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M2b Monocytes Predominated in Peripheral Blood of Severely Burned Patients

Makiko Kobayashi,†* Marc G. Jeschke,† Kenji Shigematsu,* Akira Asai,* Shohei Yoshida,* David N. Herndon,† and Fujio Suzuki*†

Severely burned patients were shown to be carriers of M2 monocytes, and all of the monocytes isolated from peripheral blood of severely burned patients (19 of 19 patients) were demonstrated as M2b monocytes (IL-12$^-$IL-10$^-$CCL17$^+$ monocytes). Low levels of M2a (IL-12$^+$IL-10$^+$CCL17$^+$ monocytes) and M2c monocytes (IL-12$^+$IL-10$^+$CXCL13$^+$ monocytes) were demonstrated in peripheral blood of severely burned patients (M2a, 2 of 19 patients; M2c, 5 of 19 patients). M2b, M2a, and M2c monocytes were not detected in peripheral blood of healthy donors. However, M2b monocytes appeared when healthy donor monocytes were cultured in media supplemented with burn patient serum (15%). CCL2 was detected in sera of all burn patients, and M2b monocytes were not generated from healthy donor monocytes cultured with media containing 15% burn patient sera that were previously treated with anti-CCL2 mAb. In addition, M2b monocytes were generated from healthy donor monocytes in cultures supplemented with rCCL2. These results indicate that M2b monocytes are predominant in peripheral blood of severely burned patients who are carriers of CCL2 that functions to stimulate monocyte conversion from resident monocytes to M2b monocytes. The Journal of Immunology, 2010, 185: 7174–7179.

A burn injury represents one of the most severe forms of trauma and affects over 2 million people in the United States per year (1). Severely burned patients are particularly susceptible to infections with various pathogens (2–4). Burn wound infections (local infection) in these patients easily escalate into sepsis (5). In our previous animal studies, as little as 50 CFU/mouse Pseudomonas aeruginosa infected beneath the burn wound was sufficient to kill severely burned mice, when $>10^7$ CFU/mouse of the intradermal pathogen was required to induce sepsis in normal mice (6). The difference in lethal doses of the pathogen (normal mice, $10^7$ CFU/mouse; burn mice, 50 CFU/mouse) is reflective of the difference in the ability of hosts to eradicate invasive pathogens. Among various immunologic abnormalities documented in severely burned patients (7–13), diminished innate immune responses contribute to the systemic dissemination of pathogens from the local infection site.

Classically activated macrophages (M1M$b$), IL-12–producing and IL-10–nonproducing macrophages, have been reported as a major effector cell in the first line of host antibacterial defense in innate immune responses (14, 15). The reason that M1M$b$ do not appear in severely burned mice is due to the appearance of alternatively activated macrophages (M2M$b$) (16, 17). M2M$b$ are IL-10–producing and IL-12–nonproducing macrophages with reduced capacity to kill bacterial pathogens (14, 15, 17). M2M$b$

Materials and Methods

Thermally injured patients

Nineteen children (15 male, 4 female; 4 mo to 17 y old) who were admitted to the Shriners Hospital for Children from February 2007 to April 2008 with burn injuries covering >30% of their total body surface area were enrolled in the study. The Institutional Review Board for human investigation at the University of Texas Medical Branch (Galveston, TX) approved all human experiments in the study. The patients were taken to the operating room within 24 h of their admission to the hospital, where complete excision of the burn was undertaken with autografting or allografting as clinically indicated. The administration of antibiotics was continued until the dressings were removed on postoperative day 4.

Reagents

Human rIL-10, rIL-12, rCCL2, rCCL1, rCCL17, and rCXCL13 were obtained from PeproTech (Rocky Hill, NJ). mAbs for human IL-10, IL-12, CCL2, CCL1, CCL17, and CXCL13 were obtained from R&D Systems (Minneapolis, MN) and BD Biosciences (San Jose, CA). Biotin-conjugated anti-CD3, anti-CD14, anti-CD19, and anti-CD56 mAbs were purchased from ebScience (San Diego, CA). Staphylococcus aureus Cowan I (SAC) strain was obtained from Calbiochem–Behring (San Diego, CA).
Preparation of monocytes and other cell populations

Whole blood was drawn into a vacutainer tube with sodium heparin. PBMCs were isolated from the heparinized whole blood by Ficoll-Hypaque density gradient centrifugation (20). To isolate monocytes, obtained mononuclear cells were adjusted to 5 × 10⁶ cells/ml in MagCellect buffer (R&D Systems) and incubated with magnetic beads coated with anti-CD14 mAb (30 min at 4˚C). CD14⁺ cells were magnetically harvested. The purity of monocytes isolated by this procedure was routinely >97%. T, B, and NK cells were isolated from the above PBMC preparation using magnetic beads coated with anti-CD3 mAb, anti-CD19 mAb, and anti-CD56 mAb, respectively. Polymorphonuclear neutrophils (PMNs) were isolated from the precipitates of the above Ficoll-Hypaque sedimentation, as previously described (21). The cells prepared above were cultured with RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics (culture media).

Production and assay of cytokines and chemokines

Monocytes obtained were adjusted to 1 × 10⁶ cells/ml in culture media and cultured for 48 h with or without stimulation. Culture fluids were harvested and assayed for IL-10, IL-12, CCL1, CCL17, and CXCL13 by ELISA, according to the manufacturer’s instructions. Also, PMNs obtained were adjusted to 1 × 10⁶ cells/ml in culture media, and stimulated with 0.0075% SAC for 18 h. Culture fluids harvested were assayed for CCL2 by ELISA. Serum specimens obtained from thermally injured patients and healthy donors were assayed for CCL2 by ELISA. The minimum detection limits for the above cytokines and chemokines in our assay system were 4–13 pg/ml.

Detection of arginase activity

Monocytes (1 × 10⁶ cells/ml) were lysed in 50 µl solution containing 0.1% Triton X-100, 0.01% antipain, 0.01% pepstatin, and 0.01% aprotinin. Then the amount of arginase in the cell lysates was determined, as previously described (22, 23).

Bactericidal activity

Monocytes obtained (1 × 10⁶ cells/ml) were suspended in antibiotic-free RPMI 1640 medium supplemented with 10% FBS, and 100 µl cell suspension (1 × 10⁵ monocytes/well) was seeded into 96-well round-bottomed microtiter plates. These cells were infected with 10⁷ CFU/ml P. aeruginosa 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, St. Louis, MO). Serial 10-fold dilutions of these fluids were plated on brain-heart infusion 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.

Statistical analyses

Comparisons between two groups were performed using an unpaired Student t test, whereas those between three groups were done by one-way ANOVA. Prism 4.0 software (GraphPad, San Diego, CA) was used for statistical analyses. The result was considered significant when p < 0.05.

Results

Severely burned patients are carriers of M2 monocytes

In the first series of experiments, severely burned patient monocytes were tested for their ability to produce IL-10 or IL-12, and to kill bacteria in vitro. Monocytes were isolated from peripheral blood of 16 severely burned patients (43–95% total body surface area burn; see Table I). These patients were randomly selected from inpatients of Shriners Burn Hospital (Galveston, TX). These monocyte preparations (1 × 10⁶ cells/ml) were tested individually for their ability to produce IL-12 in response to 0.0075% SAC. Also, these monocytes were cultured without any stimulation, and amounts of IL-10 produced into their culture fluids were measured. Five monocyte preparations similarly isolated from five healthy donors (see Table II) were used as controls. In previous papers (14, 15), IL-12-producing and IL-10–producing monocytes have been identified as M1Mφ, and IL-12–producing and IL-10–producing macrophages have been characterized as M2Mφ. In addition, it has been well documented that M1Mφ kill bacteria very efficiently, whereas such killing activities are not shown by M2Mφ (17). The average amount of cytokines detected in the culture fluids of both cultures is shown in Fig. 1A. IL-12 was not produced in cultures of unstimulated monocytes. After SAC stimulation, 0.4–1.2 ng/ml IL-12 was produced by healthy donor monocytes, whereas IL-12 was not produced by burn patient monocytes (Fig. 1A-1). In contrast, 1.8–2.6 ng/ml IL-10 was detected in the culture fluids of burn patient monocytes without any stimulation, whereas IL-10 was not produced by healthy donor monocytes in the same culture conditions (Fig. 1A-2). Both healthy donor monocytes and burn patient monocytes exhibited phagocytic activities (data not shown); however, burn patient monocytes did not kill bacteria, whereas healthy donor monocytes showed bactericidal activities (Fig. 1B). In addition, lysates of 1 × 10⁶ monocytes from burn patients contained 30–39 mU arginase, but cell lysates of healthy donor monocytes contained no arginase (Fig. 1C). These results indicate that M2 monocytes are present in burn patient monocytes. M1 monocytes are not generated in cultures of burn patient monocytes stimulated with SAC.

M2b monocytes, a subset of M2 monocytes, are detected in burn patient peripheral blood

M2 monocytes demonstrated in peripheral blood of severely burned patients were analyzed for their subtypes using their chemokine-producing profiles. Thus, monocyte preparations obtained from the above patients and healthy donors were cultured without any stimulation, and culture fluids harvested were assayed for CCL2 (a biomarker of M2aMφ), CCL1 (a biomarker of M2bMφ), and CXCL13 (a biomarker of M2cMφ). In the results, none of the

| Table I. A list of burn patients enrolled in this study |
|----------------|----------------|----------------|
| Patient No. | Age (y) | Sex |
| 1            | 1       | F   |
| 2            | 14      | M   |
| 3            | 14      | M   |
| 4            | 2       | F   |
| 5            | 0.3     | F   |
| 6            | 10      | M   |
| 7            | 7       | M   |
| 8            | 0.8     | F   |
| 9            | 12      | M   |
| 10           | 2       | M   |
| 11           | 3       | M   |
| 12           | 12      | M   |
| 13           | 4       | F   |
| 14           | 10      | M   |
| 15           | 17      | M   |
| 16           | 15      | M   |
| 17           | 0.6     | M   |
| 18           | 8       | M   |
| 19           | 7       | M   |

All patients were admitted to the Shriners Hospital for Children from February 2007 to April 2008. F, female; M, male; TBSA, total body surface area.

| Table II. A list of healthy volunteers enrolled in this study |
|----------------|----------------|----------------|
| Healthy Volunteer No. | Age (y) | Sex |
| 1              | 7       | F   |
| 2              | 4       | F   |
| 3              | 6       | M   |
| 4              | 24      | F   |
| 5              | 38      | M   |

F, female; M, male.
monocytes from healthy donors produced CCL17, CCL1, or CXCL13 (Fig. 2B). CCL1 (320–600 pg/ml) was detected in the culture fluids of monocytes isolated from all of the burn patients. However, significant amounts of CCL17 and CXCL13 were not produced by these monocytes. Small amounts of CCL17 (80–170 pg/ml) were detected in culture fluids of patient 7 and 8 monocytes. Also, small amounts of CXCL13 (35–80 pg/ml) were detected in culture fluids of patient 3, 8, 11, 12, and 14 monocytes (Fig. 2A). These results indicate that severely burned patients are carriers of M2b monocytes. M2a and M2c monocytes are shown not to be significantly present in peripheral blood of severely burned patients.

Kinetic studies on the chemokine production of an additional three patient monocytes were next examined. CCL1 production by monocytes from patient 17 (2 d postburn) was first demonstrated 12 h after cultivation, and then increased until 48 h after cultivation. However, CCL17 and CXCL13 were not produced by these monocytes. Similar results were obtained when monocytes from the same patients (14 d postburn), patient 18 (2 d and 13 d postburn), and patient 19 (3 d and 14 d postburn), were cultured under the same conditions (Fig. 3). These results indicate that M2b monocytes are continuously present in peripheral blood of severely burned patients.

CCL2 as a stimulator of the monocyte conversion to M2b monocytes

To determine why M2b monocytes, but not M2a monocytes and M2c monocytes, are predominant in peripheral blood of severely burned patients, the role of severely burned patient sera on the monocyte conversion from resident monocytes to M2b monocytes was examined. First, healthy donor monocytes were cultured without any stimulation in media containing 15% sera of burn patients or healthy donors, and culture fluids harvested were assayed for CCL1. In the results, CCL1 was not produced by healthy donor monocytes cultured with sera of healthy donors. However, this chemokine was produced by healthy donor monocytes in cultures supplemented with sera of severely burned patients (Fig. 4A). These results suggest that the conversion of resident monocytes to M2b monocytes is influenced by some factor(s) contained in the sera of severely burned patients.

When sera from 19 severely burned patients (Table I) were assayed for chemokine, 8–25 ng/ml CCL2 was detected in all of the serum specimens. However, CCL2 was not detected in sera of burned patients, the role of severely burned patient sera on the monocyte conversion from resident monocytes to M2b monocytes was examined. First, healthy donor monocytes were cultured without any stimulation in media containing 15% sera of burn patients or healthy donors, and culture fluids harvested were assayed for CCL1. In the results, CCL1 was not produced by healthy donor monocytes cultured with sera of healthy donors. However, this chemokine was produced by healthy donor monocytes in cultures supplemented with sera of severely burned patients (Fig. 4A). These results suggest that the conversion of resident monocytes to M2b monocytes is influenced by some factor(s) contained in the sera of severely burned patients.

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Healthy donor monocytes (10^6 cells/ml) were treated with rCCL2 (5 ng/ml) for 48 h, and culture fluids harvested were assayed for CCL1. In the results, after cultivation with rCCL2, CCL1-producing monocytes were generated from healthy donor monocytes. CCL17- and CXCL13-producing monocytes were not demonstrated in healthy donor monocyte cultures supplemented with rCCL2 (Fig. 4D). These results indicate that the conversion of resident monocytes to M2b monocytes is stimulated by CCL2. Furthermore, bactericidal activities of monocytes treated with rCCL2 were examined. In the results, the bactericidal activities of monocytes treated with rCCL2 against *P. aeruginosa* were not shown, whereas the pathogen was killed significantly by healthy donor monocytes (Fig. 4E). These results indicate that healthy monocytes (resident monocytes) treated with rCCL2 have no bactericidal activities.

**CCL2 production in cultures of PMNs from severely burned patients**

In the next series of experiments, burn patient PMNs were examined for their abilities to produce CCL2. PMNs were isolated from peripheral blood of 19 severely burned patients (Table I). These PMN preparations (10^6 cells/ml) were individually stimulated with a bacterial Ag (0.0075% SAC) for 18 h. As controls, PMN preparations isolated from five healthy donors were stimulated with the Ag in the same fashion. The culture fluids harvested from both groups of PMN cultures were assayed for CCL2. In response to SAC stimulation, all PMN preparations isolated from burn patients produced CCL2 (5-14 ng/ml), whereas all of healthy donor PMN preparations did not produce CCL2 (<30 pg/ml) (Fig. 5). These results indicate that PMNs isolated from peripheral blood of severely burned patients are cells with the ability to produce CCL2.

**Discussion**

Impaired IL-12 production in severely burned patients has been well documented (24–26). In the first step of this study, the properties of monocytes isolated from peripheral blood of severely burned patients were examined. After stimulation with or without staphylococcal Ag, peripheral blood monocytes isolated from severely burned patients did not produce IL-12. IL-10 was detected in all cultures of burn patient monocytes. IL-10 was not demonstrated in cultures of healthy donor monocytes. After stimulation with the Ag, IL-12 was produced by all of the monocytes isolated from healthy donors. This indicates that severely burned patients are carriers of IL-12-IL-10^+^ monocytes (M2 monocytes). Additionally, lysates of burn patient monocytes contained arginase, but cell lysates of healthy donor monocytes contained no arginase. It is well known that M2 monocytes produce arginase.

In the next step of this study, we tried to characterize the subset of M2 monocytes, demonstrated in peripheral blood of severely burned patients, based on their chemokine-producing profiles. CCL1 (a biomarker of M2b monocytes) was detected in culture fluids of peripheral blood monocytes isolated from severely burned patients, whereas culture fluids of patient monocytes contained small amounts of CCL17 (a biomarker of M2a monocytes) and CXCL13 (a biomarker of M2c monocytes). These results indicate that all of the severely burned patients tested in this study are carriers of M2b monocytes. We also measured the amount of
CCL1 produced by burn patient monocytes cultured in macrophage serum-free medium or RPMI 1640 media supplemented with heat-inactivated FBS. In the results, CCL1 was equally produced by the monocytes cultured in these two media. This indicates that the monocyte conversion from resident monocytes to M2b monocytes is not enhanced by the serum added to the culture media.

In our studies, peripheral blood specimens were obtained from burn patients within 2 d of admission to the hospital (corresponds to 1–4 d after burn injury). In the cases of patients 1 and 3, the initiation of blood drawings was delayed. All these burn patients were subjected to autografting or allografting and antibiotic treatment. Therefore, delayed enrollment of the patients to the study was a big concern for the standardization of the results shown by the experiments. In fact, plasma levels of catecholamines, steroid hormones, and acute-phase proteins are greatly changed (increased or decreased) within the first 10 d of burn injury. To decrease this concern, we have additionally measured the amounts of CCL2 in sera of three burn patients at ∼2 wk after burn injury. CCL2 was detected in sera of all these patients. These results indicate that CCL2 was consistently present, at least, in sera of severely burn patients from the day of admission to 14 d after burn injury. Further studies will be needed to clarify the kinetics of burn-associated CCL2 production in the large scale experiments.

To determine why M2b monocytes are predominantly present in peripheral blood of severely burned patients, the role of burn patient sera on the monocyte conversion from resident monocytes to M2b monocytes was examined in vitro. After cultivation in the media supplemented with burned patient sera, healthy donor monocytes converted to M2b monocytes. However, M2b monocytes were not detected in healthy donor monocyte cultures supplemented with sera of healthy donors. CCL2 was detected in all serum specimens derived from 19 severely burned patients, whereas this chemokine was not detected in serum specimens of healthy donors. These results suggest that CCL2 contained in sera of severely burned patients plays a role on the monocyte conversion from resident monocytes to M2b monocytes. In fact, the generation of M2b monocytes was greatly reduced in healthy donor monocyte cultures supplemented with anti-CCL2 mAb-treated burn patient sera. Also, the importance of CCL2 on the appearance of M2b monocytes was shown when CCL1-producing monocytes were generated in healthy donor monocyte cultures supplemented with rCCL2. All of these facts strongly indicate that CCL2 plays an important role on the monocyte conversion from resident monocytes to M2b monocytes.

In our previous studies, authentic M2aMΦ, M2bMΦ, and M2cMΦ were prepared from resident MΦ after stimulation with IL-4/IL-13, immune complex and IL-10/corticosteroid, respectively, and the abilities of these M2MΦ preparations to suppress host antibacterial resistance were examined in normal mice exposed to cecal-ligation and puncture (CLP). As controls, normal mice inoculated with resident MΦ (peritoneal MΦ from normal mice) were subjected to the same CLP. Whereas 70% of normal mice inoculated with resident MΦ survived after the CLP, all of the normal mice inoculated with M2aMΦ, M2bMΦ, or M2cMΦ died equally within 4 d of CLP (A. Asai, K. Shigematsu, M. Kobayashi, and F. Suzuki, unpublished data). In contrast, we have previously reported that three different M2MΦ subsets are demonstrated in mice following burn injuries. M2MΦ preparations obtained from mesenteric lymph nodes of mice 2 d after burn injury were shown to be a mixture of M2aMΦ and M2cMΦ, because both CCL17 and CXCL13 were detected in culture fluids of these M2MΦ preparations. M2bMΦ (CCL1-producing MΦ) were demonstrated in the mesenteric lymph nodes of mice 7–21 d after

**FIGURE 5.** CCL2 production by PMNs isolated from peripheral blood of severely burned patients. PMNs (1 × 10⁶ cells/ml) isolated from 5 healthy donors (A) and 19 severely burned patients (B) were stimulated with 0.0075% SAC for 18 h. Culture fluids harvested were assayed for CCL2. Individual data obtained in A and B were combined and shown in C as the mean ± SEM. *p < 0.001 versus healthy donor PMNs.
burn injury (27). M2b macrophages were not demonstrated when CCL2 knockout mice were exposed to burn injuries. Therefore, we examined a role of M2b macrophages on the susceptibility of burned mice to Enterococcus faecalis infection on the bases of M2b macrophage elimination by treatment with CCL2 antisense oligodeoxynucleotides. Burn mice were greatly susceptible to E. faecalis infection. However, in burn mice treated with CCL2 antisense oligodeoxynucleotides, their susceptibility to E. faecalis infection was significantly decreased (K. Shigematsu, M. Kobayashi and F. Suzuki, unpublished data). These results suggest that the host antibacterial resistance is suppressed by M2bMφ appearing in response to severe burn injuries.

In conclusion, M2 monocytes were detected in peripheral blood of patients suffering with severe burn injuries, and the majority of monocytes in these M2 monocyte populations were shown to be M2b monocytes. CCL2, persistently present in burn patient sera and possibly produced by burn-associated neutrophils, is suggested as a reason that M2b monocytes are predominant in severely burned patients, due to the ability of CCL2 to stimulate the monocyte conversion from resident monocytes to M2b monocytes.

**Disclosures**

The authors have no financial conflicts of interest.

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