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CCL1 released from M2b macrophages is essentially required for the maintenance of their properties

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ABSTRACT

Patients with 10–30 days postburn injury are greatly susceptible to infections. M1Mϕ (IL-10$^-$IL-12$^+$ Mϕ) are essential cells in host antibacterial innate immunity against MRSA infections. However, these effector cells are not easily generated in hosts who are carriers of M2bMϕ (IL-12$^-$IL-10$^+$CCL1$^+$LIGHT$^+$ Mϕ). M2bMϕ are inhibitory on M1Mϕ generation. In this study, the antibacterial resistance of mice, 10–30 days postburn injury against MRSA infection, was improved by the modulation of M2bMϕ activities. Unburned mice inoculated with Mϕ preparations from mice, 10–30 days after burn injury, were susceptible to MRSA infection, whereas unburned mice, inoculated with Mϕ preparations from the same mice that were previously treated with CCL1 antisense ODN, were resistant to the infection. M2bMϕ, isolated from Day 15 burn mice, lost their M2bMϕ properties 3 days after cultivation under frequent medium changes, whereas their M2bMϕ properties remained in the same cultures supplemented with rCCL1. In cultures, Mϕ preparations from Day 15 burn mice treated with CCL1 antisense ODN did not produce CCL1 and did convert to M1Mϕ after heat-killed MRSA stimulation. Also, Day 15 burn mice treated with the ODN became resistant against MRSA infection. These results indicate that CCL1 released from M2bMϕ is essentially required for the maintenance of their properties. The increased susceptibility of mice, 10–30 days after burn injury to MRSA infection, may be controlled through the intervention of CCL1 production by M2bMϕ appearing in association with severe burn injuries. *J. Leukoc. Biol.* 92: 859–867; 2012.

Introduction

A leading cause of morbidity and mortality in patients within 1 month of severe burn injury is infections with various pathogens [1, 2]. It has been described in many papers that the incidence of infections remains high enough for several weeks after burn injury [3–7]. Gram-negative and -positive bacteria are isolated frequently from peripheral blood of patients, 10–30 days after burn injury, and a majority of these infections develops easily into severe sepsis. In fact, the restoration of normal host defenses commonly lags behind the healing of burns for 10–30 days [3–7]. The causative agents of bacteremia are *Staphylococcus aureus* in 34% of all cases [6]. Although more than one-half of these patients with bacteremia survive, sepsis is the cause of death or prolonged hospitalization [8]. In hospitalized patients, the increasing prevalence of MRSA has been demonstrated. Vancomycin has been used to treat MRSA infection; however, the appearance of strains resistant to vancomycin raises the specter of untreatable staphylococcal infections in burn patients. Excessive antibiotic use (amounts and duration) is one of the key factors promoting emergence of untreatable staphylococcal strains [9]. These facts indicate that new strategies are needed to control septic complications caused by MRSA in thermally injured patients.

The innate immune system is the first line of host defense against MRSA infection [10, 11]. In healthy individuals, invading MRSA is eliminated rapidly at the local infection site by the innate immune responses. However, MRSA can escape easily from the local innate immune responses in thermally injured hosts who have severe, defective host antibacterial resistance [10–12]. Thus, improvement of innate immune responses is a potential strategy to prevent MRSA sepsis in severely burned hosts. Among effector cells in host anti-MRSA innate immune systems, Mϕ have been described as pivotal cells [13–16]. M1Mϕ are characterized as the main effector

Abbreviations: IACUC=Institutional Animal Care and Use Committee, LIGHT=lymphotoxin-related inducible ligand that competes for glycoprotein D binding to herpesvirus entry mediator on T cells, Mϕ=macrophage(s), MRSA=methicillin-resistant *Staphylococcus aureus*, TBSA=total body surface area

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cells in the anti-MRSA innate immunity [17, 18]. M1Mϕ kill MRSA through the production of lysozomal enzymes, reactive oxygen intermediates, reactive nitrogen intermediates, and/or antimicrobial peptides [19–23]. M1Mϕ are generated from resident Mϕ following stimulation with invasive pathogens via PRRs [24, 25].

However, M1Mϕ have not been generated in burn mice, even when they are exposed to MRSA or substances that function to induce M1Mϕ [26]. Severely burned hosts have a predominance of M2Mϕ with the reduced capacity to kill bacterial pathogens [17, 18], and soluble factors released from M2Mϕ inhibit Mϕ conversion from resident Mϕ to M1Mϕ [27]. Recently, three different subtypes of M2Mϕ (M2aMϕ, M2bMϕ, and M2cMϕ) were described [28]. These subsets can be distinguished from each other by profiles for gene expressions and chemokine production [28–32]. Thus, CCL17-producing Mϕ with mannose receptor gene expression are identified as M2aMϕ, CCL1-producing Mϕ with TNF superfamily 14/LIGHT gene expression are classified as M2bMϕ, and CXCL13-producing Mϕ with mannose receptor gene expression are recognized as M2cMϕ [29, 32]. All of the M2Mϕ subtypes express the IL-10 gene [29]. Unlike M2aMϕ and M2cMϕ, M2bMϕ produce TNF-α, IL-1, and IL-6 [29, 32]. Each M2Mϕ population shows similar activities to inhibit Mϕ conversion from resident Mϕ to M1Mϕ.

Previously, we have described that M2aMϕ appear together with M2cMϕ in mice, 1–6 days after burn injury, and both M2Mϕ subpopulations are responsible for the increased susceptibility of mice to infections early after burn injury (within 1 week of burn) [33]. M2aMϕ and M2cMϕ disappear within 10 days of burn injury. In contrast, M2bMϕ are not isolated from mice within 1 week of burn injury and present in mice 10–30 days after burn injury. In this study, therefore, the long-lived properties of M2bMϕ were examined in mice 10–30 days after burn injury, and CCL1 released from M2bMϕ was shown to be an important chemokine on the maintenance of M2bMϕ properties. M2bMϕ properties of Mϕ derived from mice 10–30 days after burn injury were intervened through the inhibition of CCL1 production by M2bMϕ. The Mϕ population in burned mice treated with CCL1 antisense ODN displayed characteristics of resident Mϕ (IL-12$^-$IL-10$^-$CCL1$^-$LIGHT$^-$ Mϕ). These Mϕ populations converted to M1Mϕ following stimulation with heat-killed MRSA. The results shown in this study may be beneficial to create a new strategy on the controlling MRSA infection, immunologically, in severely burned patients.

MATERIALS AND METHODS

Mice

Seven- to 10-week-old BALB/c male mice (The Jackson Laboratory, Bar Harbor, ME, USA) were used in these experiments. The animal studies were reviewed and approved by the IACUC of The University of Texas Medical Branch at Galveston (Galveston, TX, USA; IACUC Approval Number 0204024A).

Reagents, media, and bacteria

rCCL17 and rCCL1 and mAb for CCL17, CCL1, and CXCL13 were purchased from R&D Systems (Minneapolis, MN, USA). rCXCL13, IL-4, and IL-13 were purchased from PeproTech (Rocky Hill, NJ, USA). Biotin-conjugated anti-mouse F4/80 mAb was obtained from eBioscience (San Diego, CA, USA). Streptavidin particles plus-DM, and BD IMag buffer were purchased from BD Biosciences (San Diego, CA, USA). Single-stranded nucleic acid that inhibits the synthesis of CCL1 (CCL1 antisense ODN; 5′-GAAGC-CCGAGAACATCAT-3′) was synthesized by Sigma-Proligo (Woodlands, TX, USA). 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′-CATCACAAAT-GCGACAGG-3′) was used. For cultivation of Mϕ, RPMI-1640 medium, supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin; complete medium), was used. MRSA (BAA-44 strain), purchased from the American Type Culture Collection (Manassas, VA, USA), was cultured in tryptic soy broth for 16 h at 37°C in aerobic conditions. Heat-killed MRSA was prepared by heating bacteria at 65°C for 30 min. The inactivated cell properties of the killed MRSA were confirmed by culturing the pathogen on agar plates and stored at −80°C until needed in the experiments.

Burn injury

Thermally injured mice were created according to our protocol reported previously [33, 34]. 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 by pressing firmly with a custom-made, insulated mold (with a 4×5-cm window) against their shaved backs. 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 the TBSA for a 26-g mouse. Immediately after thermal injury, physiological saline (1 ml/mouse, i.p.) was administered for fluid resuscitation. Along with fluid resuscitation, deaths of mice with 25% TBSA flame burn were not demonstrated within 10 days of burn injury. Control mice had their back hair shaved but were not exposed to the gas flame. They also received physiological saline (1 ml/mouse, i.p.) under the same conditions. To minimize pain and distress, burned mice were treated with buprenorphine (2 mg/kg, s.c.) every 12 h during the postburn period. Sham burn animals (unburned mice) also received identical regimens of analgesics (buprenorphine) throughout the study period.

Treatment of burn mice with CCL1 antisense ODN

To deplete CCL1, mice, 13 days after burn injury, were treated s.c. with CCL1 antisense ODN (0.5–6 μg/mouse) twice/day for 2 days. As a control, scrambled ODN was administered to burn mice under the same condition. The route and schedule of ODN administration were determined by our preliminary studies.

Preparation of M2Mϕ

Mϕ, isolated from mice 1–3 days after burn injury, were used as a mixture of M2aMϕ and M2cMϕ, as in our previous studies, M2aMϕ first appeared in mice 1 day after burn injury and then disappeared within 5 days of burn injury [33]. M2cMϕ appeared in mice immediately after burn injury and disappeared gradually until 10 days after burn injury [33]. Mϕ preparations derived from mice within 1 week of burn injury did not contain M2bMϕ, which first appeared 10 days after burn injury, peaked 15 days after burn injury, and declined gradually until 35 days after burn injury [33]. Therefore, Mϕ preparations, derived from mice 15 days after burn injury, were used as M2bMϕ, and M2bMϕ preparations, derived from mice 15 days after burn injury, contained neither M2aMϕ nor M2cMϕ, as CCL17, CXCL13, and IL-12 production and mannose receptor and iNOS mRNA expression were not demonstrated in M2bMϕ preparations derived from

mice 15 days after burn injury. As described previously [27, 34], Mϕ were isolated from peritoneal exudates. Mϕ preparations were adjusted to 5×10^6 cells/ml in BD IMag buffer and then treated with anti-mouse CD3 magnetic particles to deplete T cells. After magnetically separating the T cells to the side of the tube, the remaining cells were treated with 5 μg/ml biotin-conjugated anti-mouse F4/80 mAb. After washing with BD IMag buffer, the cell suspension was mixed with streptavidin particles Plus–DM at a ratio of one cell:five beads for 40 min at 4°C. $F4/80^+$ cells were magnetically harvested. The purity of these cells was >94% when measured by flow cytometry.

Phenotypic characterization of M2bMϕ

Mϕ preparations derived from mice 5–40 days after burn injury (mainly mice 15 days after burn injury) were tested for M2bMϕ by the following biomarkers: production of CCL1 and IL-10 (no production of CCL17, CXCL13, and IL-12) and expression of LIGHT mRNA [29, 33]. For production of cytokines and chemokines, Mϕ (1×10^6 cells/ml) were cultured for 48 h without any stimulation. Culture fluids harvested were assayed for IL-10, CCL1, CCL17, CXCL13, and IL-12 using ELISA. The detection limits of our assay system for these cytokines/chemokines are 3–16 pg/ml. LIGHT mRNA expression was analyzed by RT-PCR. Total RNA was extracted from Mϕ (1×10^6 cells/ml) using an RNA isolator, following the manufacturer's recommendations. Within each experiment, each sample was normalized by the amount of isolated RNA. This RNA was then turned back into cDNA through reverse transcription of mRNA. PCR was conducted using synthesized oligonucleotide primers from Sigma-Aldrich (St. Louis, MO, USA): 5′-CTGCATCAACGTCTTGGAGA-3′ (forward) and 5′-GATACGTCAAGCCCCTCAAG-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.

Cultivation of M2bMϕ

Mϕ (1×10^6 cells/ml) from mice 15 days after burn injury were cultured for 3 days. During cultivation, culture media in each well were replaced every 12 h with fresh culture media. The amounts of CCL1 in replaced culture media were measured by ELISA. In some experiments, Mϕ were cultured for 3 days with media containing CCL1 antisense ODN (30 nmol) or under the frequent change of their culture media supplemented with 200 pg/ml rCCL1. As a control, the same Mϕ were cultured for 3 days without medium changes. Mϕ were harvested from the above cultures and tested for their M2bMϕ properties, as mentioned above.

MRSA infection

Various groups of mice were infected i.v. with 2×10^6 CFU/mouse MRSA (corresponds to five LD_{50} in burn mice and <0.1 LD_{50} in unburned mice). These groups included: (1) mice 5–40 days after burn injury (the control group is Day 0 burn mice, and others were tested groups); (2) unburned mice inoculated with Mϕ from mice 5–40 days after burn injury (the control group is mice inoculated with Day 0 burn mouse Mϕ, and others were tested groups), (3) Day 15 burn mice treated with CCL1 antisense ODN (test group) or scrambled ODN (control group), and (4) unburned mice inoculated with Mϕ from Day 15 burn mice that were treated with CCL1 antisense (test group) or scrambled ODN (control group). For adoptive transfer experiments, Mϕ preparations were adjusted to 5×10^6 cells/ml in PBS, and 0.2 ml of the cell suspension was injected i.v. to unburned mice. Two hours after inoculation, mice were infected with MRSA.

The severity of MRSA infection in test groups of mice was determined by the growth of bacteria in organs and blood and their mortality rates, as compared with appropriate control groups (unburned mice, unburned mice inoculated with Day 0 burn mouse Mϕ, Day 15 burn mice treated with scrambled ODN, or unburned mice inoculated with Mϕ from Day 15 burn mice that were treated with scrambled ODN). To measure the quantity of MRSA, liver, kidneys, and spleen of mice 1–3 days after infection were disrupted with a glass homogenizer. A serial tenfold dilution of the homogenates or blood samples were plated onto trypticase soy agar plates and incubated for 24 h at 37°C. The colonies were counted, and results obtained were expressed as bacterial numbers in a gram organ or 1 ml blood. To determine the percentage of survival, mice exposed to MRSA were monitored twice/day for 2 weeks after infection.

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 (Calabasas, CA, USA). A value of $P < 0.05$ was considered significant.

RESULTS

Host's anti-MRSA resistance of burn mice or unburned mice inoculated with M2bMϕ

After i.v. infection with 2×10^6 CFU/mouse MRSA, the mortality rates of mice various days after burn injury were compared with those of unburned mice (control mice). In the results, all of the unburned mice infected with MRSA survived, and 95–100% of mice 5–30 days after burn injury died within 7 days of the same infection (**Table 1**). In our previous studies, M2aMϕ and M2cMϕ appeared in mice 1–6 days after burn injury and were shown to be responsible in the increased susceptibility of mice early after burn injury [33, 34]. Therefore, in this study, we characterized the role of M2bMϕ on the increased susceptibility of mice 10–30 days after burn injury to MRSA infection. Mϕ (1×10^6 cells/ml) isolated from peritoneal exudates of mice 10–30 days after burn injury were cultured for 24 h without any stimulation. Culture fluids obtained were assayed for CCL17 (a biomarker of M2aMϕ), CCL1 (a biomarker of M2bMϕ), and CXCL13 (a biomarker of M2cMϕ). As shown in **Table 2**, mice, 10–30 days after burn injury, were determined as M2bMϕ carriers. M2aMϕ and M2cMϕ were not included in the Mϕ preparations from these mice (Table 2). Therefore, in the following studies, the role of M2bMϕ on the susceptibility of unburned mice to MRSA infection was examined. All of the unburned mice inoculated

TABLE 1. Host's Anti-MRSA Resistance Influenced by Burn Injuries

Days after burn injury	No. of mice	Mean survival days[a]	Survival[b] (%)
0	20	>14.0	100
5	10	4.3	0
10	20	4.8	5
15	20	4.6	0
20	8	5.1	0
25	8	5.5	0
30	8	6.1	0
35	8	>9.6	50
40	8	>14.0	100

[a]Normal mice (Day 0 burn) and mice 5–40 days after burn injury were infected i.v. with 2×10^6 CFU/mouse MRSA. [b]Survival (%) was calculated at 14 days after infection.

TABLE 2. Anti-MRSA Resistance of Unburned Mice Inoculated with Various Sources of Mϕ

Source of Mϕ[a]	Chemokine-producing profile[b]			Survival (%) of recipient mice[c]
	CCL17	CCL1	CXCL13	
0-day burn mice	−	−	−	100
5-day burn mice	+	−	+	Not done
10-day burn mice	−	+	±	0
15-day burn mice	−	+	−	0
20-day burn mice	−	+	−	0
25-day burn mice	−	+	−	0
30-day burn mice	−	+	−	0
35-day burn mice	−	±	−	80
40-day burn mice	−	−	−	100

[a]Normal mice (10 mice each) inoculated with 1×10^6 cells/mouse of various Mϕ preparations were infected i.v. with 2×10^6 CFU/mouse MRSA. [b]Chemokine-producing profiles of Mϕ preparations (1×10^6 cells/ml) were determined by ELISA. CCL17, CCL1, and CXCL13 are biomarkers of M2aMϕ, M2bMϕ, and M2cMϕ, respectively. −, Less than detection limits; ±, length of detection limits; +, produced. [c]Survival (%) was calculated at 14 days after infection.

with 1×10^6 cells/mouse of Mϕ from mice 10–30 days after burn injury died in response to the MRSA infection. However, after MRSA infection, 80–100% of unburned mice inoculated with Mϕ preparations from unburned mice (0 day burn mice) or mice 35–40 days after burn injury survived. M2bMϕ were not included in Mϕ preparations from mice 40 days after burn injury (Table 2). These results indicate that M2bMϕ, appearing in response to burn injuries, are responsible for the increased susceptibility of mice 10–30 days after burn injury to MRSA infection.

Effect of CCL1 antisense ODN on the susceptibility of mice 15 days after burn injury to MRSA infection

As M2bMϕ are CCL1 producer cells [31–33], the susceptibility of burned mice to MRSA infection was examined after the elimination of CCL1 by treatment with CCL1 antisense ODN. As carriers of representative M2bMϕ, mice, 15 days after burn injury (Day 15 burn mice), were used in these experiments. Thus, following the infection with 2×10^6 CFU/mouse MRSA, Day 15 burn mice were treated with 6 μg/mouse CCL1 antisense ODN twice/day for 2 days starting 13 days after burn injury. The effect of CCL1 antisense ODN on the MRSA infection was determined in Day 15 burn mice by mortality rates, bacteremia, and growth of pathogen in organs. In the results, all of the Day 15 burn mice treated with scrambled ODN died within 7 days of MRSA infection, whereas all mice treated with CCL1 antisense ODN survived after the same infection (**Fig. 1A**). Also, after MRSA infection, blood specimens and organs (liver, kidneys and spleen) were obtained from Day 15 burn mice treated with scrambled ODN or CCL1 antisense ODN. The number of pathogens in the blood specimens and homogenates of organs was determined by a standard colony-counting assay. In the results, MRSA was detected in the blood and organs of Day 15 burn mice treated with scrambled ODN, whereas the pathogen did not grow significantly in the blood, liver, and kidneys of these mice treated with CCL1 antisense ODN (Fig. 1B–E).

Next, the effect of Mϕ preparations, derived from Day 15 burn mice treated with CCL1 antisense ODN or scrambled ODN, on the MRSA infection was examined in unburned mice. Obtained results are shown in **Figs. 2A–F**. All of the Day 15 burn mice (Fig. 2B) and unburned mice inoculated with Mϕ from Day 15 burn mice that were treated with scrambled ODN (Fig. 2C) died within 7 days of MRSA infection. However, unburned mice inoculated with Mϕ from Day 15 burn mice that were treated with 6 μg/mouse CCL1 antisense ODN (Fig. 2D) were shown to be resistant against MRSA infection. These results indicate that CCL1 released from M2bMϕ is an important chemokine on the Mϕ-associated susceptibility of Day 15 burn mice to MRSA infection.

The properties of Mϕ from Day 15 burn mice treated with CCL1 antisense ODN or scrambled ODN were determined. Mϕ from both groups of mice were cultured for 12–48 h (for IL-10) and 48 h (for CCL17, CCL1, and CXCL13) without any stimulation. Culture fluids harvested were assayed for IL-10 (a M2Mϕ biomarker), CCL17 (a M2aMϕ biomarker), CCL1 (a M2bMϕ biomarker), and CXCL13 (a M2cMϕ biomarker). In the results, all of these soluble factors were not detected in culture fluids of Mϕ from Day 15 burn mice treated with

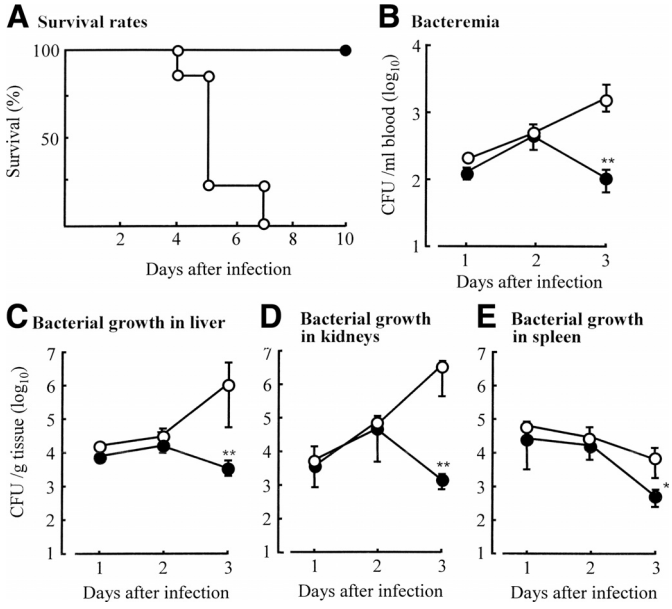

Figure 1. Effect of CCL1 antisense ODN on MRSA infection in burn mice. Burn mice were treated with 6 μg/mouse CCL1 antisense ODN (●) or scrambled ODN (○) twice/day for 2 days. All mice were infected i.v. with a lethal dose of MRSA (2×10^6 CFU/mouse). These mice were tested for survival rates (A; 10 mice each), bacteremia (B; five mice each), and growth of the pathogen in various organs (C–E; five mice each), as mentioned in Materials and Methods. The difference of survival curves was $P < 0.001$, according to the Kaplan-Meyer log rank test. The numbers of bacteria are shown as mean ± SEM. $*P < 0.01$; $**P < 0.001$ compared with control.

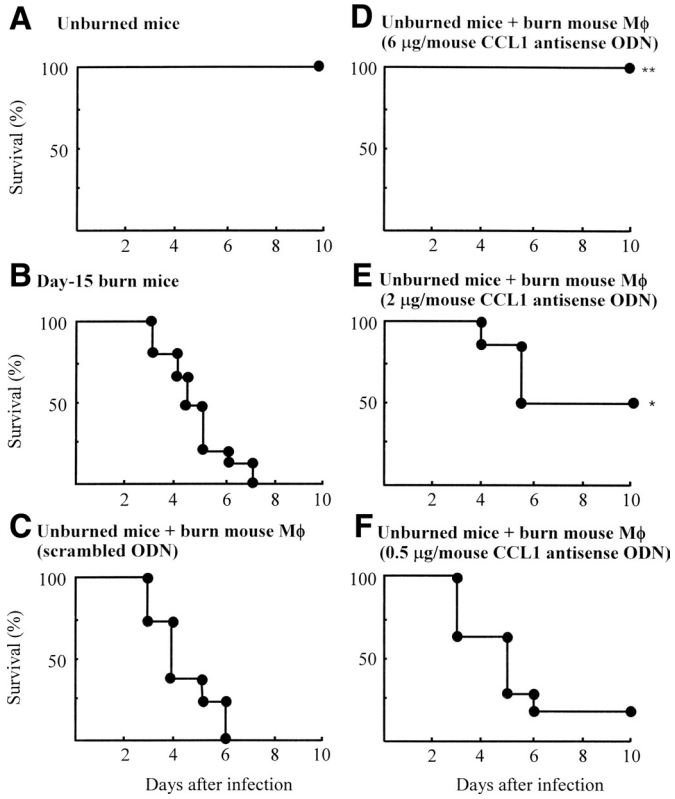

Figure 2. MRSA infection in unburned mice inoculated with various Mϕ preparations. Unburned mice (A; 10 mice), Day 15 burn mice (B; 10 mice), and unburned mice inoculated with various Mϕ preparations (1×10^6 cells/mouse) from Day 15 burn mice that were treated with scrambled ODN (C; twice/day for 2 days; 6 μg/mouse) or CCL1 antisense ODN (twice/day for 2 days: D, 6 μg/mouse; E, 2 μg/mouse; F, 0.5 μg/mouse) were infected i.v. with MRSA (2×10^6 CFU/mouse). Kaplan-Meyer log rank test: $^*P < 0.01$; $^{**}P < 0.001$ compared with Group C.

CCL1 antisense ODN, whereas IL-10 and CCL1 were produced by Mϕ from Day 15 burn mice treated with scrambled ODN (**Figs. 3A** and **B**). Also, the percentage of IL-10^+ cells and an ability to express LIGHT mRNA were tested for Mϕ preparations derived from Day 15 burn mice that were treated with scrambled ODN or CCL1 antisense ODN. In the results, IL-10^+ cells and LIGHT mRNA expression were not seen in Mϕ preparations from Day 15 burn mice treated with CCL1 antisense ODN (Figs. 3C and D). These results indicate that M2bMϕ are not included in the Mϕ preparations from Day 15 burn mice treated with CCL1 antisense ODN, whereas these mice treated with scrambled ODN are shown to be carriers of M2bMϕ.

Requirement of CCL1 released from M2bMϕ on the maintenance of their M2bMϕ properties

As M2bMϕ were not isolated from Day 15 burn mice treated with CCL1 antisense ODN, the role of CCL1 on the fate of M2bMϕ was examined in vitro. M2bMϕ (1×10^6 cells/ml) from Day 15 burn mice were cultured for 3 days under frequent changes of culture media. After recultivation, >98% of cells were shown to be viable. When M2bMϕ (1×10^6 cells/ml) were cultured without any medium changes, CCL1 was first detected in their culture fluids 6 h after cultivation and increased progressively for 12 h (**Fig. 4A**). When M2bMϕ were cultured for 3 days with frequent medium changes, CCL1, IL-10, and IL-12 were not detected in their culture fluids (Fig. 4B). LIGHT mRNA was expressed by M2bMϕ that were cultured for 3 days without any medium changes, whereas this mRNA was not expressed by the Mϕ preparations that were cultured under the frequent medium changes (Fig. 4C). From the results shown in Figs. 4B and C, M2bMϕ are shown to be converted to IL-12^-IL-10^-CCL1^-LIGHT$^-$ Mϕ during cultivation with the frequent medium changes. In the next experiments, M2bMϕ were cultured for 3 days with frequent medium changes to fresh medium supplemented with rCCL1. As shown in **Fig. 5A–C**, M2bMϕ cultured with frequent changes of media to rCCL1-containing media maintained their IL-10^+CCL1^+LIGHT$^+$ properties. These results indicate that CCL1, released from M2bMϕ, is required for the maintenance of their M2bMϕ properties.

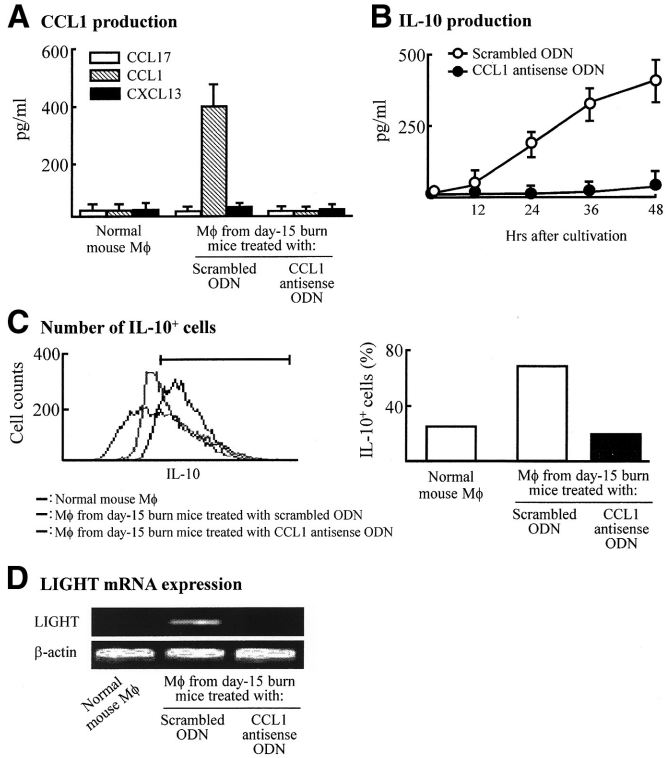

Figure 3. Characteristics of Mϕ preparations from Day 15 burn mice treated with CCL1 antisense ODN. Mϕ preparations, isolated from Day 15 burn mice that were treated with scrambled ODN or CCL1 antisense ODN (n=5–6), were cultured for 24 h (for CCL1) or 12–48 h (for IL-10). Culture fluids harvested were assayed for CCL1 (A) and IL-10 (B) by ELISA. Also, Mϕ preparations derived from Day 15 burn mice that were treated with or without CCL1 antisense ODN (n=5–6 mice) were analyzed for intracellular IL-10 (C; flow cytometry) and LIGHT mRNA expression (D; RT-PCR).

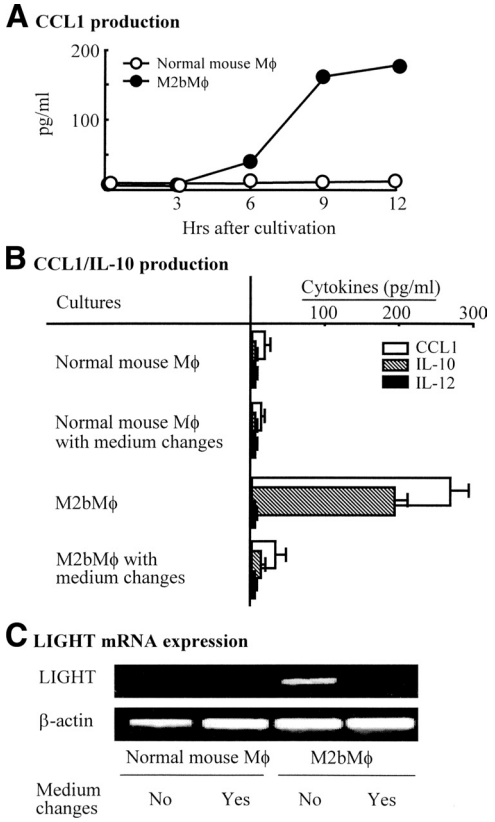

Figure 4. Characteristics of Mϕ from Day 15 burn mice after cultivation under frequent medium changes. Mϕ (1×10^6 cells/ml), isolated from peritoneal exudates of Day 15 burn mice (n=4–5 mice), were cultured for 3–12 h without any medium changes (A) or for 3 days under the frequent medium changes (every 12 h; B and C). Then, they were cultured additionally for 2 days. Mϕ populations derived from unburned mice (n=4–5 mice) were cultured in the same manner and served as a control. Culture fluids harvested were assayed for CCL1 (A) or CCL1, IL-10, and IL-12 (B). Also, cells harvested from these cultures were assayed for LIGHT mRNA expression by RT-PCR (C).

M1Mϕ induction by heat-killed MRSA from Mϕ of Day 15 burn mice treated with CCL1 antisense ODN

M1Mϕ (IL-12^+IL-10^- Mϕ, antibacterial effector cells) are not induced from M2Mϕ or in hosts whose M2Mϕ predominate [26]. M2Mϕ not only lack the ability to convert M1Mϕ but also inhibit the conversion of Mϕ from resident Mϕ to M1Mϕ [27]. In the series of experiments, therefore, we tried to induce M1Mϕ by heat-killed MRSA from Mϕ of Day 15 burn mice treated with CCL1 antisense ODN. Mϕ (1×10^6 cells/ml) from Day 15 burn mice treated s.c. with 6 μg/mouse CCL1 antisense ODN were stimulated with 10^6 heat-killed MRSA for 24 h. As a control, Mϕ, from Day 15 burn mice treated with scrambled ODN, were stimulated with the antigen in the same manner. Cells obtained were washed and recultured for 24 h with fresh medium. After termination of the culture, cells were harvested and assayed for IL-12 by flow cytometry (**Fig. 6A**) and killing activity against MRSA (Fig. 6B). In the results, 86% of Mϕ from unburned mice stimulated with heat-killed MRSA were identified as IL-12^+ cells. Similarly, after stimulation with heat-killed MRSA, 90% of Mϕ from Day 15 burn mice treated with CCL1 antisense ODN were identified as IL-12^+ cells, whereas only 13% of Mϕ from Day 15 burn mice treated with scrambled ODN were shown to be IL-12^+ cells (Fig. 6A). Furthermore, after stimulation, both groups of Mϕ were assayed for their bactericidal activities against MRSA in vitro. Thus, heat-killed MRSA-stimulated Mϕ preparations (1×10^5 cells/well) were mixed with 10^6 CFU/well MRSA. Three hours after cultivation, the numbers of total bacteria in cultures were measured by a colony-counting method. Obtained results are

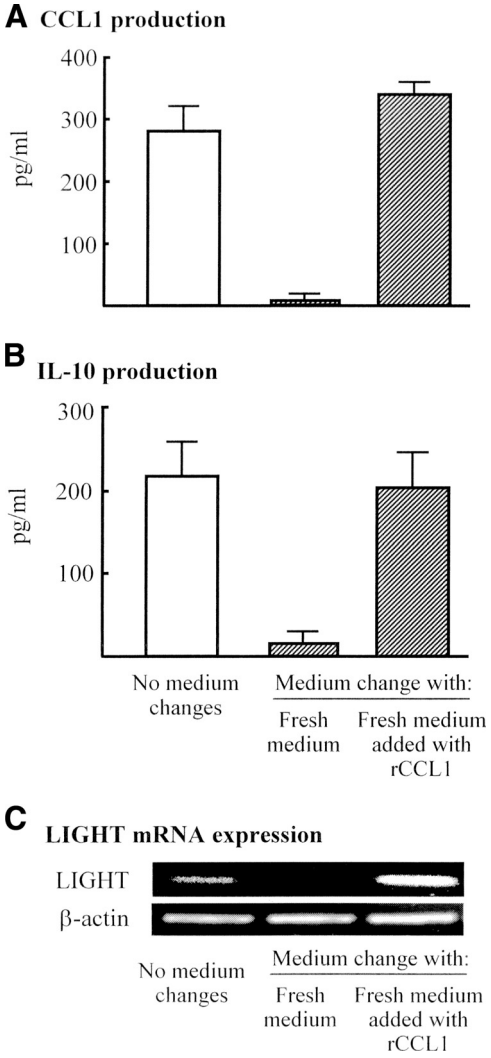

Figure 5. The properties of M2bMϕ after cultivation with frequent changes of culture media supplemented with or without rCCL1. Mϕ (1×10^6 cells/ml), prepared from mice 15 days after burn injury (n=4–5 mice), were cultured for 3 days under the frequent changes of culture media (every 12 h), supplemented with or without 200 pg/ml rCCL1. Cells harvested after the cultivation were recultured for 2 days. Culture fluids obtained were assayed for CCL1 (A) and IL-10 (B) as biomarkers for M2bMϕ, and cultured cells were tested for their LIGHT mRNA expression as a biomarker of M2bMϕ (C).

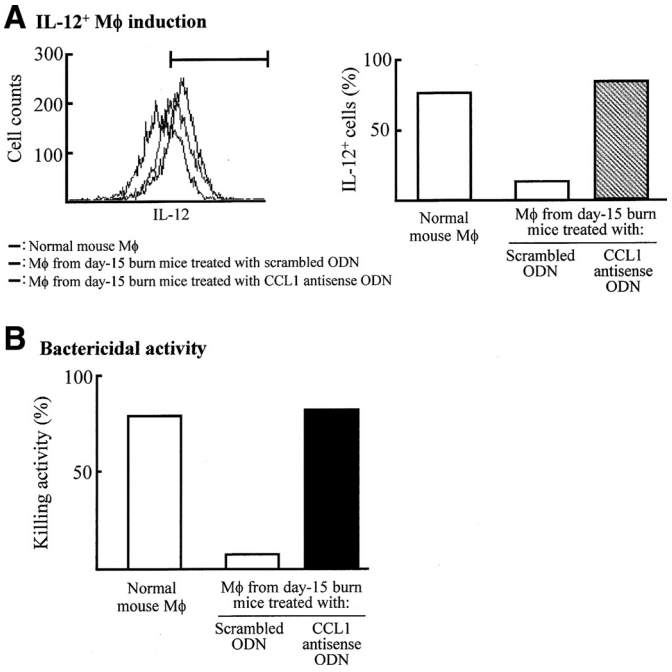

Figure 6. M1Mϕ generated in cultures of Mϕ preparations from Day 15 burn mice that were treated with CCL1 antisense ODN. Mϕ were isolated from Day 15 burn mice treated with scrambled ODN or CCL1 antisense ODN (n=5 mice). Mϕ obtained were stimulated with 10^6 heat-killed MRSA for 24 h. Mϕ harvested were washed and assayed for their intracellular expression of IL-12 (a biomarker of M1Mϕ) by flow cytometry (A) and bactericidal activity against MRSA (B). The bactericidal activity of Mϕ was analyzed by a standard colony-counting method, as shown in Materials and Methods. The killing activity (%) was calculated by the following formula: (CFU after cultivation with Mϕ)/(CFU after cultivation without Mϕ)] $\times$ 100.

shown in Fig. 6B. Mϕ from unburned mice exhibited an average of 78% killing activities against MRSA, whereas Mϕ from Day 15 burn mice treated with scrambled ODN killed only 9% of the bacteria. At this time, Mϕ from Day 15 burn mice treated with CCL1 antisense ODN killed 82% of MRSA. These results indicate that after treatment with CCL1 antisense ODN, M2bMϕ regress to resident Mϕ that possess the ability to convert M1Mϕ in response to heat-killed MRSA.

DISCUSSION

M1Mϕ (IL-12-producing and IL-10-nonproducing Mϕ) are one of the major effector cells in antimicrobial innate immune responses. M1Mϕ migrate to the infection site tissues and kill the invaded pathogens before spreading to the whole body. However, the generation of M1Mϕ is not demonstrated in severely burned patients who are greatly susceptible to various infections [35–38]. One of the reasons why M1Mϕ do not generate in severely burned mice is the appearance of IL-10-producing, IL-12-nonproducing Mϕ (M2Mϕ) with reduced capacity to kill pathogens [27, 34]. M2Mϕ strongly inhibit the conversion of Mϕ from resident Mϕ to M1Mϕ, and M2Mϕ never convert to M1Mϕ, although they are stimulated with pathogens or typical M1Mϕ inducers [26].

Recently, we have demonstrated [38] that monocytes carrying M2bMϕ properties were predominantly present in peripheral blood of severely burned patients. We have also demonstrated [33] that M2bMϕ appear in mice 10–30 days after burn injury, whereas M2aMϕ and M2cMϕ are demonstrated in mice 1–5 days after burn injury. As described previously [34], mice 1–5 days after burn injury were susceptible to MRSA infection, and the susceptibility of burn mice to MRSA infection was transferred to unburned mice by the adoptive transfer of M2aMϕ and M2bMϕ. Here, we showed that mice 10–30 days after burn injury are also susceptible to MRSA infection. The increased susceptibility of these mice to MRSA infection was transferred to unburned mice by the Mϕ preparations (M2bMϕ) isolated from mice 10–30 days after burn injury. As M2bMϕ are inhibitory on the generation of antibacterial effector cells (M1Mϕ), the intervention of M2Mϕ function may cause recovery of the impaired antibacterial resistance in severely burned hosts.

Mϕ from mice 10–30 days after burn injury were shown to be M2bMϕ, as they produced IL-10 (but not IL-12) and CCL1 (but not CCL17 and CXCL13) and expressed LIGHT mRNA. However, the Mϕ preparation derived from Day 15 burn mice that were treated with CCL1 antisense ODN did not possess the ability to produce IL-10 and to express LIGHT mRNA. CCL1 was not produced in cultures of Mϕ preparation derived from Day 15 burn mice that were treated with CCL1 antisense ODN. Also, IL-10 and CCL1 were not produced, and LIGHT mRNA was not expressed by Mϕ from Day 15 burn mice after cultivation with frequently changed culture media to fresh media. This suggests that a soluble factor(s) released from M2bMϕ to the culture fluids is involved in the maintenance of their M2bMϕ properties. In our preliminary studies, the culture fluids of M2bMϕ were assayed for cytokines and chemokines. IL-10, TNF-α, IL-1, IL-6, and CCL1 were detected in the culture fluid of M2bMϕ. When M2bMϕ (1×10^6 cells/ml) were cultured with media supplemented with mAb (5 μg/ml) directed against IL-10, TNF-α, IL-1, IL-6, or CCL1, their M2bMϕ properties (for example, LIGHT mRNA expression) were kept. However, M2bMϕ properties were not detected when these Mϕ were cultured with anti-CCL1 mAb. From this, we have considered that CCL1 is an essential chemokine on the maintenance of M2bMϕ properties. This was confirmed in the cultivation experiments of M2bMϕ under frequent changes of culture media, supplemented with or without rCCL1. Obtained results clearly showed that CCL1 was essentially required for the maintenance of M2bMϕ properties.

As M2bMϕ are inhibitory on Mϕ conversion from resident Mϕ to M1Mϕ, we next examined the role of CCL1 on the conversion of Mϕ from resident Mϕ to M1Mϕ or M2Mϕ. When the Mϕ preparation from Day 15 burn mice treated with CCL1 antisense ODN was stimulated with heat-killed MRSA in vitro, IL-12$^+$ Mϕ, with strong bactericidal activity, were generated. This indicates that M1Mϕ are inducible from the Mϕ preparation derived from Day 15 burn

mice that were treated with CCL1 antisense ODN. As M1Mϕ are generated from resident Mϕ (but not M2aMϕ, M2bMϕ, and M2cMϕ), M2bMϕ may regress to resident Mϕ in Day 15 burn mice after treatment with CCL1 antisense ODN.

The reason why M2bMϕ are predominant in mice 10–30 days after burn injury remains unclear. When resident Mϕ were cultured with media containing 100–400 pg/ml of rCCL1 for 1–3 days, LIGHT mRNA was not expressed by cultured resident Mϕ (data not shown). This indicates that CCL1 is not involved directly in the generation of M2bMϕ from resident Mϕ. Our previous murine studies [33] showed that M2bMϕ and M2cMϕ were not demonstrated in CCL2 knockout mice with severe burn injuries. In contrast, M2bMϕ appeared in CCL2 knockout mice 14 days after burn injury, although the numbers of M2bMϕ generated were diminished greatly as compared with burned WT mice [33]. These results suggest that there may be some relations between the appearance of M2aMϕ/M2cMϕ in mice early after burn injury and M2bMϕ in mice 10–30 days after burn injury. To clarify these questions, further studies will be required. It has been described that the TLR4 reactivity of Mϕ in mice 7–15 days after burn injury is increased [39, 40]. In response to LPS, these Mϕ produce IL-1, IL-6, TNF-α, as well as IL-10 [39, 40]. In our study, Mϕ from Day 15 burn mice expressed LIGHT mRNA and produced IL-1, IL-6, TNF-α, IL-10, and CCL1 [33]. These facts suggest that burn-induced, TLR4-reactive Mϕ may belong to M2bMϕ.

In our results shown here, CCL1 released from M2bMϕ was shown to be essential for the maintenance of their M2bMϕ properties. Therefore, the knockdown of CCL1 from Day 15 burn mice using CCL1 antisense ODN resulted in the improvement of their resistance against MRSA infection. Similar results have been reported in CCR8 knockout mice [41]. As CCR8 was a receptor for CCL1, CCR8 knockout mice were shown to be resistant against sepsis caused by cecal ligation and puncture. Mϕ derived from CCR8 knockout mice exhibited marked abilities to produce IL-12 and to kill bacteria. The descriptions in this report strongly support our results described in this paper that MRSA infection is controlled effectively in burned mice depleted of M2bMϕ by CCL1 gene therapy. The depletion of M2bMϕ by the knockdown of CCL1 may be a relevant approach in improving the host antibacterial resistance of severely burned patients.

AUTHORSHIP

A.A. performed experiments and analyzed data. K.N. performed experiments. M.K. designed experiments and wrote the paper. D.N.H. and F.S. designed the research and edited the paper.

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KEY WORDS:
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