NF-κB Is Involved in Regulation of CD40 Ligand Expression on Mycobacterium bovis Bacillus Calmette-Guérin-Activated Human T Cells

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Received 3 December 2002/Returned for modification 7 January 2003/Accepted 20 February 2003

Interaction between CD40L (CD154) on activated T cells and its receptor CD40 on antigen-presenting cells (APC) and its ligand (CD40L) on activated T cells plays an important role in many immune responses. CD40 is a 50-kDa type I transmembrane protein belonging to the tumor necrosis factor family (26). CD40 ligand, also known as CD154, is a 261-amino-acid-long type II transmembrane protein with a molecular mass of 33 kDa and is a member of the tumor necrosis factor family of cytokines (1, 12). Its gene is preferentially expressed in activated CD4+ T cells and mast cells, but the surface protein can also be detected on monocytes and CD8+ T cells (2, 13). CD40-CD40L interactions are known to activate APC such as macrophages for their microbicidal activity (14, 17) and play a critical role in immunity to intracellular pathogens by up-regulating the production of interferon-γ (IFN-γ) (9, 15, 16). CD40L expression in human T cells is regulated at multiple intracellular levels, beginning with transcription (39). Increased amounts of CD40L mRNA and activation of a relevant transcription factor NF-κB have been reported in human T cells after activation with anti-CD3 or phorbol myristate acetate (20, 31, 32). NF-κB is a ubiquitous dimeric transcription factor that is retained in the cytoplasm in a latent form as a heterotrimeric complex consisting of p50, p65 subunits, and an inhibitor, IκB (inhibitor of κB). The nature of the signals that lead to activation of NF-κB strongly implies that this nuclear factor plays a critical role in the activation of immune cells (3, 11, 24). It has been demonstrated that NF-κB is involved in mycobacterial infections, since expression of IL-2 receptor and activation of IL-6 and IL-8 by Mycobacterium tuberculosis are mediated by this nuclear factor (37, 38, 41, 42). Moreover, a purified protein derivative induces the activation of NF-κB in monocytes from patients with tuberculosis (40). However, little is known about the role of NF-κB in regulating CD40L expression on Mycobacterium bovis bacillus Calmette-Guérin (BCG)-activated T cells. Therefore, the present study was undertaken to determine the role of NF-κB in BCG-mediated up-regulation of CD40L expression on human CD4+ T cells. Furthermore, we evaluated the role of protein kinase C (PKC) in BCG-induced NF-κB activation and the role of phosphorylation of the IκB protein in BCG-induced activation of NF-κB.

**MATERIALS AND METHODS**

**Reagents.** N-acetyl-Leu-Leu-norleucinal (ALLN) was purchased from Boehringer Mannheim (Indianapolis, Ind.), 1-[5-isooquinolinesulfonyl]-2-methylpiperaazine (H-7), pyrrolidinedithiocarbamate (PDTC), cycloheximide (CHX), actinomycin D (Act D), and salicylate were purchased from Sigma Chemical Co. (St. Louis, Mo.). The PKC inhibitor rottlerin was purchased from Calbiochem (San Diego, Calif.). Anti-IκBα and anti-IκBβ antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, Calif.). Live M. bovis BCG Danish strain 1331 was kindly supplied by J. Ruiz-Puente (Birnex, Mexico City, Mexico). BCG was grown at 37°C in Sauton medium with stationary tissue culture flanks. Mycobacterial viability, as assessed by the number of CFU, was 60 to 70%.

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Cell culture. Blood was obtained by peripheral venipuncture of healthy adult volunteers. After isolation of peripheral blood mononuclear cells by density centrifugation over Ficoll-Hypaque gradient (d = 1.007, Histopaque; Sigma), the CD4+ T-cell subset was purified by positive selection with magnetic microbead-coated anti-CD25 antibody (Miltenyi Biotec; Gladbach, Germany). Cells were incubated with beads conjugated to monoclonal mouse anti-human CD4 antibody (Leu-3a). The purity of CD4+ T cells after positive selection was confirmed by fluorescence-activated cell sorting (FACS). One cycle of selection was sufficient to obtain ≥95% CD4+ T cells. Autologous APC were placed in 24-well flat-bottom plates and infected with M. bovis BCG. The multiplicity of infection (MOI) was 3 live, 1 dead, or 3×10^5 M. bovis BCG organisms per cell. After infection, cells were washed twice with warm RPMI 1640 medium to remove extracellular bacteria, treated with 50 μg of mitomycin C/ml, and then cultured in fresh RPMI 1640 medium supplemented with 2 mM L-glutamine, 100 U of penicillin/ml, 100 μg of streptomycin/ml, and 10% fetal calf serum in a 5% CO2 incubator at 37°C. Then, purified CD4+ T cells were added to the wells (the equivalent of 1 T cell per APC). In some experiments, T cells were precultured with medium or various inhibitors for 60 min, washed by RPMI 1640, and then cocultured with APC infected with BCG. The optimal concentrations of the inhibitors were determined in advance.

Flow cytometry. Cells were suspended in 100 μl of phosphate-buffered saline containing 1% (wt/vol) bovine serum albumin (Sigma). The cells were incubated for 30 min at 4°C with fluorescein isothiocyanate-conjugated anti-CD40L monoclonal antibody (clone TRAP1), immunoglobulin G isotype control antibody, or anti-CD69 monoclonal antibody (purchased from Becton Dickinson, San Jose, Calif.). After two washes, the cells were fixed with 1% (vol/vol) paraformaldehyde and acquired on a FACSscan (Becton Dickinson). Data were analyzed with CellQuest software (Becton Dickinson). The fluorescence of 10,000 cells was accumulated for analysis.

RNA isolation and RT-PCR. Total RNA from 10^7 T lymphocytes was isolated by the method of Chomczynski and Sacchi (7). Briefly, total RNA extracted with 1 ml of Trizol for 5 min at room temperature was transferred to a microcentrifuge tube, and 200 μl of chloroform was added, vortex mixed, and incubated for 10 min. After centrifugation for 5 min, the aqueous layer was transferred to a fresh tube and RNA was precipitated by adding an equal volume of isopropanol. After centrifugation, the RNA pellet was dried and dissolved in 50 μl of sterile water. The RNA concentration was determined by optical density measurements. RNA (2 μg) was reverse transcribed by using random hexamer primers ( Gibco BRL) and Superscript II reverse transcriptase (RT; Gibco BRL), and then amplified with Taq DNA polymerase and the appropriate primers (GIBCO BRL) and Superscript II reverse transcriptase (RT; GIBCO BRL), and Taq DNA polymerase and the appropriate primers (GIBCO BRL) and 2 μl of cDNA was used for specific PCR, for a total of 20 cycles in the standard reaction mixture with Taq DNA polymerase and the appropriate primer set. The quantity of mRNA was normalized by using B2-microglobulin (B2) primer set. The B2-microglobulin mRNA was used as a control to ensure equal loading of RNA samples. The results were expressed as a percentage of the B2-microglobulin mRNA level in each sample.

RESULTS

Inhibitors of NF-kB activation inhibit expression of CD40L levels on the surface of BCG-activated CD4+ T cells. In preliminary experiments, the kinetics of CD40L expression revealed a significant expression of CD40L after 24 h of culture. This is consistent with previous results demonstrating that strong CD40L expression occurs 24 h after stimulation (21). In order to determine whether NF-kB is involved in the regulation of CD40L expression, CD4+ T cells were pretreated with ALLN, a well-described inhibitor of NF-kB activation which prevents degradation of IkB and eventually results in a lack of translocated NF-kB in the nucleus (30). After incubation, cells were activated with autologous APC infected with BCG at an MOI of 3 or left nonactivated. The cells were harvested at 24 h, and cell surface CD40L or CD69 expression on BCG-activated CD4+ T cells was measured by FACS. As shown in Fig. 1A, high surface levels of CD40L were observed after 24 h of activation compared to nonactivated T cells (controls). However, this up-regulation is abolished by treatment with ALLN. In order to confirm these results, cells were pretreated with PDTC, another well-known inhibitor of NF-kB activation with a different mechanism of action (23), and activated with APC infected with BCG. As evident in Fig. 1A, treatment of cells with PDTC caused a significant down-regulation of CD40L expression, with significant inhibition at 89%. Cell viability, as determined by vital dye exclusion (trypan blue), was always >92% after incubation of cells for 24 h with the inhibitors at the concentrations used. In contrast, the CD69 level (used as another T-cell activation marker) was only slightly affected, indicating that the effect of the inhibitors on CD40L expression was specific (Fig. 1B).

Expression of CD40L mRNA in BCG-activated CD4+ T cells is inhibited by ALLN and PDTC. To correlate protein CD40L expression with CD40L mRNA concentrations, the levels of CD40L mRNA were measured by a semiquantitative RT-PCR method. As shown in Fig. 2, adding ALLN or PDTC to BCG-activated CD4+ T cells significantly inhibited CD40L mRNA production in contrast to the level of production obtained after BCG stimulation alone. Parallel RT-PCR showed quasi-equal amounts of β-actin mRNA in all of the RT-PCR samples, as shown in Fig. 2. The quantity of mRNA was normalized by detection of β-actin to correct for any gel-loading discrepancies. These results correlate well with the reduced surface levels of CD40L in the presence of inhibitors of NF-kB. To determine whether IkBα or IkBβ plays an important function in this pathway, we stimulated CD4+ T cells with BCG-infected APCs, and cytosolic extracts were analyzed by immunoblotting with polyclonal antibodies against IkBα and IkBβ. Immunoblotting analysis revealed that BCG led to a loss of IkBα protein, which was rapidly degraded upon stimulation (~30 min) but reappeared within 2 h (Fig. 3A). By contrast, the IkBβ protein was less affected at early time points (30 min), but then its levels decreased gradually and almost disappeared by 2 h (Fig. 3A). Taken together, these results indicate that BCG leads to the rapid but transient induction of NF-kB activity. To examine the potential effects of ALLN and PDTC on IkB at the protein levels, cytosolic extracts were prepared and analyzed by immunoblotting with polyclonal antibodies against IkBα and IkBβ. The IkBα and IkBβ proteins were detected in CD4+ T cells preincubated with ALLN or PDTC for 60 min and followed by BCG treatment for different lengths of time (0, 30, 60, and 120 min), and neither IkBα nor IkBβ was reduced in the presence of inhibitors (Fig. 3B).

Activation with BCG results in increased transcription of the CD40L gene by a mechanism which is independent of protein synthesis. We sought to determine whether or not transcriptional up-regulation of the CD40L gene is the pre-
dominant mechanism through which BCG induces CD40L expression in T cells. To test this, cells were cultured with or without 5 μg/ml Act D/ml to block CD40L gene transcription. After 1 h, cells were activated with APC infected with BCG or left nonactivated. Then cDNA was synthesized and analyzed by PCR with primers specific for CD40L or β-actin. As shown in Fig. 4, treatment of cells with Act D completely abolished the ability of BCG to induce CD40L expression, indicating that CD40L expression in this system is dependent upon transcription. Next, we analyzed whether the transcriptional effect mediated by BCG was dependent on de novo protein synthesis. To this purpose, 10 μg/ml CHX was added to cells for 1 h and cells subsequently activated with BCG. Following this, total RNA was extracted and CD40L mRNA levels were analyzed by RT-PCR. Control cultures without inhibitors were manipulated under the same conditions. The PCR products were separated in a 1% agarose gel and analyzed by staining with ethidium bromide. RNAs for CD40L and β-actin are shown from one representative of four experiments. +, present; −, absent. (B) The quantity of mRNA was determined by densitometer, with the result normalized to β-actin and expressed as a relative index.
induced NF-κB activation by treating cells with the PKC antagonist H-7. Figure 5 indicates that BCG-activated cells showed significantly increased CD40L expression compared with that in control cells. However, BCG-activated cells treated with H-7 showed significant down-regulation (Fig. 5). Supporting the hypothesis that BCG induces phosphorylation of IκB via PKC to activate NF-κB, H-7-treated uninfected cells showed no significant change in CD40L expression compared with that in untreated cells (data not shown). Thus, H-7, which blocks phosphorylation of IκB and subsequent activation of NF-κB induced by BCG activation, also blocks resultant CD40L regulation. To confirm the role of NF-κB in regulating BCG-induced CD40L expression, cells were treated with salicylate or left untreated and then were activated with autologous APC infected with BCG at an MOI of 3 or left nonactivated for 24 h and labeled with antibody to CD40L. As shown in Fig. 5, BCG-activated cells showed significantly increased CD40L expression compared with that in uninfected control cells. However, BCG-activated cells treated with salicylate showed significant down-regulation (Fig. 5), confirming that BCG-induced activation of NF-κB and the up-regulation of CD40L are blocked by inhibiting the phosphorylation of IκB. In order to confirm the role of PKC in BCG-induced CD40L expression, we examined the effect of rottlerin (a Ca²⁺-independent PKC inhibitor which interferes with IκB kinase [IKK] activation) in our system. Figure 6 shows that the presence of rottlerin inhibited CD40L mRNA expression. These data suggest that Ca²⁺-independent PKC participate in the activation of IKK complexes by the T-cell receptor after recognition of BCG.

**DISCUSSION**

Our results demonstrate that NF-κB signaling pathways modulate the surface expression of CD40L on BCG-activated CD4⁺ T cells. Using specific pharmacologic inhibitors of NF-κB activation (ALLN and PDTC), down-regulation of CD40L protein levels was observed during T-cell activation by BCG. Consistent with these findings, T cells from NF-κB- and p50-deficient mice have reduced expression of CD40L, as did T cells from wild-type mice in the presence of proteasome inhibitors (34). These results were further supported by the fact that treatment of cells with ALLN and PDTC blocked the induction of CD40L gene expression in BCG-activated CD4⁺ T cells. It is important to note that IκBα and IκBβ protein production was not affected by the chemical protease inhibitors (Fig. 3B), indicating that our data are unlikely to be due to the effect of these chemical inhibitors on the level of expression of IκBα and IκBβ protein. On the other hand, we have not ruled out decreased mRNA stability in the presence of the inhibitors used. It has recently been reported that the stability pathway of CD40L mRNA decay is regulated in part by the formation of an activation-dependent ribonucleoprotein complex on a de-
The defined region of the CD40L mRNA (4). However, these agents both inhibit NF-κB, but they do so by distinct mechanisms. PDTC is a scavenger of free radicals, which interferes with the activation of genes requiring NF-κB, whereas ALLN is a specific inhibitor of the proteasome pathway, which prevents the degradation of IκB, thus keeping the IκB–NF-κB complex intact and preventing nuclear translocation of NF-κB. Moreover, NF-κB is not known to increase mRNA stability (22). These results indicate that NF-κB activation is the critical signal for CD40L expression. A canonical NF-κB-binding site within the CD40L promoter has been identified (19). This site has been shown to be required for maximal induction of CD40L in human T cells after activation with anti-CD3 antibody. Whether this NF-κB-binding site is required by BCG remains to be elucidated.

We have demonstrated that the increase in CD40L in BCG-activated CD4+ T cells is abolished by the addition of Act D, which suggests that transcription of the CD40L gene is required for BCG-induced up-regulation of cell surface CD40L expression. Transcriptional regulation is a fundamental control mechanism in biological processes and requires the participation of several classes of proteins (28). Therefore, we investigated whether the transcriptional effect mediated by BCG was dependent on de novo protein synthesis. We have shown that CD40L mRNA induction, in CD4+ T cells activated with BCG, is not inhibited by CHX, indicating that de novo protein synthesis is not required for the induction of mRNA expression. At the same time, these results also suggest an important role of NF-κB-like transcription factors in this induction. Activation of NF-κB requires its dissociation from IκB (6, 10). A critical step in this process is the phosphorylation of IκB proteins, which then are degraded, resulting in the activation of NF-κB (36). In this study, salicylate, which also has been associated with down-regulation of NF-κB activation due to blocking the phosphorylation of IκB (18, 29), also blocked the functional activation of NF-κB in the BCG-activated CD4+ T cells, and it abolished the up-regulation of CD40L cell surface expression by BCG. This is further supported by our finding that the surface expression of CD40L was down-regulated in the presence of H-7, which blocks phosphorylation of IκB via PKC to inhibit NF-κB activation. These results suggest that the activation of PKC has a positive regulatory effect on CD40L gene expression by increasing the transcription in BCG-activated T cells. In contrast to our data, Nüsslein et al. (27) found that the PKC pathway is not the essential signal for the expression of CD40L in T cells. These apparently contradictory results are probably due to the stimulation of human T lymphocytes by different stimuli. Moreover, the requirement for PKC...
in the activation of the NF-κB cascade was further confirmed by the fact that treatment of CD4+ T cells with rottlerin, a Ca2+-independent PKC inhibitor which interferes in IKK activation, inhibits BCG-induced CD40L mRNA expression. These results are in agreement with the observation that mycobacterium-induced activation of NF-κB is inhibited by using a specific inhibitor of IKK (5). Although the importance of PKC in the activation of the NF-κB pathway in T lymphocytes has been recently demonstrated (8, 35), to our knowledge, this is the first evidence for BCG-induced association of PKC with the IKK complex in a physiologically relevant system. Since PKC family members includes the δ, ε, η, and θ isoenzymes (25), it remains to be elucidated which PKC isoenzyme is responsible for initiating the phosphorylation events. However, among the several PKC isoenzymes expressed in T cells, PKC-θ is unique in being rapidly recruited to the site of T cell receptor clustering. Therefore, it is possible that PKC-θ may play a role in IKK activation in our system.

Two main groups of transcription factors have been implicated in the expression of CD40L gene: NF-AT and NF-κB (33). Depending on the stimulus and system used, a role can be demonstrated for these transcription factors. Whether these transcription factors, separately or together, are required for BCG-induced human CD40L transcription remains to be determined.

In summary, these data show that PKC participates in NF-κB activation to increase the CD40L mRNA and protein levels in human T cells activated with BCG. Future directions should examine if these signaling pathways are found to be significant in host defense against *M. tuberculosis* infection.

ACKNOWLEDGMENTS

We thank J. Ruiz-Puente (Birmex) for providing the *M. bovis* BCG.

P.M.-S. is an EDI, COFAA, and SNII fellow. This work was financed in part by a Coordinación General de Posgrado e Investigación (CGPI) grant to P.M.-S.

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