppression (6, 7). Enterococci were viewed as a relatively avirulent endogenous flora with little potential for human infection when compared with other Gram-positive bacteria. However, *Enterococcus faecalis* has emerged as a clinically important pathogen that causes septic complications in immunocompromised hosts (11, 12). Therefore, treatment targeting the host's antibacterial immune responses seems to be critical to successfully regulate infectious complications stemming from *E. faecalis* translocation.

The innate immune system is the first line of host defense against bacterial translocation (13, 14). The important roles of polymorphonuclear neutrophils (PMN)[3] and macrophages (Mϕ) in antibacterial innate immunity have been proven in many papers (15–18). Classically activated Mϕ (M1Mϕ), characterized as major killer cells for pathogens (19), are the main effector cells in innate immunities. M1Mϕ are generated from resident Mϕ following stimulation with invasive pathogens via pattern recognition receptors (14, 18, 19). However, M1Mϕ have never been generated in burn mice whose alternatively activated Mϕ (M2Mϕ) predominated, even when they are exposed to the pathogens or stimulated with M1Mϕ inducers (16, 20). M2Mϕ lack the ability to kill bacteria, and soluble factors released from M2Mϕ inhibit the conversion of resident Mϕ to M1Mϕ following stimulation with bacteria (21). In the recent studies, SCID-beige (SCIDbg) mice depleted of PMN (SCIDbgN) were shown to be resistant to sepsis stemming from *E. faecalis* translocation, while all SCIDbgN mice depleted of Mϕ (SCIDbgMN) died after the same oral infection with *E. faecalis*. However, SCIDbgN mice became highly susceptible to infectious complications stemming from *E. faecalis* translocation when they were inoculated with PMN from thermally injured SCIDbg mice (TI-PMN). Both SCIDbgN mice and those inoculated with PMN from unburned SCIDbg mice were resistant to the same infection. SCIDbgN mice were SCID-beige mice depleted of PMN.

*Department of Internal Medicine, University of Texas Medical Branch, Galveston, TX 77555; and †Shriners Hospitals for Children, Galveston, TX 77550

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[2] Address correspondence and reprint requests to Dr. Fujio Suzuki, Department of Internal Medicine, University of Texas Medical Branch, 301 University Boulevard, Galveston, TX 77555. E-mail address: fsuzuki@utmb.edu

[3] Abbreviations used in this paper: PMN, polymorphonuclear neutrophil; Mϕ, macrophage; M1Mϕ, classically activated Mϕ; SCIDbg, SCID-beige mice; TI-PMN, PMN from thermally injured SCIDbg mice; M2Mϕ, alternatively activated Mϕ; SCIDbgN mice, SCIDbg mice depleted of PMN; SCIDbgMN mice, SCIDbgN mice depleted of Mϕ; iNOS, inducible NO synthase; SAC, *Staphylococcus aureus* Cowan I.

Among various immunocompetent cells, only intact Mϕ are present in these mice. SCIDbgMN mice were SCIDbgN mice depleted of Mϕ. Therefore, the role of TI-PMN on the antibacterial host defense against *E. faecalis* translocation was examined by focusing on the Mϕ activation.

Materials and Methods

Animals

Seven- to 11-wk-old, pathogen-free, male SCIDbg mice purchased from Taconic Farms were used in this study. These mice have been defined as immunodeficient mice without functional T, B, and NK cells. In some experiments, SCIDbg mice depleted of PMN (SCIDbgN mice) or PMN and Mϕ (SCIDbgMN mice) were used. SCIDbgN mice were SCIDbg mice treated with anti-Ly6G mAb (100 μg/mouse, i.p., every day for 5 days) plus whole body X-irradiation (4 Gy, 1 day before infection). Functional PMN were not recovered from SCIDbgN mice 1 to 7 days after the X-irradiation, even after they were exposed to pathogens (16). When bone marrow cells or peripheral blood cells taken from these mice were tested morphologically for residual PMN after Wright-Giemsa and alkaline phosphatase stainings, no PMN were detected until 7 days after the combination treatment. In addition, myelocytes (PMN precursor cells) were not demonstrated in the bone marrow of SCIDbgN mice until 7 days after the PMN depletion. SCIDbgMN mice were SCIDbgN mice treated with carrageenan (0.4 mg/mouse, i.v., once daily for 5 days, starting 5 days before X-irradiation) and trypan blue (1 mg/mouse, i.p., 1 day before, and 1 and 3 days after X-irradiation) (16). Three to 7 days after the final treatment, no functional Mϕ were found in the reticuloendothelial systems of SCIDbgMN mice. All experiments with animals were performed according to protocols approved by The Institutional Animal Care and Use Committee of University of Texas Medical Branch at Galveston.

Bacteria, reagents, and media

E. faecalis (49757 strain) was purchased from The American Type Culture Collection. *E. faecalis* was grown in brain heart infusion broth for 18 h at 37°C in aerobic conditions. Murine rIL-12, rIL-10, and rCCL2 were purchased from BD Biosciences, and murine rCCL3, rCCL5, and rCCL17 were obtained from R&D Systems. mAbs directed against CCL2, CCL5, CCL17, and Ly6G (Gr-1) were obtained from eBioscience. For cultivation of various cell preparations, RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) was used.

Burn injury

Thermally injured mice were created according to our previously reported protocol (17, 21). Thus, mice were anesthetized with pentobarbital (40 mg/kg, i.p.) and electric clippers were used to shave the hair on the back of each mouse from groin to axilla. The mice were then exposed to a gas flame for 9 s after pressing the window of the custom-made insulated mold (with a 4 $\times$ 5-cm window) firmly against the shaved back. A Bunsen burner equipped with a flame-dispersing cap was used as the source of the gas flame. This procedure consistently produced a third degree burn on ~25% of total body surface area for a 26-g mouse. Immediately after thermal injury, physiologic saline (1 ml per mouse, i.p.) was administered for fluid resuscitation. All mice remained alive >10 days after burn injury. Control mice had their back hair shaved but were not exposed to the gas flame. They also received physiologic saline (1 ml per mouse, i.p.). Buprenorphine (2 mg/kg) was given s.c. every 12 h during the postburn period. Control animals also received identical regimens of analgesics (buprenorphine) throughout the study period.

E. faecalis *oral infection*

Mice decontaminated by the oral administration of the antibiotic mixture were used in these experiments. For decontamination, mice were treated for 4 days with drinking water containing 4 mg/ml penicillin, streptomycin, and bacitracin (22). One day after decontamination, mice were exposed to flame burns, and then infected orally with 1×10^5 CFU/mouse of *E. faecalis* 4 h after burn injury. The development of infectious complications was evaluated by 1) growth of the bacteria in mesenteric lymph nodes and liver, and 2) the mortality rates of the test groups in comparison with the controls. To measure the quantity of bacteria, organ specimens (mesenteric lymph nodes 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 mesenteric lymph nodes 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 a day for 5 days after infection.

Preparation of PMN and Mϕ

As previously described (16), PMN were isolated from whole peripheral blood of normal mice and thermally injured mice (mice 18 h after burn injury) using Ficoll-Hypaque and dextran sedimentations. In brief, peripheral blood was drawn from the heart of mice with a heparinized syringe. The peripheral blood was centrifuged with Ficoll-Hypaque, and precipitates were obtained as a PMN rich fraction. Then, precipitates were suspended in 1% dextran (T-500, Pharmacia) and kept for 1 h at room temperature to allow the sedimentation of erythrocytes. The resulting PMN fraction was further treated with a mixture of biotin-conjugated anti-CD3 (T cells), anti-F4/80 (monocytes/Mϕ), and anti-CD19 (B cells) mAbs for 30 min at 4°C. Then, these cells were suspended in MagCellect buffer (R&D Systems) and incubated with magnetic beads (Dynal) coated with streptavidin. The purity of PMN, isolated in this procedure, was >98% when measured morphologically (Wright-Giemsa/alkaline phosphatase stainings).

Mϕ were prepared from the peritoneal exudates and mesenteric lymph nodes of normal mice. Peritoneal exudates were obtained by injecting 4 ml of PBS and harvesting the fluids (16, 21), and single cell suspensions of mesenteric lymph nodes were obtained. These cells were adjusted to 5×10^6 cells/ml in MagCellect buffer (R&D Systems), and then Mϕ were isolated from these 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.

Determination of M2Mϕ

Mϕ were considered to be M2Mϕ when they produced CCL17 (but not CCL5) and expressed mannose receptor (but not inducible NO synthase (iNOS)) mRNA (18, 21). For the chemokine production, Mϕ (1×10^6 cells/ml) were cultured for 48 h without any stimulation. Culture fluids harvested were assayed for chemokines using ELISA. mRNAs for mannose receptor and iNOS were analyzed by RT-PCR. Total RNA was extracted from Mϕ (1×10^6 cells) using RNA isolator, following the manufacturer's recommendations. Within each experiment, each sample was normalized by the amount of isolated RNA. Then, this RNA was turned back into cDNA through the reverse transcription of mRNA. PCR was conducted using synthesized oligonucleotide primers from Sigma-Aldrich: mannose receptor, 5′-CCATCGAGACTGCTGCTGAG-3′ (forward) and 5′-AGCCCTTGGGTTGAGGATCC-3′ (reverse); iNOS, 5′-CCCTCCAG TGTCTGGGAGCA-3′ (forward) and 5′-TGCTTGTCACCACCAGCAGT-3′ (reverse) (21). Using a thermal cycler (GeneAmp PCR Sysyem 9600), 35 cycles of PCR were performed at 94°C for 15 s, 60°C for 15 s and 72°C for 20 s. The predicted products were run on 2% agarose gels containing ethidium bromide.

Characterization of TI-PMN

TI-PMN were tested for their abilities 1) to produce CCL2, CCL3, IL-10, and IL-12, and 2) to induce M1Mϕ or M2Mϕ from resident Mϕ. To determine the cytokine/chemokine-producing profile, PMN preparations with a cell density of 1×10^6 cells/ml were cultured with 0.0075% *Staphylococcus aureus* Ag (SAC; Calbiochem) for 18 h. The harvested culture fluids were assayed for CCL2, CCL3, IL-12, and IL-10 by ELISA.

The effect of TI-PMN on the Mϕ activation was tested in dual-chamber transwells (0.4 μm micropores; Corning) (21). In brief, the resident Mϕ suspension (1×10^6 cells/ml, lower chamber) was cultured with the PMN suspension (1×10^5 cells/ml, upper chamber) in a dual-chamber transwell. Eighteen hours after cultivation, the upper chamber was removed and Mϕ in the lower chamber were examined for M2Mϕ properties, as described above.

Statistical analysis

The results obtained were analyzed statistically using an ANOVA test. Survival curves were analyzed using the Kaplan-Meier test. All calculations were performed on a computer using the program Statview 4.5 from Brain Power. A value of $p < 0.05$ was considered significant.

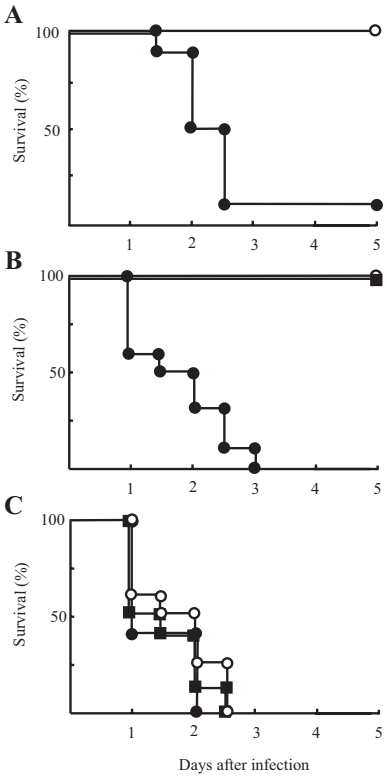

FIGURE 1. PMN from thermally injured SCIDbg mice (TI-PMN) decreased the resistance of SCIDbgN mice orally infected with *E. faecalis*. *A*, SCIDbg mice 18 h after burn injury (10 mice, ●) were infected orally with *E. faecalis* (10^5 CFU/mouse). Unburned SCIDbg mice (10 mice, ○) infected with the same pathogen served as a control. The difference of survival rates between thermally injured SCIDbg mice and unburned SCIDbg mice was $p < 0.001$ according to Kaplan-Meier log rank test. *B*, SCIDbgN mice were inoculated i.v. with 1×10^5 cells/mouse of normal PMN (12 mice, ○) or TI-PMN (12 mice, ●). Two hours after PMN inoculation, these mice were infected orally with *E. faecalis* (10^5 CFU/mouse). As a basic control, SCIDbgN mice were infected with the pathogen in the same way (12 mice, ■). The difference of survival rates between SCIDbgN mice inoculated with TI-PMN and those inoculated with normal PMN was $p < 0.0001$ according to Kaplan-Meier log rank test. *C*, SCIDbgMN mice were inoculated i.v. with 1×10^5 cells/mouse of normal PMN (12 mice, ○) or TI-PMN (12 mice, ●). Two hours after inoculation, these mice were infected orally with *E. faecalis* (10^5 CFU/mouse). As a basic control, SCIDbgMN mice were infected with the pathogen in the same fashion (12 mice, ■).

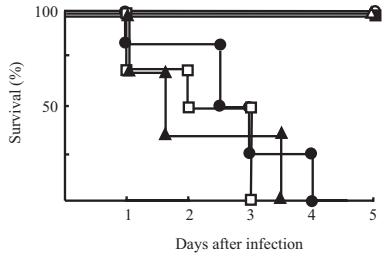

FIGURE 2. Mϕ cultured with TI-PMN failed to protect SCIDbgMN mice orally infected with *E. faecalis*. Peritoneal Mϕ or mesenteric lymph node Mϕ (5×10^6 cells/ml, lower chamber) were cultured with TI-PMN (5×10^5 cells/ml, upper chamber) for 18 h in dual-chamber transwells. Cells harvested from the lower chamber were adoptively transferred i.v. to SCIDbgMN at a number of 1×10^6 cells/mouse (peritoneal Mϕ, 12 mice, ●; mesenteric lymph node Mϕ, 12 mice, ▲). As a control, the same number of Mϕ that were cultured with normal PMN in dual chambers was transferred to SCIDbgMN mice (peritoneal Mϕ, 12 mice, ○; mesenteric lymph node Mϕ, 12 mice, △). Two hours after inoculation, these mice were infected orally with *E. faecalis* (10^5 CFU/mouse). SCIDbgMN mice (10 mice, □) and SCIDbgN mice (10 mice, ■) infected with *E. faecalis* in the same fashion served as additional controls. The difference of survival rates between SCIDbgMN mice inoculated with Mϕ that were cultured with TI-PMN and normal PMN was $p < 0.0001$ according to Kaplan-Meier log rank test.

Results

Susceptibility of TI-PMN inoculated SCIDbgN mice to E. faecalis *oral infection*

Unburned SCIDbg mice and SCIDbg mice 18 h after burn injury (thermally injured SCIDbg mice) were infected orally with 10^5 CFU/mouse of *E. faecalis*. In the results, all unburned SCIDbg mice orally infected with *E. faecalis* survived, while 90% of the thermally injured SCIDbg mice died after the same infection (Fig. 1*A*). SCIDbgN mice (Mϕ function is intact) were shown to be resistant against infections, because all of these mice survived after orally infected with 10^5 CFU/mouse of the pathogen. In contrast, the antibacterial resistance of SCIDbgN mice decreased to the level observed in thermally injured SCIDbg mice when they were inoculated with TI-PMN (Fig. 1*B*). Because all of the SCIDbgN mice orally infected with *E. faecalis* survived after inoculation with PMN from unburned SCIDbg mice (normal PMN), these results indicate that TI-PMN suppress the host antibacterial resistance against *E. faecalis* infection in SCIDbgN mice. However, the host resistance of SCIDbgN mice against *E. faecalis* oral infection was not influenced by normal PMN. In the next series of experiments, the effect of TI-PMN and normal PMN against *E. faecalis* infection in SCIDbgMN mice (SCIDbg mice depleted of Mϕ and PMN) was examined. In the results, SCIDbgMN mice were not resistant to *E. faecalis* infection after inoculation with TI-PMN or normal PMN (Fig. 1*C*). These results indicate that TI-PMN decrease the host resistance against oral infection with *E. faecalis* through the modulation of Mϕ functions intactly remaining in SCIDbgN mice.

Mϕ cultured with TI-PMN failed to protect SCIDbgMN mice orally infected with E. faecalis

To determine the effect of TI-PMN or normal PMN on the antibacterial functions of Mϕ, peritoneal Mϕ, or mesenteric lymph node Mϕ from normal mice were transwell cultured with one of the each PMN preparation (1×10^6 cells/mouse) and adoptively transferred to SCIDbgMN mice. Then, these mice were infected orally with *E. faecalis*. In the results, all of the SCIDbgMN mice treated with media (a control group) died within 3 days of infection; however, all of the SCIDbgMN mice survived after inoculation with peritoneal Mϕ previously cultured with normal PMN in dual chamber transwells. All of the SCIDbgN mice, not additionally inoculated with Mϕ preparations, survived. In contrast, all of the SCIDbgMN mice inoculated with peritoneal Mϕ previously transwell cultured with TI-PMN died within 4 days of *E. faecalis* oral infection (Fig. 2). Similar results were obtained when mesenteric lymph node Mϕ were transwell cultured with TI-PMN. In addition, numbers of bacteria in mesenteric lymph nodes and liver taken from the above groups of mice 48 h after *E. faecalis* infection were determined by colony forming assay. Bacteria were isolated from mesenteric lymph nodes and liver of SCIDbgMN mice inoculated with peritoneal Mϕ previously transwell cultured with TI-PMN (mesenteric lymph nodes, 1×10^4 CFU/g; liver, 4×10^2 CFU/g). However, none of the bacteria were detected in the same organs of SCIDbgMN mice inoculated with Mϕ previously

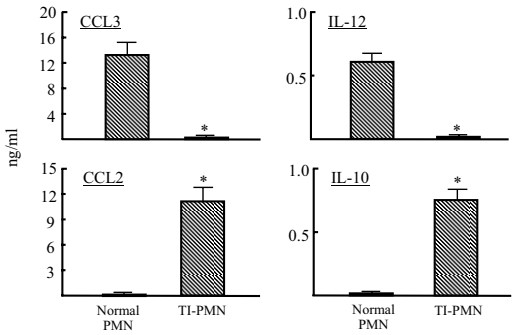

FIGURE 3. Cytokine and chemokine-producing properties of TI-PMN. TI-PMN (1×10^6 cells/ml) were cultured with 0.0075% SAC for 18 h to determine their cytokine/chemokine-producing profiles. As a control, normal PMN were cultured under the same conditions. The amounts of CCL2, CCL3, IL-10, and IL-12 in their culture fluids were measured by ELISA.

transwell cultured with normal PMN. These results indicate that Mϕ cultured with TI-PMN have no antibacterial activities against orally infected *E. faecalis*.

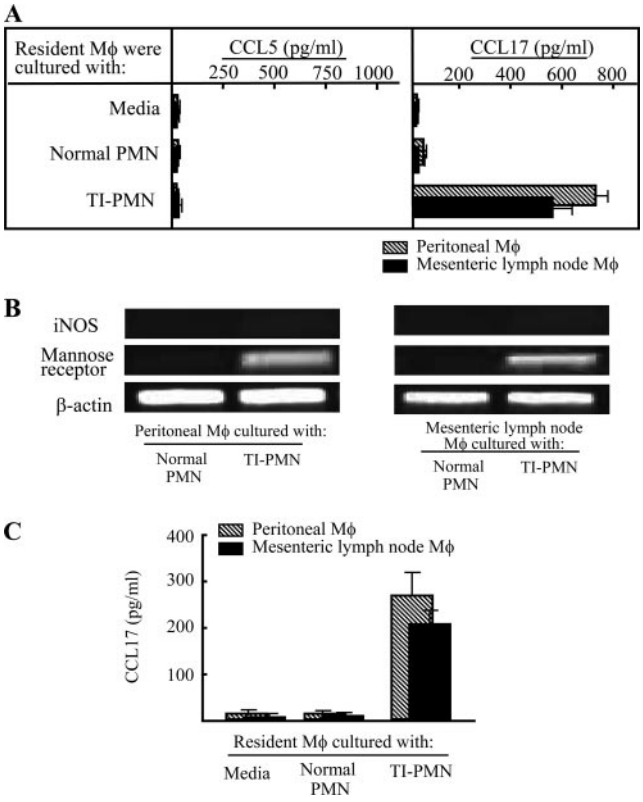

FIGURE 4. Properties of resident Mϕ after stimulation with TI-PMN. *A*, Peritoneal Mϕ or mesenteric lymph node Mϕ (1×10^6 cells/ml, lower chamber) were cultured with TI-PMN (1×10^5 cells/ml, upper chamber) for 18 h in double chamber transwells supplemented with 0.0075% SAC. As a control, Mϕ were transwell cultured with normal PMN in the same fashion. After removal of the upper chamber, Mϕ in the lower chamber were cultured for an additional 48 h, and the amounts of CCL5 (a biomarker for M1Mϕ) and CCL17 (a biomarker for M2Mϕ) in their culture fluids were measured using ELISA. *B*, The expression of iNOS and mannose receptor mRNAs by TI-PMN-stimulated Mϕ were analyzed using RT-PCR. *C*, Peritoneal Mϕ or mesenteric lymph node Mϕ were cultured with media supplemented with the culture fluids (15%, v/v) of TI-PMN (2×10^6 cells/ml, stimulated with SAC for 18 h). As a control, Mϕ were cultured with the culture fluids of normal PMN in the same fashion. Cells harvested 18 h after cultivation were cultured for an additional 48 h without any stimulation. The culture fluids obtained were assayed for CCL17.

TI-PMN were identified as PMN-II, and resident Mϕ influenced by TI-PMN converted to M2Mϕ

TI-PMN were tested for their cytokine/chemokine-producing profiles and surface Ag expressions. As shown in Fig. 3, TI-PMN produced CCL2 and IL-10 (biomarkers for PMN-II) into their culture fluids. However, IL-12 and CCL3 (biomarkers for PMN-I) were not produced by these PMN. Normal PMN did not produce CCL2 and IL-10. In the next experiments, the properties of Mϕ stimulated with TI-PMN were examined. Peritoneal Mϕ or mesenteric lymph node Mϕ from normal mice (lower chamber) were cultured with TI-PMN (upper chamber) for 18 h in a dual-chamber transwell. After removing the upper chamber, Mϕ in the lower chamber were examined for their abilities to produce CCL5 (a biomarker for M1Mϕ) and CCL17 (a biomarker for M2Mϕ). Peritoneal Mϕ transwell cultured with TI-PMN produced CCL17. However, CCL5 was not produced by these Mϕ. Peritoneal Mϕ transwell cultured with normal PMN produced neither CCL5 nor CCL17 (Fig. 4*A*). In addition, peritoneal Mϕ transwell cultured with TI-PMN expressed mannose receptor mRNA and did not express iNOS mRNA, while these Mϕ transwell cultured with normal PMN did not express either mRNA (Fig. 4*B*). Similar results were obtained when mesenteric lymph node Mϕ were transwell cultured with TI-PMN (Fig. 4, *A* and *B*). Furthermore, resident Mϕ were cultured with complete medium supplemented with the culture fluid (15%, v/v) of TI-PMN (2×10^6 cells/ml, stimulated with SAC for 18 h), and the generation of M2Mϕ was examined. M2Mϕ were generated from resident Mϕ cultures supplemented with the culture fluid of TI-PMN, while M2Mϕ were not generated from resident Mϕ stimulated with the culture fluids of normal PMN (Fig. 4*C*). These results indicate that soluble factors released from TI-PMN stimulate Mϕ conversion from resident Mϕ to M2Mϕ.

Discussion

Mϕ located in the subepithelial lamina propria and mesenteric lymph nodes are the first cells that fight translocated bacteria. Resident Mϕ (Mϕ from unstimulated healthy individuals) 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 Toll-like receptors (23) or IFN receptors (24). M1Mϕ are actual effector cells in host antibacterial innate immunities (25, 26). These Mϕ exhibit 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, and CCL5), and antimicrobial peptides (21, 27, 28). In contrast, M2Mϕ have been described to be activated by an alternative pathway involving Th2 cytokines (27). These Mϕ have been implicated in the negative regulation of both M1Mϕ and Th1 cell generation (27). M2Mϕ preferentially express mannose receptor, β-glucan receptors, and scavenger receptors, and produce arginase, IL-1 receptor antagonist, IL-10, and CCL17 (27). Recently, three different subtypes of M2Mϕ (M2aMϕ, M2bMϕ, and M2cMϕ) were described (18). These subsets can be separated by their gene expression and chemokine profiles (18, 29–31). Thus, CCL17-producing Mϕ with FIZZ1 gene are identified as M2aMϕ, CCL1-producing Mϕ with SPHK1 gene are classified as M2bMϕ, and CXCL13-producing Mϕ with FIZZ1 gene are recognized as M2cMϕ (18, 31). All of the M2Mϕ subtypes express the IL-10 gene (18). Unlike M2aMϕ and M2cMϕ, M2bMϕ produce TNF-α, IL-1, and IL-6 (18, 31). Because Th2 cytokines are able to suppress the transcriptional activation of IFN-γ- and LPS-responsive

genes in Mϕ (32), resident Mϕ cannot convert to M1Mϕ in circumstances where M2Mϕ predominate.

In the present study, we examined the role of TI-PMN (PMN from thermally injured SCIDbg mice) on the development of sepsis stemming from burn-associated *E. faecalis* translocation, using SCIDbgN mice and SCIDbgMN mice. SCIDbgN mice were SCIDbg mice depleted of PMN, and SCIDbgMN mice were SCIDbgN mice depleted of Mϕ. SCIDbgN mice were shown to be resistant against oral *E. faecalis* infection, while SCIDbgMN mice were very susceptible to this infection (Fig. 1*B*). Also, M1Mϕ were isolated from SCIDbgN mice after oral infection with *E. faecalis* (17). Because functional T, B, and NK cells and PMN are not present in SCIDbgN mice, these results indicate that M1Mϕ are key effector cells for host defense against *E. faecalis* translocation (Fig. 1, *B* and *C*). In contrast, SCIDbgN mice were shown to be susceptible to *E. faecalis* infection after inoculation with TI-PMN (Fig. 1*B*). In a dual-chamber transwell, TI-PMN (upper chamber) stimulated Mϕ conversion from resident Mϕ (lower chamber) to M2Mϕ (Fig. 4). Also, M2Mϕ were isolated from SCIDbgN mice inoculated with TI-PMN (data not shown). M2Mϕ have been previously characterized as cells that are inhibitory on the generation of M1Mϕ (21). These results indicated that TI-PMN were cells responsible for the impaired resistance of thermally injured mice to *E. faecalis* oral infection by converting resident Mϕ to M2Mϕ. Subsequently, TI-PMN were characterized as PMN-II, because they produced IL-10 and CCL2 (Fig. 3). PMN-II have been described as IL-10 and CCL2-producing Gr-1$^+$CD11b$^+$CD11c$^-$F4/80$^-$ cells with the ability to inhibit the generation of M1Mϕ (16). Also, PMN-II express TLR2, TLR4, TLR7, and TLR9 mRNAs (16).

Mϕ and PMN have been shown to accumulate in intestinal lymphoid tissues, such as the lamina propria and mesenteric lymph nodes, following gastrointestinal infections (33). We recently examined properties of F4/80$^+$ Mϕ from lamina propria and mesenteric lymph nodes of mice 1 to 3 days after burn injury. These Mϕ produced CCL17 and expressed mannose receptor mRNA, while CCL17 production and mannose receptor expression were not shown by Mϕ isolated from unburned SCIDbg mice. These results indicate that Mϕ in lamina propria and mesenteric lymph nodes of thermally injured SCIDbg mice are M2Mϕ, while those from unburned SCIDbg mice are not. As mentioned above, M2Mϕ were generated from resident Mϕ in cultures with TI-PMN in dual-chamber transwells (Fig. 4, *A* and *B*). This indicates that the cell-to-cell contact between resident Mϕ and TI-PMN is not necessary when M2Mϕ were generated from resident Mϕ under the TI-PMN stimulation. In fact, M2Mϕ appeared in the resident Mϕ cultures supplemented with TI-PMN culture fluids (Fig. 4*C*). Because IL-10 and CCL2 were detected in the culture fluids of TI-PMN (Fig. 3), the role of these soluble factors on the TI-PMN-associated M2Mϕ generation was examined. In the results, M2Mϕ were not generated from resident Mϕ stimulated with the TI-PMN culture fluids that were previously treated with a mixture of anti-IL-10 and anti-CCL2 mAbs (data not shown). This indicates that IL-10 and CCL2 released from TI-PMN act as stimulators of Mϕ conversion from resident Mϕ to M2Mϕ.

TLR reactivity of Mϕ in mice 4–10 days after thermal injury has been widely described in many papers (34, 35). In response to TLR stimulation, these Mϕ produce IL-1, TNF-α, and IL-6 as well as NO. In these papers, however, the activated Mϕ are not classified as M1Mϕ. Mϕ with abilities to produce IL-12/CCL3, to kill bacteria/tumor cells, and to induce Th1 cells are specifically designated as M1Mϕ (31, 36). It has been reported that IL-1, TNF-α, IL-6, and NO are not only produced by M1Mϕ, but also produced by a subset of M2Mϕ (18). These facts indicate that NO- and proinflammatory cytokine-producing Mϕ detected in mice 4 to 10 days postburn injury may belong to M2bMϕ. In this study, M2Mϕ generated from resident Mϕ under the TI-PMN stimulation will be classified with the M2aMϕ subset, because CCL17 is detected in culture fluids of these M2Mϕ preparations. The incidence of infections remains high in patients 1 or more weeks after burn injury. In fact, Gram-negative and positive bacteria are frequently isolated from peripheral blood of patients 1 to 3 wk after burn injury (37–39), and a majority of these infections develop into sepsis. In our recent studies, M2aMϕ and M2cMϕ appeared in mice 1 to 3 days after burn injury, and then disappeared from the mice within 5 days of burn injury (Shigematsu, K., M. Kobayashi, D.N. Herndon and F. Suzuki, unpublished data). In contrast, M2bMϕ appeared in mice ~1 wk after burn injury. These facts suggest that M2bMϕ may play a role on the susceptibility of mice late after burn injury.

In our previous studies (40), PMN displaying PMN-II properties were found in the peripheral blood of burn patients (3rd degree, >40% total body surface area burns). When PMN from five healthy donors and eight burn patients were stimulated with SAC, seven of eight patient PMN produced CCL2 and IL-10 (CCL2, 639~54,782 pg/ml; IL-10, 183~13,541 pg/ml), while none of the healthy donor PMN produced these soluble factors (CCL2, < 30 pg/ml; IL-10, < 12 pg/ml). These results suggest that burn patients are carriers of PMN-II (or TI-PMN).

Apoptotic process of PMN from humans and rodents with severe burn injuries have been described to be inhibited (41, 42). It is possible that the prolongation of PMN in burned hosts may result in the long-term stimulation of Mϕ conversion from resident Mϕ to M2Mϕ. To determine whether PMN apoptosis contributed to the results shown in this study, SCIDbgN mice were inoculated i.v. with 1×10^6 cells/mouse of normal neutrophils (A) every 12 h or (B) once and exposed orally to 1 LD_{50} of *E. faecalis*. After the infection, 50% of group A and group B mice survived equally. Also, when SCIDbgN mice were inoculated i.v. with 1×10^6 cells/mouse of TI-PMN (C) every 12 h or (D) once, and infected with the same amount of *E. faecalis*, 100% of group C and group D mice died equally. Therefore, in our experimental model, the antibacterial resistance of mice is not influenced by apoptotic frequency of PMN.

The mechanism by which normal PMN differentiate into PMN-II (TI-PMN) remains unclear. However, prostaglandin E_2 and catecholamines have abilities to induce immature myeloid cells (Gr-1$^+$CD11b$^+$ cells) (43). The increased levels of stress hormones (corticosteroids, catecholamines) and prostaglandin E_2 have been demonstrated in the plasma of burn hosts (44–47). PMN-II and Gr-1$^+$CD11b$^+$ immature myeloid cells have been demonstrated to be very similar to each other. CCL2 and IL-10, effector soluble factors of TI-PMN, have been detected in the culture fluids of Gr-1$^+$CD11b$^+$ immature myeloid cells. These descriptions suggest that prostaglandin E_2 and stress hormones may be involved in the PMN conversion to PMN-II. Further studies are required to explore the role of prostaglandin E_2 and the burn-associated PMN conversion from normal PMN to PMN-I.

Disclosures

The authors have no financial conflict of interest.

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