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CCL17 and IL-10 as Effectors That Enable Alternatively Activated Macrophages to Inhibit the Generation of Classically Activated Macrophages[1]

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Classically activated macrophages (CAMϕ) have been described as a major effector cell on the host's innate immunities. However, CAMϕ are not generated in immunocompromised hosts whose alternatively activated macrophages (AAMϕ) predominate. In this study, the mechanism by which AAMϕ suppress the ability of resident macrophages (RMϕ) to generate CAMϕ was investigated. AAMϕ were isolated from peritoneal exudates of mice 2 days after third-degree thermal injuries affecting 15% total body surface area. CAMϕ were generated from RMϕ (peritoneal Mϕ from normal mice) through stimulation with CpG DNA, a typical CAMϕ inducer. RMϕ did not polarize to CAMϕ when they were cultured with AAMϕ in a dual-chamber Transwell even when supplemented with CpG DNA. In addition, RMϕ stimulated with CpG DNA did not convert to CAMϕ when they were cultured with the culture fluids of AAMϕ (AAMϕ Culture-Sup). AAMϕ Culture-Sup contained IL-6, IL-10, CCL17, PGE_2, and TGF-β. Among these, CCL17 and IL-10 inhibited CAMϕ generation. The ability of AAMϕ Culture-Sup to inhibit CAMϕ generation was eliminated when the Culture-Sup was treated with a mixture of mAbs directed against CCL17 and IL-10. These results indicate that CCL17 and IL-10 released from AAMϕ inhibit CAMϕ generation from RMϕ stimulated with CpG DNA. ***The Journal of Immunology,*** **2004, 172: 1407–1413.**

Macrophages (Mϕ)[3] have long been recognized as heterogeneous (1). The heterogeneity of Mϕ almost certainly reflects their plasticity and versatility in response to microenvironmental signals. Recent studies have suggested five (1) or three (2) pathways of Mϕ activation in innate and acquired immunities. However, in addition to resident Mϕ (RMϕ), Mϕ have generally been divided into two populations, classically activated macrophages (CAMϕ) and alternatively activated macrophages (AAMϕ), based on their immunobiological properties (3–5). CAMϕ play an important role in the antimicrobial innate immunities of the host (6–8). CAMϕ are generated from RMϕ stimulated with a microbe, microbial product (CpG DNA, poly(I:C), and LPS) or various biological response modifiers (muramyl dipeptide and poly(styrene-maleic acid) conjugated with neocarzinostatin) (5, 9–12). CAMϕ exhibit 1) high oxygen consumption, 2) the ability to kill cells infected with intracellular pathogens, 3) cytotoxicity against tumor cells, 4) the ability to express inducible NO synthase (iNOS), and 5) the ability to secrete NO, proinflammatory cytokines (IL-1, IL-6, and TNF-α) and Th1 response-associated cytokines (IFN-γ, IL-12, IL-18, CCL3, CXCL9, and CXCL10) (4, 5). In addition, CAMϕ induce Th1 cells by producing IL-12, CCL3, and IFN-γ. In contrast, AAMϕ play a role in the negative regulation of both CAMϕ and Th1 cell generation (4, 5, 13–16). AAMϕ preferentially express receptors for foreign Ags, such as mannose receptor, β-glucan receptors, and scavenger receptors (17, 18). AAMϕ have been described as a producer cell for IL-1R antagonist, IL-10, CCL17, CCL22, and arginase (4, 5). They also inhibit IFN-γ production and developmental Th1 responses (15, 16).

Numerous studies have shown that CAMϕ generation is required for a host's defense against infections with various intracellular pathogens or the growth of tumors (1, 5). In fact, CAMϕ that appeared in mice infected with *Mycobacterium bovis* bacillus Calmette-Guérin and *Listeria monocytogenes* were shown to be powerful killer cells for these pathogens, tumor cells, and cells infected with various bacteria and viruses (1). The ability of CAMϕ to kill these targets was displayed nonspecifically (1). In other words, the host resistance of individuals exposed to foreign invasions may be effectively improved if CAMϕ can be induced. However, CAMϕ were not generated in individuals whose AAMϕ predominate, because AAMϕ inhibited CAMϕ generation (19, 20). Hosts susceptible to foreign invasions are generally recognized as individuals with a predominance of AAMϕ.

In the present study, CCL17 and IL-10 released from AAMϕ were shown to be effector molecules that inhibit CAMϕ generation from RMϕ stimulated with CpG DNA. The regulation of the production of these cytokines may improve the resistance to various opportunistic infections in patients whose AAMϕ predominate.

Materials and Methods

Animals

Eight- to 11-wk-old, pathogen-free, male BALB/c mice purchased from The Jackson Laboratory (Bar Harbor, ME) were used in this study. The Institutional Animal Care and Use Committee of the University of Texas

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[3] Abbreviations used in this paper: Mϕ, macrophages; CAMϕ, classically activated Mϕ; AAMϕ, alternatively activated Mϕ; RMϕ, resident Mϕ; iNOS, inducible NO synthase; TBSA, total body surface area; MRSA, methicillin-resistant *S. aureus*; SOCS, suppressor of cytokine signaling.

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Medical Branch approved all procedures performed in these animal experiments (Institutional Animal Care and Use Committee approval no. 02-04-024).

Reagents and medium

rIL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, IFN-γ, TNF-α, and CCL3 were purchased from PeproTech (Rocky Hill, NJ). TGF-β and mAbs for IL-1β, IL-4, IL-6, IL-10, IFN-γ, TGF-β, TNF-α, CD3, and CD28 were purchased from BD PharMingen (San Diego, CA). PGE_2 ELISA kit and CCL17 as well as mAbs for CCL3 and CCL17 were purchased from R&D Systems (Minneapolis, MN). CpG DNA (5′-TCCATGACGTTCCTGACGTT-3′) was purchased from Sigma-Genosys (Woodlands, TX). CpG DNA was used to induce CAMϕ generation, as previously described (21). Mϕ were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and antibiotics (complete medium).

Burn injury

Thermally injured mice, BALB/c mice exposed to flame-burn injuries (22), were prepared as follows. 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 the window of the custom-made insulated mold (with a 2.5 × 3.5-cm window) firmly against the shaved back. A Bunsen burner equipped with a flame-dispersing cap was used as a source of the gas flame. This procedure consistently produced a third-degree burn on ~15% of total body surface area (TBSA) for a 26-g mouse (22). Immediately after thermal injury, physiologic saline (3 ml/mouse i.p.) was administered for fluid resuscitation. Animals were then housed until use for experiments. Control mice had their back hair shaved but were not exposed to the gas flame. They also received physiologic saline (3 ml/mouse i.p.).

Preparation of RMϕ, AAMϕ, and CAMϕ

For the Mϕ isolation, 4–10 × 10^6 cells of peritoneal exudates from mice were centrifuged. The cell pellets were suspended in 2 ml of RPMI 1640 medium supplemented with 2% FBS (maintenance medium) and cultured in fibronectin-coated petri dishes (60 × 15 mm) for 15 min at 37°C (23). At the end of cultivation, the dishes were washed twice with maintenance medium warmed to 37°C. Adherent cells (Mϕ populations) were recovered from the dishes using a rubber policeman. Mϕ purity was measured as 92% or more when tested by FACSVantage (BD Biosciences, Mountain View, CA). RMϕ were Mϕ freshly isolated from peritoneal exudates of normal mice. As described previously (24), AAMϕ were isolated from peritoneal exudates of mice 2 days after severe burn injuries (third-degree flame burn, >15% TBSA). As previously described (4), standard AAMϕ were induced in cultures of RMϕ treated with a mixture of IL-4 and IL-13 (10 ng/ml each) for 48 h. CAMϕ were generated in vitro from RMϕ after stimulation with CpG DNA (24 h stimulation, 10 μg/ml) (21).

Criteria of CAMϕ and AAMϕ

Mϕ were identified as CAMϕ when the following typical properties were demonstrated: production of CCL3 and IL-12 (25, 26), expression of iNOS mRNA (27), induction of Th1 responses (25), and exhibition of killing activity against bacteria (6). Mϕ were identified as AAMϕ when the following criteria were demonstrated: production of CCL17 and IL-10 (3, 28), expression of mannose receptor mRNA (29), and induction of Th2 responses (30). For the production of cytokines, various Mϕ populations (1 × 10^6 cells/ml) were cultured for 24 h without any stimulation. Then, the culture fluids harvested were assayed for the cytokines using ELISA. The detection limits of CCL3, IL-12, IL-10, and CCL17 were 16, 14, 8, and 16 pg/ml, respectively. In addition, Mϕ populations were tested for mRNA expression by RT-PCR using primers as follows: for mannose receptor, 5′-CCATCGAGACTGCTGCTGAG-3′ (F) and 5′-AGCCCTTGGGTT GAGGATCC-3′ (R) and for iNOS, 5′-CCCTCCAGTGTCTGG GAGCA-3′ (F) and 5′-TGCTTGTCACCACCAGCAGT-3′ (R). Methicillin-resistant *S. aureus* (MRSA), biotype 21777, was isolated from a clinical specimen from a burn patient in Shriners Hospital for Children and used as target cells to test the antibacterial killing activity of Mϕ. MRSA used in this study was defined as a vancomycin-sensitive strain. Using Lissner's methods (31), MRSA-killing activities of various Mϕ populations were examined by culturing 1 × 10^6 cells/ml Mϕ with 1 × 10^7 CFU/ml MRSA for 20 min at 37°C (multiplicity of infection = 10). After unphagocytized MRSA was washed out, the cells were cultured for an additional 3 h at 37°C. The number of MRSA in the whole culture was determined using a standard colony-counting method (31). The ability of various Mϕ populations to induce Th1 or Th2 responses was determined by the methods described in previous reports (23). Naive T cells (1 × 10^6 cells/ml) were cocultured with Mϕ populations (1 × 10^6 cells/ml) for 6 days in the presence of 50 U/ml IL-2. To induce cytokines, the nonadherent cells were harvested and recultured for 12 h with a mixture of anti-CD3 and anti-CD28 mAbs (2 μg/ml each). The culture fluids were harvested and assayed for IFN-γ (a Th1 response) or IL-4 (a Th2 response) using ELISA.

Inhibition of CAMϕ generation from RMϕ stimulated with CpG DNA

The inhibitory effect of AAMϕ on CAMϕ generation was analyzed using the following procedures: 1) RMϕ stimulated with CpG DNA were cocultured with AAMϕ, 2) RMϕ were cultured with AAM0 in a dual-chamber Transwell supplemented with CpG DNA, and 3) RMϕ stimulated with CpG DNA were cultured with the culture fluids of AAMϕ (AAMϕ Culture-Sup). After cultivation by these different methods, the RMϕ were harvested and cultured for an additional 24 h to produce CCL3. CCL3 was chosen as a parameter of CAMϕ, because it has been described as such in previous studies (4, 5). Cocultivation experiments (above, procedure 1) were performed as follows. In the presence of 10 μg/ml CpG DNA, RMϕ were cocultured with AAMϕ at a ratio of 1:100 to 64:100 in 96-well microtiter plates. Twenty-four hours after cultivation, the cells were washed three times with complete medium and cultured for an additional 24 h with complete medium. The culture fluids harvested were assayed for CCL3. A Transwell culture (above, procedure 2) was performed as described below. RMϕ were cultured with AAMϕ in a dual-chamber Transwell supplemented with CpG DNA. Six hundred microliters of RMϕ suspension (1 × 10^6 cells/ml) was placed into the lower chamber of the Transwell (0.4-μm pore size; Costar, Corning, NY). One hundred microliters of AAMϕ suspension (5 × 10^6 cells/ml) was placed into the upper chamber of the Transwell. Twenty-four hours after cultivation, Mϕ harvested from the lower chamber were recultured for 24 h. The resulting culture fluids were assayed for CCL3. RMϕ were also cultured with the AAMϕ Culture-Sup (above, procedure 3). AAMϕ Culture-Sup was the culture fluids of AAMϕ (1 × 10^6 cells/ml) 24 h after cultivation. RMϕ stimulated with CpG DNA were cultured with complete medium supplemented with AAMϕ Culture-Sup (15%, v/v) for 24 h. The cells harvested were washed three times with complete medium and cultured for an additional 24 h. The resulting culture fluids were assayed for CCL3.

Determination of soluble factors from AAMϕ

To determine the active components in AAMϕ Culture-Sup, AAMϕ were cultured without any stimulation for 24–48 h, and the culture fluids harvested were assayed for IL-1β, IL-6, IL-10, TNF-α, TGF-β, CCL17, and PGE_2 using ELISA. These soluble factors have been well described as the products of Mϕ or Mϕ-related cells (4, 5, 16). The detection limits of IL-1β, IL-6, IL-10, TNF-α, TGF-β, PGE_2, and CCL17 were 20, 12, 8, 20, 22, 18, and 16 pg/ml, respectively. Then, the recombinant cytokines, specifically detected in the culture fluids of AAMϕ, were assayed for their ability to inhibit CAMϕ generation. Thus, in the presence of CpG DNA, RMϕ (1 × 10^6 cells/ml) were cultured with various doses of recombinant cytokines. Twenty-four hours after the cultivation, the cells were washed three times and then cultured for an additional 24 h. The culture fluids harvested were assayed for CCL3. After certain cytokines were determined to inhibit CAMϕ generation, the AAMϕ Culture-Sup was treated with mAbs directed against the appropriate cytokines and applied to the assay of CAMϕ generated from RMϕ stimulated with CpG DNA. Amounts of mAbs (clone 110904; clone JES5-2A5) used in the experiments were determined according to the manufacturer's protocol.

ELISPOT assay

To determine the percentage of AAMϕ in the CAMϕ preparation or the percentage of CAMϕ in the AAMϕ preparation, an ELISPOT assay was performed as previously described (32). A 96-well, Millititer HA plate (Millipore, Bedford, MA) was coated with 2 μg/ml anti-mouse CCL3 mAb or anti-mouse CCL17 mAb in 0.1 M carbonate buffer (pH 9.6) overnight at 4°C. The plates were then blocked with complete medium for 1 h. Mϕ suspensions diluted serially with complete medium were incubated in the plates for 24 h at 37°C. Next, the plates were washed with PBS containing 0.05% Tween 20 and overlaid with biotinylated anti-mouse CCL3 mAb or anti-mouse CCL17 mAb overnight at 4°C. Subsequently, the plates were washed and treated with a 1/2000 dilution of avidin-conjugated alkaline phosphatase (Sigma-Aldrich, St. Louis, MO). CCL3 or CCL17, secreted by single cells, was visualized by adding a solution of 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (Sigma-Aldrich). The colorimetric reaction was halted after 30 min by washing the plates with water, and the spots were enumerated under ×40 magnification. The dilution of the cells producing spots was used to calculate the total numbers of CCL3- or

Table I. *The properties of Mϕ populations used in this study*[a]

Properties	Assay Procedures	RMϕ	AAMϕ	CAMϕ
Mannose receptor mRNA expression	RT-PCR	Trace	Yes	No
iNOS mRNA expression	PT-PCR	No	No	Yes
Th1/Th2 polarization	Transwell culture	No	Th2	Th1
Killing against MRSA	CFU	Yes	No	Yes
Cytokine production	ELISA	Trace	IL-10	IL-12
Chemokine production	ELISA	Trace	CCL17	CCL3
CCL3-producing Mϕ:CCL17-producing Mϕ	ELISPOT	1:0.7	1:122	149:1

[a] Peritoneal Mϕ freshly isolated from normal BALB/c mice were used as RMϕ (5). Peritoneal Mϕ from mice 2 days after third-degree 15% TBSA burn were used as AAMϕ (24). RMϕ stimulated with CpG DNA (10 μg/ml, 24 h) were used as CAMϕ (21). The number of CCL3- or CCL17-producing Mϕ was determined using the ELISPOT assay as described in the text. The results are displayed as the ratio between CCL3-producing Mϕ and CCL17-producing Mϕ.

CCL17-producing Mϕ per sample. The results were displayed by the ratio between CCL3-producing Mϕ and CCL17-producing Mϕ.

Statistical analysis

Data are presented as mean ± SEM. Comparisons of the results between the experimental and control groups were made by ANOVA. Analysis was accomplished using Statview 4.5 (Abacus Concepts, Berkeley, CA). If a p value was <0.05, the result was considered to be significant.

Results

Inhibition of CAMϕ generation by AAMϕ or AAMϕ products

The inhibitory effect of AAMϕ on CAMϕ generation was examined. Three kinds of Mϕ populations were used in these experiments: RMϕ, freshly isolated peritoneal Mϕ from normal mice; CAMϕ, RMϕ stimulated with CpG DNA; AAMϕ, peritoneal Mϕ from thermally injured mice. As shown in Table I, the three Mϕ populations used in this study exhibited typical properties for RMϕ, CAMϕ, and AAMϕ, respectively. In the following studies, Mϕ with the ability to produce CCL3 are considered CAMϕ, because CCL3 has been described as a typical chemokine specifically produced by CAMϕ (4, 5). As shown in Table II, CAMϕ were not found in cultures of RMϕ stimulated with CpG DNA when they were cocultured with AAMϕ at percentages of 39% or more. In addition, no CAMϕ were generated when RMϕ (lower chamber) were cultured with AAMϕ (upper chamber) in a dual-chamber Transwell supplemented with CpG DNA (Fig. 1*A*). In the presence of CpG DNA, conversion of RMϕ to CAMϕ drastically decreased when the cultivation was performed with AAMϕ Culture-Sup (culture supernatants of 1×10^6 cells/ml AAMϕ 24 h after cultivation, 15%, v/v; Fig. 1*B*). Similar results were obtained when standard AAMϕ induced by a mixture of IL-4 and IL-13 or culture fluids of these Mϕ were subjected to the same test (Fig. 1). The results shown in Table II and Fig. 1 indicate that the ability of AAMϕ to inhibit CAMϕ generation from RMϕ stimulated with CpG DNA is evidenced by the soluble factors released from AAMϕ.

Table II. *Inhibitory effect of AAMϕ on the CAMϕ generation in cocultivation*[a]

No. of AAMϕ/ No. of RMϕ × 100[b]	CCL3 (pg/ml)	
	Media	CpG DNA
1	<18	215
4	<18	158
16	<18	64
64	<18	<18

[a] In the presence or absence of CpG DNA (10 μg/ml), RMϕ were cocultured with AAMϕ at a ratio of 1:100 to 64:100. Twenty-four hours after cultivation, cells harvested were washed three times and recultured for 24 h. Culture fluids obtained were assayed for CCL3 by ELISA.

[b] One $\times 10^5$ cells/well RMϕ were mixed with 1–64 $\times 10^3$ cells/well AAMϕ.

Determination of soluble inhibitory factors released from AAMϕ

To determine which components inhibited CAMϕ generation, AAMϕ were cultured at the cell density of 1×10^6 cells/ml for 24

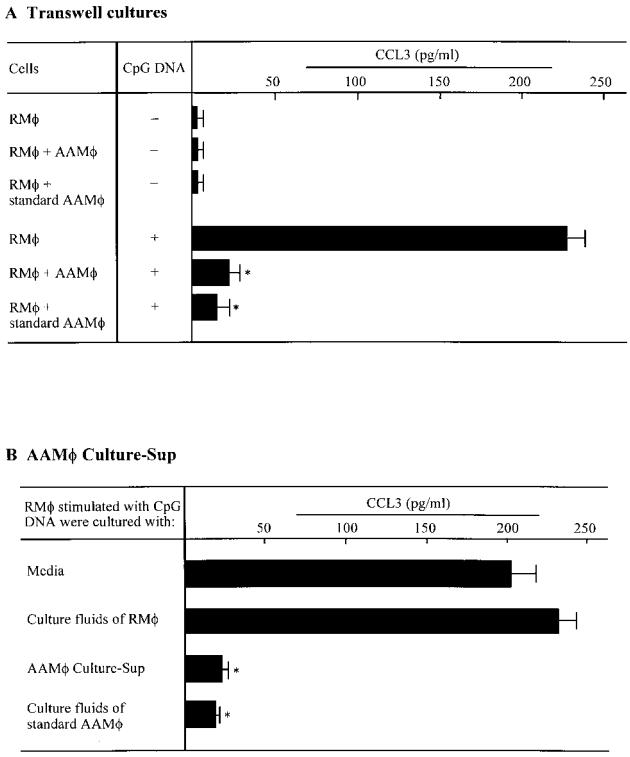

FIGURE 1. Effect of AAMϕ or AAMϕ culture fluids on CAMϕ generation from RMϕ stimulated with CpG DNA. *A*, Transwell cultures: in the presence of CpG DNA (10 μg/ml), RMϕ (6×10^5 cells/well, lower chamber) were cultured with AAMϕ from burn mice (5×10^5 cells/well, upper chamber) in a dual-chamber Transwell. As a control, standard AAMϕ induced by a mixture of IL-4 and IL-13 were added to the same assay system. Twenty-four hours after the initial cultivation, Mϕ in the lower chamber were harvested and cultured for an additional 24 h. The culture fluids were assayed for CCL3 using ELISA. *B*, AAMϕ Culture-Sup: in the presence of CpG DNA, RMϕ (1×10^6 cells/ml) were cultured with AAMϕ Culture-Sup (15%, v/v) for 24 h. AAMϕ Culture-Sup was composed of culture fluids of 1×10^6 cells/ml burn-associated AAMϕ 24 h after cultivation. As a control, culture fluids of standard AAMϕ (1×10^6 cells/ml, 24 h) were added to the same assay system. Mϕ were washed and cultured for an additional 24 h. The culture fluids harvested were assayed for CCL3. *, $p < 0.001$ compared with RMϕ cultured with CpG DNA.

or 48 h. The culture fluids were assayed for various cytokines and PGE_2, which have been described as typical products of Mϕ or Mϕ-related cells (4, 5, 16). As a control, the culture fluids of RMϕ were used for the experiments. The results are shown in Fig. 2. PGE_2, CCL17, IL-6, IL-10, and TGF-β were all found in AAMϕ culture fluids. In addition, almost the same amount of PGE_2 and TGF-β was detected in the culture fluids of RMϕ. These results suggest that CCL17, IL-6, and IL-10 all may inhibit the generation of CAMϕ from RMϕ stimulated with CpG DNA.

Therefore, the next series of experiments tested the ability of cytokines to inhibit the generation of CAMϕ from RMϕ stimulated with CpG DNA. In these experiments, various doses of murine rCCL17, rIL-10, and rIL-6 were individually added to cultures of RMϕ stimulated with CpG DNA. rIL-10 at a dose of 100 pg/ml or more inhibited CAMϕ generation (Fig. 3). CAMϕ generation was also inhibited by rCCL17 at doses ranging from 20 to 100 pg/ml. The maximum inhibition was demonstrated when 20 pg/ml rCCL17 was added to cultures of RMϕ stimulated with CpG DNA. However, rIL-6 at doses ranging from 20 to 1000 pg/ml did not inhibit CAMϕ generation. Since 500–600 pg/ml IL-10 and CCL17 were detected in AAMϕ-Culture Sup (culture fluids of AAMϕ 24 h after cultivation, Fig. 2) and 15% (v/v) of AAMϕ Culture-Sup was added to RMϕ cultures (Fig. 1*B*), the individual amounts (75–90 pg/ml) of IL-10 or CCL17 contained in this assay system were not enough to completely inhibit CAMϕ generation.

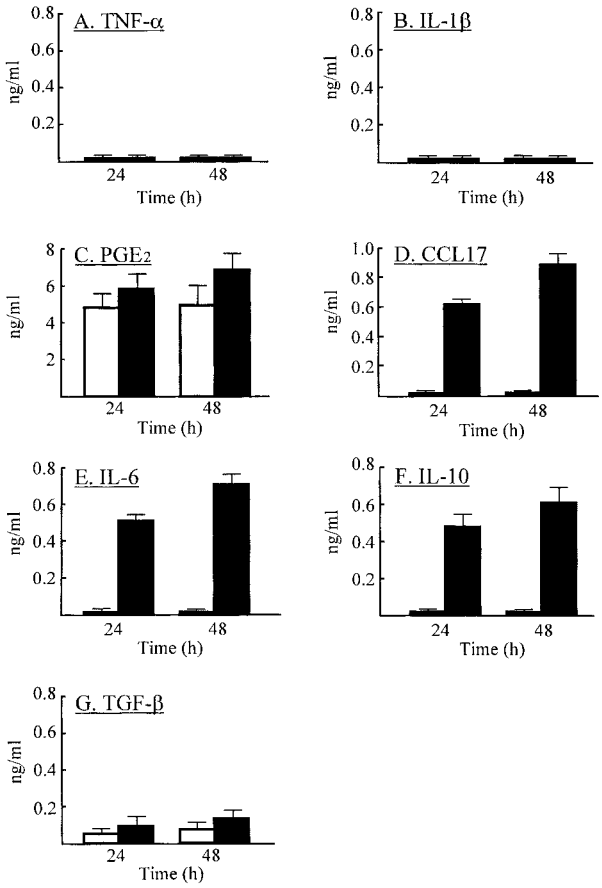

FIGURE 2. Various soluble factors detected in culture fluids of AAMϕ. At a cell density of 1×10^6 cells/ml, AAMϕ (■) or RMϕ (□) were cultured without any stimulation. The culture fluids harvested 24–48 h after cultivation were assayed for the soluble factors (listed below), that were known to be products of Mϕ or Mϕ-related cells, using ELISA. TNF-α (*A*), IL-1β (*B*), PGE_2 (*C*), CCL17 (*D*), IL-6 (*E*), IL-10 (*F*), and TGF-β (*G*).

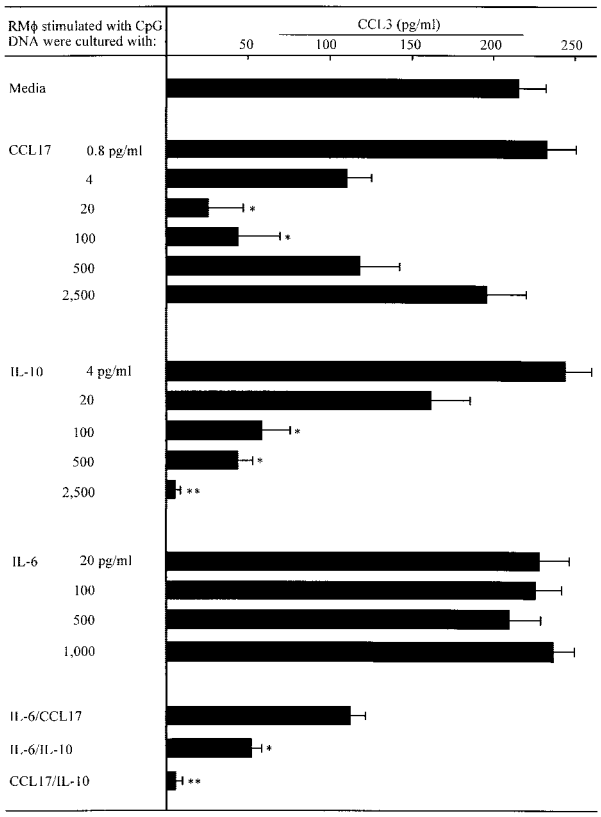

FIGURE 3. Effect on CAMϕ generation of the soluble factors released by AAMϕ. In the presence of CpG DNA, RMϕ (1×10^6 cells/ml) were cultured with various doses of rCCL17, rIL-10, and rIL-6 or a 100-pg/ml dose each of cytokine mixture for 24 h. In addition, a mixture of the cells was washed and cultured for an additional 24 h. Then, the culture fluids harvested were assayed for CCL3. *, $p < 0.05$ and **, $p < 0.001$ compared with RMϕ cultured with CpG DNA.

When a mixture of rIL-10 and rCCL17 (100 pg/ml each) was added to cultures of RMϕ stimulated with CpG DNA, CAMϕ generation was completely inhibited (Fig. 3). These results suggest that both IL-10 and CCL17 contained in AAMϕ Culture-Sup play a cooperative role in inhibiting CAMϕ generation. These results were reproduced when the experiment was performed with AAMϕ Culture-Sup and mAbs directed against CCL17 and IL-10. As shown in Fig. 4, AAMϕ Culture-Sup inhibited CAMϕ generation from RMϕ (89% inhibition). This activity of AAMϕ Culture-Sup was not completely abrogated when it was treated with anti-IL-10 mAb (clone 110904) and anti-CCL17 mAb (clone JES5-2A5) individually. Anti-IL-10 mAb treatment eliminated the activity of AAMϕ Culture-Sup by 15%. Anti-CCL17 mAb treatment eliminated the activity by 50%. AAMϕ Culture-Sup treated with isotype control Ab inhibited CAMϕ generation from RMϕ (90% inhibition). When AAMϕ Culture-Sup was treated with a mixture of mAbs for IL-10 and CCL17, the ability of AAMϕ Culture-Sup to inhibit CAMϕ generation was eliminated by 78%. These results indicate that IL-10 and CCL17 may cooperatively inhibit CAMϕ generation. Data shown in Figs. 3 and 4 suggest that small amounts of CCL17 released from AAMϕ early after cultivation may have an influence on IL-10 production from AAMϕ or induce expanded expression of IL-10R on RMϕ.

Appearance of CAMϕ or AAMϕ in various RMϕ cultures

In the presence or absence of CpG DNA, RMϕ (lower chamber) were cultured with AAMϕ (upper chamber) in a dual-chamber

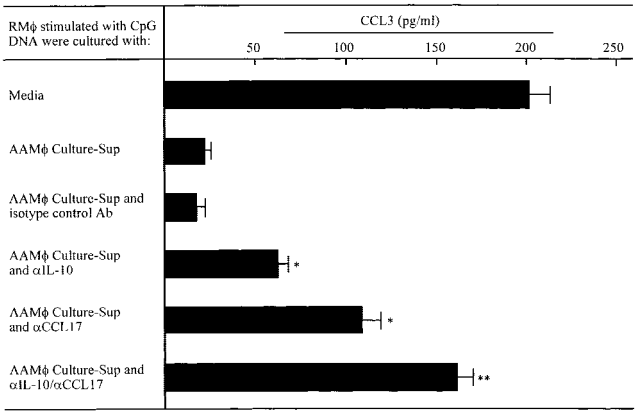

FIGURE 4. Effect of mAbs directed against IL-10 and CCL17 on the ability of AAMϕ Culture-Sup to inhibit CAMϕ generation from RMϕ stimulated with CpG DNA. RMϕ (1×10^6 cells/ml) stimulated with CpG DNA were cultured with AAMϕ Culture-Sup that had been previously treated with neutralizing mAb for IL-10 (50 ng/ml), CCL17 (500 ng/ml), or their mAb mixture. As a control, AAMϕ Culture-Sup was treated with isotype control Ab (500 ng/ml). Twenty-four hours after cultivation, the cells were washed and cultured for an additional 24 h. The culture fluids harvested were assayed for CCL3 using ELISA. *, $p < 0.05$ and **, $p < 0.01$ compared with AAMϕ Culture-Sup treated with isotype control Ab.

Cells	CpG DNA	Number of cells to produce : CCL3	Number of cells to produce : CCL17	CCL3-producing Mϕ : CCL17-producing Mϕ
RMϕ	-			1 : 0.7
AAMϕ	-			1 : 122
RMϕ + AAMϕ	-			1 : 106
RMϕ	+			149 : 1
RMϕ + AAMϕ	+			1 : 102
RMϕ + AAMϕ plus mAbs for IL-10/CCL17	+			124 : 1

FIGURE 5. Appearance of CAMϕ or AAMϕ in various RMϕ cultures. In the presence or absence of CpG DNA, RMϕ (6×10^5 cells/well, lower chamber) were cultured with AAMϕ (5×10^5 cells/well, upper chamber) in a dual-chamber Transwell supplemented with or without a mixture of mAbs for IL-10 (50 ng/ml) and CCL17 (500 ng/ml). As a control, AAMϕ (6×10^5 cells/well, lower chamber) were cultured in Transwells with medium (upper chamber). Twenty-four hours after cultivation, Mϕ were harvested from the lower chamber, and the numbers of CCL3- or CCL17-producing Mϕ in these Mϕ populations were measured using the ELISPOT assay. The results are displayed by ratios between CCL3-producing Mϕ and CCL17-producing Mϕ.

Transwell supplemented with or without a mixture of mAbs for IL-10 (50 ng/ml) and CCL17 (500 ng/ml). Mϕ harvested from the lower chamber 24 h after cultivation were assayed using ELISPOT to determine the number of CCL3-producing Mϕ or CCL17-producing Mϕ. As shown in Fig. 5, CCL3-producing Mϕ were not found in the lower chamber of the Transwell (RMϕ stimulated with CpG DNA) 24 h after cultivation with AAMϕ (upper chamber). A majority of cells in the lower chamber was shown to be CCL17-producing Mϕ. However, a majority of cells in the lower chamber (RMϕ stimulated with CpG DNA) converted to CCL3-producing Mϕ when Transwell cultures were performed with AAMϕ (upper chamber) in the presence of a mixture of mAbs directed against CCL17 and IL-10. These results indicate that CCL3-producing Mϕ and CCL17-producing Mϕ are not mixed with each other in the same cell populations.

Discussion

CAMϕ have been described as effector cells for the host's innate immunities (6–8). It has been suggested that individuals whose AAMϕ predominate, such as severely burned patients, are susceptible to various opportunistic pathogens due to impaired CAMϕ generation (5, 16, 19). Therefore, the mechanism by which AAMϕ inhibit CAMϕ generation was investigated using RMϕ (peritoneal Mϕ from normal mice), AAMϕ (peritoneal Mϕ from burn mice), and CAMϕ (RMϕ stimulated with CpG DNA). As shown in Table I, these Mϕ preparations were typical cellular populations for RMϕ, CAMϕ, and AAMϕ. When RMϕ stimulated with CpG DNA were cocultured with AAMϕ, CAMϕ generation was clearly suppressed depending on the percentage of AAMϕ added to the cocultures. In addition, RMϕ stimulated with CpG DNA did not convert to CAMϕ when they (lower chamber) were cultured with AAMϕ (upper chamber) in a dual-chamber Transwell. A recent report has noted that AAMϕ suppress mitogen-stimulated lymphocytic proliferations through cell-cell contact, implicating a receptor-mediated mechanism (33). However, the results in this study indicated that cell-cell contact between AAMϕ and RMϕ was not necessary for AAMϕ to inhibit the generation of CAMϕ. Furthermore, in the presence of CpG DNA, RMϕ did not convert to CAMϕ when they were cultured with culture fluids of AAMϕ (AAMϕ Culture-Sup). Since IL-6, IL-10, and CCL17 were specifically detected in AAMϕ Culture-Sup, recombinant murine preparations of these cytokines were tested for their ability to inhibit CAMϕ generation. The results showed that only IL-10 and/or CCL17 were shown to inhibit CAMϕ generation from RMϕ stimulated with CpG DNA, while IL-6 did not. TGF-β was found in the culture fluids of RMϕ and AAMϕ. The various immunosuppressive activities of TGF-β have already been documented (34, 35). NO production and arginase induction by Mϕ were inhibited when >1 ng/ml TGF-β was added to the cultures (36). In these results, however, the amount of TGF-β detected in the culture fluids of AAMϕ was <100 pg/ml. This amount of TGF-β may not represent inhibitory activity on CAMϕ generation. Together, all of these results indicate that AAMϕ inhibit the generation of CAMϕ from RMϕ by producing IL-10 and CCL17.

Recently, suppressor of cytokine signaling (SOCS) 1 and SOCS3 were described as key inhibitors of Mϕ activation (37, 38). Especially, IL-10 has been described as an activator of SOCS3, which inhibits the Mϕ activation stimulated by LPS (39). In addition, IL-6 has been identified as an activator of SOCS3 (37, 38). In this study, however, IL-6 did not inhibit CAMϕ generation. These findings appear to stem from the different sensitivities of IL-10- and IL-6-induced signal transduction toward inhibitory mechanisms. SOCS3 inhibits IL-6-induced signal transduction; however, IL-10-induced signal transduction is not inhibited by SOCS3 (38, 40). The role played by CCL17 in the induction of

SOCS3 is not known. The effect of this chemokine on the various functions of Mϕ is also unknown. However, the results of this study show that CCL17 may inhibit the immunobiological functions expressed by CAMϕ. CCL17 has been suggested as an effector molecule on Th2 responses associated with AAMϕ (41, 42). CCL17 migrates Th2 cells into inflammatory sites through the CCR4 displayed by Th2 cells (43, 44). Th2 responses enhanced by AAMϕ stimulate further generation of AAMϕ by producing IL-4, IL-10, and IL-13. These reactions severely impair the generation of CAMϕ and Th1 cells. Actually, in an OVA-induced murine asthma model, anti-CCL17 mAb treatment reduced the production of Th2 cytokines (IL-4 and IL-13) in bronchoalveolar lavage fluids (45). Further studies are required to explore the inhibitory mechanism of CCL17 on CAMϕ generation.

CpG DNA has been shown to activate dendritic cells, B cells, and NK cells as well as Mϕ (46, 47). In this study, we used CpG DNA as an inducer of CAMϕ, because RMϕ treated with CpG DNA produced CCL3, CCL5, and IL-12, expressed iNOS mRNA, showed strong killing activity against *S. aureus*, and induced Th1 responses (24). We have also reported that all of these parameters shown by CAMϕ were not significantly demonstrated when RMϕ were cultured with AAMϕ (peritoneal Mϕ from burn mice, RMϕ treated in vitro with a mixture of IL-4 and IL-13) (24). Among these properties shown by CAMϕ, in this article we presented CCL3 production as a representative maker of CAMϕ. In addition to CpG DNA, LPS, dsRNA, and IFN-γ have been reported to induce CAMϕ generation (5, 11, 48). In our preliminary studies, when RMϕ were cultured with AAMϕ in the presence of LPS (10 ng/ml), dsRNA (2 μg/ml), or IFN-γ (1 ng/ml), CAMϕ were not generated. In contrast, AAMϕ, induced by α_1 acid glycoprotein (15), a mixture of IL-4 and IL-13 (24) or glucocorticoids (49), equally inhibited CAMϕ generation from RMϕ stimulated with CpG DNA. These results indicate that the AAMϕ, which appeared in various patients, might randomly inhibit the generation of CAMϕ from RMϕ. Thus, individuals with AAMϕ predominating are nonspecifically susceptible to wide-ranging foreign invasions.

In this article, TNF-α, IL-1β, IL-6, PGE_2, IL-10, CCL17, and TGF-β were measured as possible soluble factors released from AAMϕ. TNF-α, IL-1β, and IL-6 have been described as the products of Mϕ or Mϕ-related cells (4, 5, 16). PGE_2, IL-10, and TGF-β have also been described as Mϕ products with immunosuppressive activities (16). Therefore, we tested these soluble factors as possible effector molecules of AAMϕ. Also, we tested CCL17 as a possible effector molecule of AAMϕ. CCL17 and CCL22 have been described as chemokines equally associated with the induction of Th2 responses (1). This means there is a possibility that these two chemokines may be equally produced by AAMϕ and equally cooperate with IL-10 on inhibiting CAMϕ generation. However, due to availability, in this study we only measured CCL17. Further studies for CCL22 will be required.

In conclusion, CCL17 and IL-10 released from AAMϕ were shown to inhibit CAMϕ generation from RMϕ stimulated with CpG DNA. The impairment of the host's innate immunities associated with the function of CAMϕ may be remedied by controlling AAMϕ or AAMϕ products (CCL17 and IL-10).

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