CCL16/LEC powerfully triggers effector and antigen-presenting functions of macrophages and enhances T cell cytotoxicity

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Abstract: The human CC chemokine CCL16, a liver-expressed chemokine, enhances the killing activity of mouse peritoneal macrophages by triggering their expression of tumor necrosis factor α (TNF-α) and Fas ligand. Macrophages also respond to CCL16 by enhancing their production of monocyte chemoattractant protein-1, regulated on activation, normal T cells expressed and secreted chemokines, and interleukin (IL)-1β, TNF-α, and IL-12. The effect of CCL16 is almost as strong as that of lipopolysaccharide and interferon-γ, two of the best macrophage activators. Moreover, CCL16-activated macrophages overexpress membrane CD80, CD86, and CD40 costimulatory molecules and extensively phagocytose tumor cell debris. On exposure to such debris, they activate a strong, tumor-specific, cytolytic response in virgin T cells. Furthermore, cytolytic T cells generated in the presence of CCL16 display a higher cytotoxicity and activate caspase-8 in tumor target cells. This ability to activate caspase-8 depends on their overexpression of TNF-α and Fas ligand induced by CCL16. These data reveal a new function for CCL16 in the immune-response scenario. CCL16 significantly enhances the effector and the antigen-presenting function of macrophages and augments T cell lytic activity. *J. Leukoc. Biol.* 75: 135–142; 2004.

Key Words: *tumor antigen uptake · T cell priming · costimulatory molecules*

INTRODUCTION

Chemokines are structurally related polypeptides. They were initially interpreted as leukocyte chemoattractants promoting endothelial cell adhesion and transmigration of various leukocyte subsets [1] but have since been shown to modulate a larger array of biological functions, including enzyme release, cell–cell adhesion, cytotoxicity, and tumor cell growth [2–6]. They are divided into four classes, namely CC, CXC, C, and CXXXC chemokines, according to the position of the first two cysteines in their protein sequence. The human CC chemokine CCL16/liver-expressed chemokine was originally found in an expressed sequence tag library, and its gene was located in the CC chemokine cluster on chromosome 17. Its genomic organization [7] and amino acid sequence [8] have been determined. Two sizes of CCL16 mRNA, 1.8 and 0.8 kb, are expressed by unstimulated liver [9]. The shorter messenger is detected in most lymphoid tissue, whereas the larger only appears after stimulation with interleukin (IL)-10 [10]. Both messengers encode a 120 amino acid (aa) protein, including a signal peptide that shows 19–38% identity with other human CC chemokines [8]. Thus, CCL16 is an unusually large chemokine expressed by a relatively restricted kind of cell, and its functional role cannot be readily predicted. There are few reports on its biological activity. It is chemotactic for human monocytes, lymphocytes, and dendritic cells [10–12]. CCR1 has been identified as a functional CCL16 receptor, although CCL16 also interacts with CCR8 [13] or CCR2 and CCR5 [14]. We have shown that CCL16 elicits a potent in vivo activity against a poorly immunogenic adenocarcinoma (TSA) of spontaneous origin [15, 16]. TSA cells have been engineered to release many distinct cytokines and chemokines [17, 18]. CCL16 was easily the best elicitor of an effective reaction that led to tumor rejection and very rapid induction of a TSA-specific immune response of peculiar efficacy [15]. To dissect the mechanisms of this powerful action, we studied its effect on macrophage effector functions, antigen presentation, and cytotoxic T lymphocyte (CTL) induction. We report here that recombinant CCL16 induces murine macrophages to become more potent killers, secrete downstream chemokines and cytokines, and express costimulatory molecules on their membrane and enhances their antigen uptake and ability to specifically activate CTLs. It also enhances CTL activity by inducing the overexpression of tumor necrosis factor (TNF)-α and Fas ligand (FasL) on the CTL membrane.

Its recruitment and maturation of antigen-presenting cells (APCs) in vivo [15], combined with its enhancement of many crucial macrophage functions, account for the unique ability of

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CCL16 to positively modulate effector functions and induce a marked memory response.

MATERIALS AND METHODS

Mice

Seven-week-old female BALB/cAnCr (H-2d) mice (Charles River Laboratories, Calco, Italy) were treated in accordance with the European Union guidelines.

Tumor cells

TSA is an aggressive and poorly immunogenic cell line established from a moderately differentiated mammary adenocarcinoma that arose spontaneously in a multiparous BALB/c mouse [19]. TSA cells express major histocompatibility complex class I (MHC I) but not class II molecules and secrete granulocyte-colony stimulating factor (G-CSF) and granulocyte macrophage-CSF, transforming growth factor-β [16], basic fibroblast growth factor, and vascular endothelial growth factor [20] but not CCL16 (not shown). They do not stimulate a syngeneic antitumor response in vivo nor a proliferative and cytokine release in mixed lymphocyte-tumor cell interaction (MLTI) [16]. F1-F is a newborn BALB/c mouse-derived skin fibroblast cell line that spontaneously transformed after the 15th in vitro passage [15]. Its cells do not immunologically cross-react with TSA and were therefore used as the control target in cytotoxic assays.

CCL16 source

The human recombinant CCL16 used in these experiments (a gift from PeproTech Inc., Rocky Hill, NJ) is an 11.2-kDa protein of 97 aa residues produced in *Escherichia coli* from a DNA sequence encoding the mature human CCL16 protein sequence (Q26–Q120) [10]. Its endotoxin level is less than 0.1 ng per μg, as determined by the limulus amoebocyte lysate method. In a few experiments, it was compared with another commercial preparation from R&D Systems (Minneapolis, MN). As the results were identical, only the data obtained with CCL16 from PeproTech Inc. are shown for the sake of simplicity.

Killing activity by macrophages

To evaluate the effect of CCL16 on the killing ability of resident macrophages, the peritoneal cavity of normal mice was washed three times with 5 ml RPMI-1640 medium (BioWhittaker, Walkersville, MD) supplemented with 10 U/ml heparin (Parke-Davis, Lainate, Italy). Differential cell counts on Diff-Quick (Hartleco, Gibbstown, NY)-stained smears showed that this population consists of 55–65% macrophages. To obtain macrophage enrichment, 50 μl of a peritoneal cell suspension containing 2×10^5 cells was plated in the first three wells of a round-bottom, 96-well plate, diluted, and allowed to adhere for 2 h at 37°C in a 5% CO_2 atmosphere. Nonadherent cells were removed by vigorous washes with RPMI-1640 medium. Adherent cells (peritoneal macrophages) were incubated with 5×10^3 ^{3}H thymidine (Amersham, Milan, Italy)-labeled TSA target cells at 20:1, 10:1, 5:1, and 2.5:1 effector:target (E:T) ratios in a final volume of 200 μl RPMI 1640–10% fetal bovine serum (FBS; Hyclone serum-defined, Celbio, Milan, Italy) in the absence or presence of different concentrations of recombinant human CCL16 (PeproTech Inc.), interferon (IFN)-γ (10 ng/ml; PeproTech Inc.), and lipopolysaccharide (LPS) (1 μg/ml; *E. coli* 0111:B4, Sigma, Milan, Italy) as positive control.

In some experiments, neutralizing anti-human CCL16 (1 μg/ml; R&D Systems), monoclonal antibody (mAb), anti-human FasL (1.5 μg/ml; Alexis c/o Vinci-Biochem, Florence, Italy), and anti-mouse TNF-α (0.4 μg/ml; R&D Systems) mAb were added during the assay. After 48 h, specific lysis was determined, and the values were expressed as percentages of specific lysis and as lytic unit $(LU)_{20}/10^7$ effector cells, calculated as described previously [16].

To assess the purity of macrophages in adherent cell populations, 4×10^6 peritoneal cells in 1 ml RPMI-1640 medium were allowed to adhere to glass-base dishes (Iwaki, Bibby Sterilyn, Milan, Italy) for 2 h at 37°C in a 5% CO_2 atmosphere. Nonadherent cells were removed by vigorous washes with cold RPMI-1640 medium, and adherent cells were stained with phycoerythrin (PE)-labeled anti-mouse CD14 mAb (PharMingen, Milan, Italy) or fluorescein isothiocyanate (FITC)-labeled anti-mouse CD11c (Miltenyi Biotech, Calderara di Reno, Bologna, Italy) for 30 min at 4°C after a 20-min preincubation with an excess of mouse immunoglobulin G (DakoCytomation, Milan, Italy) and were examined with a confocal microscope (LFM 310, Zeiss, Jena, Germany; 488 nm argon laser and 543 nm helium-neon laser). Images were recorded as TIF files and processed (LSM Image Examiner, Zeiss) to subtract background and enhance lower-middle intensity fluorescence. Over 99% of these adherent cells were CD14$^+$-positive and hence, classed as peritoneal macrophages. There were no CD11c$^+$ cells.

Chemokine and cytokine production by peritoneal macrophages

Peritoneal macrophages (4×10^6) were incubated in a final volume of 2 ml RPMI 1640–10% FBS in the absence or presence of CCL16 (1–100 ng/ml), IFN-γ (10 ng/ml, PeproTech Inc.), and LPS (1 μg/ml, Sigma) for 72 h at 37°C in a 5% CO_2 atmosphere. Supernatants were then analyzed by enzyme-linked immunosorbent assay (ELISA) for monocyte chemoattractant protein (MCP)-1/CCL2, macrophage inflammatory chemokine-1α/CCL3, regulated on activation, normal T cells expressed and secreted/CCL5, IL-12, IL-18, IL-1β, and TNF-α (all from R&D Systems), following the manufacturer's instructions.

Immunocytochemistry

Peritoneal macrophages (4×10^6) were incubated in RPMI 1640–10% FBS in the absence or presence of 100 ng/ml CCL16 for 72 h at 37°C in a 5% CO_2 atmosphere. The slides were washed with phosphate-buffered saline (PBS) and incubated with normal goat serum (1:200, Cedarlane, Celbio) for 20 min at 4°C. Unwashed slides were incubated with various FITC- or PE-conjugated mAb for 30 min at 4°C. After several washes with PBS, the slides were mounted and observed with a confocal microscope (Zeiss). mAb used were anti-mouse CD11b PE, anti-mouse CD80 FITC, anti-mouse CD86 FITC, anti-mouse CD40 FITC, and anti-mouse FasL FITC (all purchased from PharMingen).

To evaluate Fas expression by TSA cells, fresh cells were washed in PBS after trypsin detachment, stained with a FITC-conjugated anti-mouse Fas mAb (PharMingen), and then examined with the confocal microscope.

Apoptotic tumor cell uptake by peritoneal macrophages

To assess this uptake, TSA cells were stained with 5 μM lipophilic fluorochrome 5,6-carboxyfluorescein diacetate succinimidyl ester (CFSE; Molecular Probes, Poorgebouw, Netherlands) for 20 min in PBS at 37°C in a 5% CO_2 atmosphere and were washed four times in RPMI-1640–10% FBS medium before induction of apoptosis. After 24 h of incubation in complete medium to allow tumor cells to undergo apoptosis, 4×10^6 apoptotic TSA cells were cocultured with 4×10^6 peritoneal macrophages in the absence or presence of 100 ng/ml CCL16 in glass-base dishes (Iwaki). Two, 18, and 72 h later, peritoneal macrophages were abundantly washed and stained with PE-labeled anti-mouse CD11b mAb (PharMingen) for 30 min at 4°C before examination with the confocal microscope.

Apoptosis induction

TSA cells cultured in RPMI-1640–10% FBS were irradiated with 1500 $\mu W/cm^2$ ultraviolet B (UVB; 6W, Vilber Lourmat, Marne La Vallee, France) for 15 min. To minimize the UVB-absorbing effect of phenol red in RPMI 1640, the medium level was reduced to a minimum during irradiation. Apoptosis was detected by propidium iodide (10 μg/ml, Sigma) staining after 24 h of incubation in complete medium to allow cells to undergo apoptosis. Ten thousand events were acquired and analyzed by flow cytometry (FACScan, Becton Dickinson, Milan, Italy). TSA cells (98–99%) were apoptotic.

Priming of naive T cells

Peritoneal macrophages (4×10^6) were incubated with 4×10^6 apoptotic TSA cells (as described above) in the absence or presence of 100 ng/ml CCL16 for 72 h at 37°C in a 5% CO_2 atmosphere. After washing with RPMI-1640 medium, pulsed peritoneal macrophages were overlaid with 12×10^6 spleen cells (Spc) from normal, syngeneic BALB/c mice. After 6 days of culture, CTL were recovered and assayed for cytotoxic activity as described below.

T cell cytotoxic activity

Effector lymphocytes were generated from MLTI by culturing 1×10^7 responder Spc with 5×10^5 mitomycin-C (Mit-C; Sigma)-treated stimulator TSA cells for 6 days in the absence or presence of scalar doses (1–100 ng/ml) of CCL16. CTL activity was assayed in a 48-h ^{3}H thymidine-release assay [16] by mixing effector lymphocytes with 5×10^3-labeled target cells at 50:1, 25:1, 12:1, and 6:1 E:T ratios in round-bottom, 96-well plates in triplicate. Values were expressed as percentages of specific lysis, and $LU_{20}/10^7$ effector cells were calculated as described previously [16]. In some experiments, neutralizing anti-mouse TNF-α (0.4 μg/ml, R&D Systems) and anti-human FasL (1.5 μg/ml, Alexis) mAb were added to the effectors to evaluate their involvement in the cytotoxicity.

Caspase activation-detection assay

To determine whether CCL16 improves CTL activity generation by increasing the proapoptotic arms, the activation of caspase-8 in target cells was examined. Lymphocytes (50×10^6) from 6-day MLTI generated in the absence or presence of 10 ng/ml CCL16 were incubated with TSA target cells in six-well plates in RPMI-1640–10% FBS at an E:T ratio of 25:1 for 3 h at 37°C in a 5% CO_2 atmosphere. Next, CTL were removed, and activation of caspase-8 in TSA cells was assayed colorimetrically with a commercial kit (R&D Systems). Briefly, TSA cells were treated with trypsin and collected by centrifugation. The pellets were lysed through a buffer for 15 min at 4°C. Protein extracts (200 μg) were incubated with the substrate in flat-bottom, 96-well plates for 1 h at 37°C, and caspase-8 activation was evaluated at 405 nm with an ELISA microplate reader (Bio-Rad, Richmond, CA). Changes in activation are expressed as fold increases over that of TSA cells cultured in medium only.

Statistical analysis

The significance of differences in LU obtained from the ^{3}H thymidine-release assay, chemokine secretion, and fold increase in caspase-8 activation was evaluated with a two-sample Student's *t*-test.

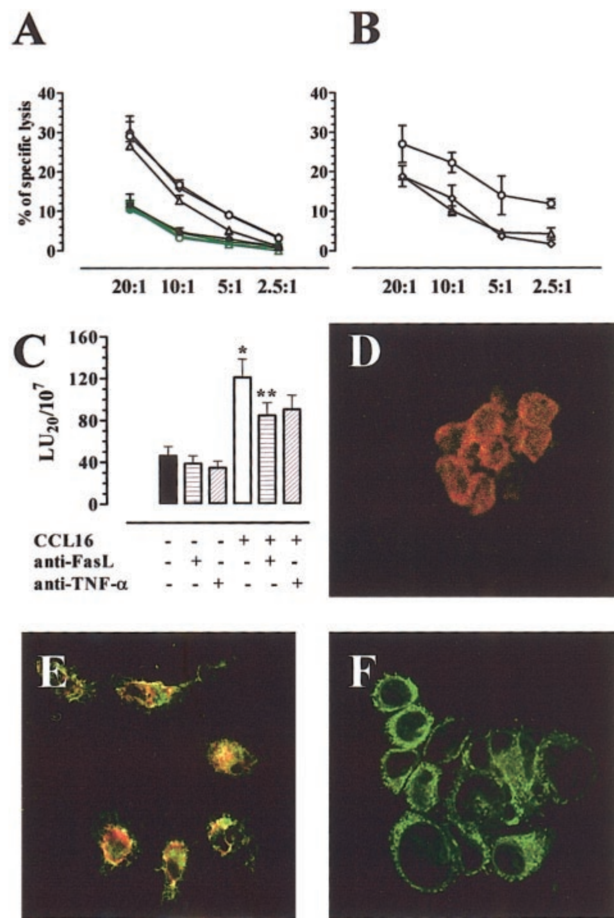

Fig. 1. Effector functions of peritoneal macrophages in the presence of CCL16. (A) Killing ability of peritoneal macrophages from normal BALB/c mice was assessed against ^{3}H thymidine-labeled TSA target cells in only medium (●) and with 1 (△), 10 (○), or 100 ng/ml (◇) CCL16 in the absence (black line) and in the presence (green line) of anti-human CCL16 polyclonal Ab. (B) As positive control, 10 ng/ml IFN-γ (△), 1 μg/ml LPS (◇), and both (○) were added to macrophage culture assayed against ^{3}H thymidine-labeled TSA target cells. The percentages of specific lysis are expressed as mean ± SEM from three independent experiments. Where vertical bars are not appreciable, the SEM is smaller than the size of the symbols. (C) Peritoneal macrophages were incubated during a ^{3}H thymidine release assay with neutralizing anti-human FasL (horizontal-lined bars) or anti-mouse TNF-α (diagonal bars) mAb in the absence and presence of 10 ng/ml CCL16. The values of their killing activity are expressed as a mean ± SEM $LU_{20}/10^7$ effector cells obtained from two independent experiments. *, Values significantly different from those evaluated by peritoneal macrophages cultured in the absence of CCL16 ($P<0.01$); **, in the presence of CCL16 ($P<0.05$). (D and E) Peritoneal macrophages from normal BALB/c mice were cultured for 72 h in the absence or presence of 100 ng/ml CCL16, respectively, and then processed for confocal analysis. Positivity to anti-mouse CD11b mAb and anti-mouse FasL mAb is shown in red and green, respectively. (F) Fresh TSA cells were analyzed with confocal microscope for Fas expression.

RESULTS

CCL16 enhances the killing ability of peritoneal macrophages

Peritoneal macrophages cultured for 48 h in medium only killed TSA tumor-target cells at low levels. Their killing ability was doubled or tripled when the medium was supplemented with 1–10 ng/ml CCL16 (**Fig. 1A**). No further enhancement was seen with tenfold more CCL16, as is usual with many cytokines and chemokines that display a bell-shaped stimulation profile. A substantial contribution on the part of contaminating endotoxin can be ruled out, as no enhancement was found when CCL16 was admixed with anti-human CCL16 mAb (Fig. 1A) or boiled (data not shown) before its addition.

To calibrate this strong potentiation of macrophage killing ability, enhancement by CCL16 was compared with that provided by IFN-γ and LPS, two of the best activators of macrophage functions [21, 22]. Addition of 10 ng/ml IFN-γ or 1 μg/ml LPS enhanced macrophage killing ability to the same extent as 1 ng/ml CCL16 (Fig. 1B). The strongest enhancement was observed when the medium was supplemented with IFN-γ plus LPS, although it was similar to that provided by 10–100 ng/ml CCL16 when macrophage killing ability was tested at higher E:T ratios (Fig. 1, A and B).

mAb against TNF-α and FasL were added to the culture medium during the 48-h killing assay to test their involvement in CCL16 enhancement. Addition of anti-TNF-α and FasL mAb did not impair the lytic activity of macrophages cultured in medium only but markedly reduced the enhanced activity observed in the presence of 10 ng/ml CCL16 (Fig. 1C).

To assess whether the inhibitory activity of FasL mAb agreed with an enhanced FasL expression induced by CCL16, macrophages were cultured for 72 h in the absence or presence of CCL16, and their expression of FasL was assessed with the confocal microscope. Macrophages cultured in medium only were not stained by specific anti-FasL mAb (Fig. 1D), whereas

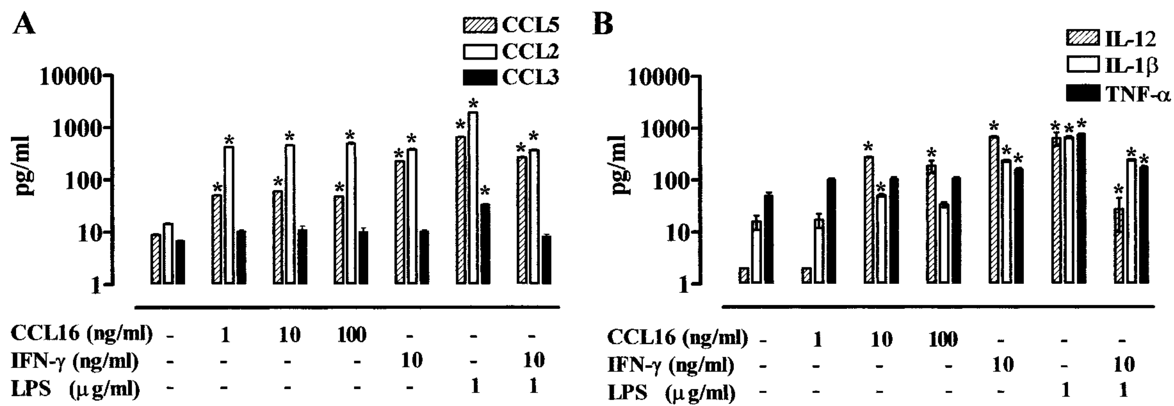

Fig. 2. Chemokine and cytokine release by peritoneal macrophages in the presence of CCL16. Peritoneal macrophages were cultured for 72 h in the absence or presence of 1–100 ng/ml CCL16, IFN-γ (10 ng/ml), LPS (1 μg/ml), or both. Supernatants were assayed by ELISA for CCL3, CCL5, CCL2, IL-1β, IL-12, and TNF-α release. The results are expressed as means from three independent experiments ± SEM. Vertical bars are not always appreciable, as smaller than 10%. *, Values significantly different from those evaluated from supernatants of peritoneal macrophages cultured in medium only ($P<0.01$).

those cultured in the presence of CCL16 were greatly stained for FasL, especially on their surface (Fig. 1E). TSA tumor-target cells were positive to Fas by cytofluorimetric (data not shown) and confocal analysis (Fig. 1F).

CCL16 enhances the production of downstream chemokines and cytokines by peritoneal macrophages

When the ability of peritoneal macrophages cultured for 72 h in the absence or presence of CCL16 (1–100 ng/ml) to release chemokines was assessed, it was found that as little as 1 ng/ml CCL16 increased the release of CCL5 fivefold and that of CCL2, 30 twofold. This effect persisted at 10 and 100 ng/ml. CCL16 enhancement of chemokine release was also compared with that of IFN-γ and/or LPS. Although CCL16 was effective as IFN-γ alone and IFN-γ plus LPS in promoting the release of CCL2, IFN-γ and/or LPS more markedly promoted CCL5 release. Conversely, the release of CCL3 was enhanced only by the presence of LPS (**Fig. 2A**). Although the highest chemokine release was obtained with only 1 ng/ml CCL16, a tenfold higher dose was needed to enhance the release of IL-1β and IL-12. IL-12 was markedly induced by the presence of CCL16, whereas TNF-α was only slightly increased by CCL16 but significantly affected by the presence of IFN-γ and LPS, which also strongly induced IL-1β and IL-12 (Fig. 2B). IL-18 release was never observed (data not shown).

CCL16 induces costimulatory function in peritoneal macrophages

As the ability to prime virgin T cells rests on the expression of costimulatory signals CD80 [23], CD86 [24], and CD40 [25] by APCs, modulation of their expression was evaluated by confocal microscopy on peritoneal macrophages cultured for 72 h in the absence or presence of 100 ng/ml CCL16. The marked overexpression of these signals displayed by $CD11b^+$ cells cultured in the presence of CCL16 (**Fig. 3**) may fully account for their ability to prime naive T cells and generate a strong CTL activity against TSA cells.

CCL16 enhances antigen uptake and presentation by peritoneal macrophages

To evaluate whether CCL16 increases the ability of peritoneal macrophages to process and present tumor antigens to T cells, 4×10^6 adherent peritoneal macrophages were cultured with CFSE-labeled apoptotic TSA cells in the absence or presence of CCL16. After 2, 18, and 72 h of culture, peritoneal macro-

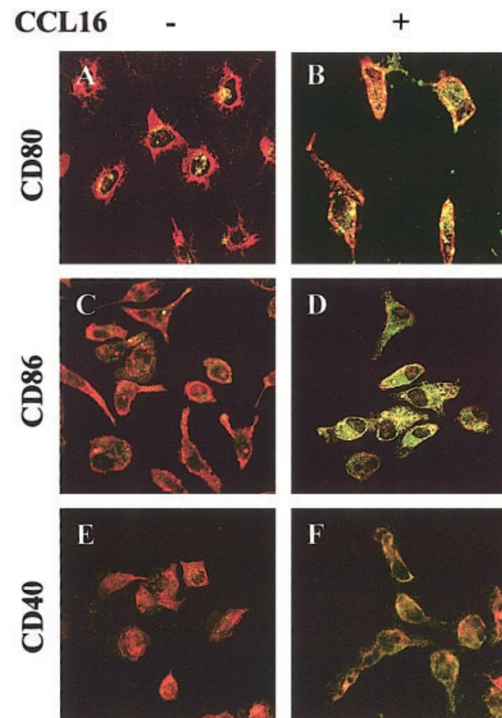

Fig. 3. Costimulatory molecule expression by peritoneal macrophages in the presence of CCL16. Peritoneal macrophages were cultured for 72 h in the absence (left lane) and presence (right lane) of 100 ng/ml CCL16 and were then processed for confocal microscopy. Peritoneal macrophages were stained with PE-conjugated anti-mouse CD11b (red) and FITC-conjugated anti-mouse CD80 (top line), anti-mouse CD86 (middle line), and anti-mouse CD40 (bottom line; green) mAb.

phages were stained with PE-anti-mouse CD11b mAb. Confocal microscopy showed that although few, if any, apoptotic bodies were detectable in the cytoplasm of macrophages cultured in the absence of CCL16, numerous apoptotic bodies were evident in almost all those cultured in its presence (**Fig. 4, A–D**). Moreover, after 72 h, macrophages cultured in the presence of CCL16 presented more small green spots at their surface than those cultured in medium only (Fig. 4, F and E, respectively). This suggests that CCL16 enhances the endocytosis of tumor debris and the processing of tumor cells themselves.

CCL16 enables peritoneal macrophages to prime T cells

Peritoneal macrophages pulsed with apoptotic TSA cells, as described previously, were cultured with fresh Spc to assess their ability to prime T cells. After 6 days of coculture in medium only, CTL activity was evaluated. A significantly enhanced, TSA-specific CTL activity was displayed by lymphocytes primed by macrophages pulsed with TSA apoptotic cells in the presence of CCL16 (**Fig. 5**).

CCL16 increases the generation of CTL

Normal Spc was cultured for 6 days with Mit-C-treated TSA cells at a 40:1 ratio in the absence or presence of 1–100 ng/ml CCL16. Lymphocytes recovered from MLTI in the presence of up to 10 ng/ml CCL16 showed significantly enhanced, specific lysis of TSA cells but not that of antigenically unrelated F1-F cells in a 48-h ^{3}H thymidine-release assay (**Fig. 6A**).

To evaluate whether CCL16-induced CTL activity was a result of an enhanced proapoptotic activity acquired by T cells

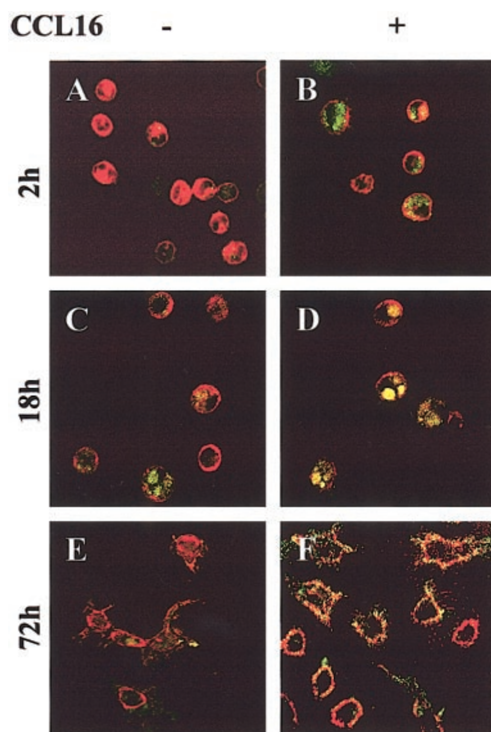

Fig. 4. Apoptotic tumor-cell uptake by peritoneal macrophages in the presence of CCL16. Peritoneal macrophages from normal BALB/c mice were cultured with apoptotic CFSE-labeled TSA cells (green) in the absence (left lane) or presence (right lane) of 100 ng/ml CCL16 for 2, 18, and 72 h. They were then stained with PE-conjugated anti-mouse CD11b (red) mAb and analyzed by confocal microscopy.

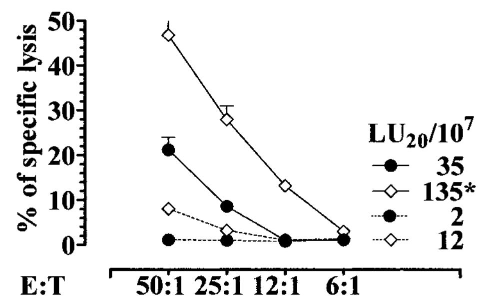

Fig. 5. Priming of naive T cells by peritoneal macrophages pulsed in the presence of CCL16. Peritoneal macrophages were cultured for 72 h with apoptotic TSA cells in the absence or presence of 100 ng/ml CCL16 and after washing, cultured with normal Spc for 6 days. CTLs, recovered from cocultures with peritoneal macrophages pulsed in the absence (●) or presence (◇) of CCL16, were assayed for their cytotoxic activity against TSA (solid lines) and F1-F (dotted lines) cells. The percentages of specific lysis are expressed as mean ± SEM from two independent experiments. Where vertical bars are not appreciable, the SEM is smaller than the size of the symbols. On the right, the mean of $LU_{20}/10^7$ is expressed. *, Values significantly different from those calculated by Spc, incubated with peritoneal macrophages, pulsed in the absence of CCL16 ($P<0.005$).

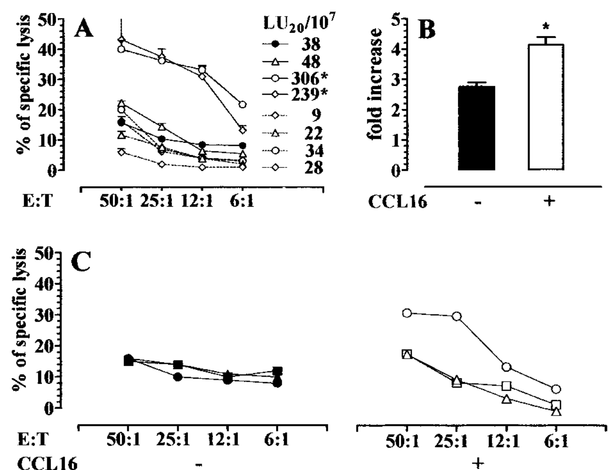

Fig. 6. Cytotoxic activity of CTL generated in MLTI in the presence of CCL16. (A) Spc obtained from normal BALB/c mice were stimulated with Mit-C-treated TSA cells in the absence (●) or presence of 1 ng/ml (△), 10 ng/ml (○), and 100 ng/ml (◇) CCL16. After 6 days, CTL were assayed in a 48-h ^{3}H thymidine-release assay against TSA (solid lines) and F1-F (dotted lines) target cells. The percentages of specific lysis are expressed as mean ± SEM from three independent experiments. Where vertical bars are not appreciable, the SEM is smaller than the size of the symbols. On the right, the mean of $LU_{20}/10^7$ is expressed. (B) The recovered CTL generated as above in the absence (solid bar) or presence (open bar) of 10 ng/ml CCL16 was incubated with TSA cells at a 25:1 E:T ratio. After 3 h, lymphocytes were removed, and tumor cells lysed to analyze caspase-8 activation. The results are expressed as a mean ± SEM from two independent experiments of fold increase related to caspase activation found in TSA cells cultured in medium only. *, Values significantly different from those obtained by CTL generated in the absence of CCL16 ($P<0.01$). (C) CTL, recovered after generation in the absence (left, solid symbols) or presence (right, open symbols) of 10 ng/ml CCL16, was incubated during lysis with neutralizing anti-human FasL (triangles) or anti-mouse TNF-α (squares) mAb. A representative experiment of three is shown.

during MLTI, their ability to activate caspase-8, a death effector domain caspase in tumor target cells, was tested. Spc from 6-day MLTI, generated in the absence or presence of 10 ng/ml CCL16, was removed and overlaid on fresh TSA target cells. After 3 h, lymphocytes were washed away, and the activation of caspase-8 in target cells was evaluated. The presence of CCL16 during MLTI enabled CTL to induce 31% more activation than that induced by CTL generated in its absence (Fig. 6B).

TNF-α and FasL equipment by CCL16-generated CTL

mAb against TNF-α or FasL were added during the CTL assay to test their involvement in the increased cytotoxicity displayed by CTL activated in the presence of CCL16. Neither mAb impaired the cytotoxic activity of CTL generated in the absence of CCL16, whereas both markedly decreased that of CTL generated in its presence. This suggests that CCL16-increased CTL activity also rests on the induction of TNF-α and FasL, in agreement with the enhanced caspase-8 activation in target cells (Fig. 6C).

DISCUSSION

These findings show for the first time that CCL16 powerfully enhances several functions of macrophages and the cytolytic activity of T cells. The intensity of CCL16 enhancement of macrophage functions is comparable with that provided by LPS, a bacterial product that is extremely powerful in the induction of inflammatory responses [22], and by IFN-γ, a cytokine that plays a central role in natural and specific immunity [21]. In human monocytes, CCL16 mainly binds CCR1 [13, 14], a receptor distinct from that of LPS and IFN-γ. CCL16 stimulation enables macrophages to efficiently present tumor antigen and activate an efficient antitumor response in resting T lymphocytes. The TSA mouse tumor used in these studies is a transplantable mammary carcinoma of spontaneous origin [19]. Although the dominant antigen expressed by TSA tumor cells is a gp70 protein coded by Moloney murine leukemia virus endogenous retrovirus [26, 27], in syngeneic mice, TSA cells are poorly immunogenic and unable to trigger any reactivity in resting peritoneal macrophages and virgin T cells [16]. By contrast, the presence of 10–100 ng CCL16 per ml culture medium triggers the killing activity and enhances the antigen-presenting function of peritoneal macrophages. The stronger ability of macrophages to kill TSA cells appears to rest on the increased expression of TNF-α and FasL.

The ability of CCL16 to activate this antigen-presenting function also results in efficient priming of anti-TSA cytotoxicity in virgin T cells. In general, cells of the macrophage lineage are professional "scavengers" but poor stimulators of naive or resting T cells [28]. Although more efficient in phagocytosis, monocytes or macrophages do not elicit an efficient cytolytic activity in CD8 cells [29]. This failure has been attributed to their inability to generate the threshold number of peptide-MHC I complexes to activate specific T cells and express enough levels of costimulatory molecules. As very little has been done to evaluate the role of chemokines in enhancing phagocytosis and antigen presentation by macrophages, our data provide probably one of the first demonstrations of chemokine modulation of the induction of an efficient T cell response by macrophages. Indeed, CCL16 enhanced the ability of peritoneal macrophages to phagocytose and process fluorescein-labeled apoptotic tumor cells. After only 2 h of culture, confocal microscopy showed that macrophages display more apoptotic tumor bodies in their cytoplasm as compared with those cultured in the absence of CCL16. Moreover, after 3 days, macrophages displayed more fluorescein spots, possibly corresponding to processed TSA cells, on their surface. Since the description of monocyte chemotactic and activating factor/MCP-1/CCL2 by Matsushima and co-workers [30], the effects of chemokines on macrophage functions have been investigated. With occasional exceptions, these studies have shown that CC chemokines are poor activators of macrophage functions. CCL2, CCL3, and CCL5 chemokines have been shown to costimulate the proliferative response of T helper cell type 1 (Th1) and Th2 clones to alloantigens and peptides through their activity on T cells and APCs. An increase of $B7.1^+$ cells was observed in chemokine-treated murine Spc [3]. By contrast, our present data show that CCL16 increases the uptake and the processing of tumor cell debris by macrophages and induces their ability to express CD80, CD86, and CD40 costimulatory molecules. CD80 and CD86 are inducible molecules that play a critical role in antigen-specific T cell activation and proliferation [31]. Moreover, following T cell receptor triggering, T cells express CD40L on their membrane. The binding of CD40L to CD40 on APCs further enhances their expression of CD80, CD86 costimulatory molecules.

The enhanced phagocytosis, antigen presentation, and expression of costimulatory molecules induced by CCL16 enable peritoneal macrophages to efficiently prime resting virgin T cells and induce specific antitumor cytotoxicity. CTLs, recovered from cultures with peritoneal macrophages pulsed with apoptotic tumor cells in the presence of CCL16, kill target tumor cells much better than those induced by macrophages pulsed in its absence. Moreover, CCL16 induced macrophages to release downstream CCL2 and CCL5 chemokines and proinflammatory cytokines, such as IL-1β, TNF-α, and markedly, IL-12. The singularly high levels of CCL2 secreted are in agreement with data showing that the APCs, which most effectively induce a T cell response, are those that produce elevated amounts of CCL2 [32]. The enhanced release of chemokines and cytokines induced by CCL16 in macrophages may stimulate and protract the inflammatory response and thus enhance acquired and innate immunity. It is intriguing that it has been reported that IL-10 increases expression of CCL16 in human monocytes activated by LPS plus IFN-γ [10]. We demonstrated that CCL16 up-regulates APC function, and IL-10 is known to suppress the proinflammatory functions of APCs by antagonizing expression of costimulatory molecules, the release of proinflammatory cytokines, and in general, APC maturation [33–35]. However, if the pathogen is not cleared, inflammation progresses and provides additional signals, including CCL16, which leads to further APC maturation and recruitment of adaptative-immune effectors [36–38]. Moreover IL-10-mediated inhibition of APC functions is antagonized by TNF-α and

CD40 ligation, suggesting that depending on the activation stimuli, the presence of IL-10 does not necessarily result in T cell anergy [38]. Studies on the role of CCL16 in diseases characterized by inflammation associated with high levels of IL-10 are required to elucidate the correlation between CCL16 functional response and IL-10 secretion. CCL16 is highly expressed in the liver [9], where it may play a role in leukocyte trafficking and activation of the immune response against pathogens infecting the liver. We suggest that substantial tissue secretion of IL-10 in the presence of CCL16 increases inflammation as a result of CCL16 expression and hence, massive enhancement of the release of CCL2. Many clinical [39, 40] and experimental [41] studies have demonstrated that CCL2 levels in the liver are markedly enhanced during various types of hepatic injury, and in this microenvironment, IL-10 synergizes with CCL16 to enhance its expression. CCL2 is also involved in the regulation of cytokine homeostasis within the liver [42]. Thus, CCL16 expression in the liver could provide a hepatoprotective effect through CCL2 induction.

CCL16 also enhances the ability of T cells to kill target cells in a 48-h assay. T cells recovered from MLTI between virgin Spc and TSA cells in the presence of CCL16 displayed a strong and specific lytic activity. Enhancement of the T cell killing rate was associated with an increased ability to activate caspase-8 in tumor target cells. Caspase-8 is involved in the initiation of apoptosis induced by members of the TNF superfamily [43]. Addition of neutralizing anti-FasL or anti-TNF-α mAb strongly reduced the lytic activity of CTL generated in the presence but not in the absence of CCL16. These findings suggest that the enhanced CTL activity mostly rests on the enhanced expression of FasL and TNF-α by CTL induced in the presence of CCL16. CCL5 has also been shown to regulate FasL expression and killing by human immunodeficiency virus-specific CD8 CTLs [44]. Likewise, stromal derived factor-1/CXCL12 and CCL5 induce membrane-bound TNF-α on primary human macrophages [45]. Taub et at. [3] showed that CCL2, CCL5, and CCL3 enhance the effector activity of T cell clones, already functionally cytotoxic, by regulating lymphocyte degranulation upon conjugation with target cells. Our data differ from these observations, as we have studied the effect of CCL16 on the generation of CTL from normal lymphocytes and on the induction of TNF-α and FasL overexpression.

In conclusion, our results suggest that CCL16 induces macrophages to directly kill tumor cells and differentiate into efficient APCs able to stimulate a strong T cell response. CCL16 enhances the differentiation of T cells into efficient tumor-specific CTLs by macrophage activation and by inducing the overexpression of TNF-α and FasL on CTLs. The concurrence of these activities makes CCL16 an attractive candidate for inclusion in antitumor immunotherapy protocols.

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