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CD40 Is Expressed on Human Peritoneal Mesothelial Cells and Upregulates the Production of Interleukin-15 and RANTES

ANNA BASOK, ALLA SHNAIDER, LIMOR MAN, CIDIO CHAIMOVITZ, and AMOS DOUVDEVANI

Department of Nephrology, Soroka University Medical Center, Ben-Gurion University of the Negev, Faculty of Health Sciences, Beer-Sheva, Israel.

Abstract. Limited data are available concerning the interaction between lymphocytes and human peritoneal mesothelial cells (HPMC) during peritonitis. CD40 is a member of the tumor necrosis factor (TNF) family of receptors whose ligand (CD154) is mainly expressed on the membrane of activated CD4-positive lymphocytes. CD154-CD40 cross-linking is a central event in antigen presentation, B-cell activation by T cells, and regulation of cytokine secretion from various types of cells. The goal of this study was to demonstrate *in vitro* the presence of CD40 on HPMC and to test its functionality in inducing interleukin-15 (IL-15) and RANTES. We assayed the levels of CD40 by reverse transcription-PCR and flow cytometry and IL-15 and RANTES by enzyme-linked immunosorbent assay. Genetically modified L cells that express elevated levels of CD154 (CD40L cells) were used to stimulate CD40. HPMC express CD40 mRNA and protein. After stimulation with interferon-γ (IFNγ, 5 U/ml) or TNFα (1 ng/ml), there was a small increase in CD40 mRNA and protein levels; when both cytokines were applied, the increase in CD40 levels was more than threefold. CD40 ligation induced IL-15 production by HPMC and was additive to IFNγ stimulation. CD40 ligation was strongly synergistic with IFNγ in induction of RANTES (20-fold as compared with unstimulated HPMC), whereas neither ligation nor IFNγ alone could induce RANTES. Pretreatment of HPMC with TNFα and IFNγ increased the response to CD40 ligation in magnitudes that correlated with the elevation of CD40 levels induced by the pretreatment. To conclude, the presence of a functional CD40 on HPMC whose ligation induced IL-15 and RANTES production was detected. It is possible that this receptor acts as a major mediator of T-cell–regulated immune and inflammatory response during peritonitis.

The peritoneal cell population consists mainly of mesothelial cells. It is now clear that in addition to its structural function, this cell layer plays an important role in peritoneal inflammatory and immune responses. The relatively large surface (approximately 2 m^2) of uninfected peritoneum is usually under the surveillance of a limited number of leukocytes consisting mainly of macrophages (1). To mount an effective immune response against invading pathogens, a large number of leukocytes are recruited to the peritoneum from the blood by a process in which human peritoneal mesothelial cells (HPMC) play important roles. HPMC express the adhesion molecules ICAM-1, ICAM-2, and VCAM-1, which play an important role in leukocyte adhesion and migration (2,3). HPMC have been shown to secrete the proinflammatory cytokines interleukin-1 (IL-1) and IL-6 and the chemokines IL-8, MCP-1, and RANTES, in response to bacterial and inflammatory mediators (*e.g.* lipopolysaccharide, IL-1, tumor necrosis factor-α [TNFα], and interferon-γ [IFNγ]), thus amplifying the inflammatory signals and inducing effective endothelial activation, which are required for migration of leukocytes to the peritoneal cavity (4–9). Li *et al.* (2) demonstrated that the chemokines IL-8, MCP-1, and RANTES are mainly secreted from a monolayer of HPMC into the apical compartment and suggested that this mechanism is important for the recruitment of leukocytes from blood vessels into the peritoneum. We recently demonstrated that HPMC support T-cell activation by presenting antigen and by secreting IL-15, a T-cell growth factor and activator (10). In the current study, we further investigated lymphocyte-mesothelial cross talk by examining the possibility that the CD40 activation pathway functions in mesothelial cells. CD40 is a cell surface receptor that belongs to the tumor necrosis factor receptor family. It was originally identified and functionally characterized on B lymphocytes. Its critical role in T-cell–dependent humoral immune responses has been demonstrated in patients with hyper-IgM syndrome, as well as by gene targeting in mice (11). In recent years, however, it has become clear that CD40 is expressed also on monocytes, dendritic, endothelial, and epithelial cells (11). The CD40 ligand (CD154), a member of the TNF family, is mainly expressed on activated CD4-positive T cells. Using CD154, Th cells activate CD40 on their target cells by direct cell-to-cell contact. Activation of CD40 induces the secretion of cytokines and chemokines and upregulates the expression of adhesion and accessory molecules to increase the efficiency of antigen

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Correspondence to Dr. Amos Douvdevani, Nephrology Laboratory, Soroka Medical Center, P.O. Box 151, Beer-Sheva 84101, Israel. Phone: ++972-7-6403214; Fax: ++972-7-6281361; E-mail: amosd@bgumail.bgu.ac.il

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presentation (11). The central role of CD40 in immune response is best illustrated by its role in graft rejection. Blocking CD154 molecules effectively reduced graft rejection in various experimental models and, as recently demonstrated, prevents acute rejection of renal graft in nonhuman primates (12).

There are no data concerning the expression of CD40 on mesothelial cells. However, from the data accumulated on the role of CD40 in other tissues, we hypothesized that CD40 ligation on HPMC might be a pathway used by T helper cells to regulate an inflammatory and immune response in the peritoneum. We therefore tested mesothelial cells for the presence of CD40 and for its functionality in inducing the secretion of IL-15 and RANTES. Another reason for testing the effect of CD40 ligation on those factors is that the authors previously found that CD40 ligation upregulates IL-15 in tubular epithelial cells (TEC) in addition to others' observation that CD40 induces IL-15 upregulation on dendritic cells and upregulates RANTES secretion by TEC and endothelial cells (13–16). We were especially interested in IL-15 and RANTES because both are involved in T-cell recruitment and activation. IL-15 is a T-cell chemoattractant and a potent T-cell activator with biologic functions similar to those of IL-2 (17). IL-15–deficient mice (IL-15 −/−) are lymphopenic and specifically lack natural killer (NK) cells and memory CD8-positive T cells and have a compromised immune response to various pathogens (18). We previously reported that HPMC produce IL-15 and that IFNγ upregulates its production (10). RANTES is a chemotactic cytokine for T lymphocytes, monocytes, and eosinophils and belongs to the CC subfamily of chemokines. Secretion of RANTES and other chemokines from HPMC in response to cytokines or bacterial stimulation have also been reported (2,4,9,19). On the basis of these observations, the aim of this study was to show whether mesothelial cells express CD40 and to demonstrate the role of CD40-CD154 ligation in upregulation of IL-15 and RANTES synthesis.

Materials and Methods

Cell Preparation

Human Peritoneal Mesothelial Cells. Omentum-derived HPMC were isolated and characterized for their specific morphology and markers, as previously reported (20). Experiments were performed on cells from the second to fourth passages.

CD154 Expressing L Cells. For CD40 activation, we used a cell line of mouse fibroblast L cells, transfected with the complete human CD154 coding sequence and a neomycin resistance gene for selection of stable transfectants. Both types of cells were cultured in RPMI and supplemented with 10% heat-inactivated fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin (Biological Industries, Bet Haemek, Israel). The parental nontransfected cells (L cells) were used as the negative control. Both parental and CD154-transfected cells were kindly supplied by Schering-Plough Corporation (Dardilly, France) and have been described (21). CD40 activation using CD154-expressing cells is commonly used to activate CD40 on various cell types in different experimental models (16,21,22).

HPMC Activation Protocols

Induction of CD40 for RNA Analysis and Flow Cytometry. For RNA analysis, HPMC were seeded in 24-well plates (2×10^4 cells/well) and cultured to confluence. HPMC were washed twice with medium and incubated for 6 h with medium alone or with medium containing IFNγ (5 U/ml; Peprotech, Rocky Hill, NJ) or TNFα (1 ng/ml; R&D System, Minneapolis, MN) or with a combination of both cytokines. After stimulation, supernatants were removed and RNA was extracted from the cells. Preliminary experiments indicated that CD40 mRNA reach peak levels at approximately 6 h. For flow cytometry, HPMC were grown in 75 cm^2 flasks and incubated for 16 h with medium or with cytokines in the same concentrations and combination as for RNA analysis.

Activation of HPMC for the Production of IL-15 and RANTES. HPMC were seeded in 12-well plates (4×10^4 cells/well) for IL-15 analysis or 24-well plates (2×10^4 cells/well) for RANTES and cultured to confluence. HPMC were washed twice with medium and incubated with IFNγ in various concentrations, or with CD40L cells (expressing CD154) or control L cells (3×10^5 in 12-well plates or 5×10^4 in 24-well plates) or with a combination of IFNγ and the cells. For IL-15 analysis, the final incubation volume was 0.75 ml and the incubation time was 72 h and for RANTES, 0.5 ml and 24 h. To prevent overgrowth of L cells and CD40L cells in the co-culture experiments, were grew them in the M-199 medium, which inhibits fibroblast growth, for the last 24 h before harvesting. For total IL-15 (secreted and cell associated) analysis at the end of incubation, plates were placed on ice and cells were lysed by adding NP40 to a final concentration of 0.1%. Lysates were collected and stored at −20°C. For RANTES assay, supernatants from 24-well plates were collected and stored at −20°C. Cells from two wells were counted in each plate for definition of cell number per well. All tissue culture reagents contained less than 0.025 ng/ml of endotoxin.

Increase of CD40 Expression. To increase CD40 expression on HPMC, we performed several experiments after 16 h of preincubation of HPMC with TNFα (1 ng/ml) or with IFNγ (5 U/ml) or with a combination of both and then activated with IFNγ and CD40L cells for 24 h.

CD154 Blocking. In some experiments, we blocked CD154 on CD40L cells by pretreating these cells for 60 min with anti-CD154 monoclonal antibodies (1 μg/ml, clone 24 to 31; Ancell, Bayport, MA). An isotype-matched antibody at the same concentration was used as a control.

Transwell Experiments. A six-well Transwell system (Corning Costar Corporation, Cambridge, MA) was used to conduct co-culture experiments. The system consists of two compartments: a top well with a porous matrix (0.4-μm pore size) and a bottom well. This set-up allows co-culture of two types of cells to grow in the same medium with soluble factors exchanged through the pores while preventing direct contact between them. HPMC were grown to confluence in the bottom well, and L cells were added either to the same well—allowing contact—or in the top well—avoiding contact.

Flow Cytometry Analysis of CD40

For flow cytometry analysis, HPMC were harvested by ethylenediaminetetraacetate solution (1:2000, Biological Industries), then washed and incubated in 100 μl of phosphate-buffered saline containing 0.5% bovine serum albumin and 0.05% sodium azide for 1 h on ice with anti-CD40 monoclonal antibodies (clone EA-5; Ancell). The cells then were washed and incubated with fluorescein (FITC)-conjugated F(ab') anti-mouse IgG (Jackson, West Grove, PA). The antibodies were diluted to recommended concentrations according to

the manufacturer's instructions. Background fluorescence was adjusted with cells labeled with matching isotype control antibodies as the first antibody. HPMC had no affinity to control antibodies, and fluorescence levels were always similar to that seen in nonstained cells. Analyses were done on a Coulter flow cytometer (Epics XL-MCL, Miami, FL).

mRNA Analysis

CD40 mRNA was determined by reverse transcription-PCR (RT-PCR) of total RNA extracted from HPMC. HPMC were incubated as described above. At the end of each experiment, total RNA was extracted from cells using the RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA suspension was converted immediately to cDNA.

For cDNA generation, to each 7 μl of reverse transcriptase reaction mixture, 13 μl of RNA sample was added. The reaction mixture contain 1 μl of Moloney murine leukemia virus-reverse transcriptase (MMLV-RT; 200 U/μl; Life Technologies BRL, Gaithersburg, MD), 4 μl of 5× reverse transcriptase buffer (Life Technologies BRL), 0.5 μl DTT (0.1 M, Life Technologies BRL), 0.5 μl RNase inhibitor (40 U/μl; Sigma, Rehovot, Israel), 1 μl of oligo-d(T) 12 to 18 mer (40 pmol/μl, Roche), and 1 μl of dNTP (2.5 nmol/μl each nucleotide; Sigma). The reaction tube was incubated for 1 h at 37°C, then the volume of each sample was adjusted to 60 μl and the enzyme was inactivated by incubation for 10 min at 65°C.

CD40 and β-actin cDNA were then amplified by PCR using specific primers: CD40 sense, AGAGTTCACTGAAACGGAAT-GCC; CD40 antisense, ACAGGATCCCGAAGATGATGG; β-actin sense, ATGGATGATGATATCGCCGCG; β-actin antisense, CTA-GAAGCATTTGCGGTGGACGATGGAGGGGCC. Five μl of reverse transcription product was added to 45 μl of PCR reaction mixture containing 32.75 μl of H_2O, 2.5 μl of 5' primer (20 μM), 2.5 μl of 3' primer (20 μM), 2 μl of dNTP (2.5 nmol/μl each nucleotide; Sigma), 5 μl of 10× reaction buffer, and 0.25 μl *Taq* DNA polymerase (Sigma). A negative control consisting of the reaction mixture without cDNA was included in each run. PCR was run for 20 to 25 cycles with β-actin primers under the following conditions: 90 s at 95°C, then 5 to 10 cycles of 45 s each at 95°C, 90 s at 60°C, and 60 s at 72°C. The last 15 cycles were run under the same conditions but at 72°C; incubation was prolonged by 5 s in each cycle. PCR with CD40 primers was run with the same protocol except that the annealing temperature was 55°C instead of 60°C. Every experiment was amplified with at least two different numbers of cycles to ensure that amplification was in the exponential phase of PCR.

We found that 30 to 35 cycles for CD40 and 20 to 25 cycles for β-actin were in the exponential phase of amplification, thus permitting comparison of mRNA levels in different samples. Under these conditions, we also found a linear dose response of the PCR product to increasing doses of cDNA.

Eight μl of each sample containing amplified cDNA were loaded on an agarose gel (1.5%) containing ethidium bromide (0.5 μg/ml). A DNA size marker was run on the same gel ("100 bp ladder"; Life Technologies BRL). PCR products were quantified by video densitometry of agarose gel with the ImageMaster VDS-CL (Amersham Pharmacia Biotech, Freiburg, Germany). To correct for differences in loading, we corrected densitometric values of CD40 cDNA with corresponding values of β-actin cDNA. To allow comparison of different gels, we normalized the densitometric CD40/β-actin ratios for the IL-15/β-actin ratio of the unstimulated HPMC run in each gel.

Enzyme-Linked Immunosorbent Assay of IL-15 and RANTES

IL-15 and RANTES in supernatants and cell lysates were measured in duplicate using enzyme-linked immunosorbent assay (ELISA) to human IL-15 or RANTES made with commercially available antibodies and standards (R&D Systems). ELISA was performed according to the manufacturer's protocol; for IL-15, we used monoclonal anti-human IL-15 antibody (type MAB647) and biotinylated monoclonal anti-human IL-15 antibody (type BAM247). The range of the IL-15 ELISA was 3 to 300 pg/ml, and the sensitivity was 2.0 pg/ml. For RANTES, we used monoclonal anti-human RANTES (type MAB678) and biotinylated monoclonal anti-human RANTES antibody (type BAF278). The range of the RANRES ELISA was 39 to 2500 pg/ml, and the sensitivity was 10 pg/ml. For detection, we used streptavidin horseradish peroxidase (type 43-4323; Zymed, San Francisco, CA; 1:20,000) and tetramethylbenzidine solution (TMBSingle Solution; Zymed). ELISA reaction was stopped with H_2SO_4 (2 N). Optical density was read with an ELISA reader at 450 nm and at 550 nm for reference.

Statistical Analyses

Results are expressed as mean ± SEM. To compare levels between groups, either Dunnet's method of multiple analysis and a one-way ANOVA or a two-way ANOVA was used. *P* values below 0.05 were considered significant.

Results

CD40 Expression on Mesothelial Cells

Because CD40 is widely expressed in various cells and tissues (11), we hypothesized that HPMC can express this important receptor. We first analyzed CD40 mRNA levels. As shown in Figure 1, detectable CD40 mRNA levels were seen in quiescent HPMC. We found that CD40 mRNA levels were slightly increased (32 or 46%) after stimulation with TNFα (1 ng/ml) or IFNγ (5 U/ml), and when both cytokines were applied, a synergistically fourfold increase in CD40 mRNA levels was observed. As shown in Figure 2, CD40 protein levels, as analyzed by flow cytometry, correlated with mRNA levels. Similar to mRNA levels, we found a constitutive expression of CD40 on unstimulated cells, a small increase in the mean (28 or 61%) after TNFα or IFNγ stimulation, and a synergism between TNFα and IFNγ that increased CD40 protein by threefold.

Activation by CD40L Cells

To demonstrate CD40 functionality, we assayed the effect of CD40 activation by CD154 on IL-15 and RANTES production. The interaction between HPMC and activated T cells involves many molecules apart from CD40/CD154. To evaluate the isolated effect of CD40 ligation on HPMC, we co-cultured the HPMC with a murine fibroblast cell line (L cells) genetically engineered to express human CD154 (CD40L cells). As a control, we used parental unmodified L cells. We added CD40L cells to the HPMC monolayer, and several hours after addition of the cells, they formed aggregates on HPMC indicating cell-to-cell contact. When the parental L cells were added, we observed less contact with HPMC (not shown).

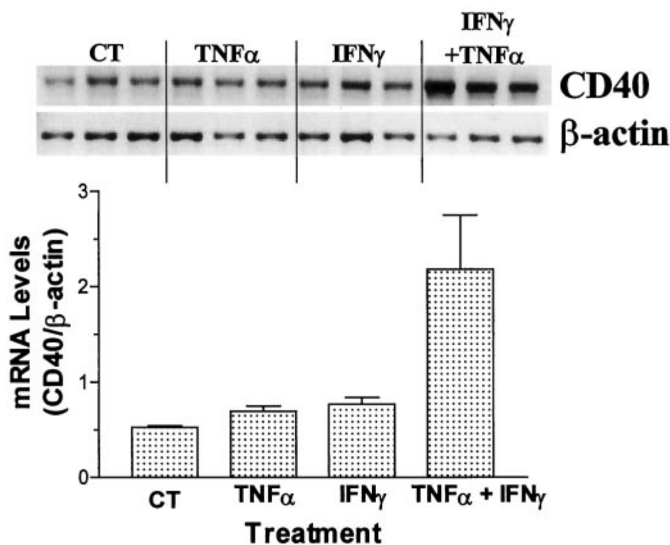

Figure 1. CD40 mRNA levels on human peritoneal mesothelial cells (HPMC). HPMC in 24-well plates were stimulated for 6 h in triplicate with tumor necrosis factor-α (TNFα; 1 ng/ml) or interferon-γ (IFNγ; 5 U/ml) or with a combination of both. Total RNA was isolated, and reverse transcription-PCR (RT-PCR) was preformed to amplify CD40 and β-actin cDNA. Triples of the various samples were run on a 1.5% agarose gel containing ethidium bromide (upper panel). The intensity of the bands was analyzed by video densitometry and is represented graphically as the IL-15/β-actin ratio (lower panel). The figure is representative of two experiments performed on cells from different donors with similar results.

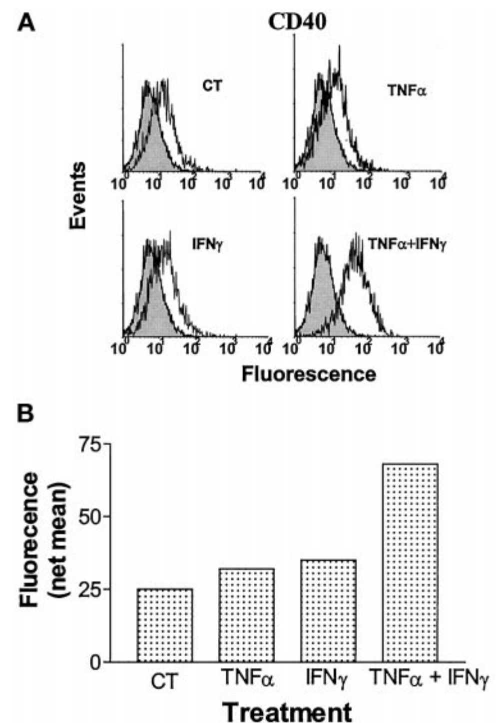

Figure 2. Flow cytometry analysis of CD40 molecules on HPMC. HPMC were stimulated for 16 h with TNFα (1 ng/ml) or IFNγ (5 U/ml) or with a combination of both. HPMC were labeled by a primary antibody to CD40 and then stained with FITC-labeled secondary-antibody. (A) The shaded histogram indicates the background fluorescence of cells labeled with an isotype-matched control antibody; the plain wide line indicates fluorescence of cells labeled with anti-CD40. (B) The bar indicates the net mean fluorescence (mean CD40 minus mean isotype control fluorescence). The figure is representative of three experiments performed on cells from different donors with similar results.

IL-15 Production

As depicted in Figure 3, IL-15 production by mesothelial cells is significantly enhanced in a dose-dependent manner by CD40 activation by CD40L cells as compared with the low effect of unstimulated HPMC or HPMC treated with L cells. In some experiments, unmodified L cells had a nonspecific stimulatory effect on HPMC, but it never reached the specific effect of CD40L cells. Because we previously found that IFNγ enhances IL-15 production by HPMC and TEC (10,15), we compared the effect of IFNγ to CD40 activation and combined these two treatments (Figure 4). The effect of CD40 activation was similar in magnitude to that observed with IFNγ treatment, and the combination of IFNγ and CD40 activation resulted in an additive increase of IL-15 production. Exposure of HPMC to CD40L cells preincubated with a blocking antibody to CD154 significantly decrease IL-15 production as compared with incubation with CD40L cells preincubated with a nonspecific isotype control antibody in the same concentration. Inhibition of CD154 was effective when HPMC were stimulated by CD40L cells alone or in combined activation with IFNγ.

RANTES Secretion

We tested the dose response of HPMC to IFNγ (Figure 5). At all tested doses (0.1 to 1000 U/ml), stimulation with IFNγ alone had no significant effect on RANTES secretion. However, the combination of CD40 ligation and IFNγ was synergistic for induction of RANTES from a concentration of 0.5

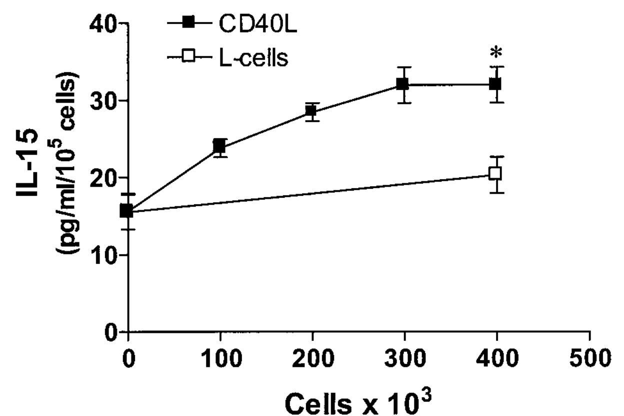

Figure 3. Induction of interleukin-15 (IL-15) by CD40 ligation. HPMC in a 12-well plate were incubated as indicated with increasing numbers of L cells or CD40L cells (L cells genetically modified to express CD154). At 72 h, cells were lysed by 0.1% NP40 and lysates were collected for total IL-15 production (secreted and cell associated). IL-15 levels were determined by enzyme-linked immunosorbent assay (ELISA). The figure is representative of three experiments performed on cells from different donors with similar results. Results are expressed as mean ± SEM. *, $P < 0.05$ for IL-15 levels higher than HPMC treated with the same number of L cells.

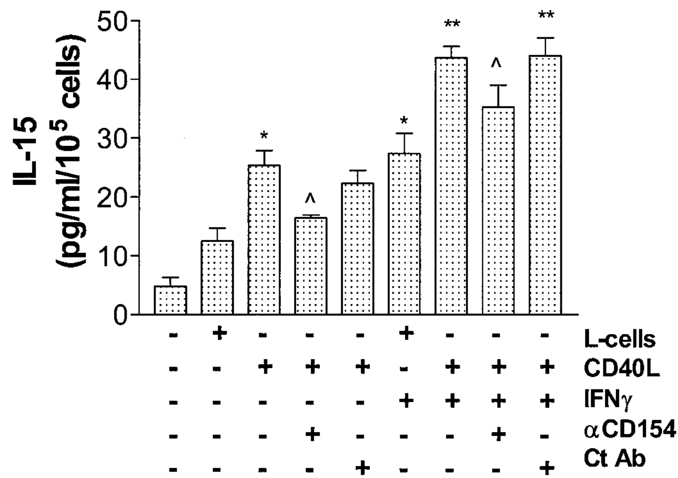

Figure 4. Induction of IL-15 by CD40 ligation and IFNγ treatment. HPMC in a 12-well plate were incubated as indicated with L cells (3×10^5 cells/well), CD40L cells (3×10^5 cells/well), IFNγ (5 U/ml) CD40L cells pretreated for 60 min with a monoclonal αCD154 or isotype control antibodies (Ct-Ab). At 72 h, cells were lysed by 0.1% NP40 and lysates were collected for total IL-15 production. IL-15 levels were determined by ELISA. The figure is representative of three experiments performed on cells from different donors with similar results. Results are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ for IL-15 levels higher than HPMC treated with L cells alone; ^, $P < 0.05$ for inhibition as compared with treatment with Ct-Ab.

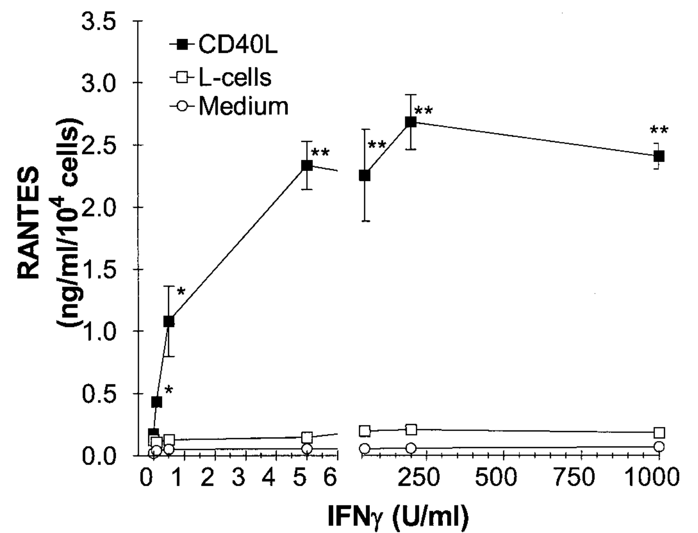

Figure 5. The effect of CD40 ligation on RANTES secretion; synergistic response with increasing doses of IFNγ. HPMC in a 24-well plate were incubated with increasing doses of IFNγ (0.1 to 1000 U/ml) and as indicated, with medium, L cells (5×10^4 cells/well) or CD40L cells (5×10^4 cells/well). At 24 h, supernatants were collected and assayed for RANTES by ELISA. The figure is representative of three experiments performed on cells from different donors with similar results. Results are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.001$ for RANTES levels higher than HPMC treated with L cells alone.

U/ml and reached a plateau at 5 U/ml IFNγ. RANTES accumulation over time is depicted in Figure 6. Accumulation after IFNγ and CD40 ligation was at a fairly invariable rate of approximately 2 ng/ml per 10^4 cells per day up to 72 h. In contrast, no significant accumulation of RANTES (when compared with L-cell treatment) was found after IFNγ or CD40 ligation. As shown in Figure 7, the induction of RANTES stimulated by exposing HPMC to IFNγ and CD40L cells was blocked by preincubation of the CD40L cells with anti-CD154 antibodies before adding them to HPMC. In contrast, an isotype-matched control antibody had no effect. The effect of CD40L cells on HPMC was contact dependent and not dependent on soluble mediators, as clearly demonstrated by the Transwell experiments depicted in Figure 8. CD40L cells separated by a porous membrane (0.4-μm pore size) in the Transwell system had no stimulatory effect on HPMC in contrast to the marked effect of CD40L cells co-cultured with HPMC in the same compartment.

Effect of CD40 Upregulation on RANTES Secretion

Because we found that treatment of HPMC with TNFα and IFNγ increase the levels of CD40 (Figures 1 and 2), HPMC were pretreated with these cytokines and then exposed to CD40L cells or to CD40L cells and IFNγ to discover whether CD40 upregulation has an effect on the response to CD40 stimulation (Figure 9). Without pretreatment, we observed that CD40L cells induced only low RANTES secretion. This effect was not higher than that seen in the nonspecific induction of L cells.

Pretreating HPMC with TNFα and IFNγ significantly increased the response to CD40L cells. This effect was significantly higher (four- to sixfold) when HPMC were pretreated with both cytokines together, as compared with pretreatment with TNFα or IFNγ alone. As compared with nonpretreated HPMC, a twofold increase in RANTES secretion was found after pretreatment with TNFα or IFNγ alone and stimulation with CD40L cells and IFNγ, and a more than sevenfold increase was seen after pretreatment with both cytokines and by the same stimulation. When the RANTES levels were compared after pretreatment with one cytokine with pretreatment with two cytokines, the increase was approximately threefold, similar to that found in CD40 levels after pretreatment with the two cytokines.

Discussion

This study is the first to demonstrate the presence of CD40 on HPMC. It is widely accepted that CD40 plays a central role in mediating T-cell–dependent immune and inflammatory reaction. We found that CD40 is expressed on mesothelial cells and is upregulated by TNFα and IFNγ. CD40 was first demonstrated on B cells, but its presence on HPMC was anticipated because besides being expressed on B cells, CD40 has been detected on endothelial cells, fibroblasts, and various epithelial cells, including kidney tubular cells (11). CD40 ligation induces expression of adhesion molecules as well as secretion of

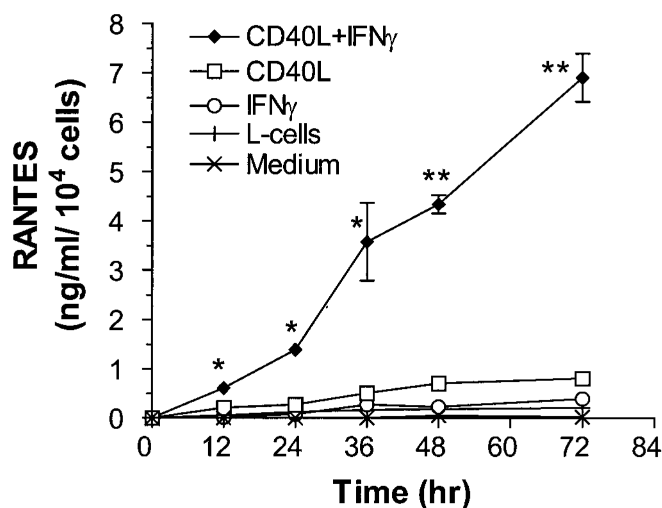

Figure 6. RANTES accumulation over time. HPMC in a 24-well plate were incubated as indicated with medium, L cells (5×10^4 cells/well), CD40L cells (5×10^4 cells/well), or IFNγ (5 U/ml) or with CD40L cells and IFNγ for various periods (0 to 72 h). Supernatants were collected and assayed for RANTES by ELISA. The figure is representative of three experiments performed on cells from different donors with similar results. Results are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ for RANTES levels higher than HPMC treated with L cells alone.

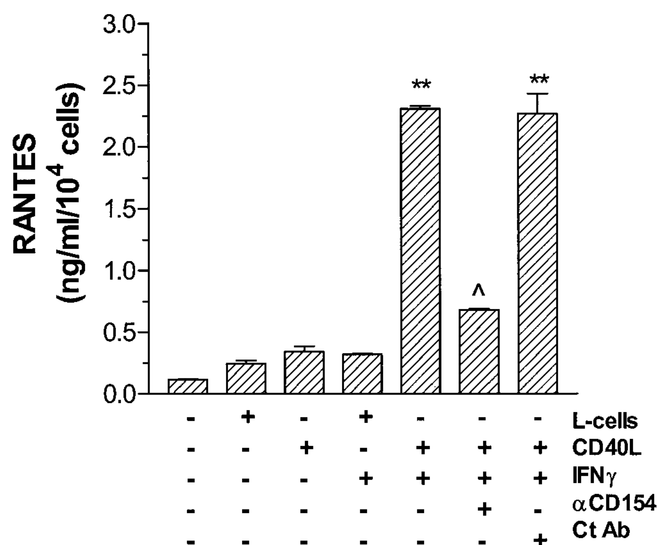

Figure 7. Antibodies to the CD40 ligand (CD154) block the stimulatory effect of CD40L cells. HPMC in a 24-well plate were incubated as indicated with L cells (5×10^4 cells/well), CD40L cells (5×10^4 cells/well), IFNγ (5 U/ml), or CD40L cells pretreated for 60 min with a monoclonal αCD154 or Ct-Ab. At 24 h, supernatants were collected and assayed for RANTES by ELISA. The figure is representative of three experiments performed on cells from different donors with similar results. Results are expressed as mean ± SEM. *, $P < 0.05$; **, indicates $P < 0.01$ for RANTES levels higher than HPMC treated with L cells alone; ^, $P < 0.05$ for inhibition as compared with treatment with Ct-Ab.

various cytokines and chemokines on endothelial and epithelial cells. Therefore, we decided to demonstrate the effect of CD40 ligation by measuring IL-15 and RANTES production.

To isolate the role of the CD40 ligand/CD154 from other lymphocyte signals, we activated CD40 on HPMC with a murine cell line genetically modified to express human CD154 (CD40L-cells). In this way, nonrelevant signals were avoided and CD40 was uniquely activated.

After CD40 ligation, both IL-15 and RANTES production by HPMC were significantly affected. CD40 ligation induced a significant increase of IL-15 production by HPMC, whose effect was additive to IFNγ stimulation. These findings are similar to the additive effect seen in TEC, dendritic cells, and muscle cells derived from polymyositis dermatomyositis patients (16,23,24).

The effect of CD40 ligation on RANTES secretion was different from the effect on IL-15 levels. When HPMC were activated by one inducer, either CD40 ligation or IFNγ stimulation, it did not induce significant RANTES secretion. Similar to our findings, Visser *et al.* (4) showed that IFNγ (0 to 3000 U/ml) does not induce RANTES secretion from HPMC. However, the combination of CD40 ligation with IFNγ was highly synergistic for inducing RANTES secretion. Similar observations corresponding to our findings were made by Altenburg *et al.* (25) and by Sugiura *et al.* (24). These authors described a synergism between CD40 ligation and IFNγ for inducing RANTES and other chemokines in cervical carcinoma cells and in muscle cells.

To ascertain that the effect induced by the CD40L cells was specifically due to expression of CD154 and not by nonspecific cell contact, we used the parental L cells as a control for nonspecific activation. L cells had only a low stimulatory effect on HPMC. This effect was usually less than 40% of that of CD40L cells on the production of IL-15 and less than 10% of the synergistic effect of CD40 L cells with IFNγ for the production of RANTES. The nonspecific stimulation of the L cells could be related to cytokine secretion or perhaps is due to irritation from cell contact. We ascertained the specificity of CD40L cells to activate CD40 by blocking the CD154 molecules that they express with anti-CD154 antibodies. Pretreating CD40L cells with anti-CD154 effectively blocked the induction of IL-15 and RANTES.

CD154 molecules are mainly active as membrane-bound molecules, not as soluble molecules. Therefore, to demonstrate that HPMC activation by CD40L cells is contact dependent, we used the "Transwell" system. This experiment clearly demonstrated that RANTES production is suppressed when contact is avoided by separating the CD40L cells from the HPMC with a membrane of 0.4-μ pore size. This experiment also demonstrated that soluble factors secreted from CD40L cells, which diffuse freely through the porous membrane, have no effect on HPMC.

Visser *et al.* (4) demonstrated that TNFα and IL-1β synergizes with IFNγ in inducing the chemokines RANTES, MCP-1, and IP-10. It is therefore suggested that an additional signal to IFNγ is needed to achieve a significant secretion of these chemokines by HPMC. The second signal for RANTES

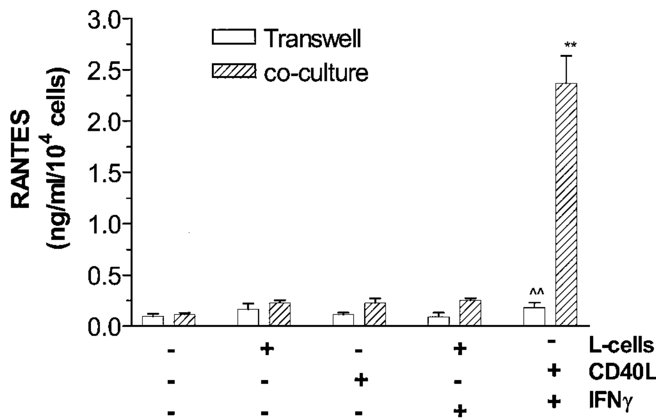

Figure 8. The effect of cell contact on CD40 activation. HPMC were grown to confluence in the lower chamber of Transwell plates and then were added to the same chamber (co-culture) or in the upper chamber (Transwell) L cells or CD40L cells. The separating membrane of the Transwell allows diffusion of solutes but prevents contact between the cells in the lower and the upper chambers. IFNγ (5 U/ml) was added as indicated. The figure is representative of two experiments performed on cells from different donors with similar results. Results are expressed as mean ± SEM. **, $P < 0.01$ for RANTES levels higher than HPMC co-cultured with L cells and IFNγ; ^^, $P < 0.01$ for lower levels as compared with co-cultured CD40L cells and IFNγ.

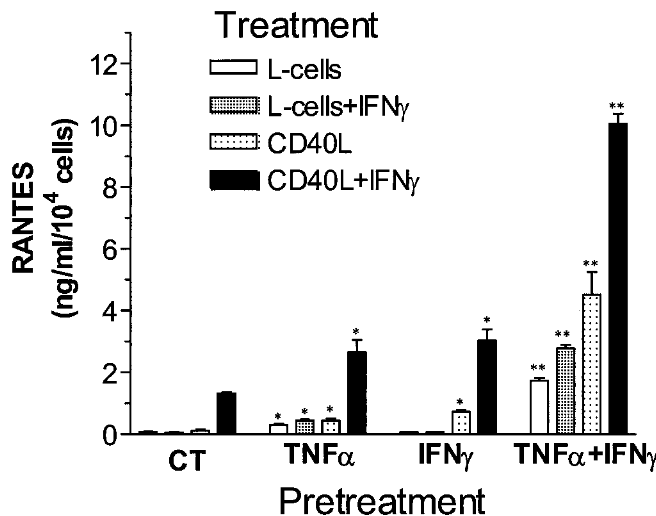

Figure 9. The effect of TNFα and IFNγ pretreatment on RANTES secretion. HPMC in a 24-well plate were pretreated for 24 h with TNFα (1 ng/ml) or IFNγ (5 U/ml) or with a combination of both as indicated. Then the cells were washed and exposed as indicated to L cells or CD40L cells with or without IFNγ (5 U/ml) for 24 h. The figure is representative of two experiments performed on cells from different donors with similar results. Results are expressed as mean ± SEM. *, $P < 0.05$; **, $P < 0.01$ for RANTES levels higher than HPMC treated by the same treatment but not pretreated (CT).

secretion could be provided by a proinflammatory cytokine or by CD154. CD154 and IFNγ are mainly expressed by T helper cells, and we found that a combination of both upregulates the production of IL-15 and RANTES. Because IL-15 and RANTES are potent T-cell modulators, this effect might be part of a positive feedback loop that enhances T-cell recruitment and activity in the peritoneum.

Our RT-PCR and flow cytometry data indicate that TNFα and IFNγ upregulate the expression of CD40 on HPMC. Similarly, Albanesi *et al.* (26) and Sugiura *et al.* (24) demonstrated that combined treatment of human keratinocytes or muscle cells with TNFα and IFNγ upregulates CD40. Our results suggest that the rise in CD40 levels is functional because pretreatment of HPMC with TNFα and IFNγ resulted in an increase in RANTES production. The augmented RANTES production was in clear correlation with the increase in CD40 levels; low increase in RANTES production was obtained after preincubation with any one of the cytokines, and combined pretreatment with TNFα and IFNγ was synergistic in its effect on the increase in RANTES secretion. We therefore suggest that TNFα and IFNγ pretreatment increase the effect of CD40 ligation by increasing the number of CD40-signaling receptors.

We have demonstrated the presence of CD40 on primary culture of HPMC, although *in vivo* expression and functionality of this receptor still remain to be established. However, it is reasonable to consider that in the peritoneum, CD40 and its ligand will have a role in central inflammatory and immune reactions such as leukocyte chemoattraction and antigen presentation, activities that were previously demonstrated for CD40 (11,13,14,24,25,27,28). CD40 expressed on mesothelial cells may synergize in the peritoneum with proinflammatory cytokines such as TNFα and IFNγ, which have been detected in the peritoneal effluent during peritonitis (5). This mechanism may play a significant role in the generation of an effective immune response in the peritoneal cavity.

In conclusion, our data indicate that a functional CD40 is expressed on HPMC that is regulated by TNFα and IFNγ. Ligation of CD40 increased the production of IL-15 and was highly synergistic to IFNγ in RANTES secretion. Our data suggest that CD40 plays a role in T-cell regulation of immune and inflammatory responses in the peritoneum.

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