The Role of the p38 Mitogen-Activated Protein Kinase, Extracellular Signal-Regulated Kinase, and Phosphoinositide-3-OH Kinase Signal Transduction Pathways in CD40 Ligand-Induced Dendritic Cell Activation and Expansion of Virus-Specific CD8+ T Cell Memory Responses

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The role of the p38 mitogen-activated protein kinase, extracellular signal-regulated kinase, and phosphoinositide-3-OH kinase signal transduction pathways in CD40 ligand-induced dendritic cell activation and expansion of virus-specific CD8+ T cell memory responses

Qigui Yu,* Colin Kovacs,† Feng Yun Yue,* and Mario A. Ostrowski‡*‡

Mature dendritic cells (DCs) are central to the development of optimal T cell immune responses. CD40 ligand (CD40L, CD154) is one of the most potent maturation stimuli for immature DCs. We studied the role of three signaling pathways, p38 mitogen-activated protein kinase (MAPK), extracellular signal-regulated kinase (ERK), and phosphoinositide-3-OH kinase (PI3K), in CD40L-induced monocyte-derived DC activation, survival, and expansion of virus-specific CD8+ T cell responses. p38 MAPK pathway was critical for CD40L-mediated up-regulation of CD83, a marker of DC maturation. CD40L-induced monocyte-derived DC IL-12 production was mediated by both the p38 MAPK and PI3K pathways. CD40L-mediated DC survival was mostly mediated by the PI3K pathway, with smaller contributions by p38 MAPK and ERK pathways. Finally, the p38 MAPK pathway was most important in mediating CD40L-stimulated DCs to induce strong alloimmune responses as well as expanding virus-specific memory CD8+ T cell responses. Thus, although the p38 MAPK, PI3K, and ERK pathways independently affect various parameters of DC maturation induced by CD40L, the p38 MAPK pathway within CD40L-conditioned DCs is the most important pathway to maximally elicit T cell immune responses. This pathway should be exploited in vivo to either completely suppress or enhance CD8+ T cell immune responses. The Journal of Immunology, 2004, 172: 6047–6056.

Strong T cell immune responses are instrumental in controlling a number of viral infections including EBV, CMV, and HIV-1 infection (1–3). Mature dendritic cells (DCs) are central to the development of optimal T cell immune responses (4). A number of stimuli, including many of the TNF family members, LPS, influenza infection, CpG DNA motifs, dsRNA, and pox viruses have been shown to facilitate the maturation of immature DCs resulting in their enhanced ability to present Ag to T cells and activate them (5–10). Of these, we and others have shown that the TNF family molecule CD40 ligand (CD40L, CD154) is one of the most potent maturation stimuli for immature DCs (11–14). CD40 ligation of immature DCs results in a cascade of morphologic and functional effects that include the following: 1) increased expression of the dendritic cell maturation marker CD83 and down-regulation of surface CD1a; 2) up-regulation of surface MHC class I and class II molecules, the costimulatory molecules CD80 (B7-1) and CD86 (B7-2), and the adhesion molecules CD54 (ICAM-1) and CD58 (LFA-30); 3) the production of proinflammatory cytokines IL-1, IL-6, and TNF-α; 4) the production of the T cell growth and differentiation factors, IL-12 and IL-15; and 5) enhanced DC survival (6, 11, 12, 14–20). Taken together, these effects have been shown to be important for optimally priming CD8+ T cell responses as well as for maintaining CD8+ T cell memory. CD154 is expressed predominantly on activated CD4+ T cells, and signaling through CD40 on DCs has been shown to be one of the primary mechanisms for CD4+ T cell responses (21–24). Although some cellular immune responses can occur without CD154/CD40 interactions, greatly impaired CD4+ T cell as well as CD8+ T cell priming is often observed in the absence of this receptor/ligand interaction (25–28).

The cellular signaling pathways associated with CD154/CD40 interactions have been elucidated mainly in studies with nonlymphoid cells or transformed B cell lines. Very few research groups have directly studied CD40 signaling pathways in DCs. Interestingly, CD40 itself lacks intrinsic kinase activity and thus mediates its intracellular function primarily through cytoplasmic tail-mediated recruitment of TNFR-associated factor (TRAF) signaling adaptors, a characteristic of members of the TNFR family (29–31). For CD40, TRAF2, 3, and 6 have been shown to bind its cytoplasmic tail. Because TRAFs themselves have no known enzyme activity they serve as adapter proteins for downstream signaling pathways. Engagement of CD40 on DCs has been shown to result in activation of the following signaling pathways: the Src family kinase, Lyn (32), NF-κB (33), the three members of the mitogen-activated protein kinase (MAPK) family including the extracellular signal-regulated kinases (ERKs), the c-Jun N-terminal kinases (JNKs), and the p38 MAPK (34), as well as the phosphoinositide-3-OH kinase (PI3K) pathway (35).

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Abbreviations used in this paper: DC, dendritic cell; CD40L, CD40 ligand; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; PI3K, phosphoinositide-3-OH kinase; MDSC, monocyte-derived DC; TRAF, TNFR-associated factor; JNK, c-Jun N-terminal kinase.
The MAPKs have been shown to be involved in all aspects of the immune response, including the activation of innate and adaptive immune responses and regulation of apoptosis (36). Among the MAPKs, p38 MAPK and ERK have both been shown to induce the IL-12 p40 subunit in DCs, with p38 MAPK being responsible for the majority of IL-12 p40 induction (34, 37). IL-12 production by DC is critical for the differentiation of IFN-γ-producing Th1 T lymphocytes (38). In a murine model, CD40-induced p38 MAPK and ERK activation were shown to be regulated upstream by TRAF6 (37). The p38 MAPK was also shown to up-regulate the antiapoptotic protein, cAP2, which is the cellular inhibitor of apoptosis protein 2, thus implying a role for maintaining DC survival. In LPS-stimulated murine DC, ERK was also shown to be very important for DC survival after maturation (39).

Activation of NF-κB in DC has been shown to up-regulate the DC costimulatory molecules, CD80, CD86, and HLA-DR as well as the maturation marker CD83 but has very little effect on DC survival (39, 40).

The PI3K-Akt pathway has been shown to play a major role in many cell survival pathways (41). In murine DC, TRAF6 has been shown to phosphorylate c-src, which in turn phosphorylates PI3K. Activated PI3K converts plasma membrane lipid phosphatidylinositol-4,5-bisphosphate to phosphatidylinositol-3,4,5-trisphosphate. The serine-threonine kinase Akt is then recruited to the membrane and is phosphorylated by phosphoinositide-dependent kinase 1. In various cell types, activated Akt has been shown to promote cell survival and protect against apoptosis through multiple mechanisms, including suppressing caspase 3 and caspase 9 activation, promoting production of Bcl-xL, repressing the activity of the proapoptotic factor BAD, and suppressing the production of Fas ligand (41–45).

Thus, engagement of CD40 on DC results in the activation of a number of signaling pathways, with the end result being a mature apoptotic factor BAD, and suppressing the production of Fas ligand (39, 40).

Materials and Methods

Study subjects

Three HIV-1-infected individuals (participants 1–3), who were HLA-A*0201-positive and had detectable EBV-specific CD8+ T cell IFN-γ responses by ELISPOT assay (data not shown), were recruited for leukopheresis to obtain large amounts of PBMCs. Three HIV-1-seropositive individuals were also studied (participants 4–6). This group included a long-term nonprogressor (participant 6), who was HIV-1-infected for 10 years, had stable CD4+ T cell counts >500 μL, and a viral load <50 copies/mL by bDNA, and two asymptomatic chronic progressors, the first of whom (participant 4) was HIV-1-infected for 5 years, CD4+ T cell count 410 μL, viral load 45,000 copies/mL, and the second (participant 5), who was HIV-1-infected for 5 years, CD4+ T cell count 700 μL, viral load 44,514 copies/mL. All HIV-1-infected individuals were not taking antiretroviral drugs during the study. Before the study, individuals were class I HLA typed and screened for EBV- or HIV-1-specific CTL by culturing PBMCs with HLA-restricted EBV or HIV-1 peptides and detecting IFN-γ-producing CD8+ T cells by ELISPOT assay as previously described (14). Participants 1–3 responded to the HLA-A*0201-restricted MBLF1 region of EBV, (GLCVLAML); participant 4 demonstrated responses to an HLA-A24-restricted epitope (KYLKLHIVW) of HIV-1 p17 region of gag; participant 5 showed responses to the HLA-A*0201-restricted LMP-2 region of EBV (CLGLLTMV); and participant 6 showed responses to an HLA-B27-restricted epitope (KRWIIGLNK) of HIV-1 p24 region of gag.

Informed consent was obtained from participants in accordance with the guideline for conduction of clinical research at the University of Toronto and St. Michael’s Hospital (Toronto, Ontario, Canada). All investigational protocols were approved by the University of Toronto and St. Michael’s Hospital institutional review boards.

Abs and reagents

The following anti-human mAbs or polyclonal Abs conjugated with fluorochrome were purchased from BD PharMingen (San Diego, CA): Anti-CD3-allophycocyanin, Anti-CD3(PE)–anti-CD4(PE)–anti-CD14(PE), and anti-CD1a(PE). Anti-CD3(PerCP), anti-CD8(PerCP), and anti-CD2(PerCP) Abs were purchased from PharMingen (San Diego, CA). Anti-CD11c(PerCP), and anti-CD86(PerCP) were purchased from Becton Dickinson (San Diego, CA). Anti-CD40(PerCP), and anti-CD40L(PerCP) were purchased from R&D Systems (Minneapolis, MN). Anti-CD83(PerCP) was purchased from Serotec (Rochester Hills, MI). Anti-CD1a(PerCP), anti-CD2(PerCP), and anti-CD14(PerCP) were purchased from BD Pharmingen (San Diego, CA). Anti-CD3(PerCP), anti-CD8(PerCP), and anti-CD14(PerCP) were purchased from Becton Dickinson (San Diego, CA).

Generation of MDDCs

MDDCs were generated by a modification of a method previously described (9, 14). Briefly, PBMCs obtained by the Ficol-Paque gradient centrifugation (Amersham Pharmacia Biotech, Uppsala, Sweden) were separated on multistep Percoll gradients (Sigma-Aldrich). PBMCs obtained by the Ficoll-Paque gradient centrifugation were used as responders and allogeneic MDDC were used as stimulators. PBMCs were depleted of contaminating B cells, T cells, NK cells, and granulocytes using Ab-conjugated magnetic beads from the Monocyte Negative Selection kit (Dynal Biotech, Oslo, Norway). Purified monocytes were cultured at 1 × 10^6 cells/mL in complete RPMI 1640 medium consisting of 10% FCS, 2 mM glutamine, 25 mM HEPES, and antibiotics in the presence of 50 ng/mL recombinant human GM-CSF and 100 ng/mL recombinant human IL-4. GM-CSF and IL-4 were added again on days 3 and 5 with the fresh complete RPMI 1640 medium. After 7 days of culture, over 90% of the cells were CD41a+, MHC class II+, CD80+, and CD14+, which represents an immature DC phenotype.

Allogeneic mixed lymphocyte proliferation

Primary MLRs were set up in 96-well round-bottom plates (Costar, Corning, NY), in a 0.2 mL of RPMI 1640 medium with 10% FCS in triplicate wells. PBMCs were used as responders and allogeneic MDDC were used as stimulators. The ratio of responder to stimulator (T cell to APC) was 20:1 for PBMCs and allogeneic MDDCs. The culture period was 3 days and were pulsed with [3H]thymidine (2 μCi/well; PerkinElmer Life Sciences, Boston, MA) for the last 6 h of culture. After harvesting cells, incorporated radioactivity was measured in an LS 6500 Multipurpose Scintillation Counter (Beckman Coulter Instruments, Fullerton, CA).

Induction of peptide-specific CTL

The protocol for expanding circulating memory CD8+ CTL ex vivo was previously described (14). Immature MDDCs that were stimulated for 3 days with trimeric CD40L were pulsed with the specific HLA class I-restricted peptide at 40 μg/mL for 1 h at 37°C, then plated in 24-well plates (5 × 10^6 peptide pulsed or nonpeptide pulsed MDDCs/well) in RPMI 1640 medium supplemented with 10% FCS, 2 mM glutamine, 25 mM HEPES, and antibiotics. Freshly isolated or thawed autologous PBMCs were added to MDDCs at a ratio of 1:10 (5 × 10^6 cells/well in 2 mL medium). On day 3 and day 5, the medium was changed. No cytokines were added. On day 7, duplicate wells were pooled and cells were harvested and tested for CTL activity by intracellular IFN-γ staining. Experiments were repeated in HIV-1-negative participant 1 and HIV-1-positive participant 3.

Flow cytometric analysis of cell surface and intracellular molecules

PBMCs or MDDCs were stained in PBS/1% FCS/0.02% NaN₃ using allophycocyanin, anti-caspase-3 FITC, anti-Bcl-2 PE, and matched-isotype control Abs conjugated with FITC, PE, PerCP, or allophycocyanin. Annexin V-FLUOS Staining kit containing Annexin V-FLUC was purchased from Boehringer Mannheim (Roche Diagnostics, Indianapolis, IN). Anti-Akt Ab, anti-ERK1/2 Ab, and anti-phospho-Akt Ab, anti-phospho-p38 MAPK Ab, anti-phospho-ERK1/2 Ab were purchased from Cell Signaling Technology (Pikering, Ontario, Canada). PI3K inhibitor LY294002, p38 MAPK inhibitor SB203580, and ERK inhibitor PD98059 were purchased from Sigma-Aldrich (Ontario, Canada) and dissolved in DMSO (Sigma-Aldrich). Recombinant human GM-CSF and IL-4 were purchased from ProTech (Rocky Hill, NJ). Trimeric CD40L was obtained as a gift from Immunix (Seattle, WA).

Flow cytometry

PBMCs or MDDCs were stained in 1× PBS with 1% FCS using allophycocyanin, Anti-CD3PerCP, anti-CD8 FITC, anti-CD14 FITC, and matched-isotype control Abs conjugated with FITC, PE, PerCP, or allophycocyanin. Annexin V-FLUOS Staining kit containing Annexin V-FLUC was purchased from Boehringer Mannheim (Roche Diagnostics, Indianapolis, IN). Anti-Akt Ab, anti-ERK1/2 Ab, and anti-phospho-ERK1/2 Ab were purchased from Cell Signaling Technology (Pikering, Ontario, Canada). PI3K inhibitor LY294002, p38 MAPK inhibitor SB203580, and ERK inhibitor PD98059 were purchased from Sigma-Aldrich (Ontario, Canada) and dissolved in DMSO (Sigma-Aldrich). Recombinant human GM-CSF and IL-4 were purchased from ProTech (Rocky Hill, NJ). Trimeric CD40L was obtained as a gift from Immunix (Seattle, WA).

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the test Ab. For intracellular staining, cells were permeabilized using Cytofix/ Cytoperm Plus kit (BD Pharmingen) according to the manufacturer’s instructions. Intracellular staining was performed for detecting IL-12, caspase 3, and Bcl-2 using anti-human IL-12 (p40/p70, clone C115), anti-human active caspase-3155, and anti-human Bcl-2156, respectively. Intracellular staining was also performed to enumerate the number of IFN-γ-producing CD8- T cells, as previously described (14). Briefly, for peptide-specific IFN-γ staining, 2.5 × 105 cells were cultured in U-bottom 96-well plates in the presence of peptide-pulsed (~1–10 μM) autologous B lymphoblastoid cell lines or autologous T cell-depleted PBMCs as stimulator cells; nonpeptide-pulsed stimulator cells were used as background controls. Positive control cells were stimulated with the bacterial superantigen staphylococcal enterotoxin B (1 μg/ml; Sigma-Aldrich). Cells were incubated with peptide-pulsed or nonpeptide-pulsed stimulator cells for 6 h at 37°C in 5% CO2. Monensin was added for the duration of the culture period to facilitate intracellular cytokine accumulation. For IFN-γ staining, we used clone 4S.B3 to identify IFN-γ-containing cells and clone MOPC-21 as the isotype control Ab (BD Pharmingen).

Cell survival analysis and inhibitor treatment
Duplicate or triplicate wells containing 0.1 × 105 immature MDDCs per well were cultured for 48 h in round-bottom 96-well plates in complete RPMI 1640 medium in the presence or absence of 2 μg/ml trimeric CD40L. Cell apoptosis was assessed for the phosphatidylinerine on the outer leaflet of apoptotic cell membranes using annexin V-fluorescein staining (Boehringer Mannheim) according to the manufacturer’s protocol. Cell apoptosis was also determined by detecting intracellular active caspase 3 using a FITC-conjugated-specific Ab for active caspase 3. Bcl-2 was also assessed by intracellular staining of MDDCs with PE-conjugated Bcl-2. For signaling kinase inhibitor experiments involving the PI3K inhibitor LY294002, the MAP/ERK inhibitor PD98059, or the p38 MAPK inhibitor SB203580, cells were preincubated with the indicated inhibitors for 30 min, then incubated with 2 μg/ml trimeric CD40L. DMSO was used as background control, as the inhibitors were dissolved in DMSO. Inhibitors were titrated at concentrations ranging from 5 to 50 mM. A dose of 25 mM for all inhibitors was used for all experiments as this was the lowest concentration that could completely inhibit kinase activity. Inhibitors were removed by twice washing MDDCs with 2%FCS/PBS before coculturing with T cells.

Western blot analysis
Immature MDDCs (5–10 × 105 cells) were incubated with 2 μg/ml trimeric CD40L or medium alone and harvested at indicated time points. Cells were washed once with ice-cold PBS, then resuspended in 100 μl of cell lysis buffer (Cell Signaling Technology). Cellular debris was removed by centrifugation and protein-containing supernatant was stored at −80°C until further use. Equal amounts (15 μl per lane) of the protein-containing supernatants were mixed with 5 μl of 4X NuPAGE SDS Sample buffer (Invitrogen, Carlsbad, CA) and boiled for 5 min, then subjected to NuPAGE Novex high-performance, precast gel (Invitrogen) and blotted onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). Membranes were blocked with blocking buffer (5% nonfat dry milk in TBST buffer, 50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20, pH 7.61) for 1 h at room temperature and incubated at 4°C overnight with primary Ab specific for the phospho-p38 MAPK and total p38 MAPK, phospho-Akt and total Akt, phospho-ERK and total ERK. They were visualized with appropriate HRP-conjugated secondary Abs (Southern Biotechnology Associates, Birmingham, AL) and an ECL detection system (Pierce, Rockford, IL). When inhibitors were used, cells were incubated for 30 min before addition of trimeric CD40L.

Statistical analysis
Data were compared using the Student t test.

Results

CD40L activates p38 MAPK, ERK, and Akt in immature MDDCs
Signal transduction via the MAPK such as ERKs and the p38 MAPK or Akt kinase, a downstream target of the PI3K, play important roles in cellular responses including cell proliferation, differentiation, and survival. CD40 ligation of immature MDDCs results in a potent maturational and survival stimulus. We therefore looked for activation of these signaling pathways in human immature MDDCs treated with trimeric CD40L (CD154). We elected to study MDDCs obtained from individuals with detectable viral-spe-
cific CD8+ T cell responses so that we could also assess the functional activity of these DC on autologous CTL responses (see below). These included healthy human volunteers with CTL directed against EBV epitopes (participants 1–3) and HIV-1-infected individuals with CTL responses directed against HIV-1 and EBV epitopes (participants 4–6, as described in Materials and Methods). In addition, we have previously shown that immature MDDC obtained from HIV-1-infected and HIV-1-uninfected individuals undergo maturation to a similar degree when treated with trimeric CD40L. Thus, the PI3K, ERK, and p38 MAPK results in their phosphorylation. We found that within 30 min of CD40L conditioning of immature MDDCs, PI3K, ERK, and p38 MAPK were activated (Fig. 1). It has previously been shown that, the PI3K can be specifically inhibited by the chomone LY294002 (49), the ERK pathway can be specifically inhibited by the flavonoid, PD98059 (50), and the p38 MAPK can be specifically inhibited by the imidazolide SB203580 (51). Pretreatment of MDDCs with the PI3K inhibitor LY294002, ERK inhibitor PD98059, or p38 MAPK inhibitor SB203580 blocked phosphorylation of PI3K, ERK, or p38 MAPK, respectively, in response to CD40L stimulation (Fig. 1). Thus, the PI3K, ERK, and p38 MAPK signaling pathways are activated after CD40 signaling of human immature MDDCs.

FIGURE 1. CD40L induces the phosphorylation of p38 MAPK, ERK, and Akt. Immature MDDCs from participant 1 were exposed to trimeric CD40L (2 μg/ml) for indicated time intervals at 37°C and then subjected to Western blot analysis with specific Abs. In some cases, the immature MDDCs were preincubated with signal transduction pathway inhibitors for 30 min before exposure to CD40L. The left side shows the detection of phosphorylated forms of p38 MAPK, Akt, and ERK, and the right side shows the same plots probed for total p38 MAPK, Akt, and ERK proteins, respectively, to demonstrate equal loading of samples. Phosphorylation of p38 MAPK, Akt, and ERK induced within 30 min and persisted for 60 min after exposure of immature MDDCs to CD40L. The phosphorylation of p38 MAPK, Akt, or ERK are inhibited if immature MDDCs are preincubated with their corresponding inhibitor SB203580, LY294002, or PD98059, respectively. SB203580, LY294002, and PD98059 were all dissolved in DMSO; therefore, immature MDDCs that had been incubated with DMSO alone were used as controls. Similar results were obtained in three separate experiments. d, DMSO control.
Roles of p38 MAPK, ERK, and PI3K pathways in MDDCs maturation and activation

Maturation of MDDC with CD40L leads to up-regulation of cell surface CD80, CD83, CD86, and HLA-DR, and down-regulation of CD1a. We examined whether p38 MAPK, ERK, and PI3K pathways are necessary for CD40L-induced surface phenotype changes on MDDCs. Inhibition of ERK activation with inhibitor PD98059 slightly reduced HLA-DR and CD86, which was not statistically significant, and had no effect on any other surface phenotype measures of MDDC maturation in response to CD40L stimulation (Fig. 2). Blocking the p38 MAPK pathway with the inhibitor SB203580, however, significantly inhibited the CD40L-induced up-regulation of CD83 and CD86 ($p < 0.05$). Inhibition of p38 MAPK slightly reduced CD40L induction of HLA-DR and CD80 (not significant), and did not affect the CD40L-induced down-regulation of CD1a (Fig. 2). Blocking of the PI3K pathway minimally inhibited CD40L-induced CD1a down-regulation and minimally inhibited CD40L-induced up-regulation of CD80, CD83, CD86, and HLA-DR but this was not statistically significant (Fig. 2). These results show that only certain features of MDDC maturation, such as CD83 and CD86 expression, are regulated by p38 MAPK. Because inhibition of p38 MAPK did not entirely abrogate CD83/CD86 expression after CD40L treatment, as observed with the DMSO alone condition, this suggests that other signaling pathways also play a role in CD83/CD86 up-regulation. Although, inhibiting ERK and PI3K slightly alters CD40L-induced surface marker changes, they do not appear to play a significant role in their regulation.

Roles of p38 MAPK, ERK, and PI3K pathways in DC-induced allogeneic T cell responses

Activated DCs are potent APCs that sensitize T lymphocytes to alloantigens and normal Ags. We therefore tested the ability of CD40L-activated MDDCs to stimulate T lymphocytes in allogeneic MLRs. As reported previously, CD40L-stimulated MDDCs strongly enhanced allogeneic T cell proliferation when compared with immature MDDCs (52). Blocking the ERK pathway with PD98059 or PI3K pathway with LY294002 slightly reduced the capacity of CD40L-induced allogeneic T cell proliferation (Fig. 3). Inhibition of p38 MAPK pathway with SB203580, however, strongly reduced the capacity of CD40L-induced allogeneic T cell proliferation (Fig. 3) to the level of allogeneic responses with unconditioned immature MDDCs (Fig. 3, $p < 0.05$). Thus, the p38 MAPK pathway plays an important role in the allostimulatory capacity of CD40L-activated MDDCs.

MDDCs were harvested and expression of surface molecules was assessed by flow cytometric analysis using mouse anti-human mAbs: PE-conjugated CD80 and CD83, FITC-conjugated CD1a and CD86, and allophycocyanin-conjugated HLA-DR. Appropriate fluorochrome-conjugated isotype was used as control for each stain. Values represent the mean fluorescence intensity subtracted from the value of matched isotype control mouse mAbs (dotted histogram). Data are analyzed using FlowJo software. B, Summary data from experiments performed on MDDCs obtained from all four participants are shown. Bars represent SE on the mean. Comparisons were performed between all conditions and CD40L+/DMSO. Asterisk indicates statistically significant ($p < 0.05$) associations between signaling inhibitor and CD40L conditions. In all experiments, CD40L+/DMSO condition was always statistically significant ($p < 0.05$) from DMSO alone conditions (unconditioned MDDC). MFI, mean fluorescent intensity.
Roles of p38 MAPK, ERK, and PI3K pathways in the expansion of viral-specific memory CTL responses induced by CD40L-activated MDDCs

To determine the role of the signal transduction via p38 MAPK, ERK, or PI3K in the expansion of virus-specific memory CD8⁺ CTL responses induced by CD40L-activated MDDCs, we used an in vitro coculture method in which peptide pulsed conditioned MDDCs are cocultured with CD8⁺ T cells for 7 days in the absence of exogenous cytokines as previously described (14). Three healthy HIV-1-uninfected (participants 1–3) and three asymptomatic HIV-1-infected (participants 4–6) individuals who previously demonstrated EBV- or HIV-1-specific memory responses to well-described HLA-restricted CTL epitopes were studied (also described in Materials and Methods). Circulating virus-specific IFN-γ-producing memory cells within PBMCs, as determined by ELISPOT assay, ranged from 250 to 1000 spot forming cells/10⁶ PBMC in these six study individuals (data not shown). Immature MDDCs derived from these individuals were conditioned with trimeric CD40L for 3 days, then pulsed with the HLA-restricted peptides that they responded to previously (see Materials and Methods) and cocultured with autologous PBMCs for 7 days. For signaling inhibitor experiments, MDDCs were preincubated with indicated inhibitors for 30 min, and then incubated with 2 μg/ml trimeric CD40L. DMSO was used as a control because the inhibitors were dissolved in DMSO. After 7 days of coculture, CTL effector activity was assessed by measuring intracellular IFN-γ production after exposure to peptide-pulsed targets (autologous B lymphoblastoid cell lines or autologous T cell-depleted PBMCs). It should be noted that only MDDCs were exposed to inhibitors before conditioning and that effector CD8⁺ T cells from PBMCs were added later, in the absence of any inhibitors. A representative experiment measuring CTL by IFN-γ by intracellular flow cytometry from HIV-1 seropositive participant 6 is illustrated in Fig. 4A. A summary of the IFN-γ flow cytometric data from participants 1–5 are illustrated in Fig. 4B. Summary pooled data from all participants are shown in Fig. 4C. Immature MDDCs treated with CD40L significantly enhanced EBV- or HIV-1-specific memory CTL responses when compared with medium-treated immature MDDCs (p < 0.0009) (Fig. 4). Blocking the PI3K pathway with LY294002 or ERK pathway with PD98059 slightly reduced the expansion of EBV- or HIV-1-specific viral memory CTLs in response to CD40L-activated MDDCs by ~10–20%, which was statistically significant (p < 0.03 and p < 0.04, respectively; Fig. 4C). Inhibition of the p38 MAPK pathway with SB203580, however, markedly inhibited the capacity of CD40L-activated DCs in the expansion of EBV- or HIV-1-specific viral CTL responses (p < 0.007; Fig. 4C). The effects of SB203580 were not due to nonspecific cellular toxicity as viability of PBMCs treated with SB203580 was comparable to untreated cells by trypan blue staining (data not shown). Inhibition of p38 MAPK within MDDCs almost completely reversed the effects of trimeric CD40L to expand CTL as the CTL response reached to the level of that using unconditioned immature MDDCs (Fig. 4C). Thus, p38 MAPK pathway plays a critical role in the expansion of EBV- or HIV-1-specific viral CTL responses induced by CD40L-activated MDDCs.

Inhibition of p38 MAPK, ERK, or PI3K leads to decreased survival of CD40L-activated MDDCs

Consistent with previous reports, immature MDDCs undergo spontaneous apoptosis in a 48-h culture (39, 40, 53), which can be inhibited by the addition of trimeric CD40L (11). Accordingly, we addressed whether the activation of p38 MAPK, PI3K, or ERK is involved in CD40L-mediated MDDC survival. As shown in Fig. 5, A and B, trimeric CD40L significantly inhibited early MDDC spontaneous apoptosis, as defined by annexin V or activated caspase 3 expression. Inhibition of either p38 MAPK, PI3K, or ERK significantly reversed the CD40L-induced MDDCs survival (pooled data, all p < 0.05, also Fig. 5D), albeit to differing degrees. Whereas p38 MAPK and ERK inhibitors had moderate effects on cellular survival, the PI3K inhibitor completely blocked the CD40L-induced inhibition of apoptosis, indicating that PI3K/Akt is a major signaling pathway involved in CD40L-induced cellular survival (Fig. 5). These effects were seen for both annexin V and activated caspase 3 expression. In fact, blocking PI3K signaling pathway not only completely reversed CD40L-induced inhibition of apoptosis, but tended to produce more apoptosis than unconditioned immature MDDCs (Fig. 5). To ensure that the effects of PI3K inhibitor LY294002 were not from nonspecific cellular cytotoxicity, we found that BMTC treated with or without LY294002 showed no statistical difference in cell apoptosis identified by annexin V (data not shown).

To understand the potential mechanisms by which CD40L-promoted MDDC survival, we examined Bcl-2 expression, a negative regulator of apoptosis. We found that treatment with CD40L significantly enhanced expression of Bcl-2 in MDDCs (Fig. 5C). Bcl-2 up-regulation by CD40L stimulation could be significantly blocked by p38 MAPK, ERK, or PI3K inhibitor, but to the greatest degree by the PI3K inhibitor (Fig. 5C, pooled data, all p < 0.05).

In summary, CD40L-induced suppression of apoptosis of MDDCs is mediated predominantly by the PI3K/Akt pathway, but can also to a lesser degree be mediated by the ERK and p38 MAPK pathways. CD40L-induced suppression of MDDC apoptosis can, in part, be explained by inhibition of caspase 3 activation and an enhancement of Bcl-2 expression, all of which are regulated by the PI3K/Akt, ERK, and p38 MAPK pathways.
FIGURE 4. Effect of p38 MAPK, ERK, and PI3K pathway inhibitors in the expansion of viral-specific memory CTL responses induced by CD40L-activated MDDCs. A, PBMCs from an HIV-1-infected individual (participant 6) were cocultured with MDDCs that were either non-pulsed (far left) or pulsed with HLA-restricted peptides of HIV-1 proteins. In certain conditions, MDDCs were previously treated with CD40L (2 μg/ml) for 72 h in the presence or absence of indicated inhibitors. On day 7, HIV-1-specific CTL activity was assessed by intracellular flow cytometric analysis of IFN-γ-producing CD8+ T cells. The experiment with participant 6 was repeated with similar results. In all conditions with CD40L, DCs were always pulsed with peptide. B, Summary intracellular flow cytometric analysis of EBV-specific CTL from three healthy individuals (participants 1, 2, and 3) and one HIV-1-infected individual (participant 5) and HIV-1-specific CTL from an HIV-1-infected individual (participant 4) are graphically depicted. C, Pooled data from all participants are shown comparing all conditions (except DC without peptide) to the CD40L alone condition, as this latter condition gave maximal CTL. *p < 0.05. Statistical comparisons among data pooled from all participants were performed using the following culture conditions: DC vs DCp, p < 0.0006 (data not shown); DCp vs CD40L, p < 0.0009; CD40L vs CD40L + LY, p < 0.03; CD40L vs CD40L + PD, p < 0.04; CD40L vs CD40L + SB, p < 0.007; CD40L + SB vs DCp, p = not significant. DC, nonpeptide-pulsed DC; DCp, DC pulsed with peptide; CD40L, trimeric CD40L; LY, PI3K inhibitor LY294002; PD, ERK inhibitor PD98059; SB, p38 MAPK inhibitor SB203580. All inhibitors were used in a final concentration of 25 μM.
Effects of p38 MAPK, ERK, or PI3K inhibitors on CD40L-induced IL-12 production from MDDCs

IL-12 is a pleiotropic cytokine that is secreted by activated professional APCs. IL-12 can induce Th1-type cellular responses, T cell proliferation, and IFN-γ secretion from activated T cells and NK cells. In addition, it has been reported that IL-12 prevents apoptotic cell death of the activated human and mouse peripheral T cells (12, 17, 38, 54, 55). We and others previously have demonstrated that MDDCs could be induced to produce IL-12 after CD40 ligation (11, 12, 14). We thus evaluated the relative roles of PI3K, p38 MAPK, and ERK in IL-12 production of CD40L-stimulated MDDCs. Immature MDDCs were stimulated for 16 h with CD40L and IL-12 induction was measured by intracellular flow cytometric analysis. A representative experiment is illustrated in Fig. 6A and summary data from all experiments is shown in Fig. 6B. CD40L stimulation induced significant amounts of IL-12 production by MDDCs. Blocking the ERK signaling pathway with PD98059 had no effects on the IL-12 production whereas blocking either PI3K with LY294002 or p38 MAPK with SB203580 significantly reduced the effect of CD40L on IL-12 production by ~50%. Blocking the p38 MAPK signaling pathway, however, gave the strongest inhibition of IL-12 production. Thus, both the p38 MAPK and PI3K pathways play a critical role in the induction of IL-12 through CD40 ligation of MDDC.

Discussion

During the development of an ongoing immune response, activated CD4+ T cells express CD40 ligand which in turn stimulate immature DCs through CD40 to become superactivated and optimally prime CD8+ T cells as well as help B cells. Our data show

FIGURE 5. Inhibition of ERK, PI3K, or p38 MAPK leads to decreased survival of trimeric CD40L-conditioned MDDCs. A representative experiment from participant 6 is shown. Immature MDDCs, not pretreated or pretreated with indicated inhibitors for 30 min, were incubated for 48 h with 2 μg/ml trimeric CD40L or medium alone. Cells from each condition were harvested and aliquoted for flow cytometric analysis for Annexin V- or HLA-DR-allophycocyanin/caspase-3- or Bcl-2 PE staining. A, Apoptotic cells were assessed for surface phosphatidylserine on the outer leaflet of apoptotic cell membranes using annexin V-fluorescein staining. The numbers (inset right) represents percentage of Annexin V-positive apoptotic MDDCs per total MDDCs. B, Caspase 3 activation of DC was determined by gating on HLA-DR-positive cells (as DC strongly express HLA-DR). The number in each box represents percentage of positive caspase 3 MDDCs per total HLA-DR-positive cells. C, Intracellular Bcl-2 expression is represented. The number (inset in box) in each condition represents percentage of positive Bcl-2 cells per total HLA-DR-positive cells. A representative experiment of four experiments for annexin V, caspase 3, and Bcl-2 analysis is shown. D, Pooled data from four participants studied is shown for annexin V staining. *, p < 0.05. Statistical analyses of pooled data were performed on the following conditions: DMSO vs DMSO+CD40L, p < 0.05; DMSO+CD40L vs CD40L+LY294002, p < 0.004; DMSO+CD40L vs CD40L + PD098059, p < 0.009; DMSO+CD40L vs CD40L + SB203580, p < 0.003.
that at least three signaling pathways are activated within immature MDDCs after CD40 ligation; the PI3K, ERK, and p38 MAPK pathways. It was surprising that surface markers of activation and maturation were not greatly affected by the inhibition of these pathways. Only the p38 MAPK pathway played a significant role in CD38 and CD86 up-regulation, although inhibiting p38 MAPK did not completely abrogate CD83 and CD86 expression. This indicates that other signaling pathways, likely are involved with regulation of CD1a, CD80, and HLA-DR, as well as CD83 and CD86. The role of CD83 in inducing immune responses is beginning to emerge. Recently, Ledbetter and colleagues (56) showed that, although the p38 MAPK pathway is instrumental for DC functionality. Our observations indicate that p38 MAPK is required for a number of features involved in DC maturation including up-regulation of CD83 and CD86, IL-12 production, and inhibition of apoptosis. It is probable that this combination of events is necessary to optimally prime T cells, rather than one feature alone. It was somewhat surprising to find that, although the PI3K pathway was most critical in the prevention of MDDC apoptosis, inhibition of this pathway could still activate T cells to varying degrees. Because our MDDC-T cell coculture system only stimulated cells for a 7-day period, it is possible that the effects of PI3K inhibition might have been more dramatic on T cell expansion with longer culture periods or in in vivo systems, in which the longevity of DCs becomes more important in maintaining immune responses.

In summary, the p38 MAPK pathway is necessary for CD40L to condition DCs to become competent Ag presenters to T cells. Recently, a number of small molecule inhibitors of the p38 MAPK pathway have been proposed for use in the clinical setting to prevent inflammatory responses (59, 60). It is likely that these inhibitors will partly work by preventing the necessary signaling pathways provided by CD40L to optimally mature and activate DCs.
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References


