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

Yasuhiro Tsuda,* Kenji Shigematsu,* Makiko Kobayashi,*† David N. Herndon,† and Fujio Suzuki2*†

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.

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The innate immune system is the first line of host defense against bacterial translocation (13, 14). The important roles of polymorphonuclear neutrophils (PMN) 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.

* 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.

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Among various immunocompetent cells, only intact Mφ are present in these mice. SCIDbgM mice were SCIDbgN mice depleted of Mφ. Therefore, the role of TI-PMN on the antibacterial host defense against \textit{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 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 staining, 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 X-irradiation and trypan blue (1 µg/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**

\textit{E. faecalis} (94757 strain) was purchased from The American Type Culture Collection. \textit{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 purified from the culture of normal 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.

**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 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 × 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 animals 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.

\textit{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 the gas flame, and then infected orally with 1 × 10³ CFU/mouse of \textit{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, liver, and spleen) were weighed and homogenized in 2 ml PBS using a Bruker 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 × 10⁵ 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 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 × 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 iNOS were analyzed by RT-PCR. Total RNA was extracted from Mφ (1 × 10⁶ 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 synthetic oligonucleotide primers from Sigma-Aldrich: mannose receptor, 5′-CCATCGAGACTGCTGCTGAG-3′ (forward) and 5′-AGCCCTTGGGTGGAGGATCC-3′ (reverse); iNOS, 5′-CCCTCCAG TGTCTGGGAGCA-3′ (forward) and 5′-TGGCTGGTACCAACGCAGCT-3′ (reverse) (21). Using a thermal cycler (GeneAmp PCR System 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 × 10⁶ 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 efficiency of the 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 × 10⁶ cells/ml, lower chamber) was cultured with the PMN suspension (1 × 10⁶ cells/ml, upper chamber) in a dual-chamber transwell. Eighteen hours after culture, 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, ■).

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. 1A). 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 SCIDbgMN mice decreased to the level observed in thermally injured SCIDbg mice when they were inoculated with TI-PMN (Fig. 1B). 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 SCIDbgMN mice. However, the host resistance of SCIDbgMN 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. 1C). 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 × 10^5 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 SCIDbgMN 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 × 10^6 CFU/g; liver, 4 × 10^5 CFU/g). However, none of the bacteria were detected in the same organs of SCIDbgMN mice inoculated with Mφ previously.
transwell cultured with normal PMN. These results indicate that MΦ cultured with TI-PMN have no antibacterial activities against orally infected *E. faecalis*.

**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. 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. 4A). 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. 4B). 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. 4C). These results indicate that soluble factors released from TI-PMN stimulate MΦ conversion from resident MΦ to M2MΦ.

**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 × 10^6 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.

**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 immunity (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. 1B). 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. 1B). 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 node, 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 lymphoid nodules 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. 4C). 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 × 10⁶ cells/mouse of normal neutrophils (A) every 12 h or (B) once and exposed orally to 1 LD₅₀ 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 × 10⁶ 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 E₂ and catecholamines have abilities to induce immature myeloid cells (Gr-1⁺CD11b⁺ cells) (43). The increased levels of stress hormones (corticotestosterone, catecholamines) and prostaglandin E₂ 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 E₂ and stress hormones may be involved in the PMN conversion to PMN-II. Further studies are required to explore the role of prostaglandin E₂ and the burn-associated PMN conversion from normal PMN to PMN-I.

Disclosures
The authors have no financial conflict of interest.

References


