CEACAM1 (CD66a) Promotes Human Monocyte Survival via a Phosphatidylinositol 3-Kinase- and AKT-dependent Pathway*

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CEACAM1 (also known as CD66a) is a transmembrane glycoprotein that mediates homophilic intercellular interactions that influence cellular growth, immune cell activation, and tissue morphogenesis. Various studies have suggested a link between CEACAM1 and cellular apoptosis, including a recent demonstration that ERK1/2 signaling is triggered downstream of CEACAM1. In this study, we reveal that CEACAM1-long binding confers survival signals to human peripheral blood mononuclear cells. CEACAM-specific antibodies effectively protected peripheral blood mononuclear cells from apoptosis, with this effect being particularly dramatic for primary monocytes that undergo spontaneous apoptosis during culture. This protective effect was reiterated when using soluble CEACAM1, which binds to cell-surface CEACAM1 via homophilic interactions. Monocyte survival correlated with a CEACAM1-dependent up-regulation of the cellular inhibitor of apoptosis Bcl-2 and the abrogation of caspase-3 activation. CEACAM1 binding triggered a phosphatidylinositol 3-kinase-dependent activation of the protein kinase Akt without influencing the activity of extracellular signal-related kinase ERK, whereas the phosphatidylinositol 3-kinase-specific inhibitor LY294002 effectively blocked the protective effect of CEACAM1. Together, this work indicates that CEACAM1 confers a phosphatidylinositol 3-kinase- and Akt-dependent survival signal that inhibits mitochondrial-dependent apoptosis of monocytes. By controlling both ERK/MEK and PI3K/Akt pathways, CEACAM1 functions as a key regulator of contact-dependent control of cell survival, differentiation, and growth.

The carcinoembryonic antigen-related cell adhesion molecule (CEACAM1; also known as CD66a and the biliary glycoprotein) is a highly abundant and broadly expressed glycoprotein of the immunoglobulin superfamily. It consists of a single Ig variable domain-like amino terminus, from one to three Ig constant domain-like regions, and a single membrane-spanning segment followed by either a short (CEACAM1-S) or long (CEACAM1-L) cytoplasmic domain. The amino-terminal domain facilitates homophilic (CEACAM1-CEACAM1) intercellular binding that influences a broad spectrum of cellular processes related to cellular activation and/or cell cycle progression (reviewed in Ref. 1). It is also targeted by the adhesins of viral (i.e. murine hepatitis virus (2)) and bacterial (i.e. Neisseria gonorrhoeae and Neisseria meningitidis (3), Moraxella catarrhalis (4) and Haemophilus influenzae (5)) pathogens, allowing their infection of the diverse array of CEACAM1-expressing human cells and tissues in vivo (3).

CEACAM1 is expressed on a myriad of cell types, including epithelial, endothelial, and hematopoietic cells. In immune cells, CEACAM1 influences cellular responses to various stimuli. For example, CEACAM1 binding amplifies proliferation and immunoglobulin secretion in response to B cell receptor ligation without increasing their differentiation into plasma cells or immunoglobulin class switching (6). In T lymphocytes, cellular activation leads to increased CEACAM1 expression at the cell surface, both through the rapid mobilization of intracellular receptor stores and de novo protein synthesis (7–9). Although CEACAM1-specific antibodies may stimulate T cells under certain conditions (7), CEACAM1 binding generally inhibits T cell responses. For example, in vitro studies have revealed that CEACAM1-specific antibodies reduce proliferation, expression of activation markers, and cytotoxic function in response to various stimuli (9–11). Remarkably, CEACAM1 binding by the N. gonorrhoeae Opa proteins also inhibits T cell activation, providing this pathogen with an effective strategy by which to avoid the development of specific immunity (9).

Recent evidence suggests that the effect of CEACAM1 on immune cell function depends upon the isoform expressed. Differential splicing of CEACAM1 transcript generates either a long (73 amino acids) cytoplasmic domain-containing CEACAM1-L, which contains two immunoreceptor tyrosine-based inhibitory motifs (ITIMs)3 that inhibit cellular growth
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and activation (12–14), or the CEACAM1-S isoform, which contains a short (10 amino acids) cytoplasmic tail that lacks the inhibitory ITIMs. As in epithelial cells (15), the inhibitory effect of CEACAM1-L in lymphocytes involves SHP-1 phosphatase recruitment to the phosphorylated ITIM (9, 13, 14). The cellular response to binding thus depends upon the relative level of splice variants expressed. In fact, the demonstration that the ratio of CEACAM1-L versus CEACAM1-S isoforms expressed changes from 1:1 to 3:1 upon B cell activation (6) suggests an increasing potency of the inhibitory signals of the receptor upon B cell activation.

CEACAM1 has been demonstrated to influence apoptosis by epithelial cells. During normal mammary alveolar morphogenesis in extracellular matrix-containing Matrigel, CEACAM1-blocking antibodies inhibit the apoptotic death of cells within the forming acini, thereby preventing normal lumen formation (16). The isoform of CEACAM1 expressed can dramatically affect this process, as CEACAM1-S is required for normal acinar formation, whereas CEACAM1-L blocks it (16, 17). Interestingly in this regard, the CEACAM1-L cytoplasmic domain is cleaved by pro-apoptotic caspase-3 to liberate a truncated derivative that lacks the tyrosine-containing ITIMs and resembles CEACAM1-S (18). This makes it tempting to speculate that, in contrast to CEACAM1-S, signals emanating from the CEACAM1-L cytoplasmic domain oppose the apoptotic process. Indeed, Singer et al. (19) have recently demonstrated that CEACAM1 binding affects survival of rat neutrophils by triggering ERK kinase-dependent signals.

Here, we establish that CEACAM1 binding confers potent protection of primary human monocytes from apoptosis via a process that involves a phosphatidylinositol 3-kinase- and Akt-dependent induction of Bcl-2 expression that prevents the activation of caspase-3. Together with previous work, this reveals a central role for CEACAM1 in sensing intercellular contact to regulate survival, cell cycle progression, and cellular differentiation through the distinct but interacting ERK/MEK and PI3K/Akt signaling cascades.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—The mouse monoclonal antibodies (mAbs) D14HD11 (IgG1, cross-specific for human CEACAM1, CEACAM3, CEACAM5, and CEACAM6) and 9A6 (specific for human CEACAM6) were kind gifts of Dr. F. Grunert (University of Freiburg, Germany). Col-1 (cross-specific for human CEACAM3 and CEACAM5) was from Zymed Laboratories Inc.. The murine MOPC-21 IgG1 mAb, used as an isotype control throughout this study, was purchased from Sigma. Unless otherwise indicated, anti-human mAbs or polyclonal Abs conjugated with fluorochrome were purchased from Pharmingen. Anti–Akt antibody, anti-phospho–Akt antibody, Akt and phosphorylated Akt control cell extracts, anti-MEK1/2 antibody, anti-phospho-MEK1/2 antibody, MEK1/2, and phosphorylated MEK1/2 control cell extracts were from Cell Signaling Technology (Pickering, Ontario, Canada). The phosphatidylinositol 3-kinase (PI3K)-specific inhibitor LY294002 and mitogen-activated protein/ERK kinase-specific inhibitor PD98059 were from Sigma and were dissolved in dimethyl sulfoxide (Sigma) before use. Soluble CEA (CEACAM5) isolated from a liver metastasis of human colon carcinoma was purchased from Fitzgerald Industries International (Concord, MA). Recombinant CEACAM1-Fc protein, consisting of the extracellular domains of CEACAM1 fused to the Fc portion of human IgG1, was purified from transiently transfected COS-7 cells using FuGENE 6 reagent (Roche Applied Science), as described previously (20). FLAG-tagged Fas ligand is a recombinant protein containing an extracellular domain of human Fas ligand (amino acids 103–281) fused at the amino terminus to a linker peptide (26 amino acids) and a FLAG tag (Kamiya Biomedical, Seattle, WA).

Cell Preparations—Heparinized human blood was obtained from healthy blood donors, and peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation on Ficoll-Paque (Amersham Biosciences) and then frozen at −152 °C until use. CD3+, CD4+, and CD8+ T cells and CD19+ B cells were separated from PBMCs by single cell sorting in a FACSCalibur flow cytometer (BD Biosciences). Monocytes were separated from the PBMCs using multistep Percoll (Sigma) gradient centrifugation and then purified by depletion of contaminating B cells, T cells, NK cells, and granulocytes using antibody-conjugated magnetic beads in the monocyte negative isolation kit (Dynal, Oslo, Norway) according to the manufacturer’s guidelines. The resulting cell preparations contained more than 95% monocytes and virtually no residual T and B lymphocytes, as assessed by CD14, CD4, CD8, and CD19 staining and flow cytometric analysis. C1R B cell lines were the kind gifts of Dr. K. S. MacDonald (University of Toronto). Epstein-Barr virus-transformed human B cell lines were established from the PBMCs of healthy blood donors (21). Procedures involving isolation and handling of primary blood cells have been approved by the University of Toronto Ethics Review Unit.

Flow Cytometric Analysis—Flow cytometric analysis and fluorescence-activated cell sorting (FACS) were performed using a FACSCalibur (BD Biosciences) after staining by standard methods, and all data were analyzed using FlowJo software (Tree Star Inc., San Carlos, CA). Forward and side scatter characteristics were used to localize monocyte and lymphocyte populations, with confirmation that gates contained these cell types by staining with CD14+ (expressed by monocytes) and CD3+ (a T cell marker), respectively. For intracellular staining, cells were permeabilized using the Cytofix/Cytoperm Plus kit (Pharmingen). Intracellular staining was performed using FITC- and PE-conjugated antibodies specific for active human caspase-3 or Bcl-2, respectively. Control staining with an irrelevant isotype-matched Ab was performed during every FACS analysis. To analyze the surface expression of CEACAM1 on PBMCs, the cell suspension was first stained with mAb D14HD11 or a nonspecific isotype control as primary Ab, followed by goat anti-mouse IgG1 APC, and then stained with anti-human CD3PerCP, CD4PE, CD8FITC, CD14FITC, or CD19FITC as indicated. HeLa cells transfected with either a human CEACAM1 cDNA-encoding plasmid or the empty pRC/CMV-megaviral expression vector were used as positive and negative controls, respectively.

Cell Survival Analysis and Inhibitor Treatment—Duplicate or triplicate wells containing 0.1 × 10^6 PBMCs or isolated monocytes were cultured for 48 h in flat-bottomed 96-well
plates in complete RPMI 1640 medium (containing 10% fetal calf serum, 2 mM glutamine, 25 mM HEPES, and antibiotics) with or without mAb D14HD11 or an isotype-matched control mAb at the indicated concentrations. Where indicated for antibody immobilization, 10 μg/ml mAb or isotype-matched control antibody in plain RPMI 1640 medium was adsorbed to the wells of a flat-bottom 96-well tissue culture plate by incubation at 37 °C for 2 h and then washed three times with plain RPMI 1640 immediately before use. Apoptotic cell death and cellular apoptosis were determined by staining with annexin-V-FITC and PI from the annexin-V-FLUOS staining kit (Roche Applied Science) according to the manufacturer’s protocol. Apoptosis was also monitored by measuring active intracellular caspase-3.

When PI3K inhibitor LY294002 or the mitogen-activated protein/ERK inhibitor PD98059 was used, cells were preincubated with these inhibitors or the same dilutions of the dimethyl sulfoxide solvent for 30 min prior to the addition of mAb D14HD11 or isotype control.

**Western Blot Analysis**—Monocytes (5–10 × 10^6^ cells) were incubated with medium alone, 1 μg/ml mAb D14HD11, 1 μg/ml isotype-matched control mAb, or 10 μg/ml LPS and then harvested at the indicated time points. Cells were washed once with ice-cold phosphate-buffered saline prior to their resuspension in 100 μl of cell lysis buffer (Cell Signaling Technology, Pickering, Ontario, Canada) containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM Na3VO4, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na4VO4, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride. Cells were incubated for 15 min at 4 °C on an orbital shaker and then centrifuged to pellet the cellular debris. The protein-containing supernatant was stored at −80 °C until use. Equal amounts (15 μl per lane) of the protein-containing supernatants were mixed with 5 μl of 4× NuPAGE LDS Sample Buffer (Invitrogen), boiled for 5 min, subjected to NuPAGE Novex high performance gel electrophoresis (Invitrogen), and then blotted onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked with 5% nonfat dry milk in TBS-T buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05% Tween 20 (pH 7.6)) for 1 h at room temperature and then incubated at 4 °C overnight with primary antibody specific for phosphorylated Akt versus total Akt, or phosphorylated ERK versus total ERK. These were detected using horseradish peroxidase-conjugated secondary antibodies (Southern Biotechnology Associates, Birmingham, AL) and visualized using the ECL detection system (Pierce).

**RT-PCR**—Total RNA was extracted from the indicated cells or cell lines using TRIzol reagent (Invitrogen). RNA yield was quantified photometrically, and 1.5 μg of total RNA in a final volume of 20 μl was reverse-transcribed into first strand cDNA by Moloney murine leukemia virus reverse transcriptase using the SuperScript first strand synthesis system (Invitrogen) according to the manufacturer’s recommendation. To amplify CEACAM1, we employed a previously described triple primer RT-PCR in which one common sense primer was used together with two antisense primers that selectively recognize the CEACAM1 splice variants encoding either a long (73 amino acids) or short (10 amino acids) cytoplasmic domain. The oligonucleotide primer that specifically hybridizes to the 3′ regions of each human CEACAM1 splice variant, 5′-GTT-GTTTCTGTCCC-3′, was used for initial cDNA synthesis. A common sense primer FP49 (5′-GCAACAGGACCACAGT- CAAGACGA-3′) that recognizes both CEACAM1 long and short splice variants equally was used along with the antisense primers BP60 (5′-GTTGTTGAGACTGAGGTTT-3′) and BP59 (5′-TGGAGTGTTGCTAGTGC-3′), which are specific for the two differentially spliced isoforms. Amplification of transcript encoding the constitutively expressed housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) with primer mix 5′ primer TACCATCTCCAGAGGG and 3′ primer CTGCTCACCACCTTCTTGA used as a control (22). Oligo(dT)12–18 primer (Invitrogen) was used for GAPDH cDNA synthesis. PCR was performed in a total volume of 50 μl containing 2.5 μl of first strand cDNA solution, 0.2 mM dNTPs, 3 units of TaqDNA polymerase (Amersham Biosciences), 5 μl of 10× PCR buffer, and 0.6 μM concentrations of each of PCR primers. The reactions were initiated by heating the samples to 95 °C for 60 s, followed by 30 cycles of 94 °C for 30 s, 64 °C for 60 s, and 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. The products were analyzed on 2% agarose gels and visualized by ethidium bromide staining. The relative amounts of the PCR products were analyzed by scanning the gels and determining the intensities in the ethidium bromide-stained bands using Scan analyses software (Biosoft, Milltown, NJ).

**Statistical Evaluation**—For comparisons between groups, analysis of variance with Fisher’s post hoc testing was performed. Statistical significant was defined by p ≤ 0.05.

**RESULTS**

**CD66 Epitope-specific Antibodies Inhibit Spontaneous Apoptosis of PBMCs and Purified Monocytes**—Human PBMCs and monocytes cultured in vitro undergo spontaneous apoptosis without requiring additional external stimuli (23–25). This process can be accelerated and enhanced by the removal of serum. However, even in the presence of 20% serum, the majority of monocytes will undergo apoptosis over several days (25), whereas the surviving monocytes differentiate to become macrophages. Consistent with previous observations (23–25), we observe that significant death of PBMCs occurs following 48 h of culture in vitro. Apoptotic and necrotic cells are apparent by flow cytometry as a population exhibiting reduced forward light scatter (FSC) as a result of gross changes in cell size and refractive index, and increased side scatter (SSC) as a result of chromatin condensation (Fig. 1A, indicated as population within gate A for FSC versus SSC plot for sample cultured in medium alone). The apoptotic nature of these cells is supported by their staining with annexin V, which binds to phosphatidylserine in depolarized membranes, and the expression of active caspase 3 (see below). In clear contrast, relatively few cells were present within this apoptotic gate (A) when PBMCs were cultured in the presence of the mAb D14HD11, which defines the CD66 epitope-expressing subset of the CEACAM family of receptors (26). This change was not attributable to the simple addition of antibody, as PBMCs cultured in the presence of the isotype control mAb MOPC-21 were indistinguishable from those grown in medium alone (Fig. 1A, compare FSC versus SSC.
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The protection of monocytes present in mixed PBMC populations may either be attributable to the CD66-specific antibodies binding monocytes directly, or the antibodies could bind to other cells that can promote survival of monocytes in vitro. To assess these possibilities, we purified monocytes from other PBMCs by negative selection with magnetic beads, and then cultured these in the presence or absence of immunoglobulin. The survival of isolated monocytes cultured with CD66-specific versus control antibodies or medium alone (Fig. 1D) was indistinguishable from that observed for monocytes cultured with other PBMCs (Fig. 1A, SSC versus annexin V plot of cells within gate M). Given the reproducibility of results obtained whether the antibodies were added to PBMC cultures or purified monocytes, we interpret them to indicate that the CD66-specific antibodies function by binding to monocytes directly.

CEACAM Expression by ex Vivo Human PBMCs—The CD66 epitope-specific mAb D14HD11 recognizes a subset of the human CEACAM family of receptors, which are differentially expressed by various human leukocytes (7, 27). Consistent with these previous reports, we detected D14HD11 binding to ex vivo CD4+ and CD8+ T cells and CD19+ B cells by surface staining (Fig. 2A). Ex vivo CD14+ monocytes also expressed the CD66 epitope, albeit this was lower than that detected on the lymphocytes. Flow cytometric analysis revealed no apparent change in CEACAM1 expression during 48 h of in vitro culture of the monocytes (data not shown).

Given that mAb D14HD11 recognizes CEACAM1 (CD66a), CEACAM3 (CD66d), CEACAM5 (CD66e), and CEACAM6 (CD66c), we performed immunoblot analysis of purified monocytes using mAbs that recognize various combinations of these receptors. The mAb D14HD11 revealed a doublet of ~140 and 160 kDa, neither of which was detected by the CEACAM6-specific mAb 9A6 or CEACAM3 and CEACAM5 cross-specific mAb Col-1 (Fig. 2B), confirming previous reports that monocytes express exclusively CEACAM1 (28). CEACAM1 frequently appears as two or more bands by immunoblot analysis, a characteristic that is presumably attributable to the expression of multiple splice variants (26) and/or to differential glycosylation of this receptor (29). Although the role of carbohydrate moieties on CEACAM1 remains poorly understood, the differential splicing of CEACAM1 transcripts has significant consequences to the cell. The full-length CEACAM1 protein contains from one to four extracellular immunoglobulin-like domains that mediate intercellular homophilic (CEACAM1-CEACAM1) binding, followed by transmembrane and cytoplasmic domains, the latter of which contain the ITIMs that control cellular growth (15, 30), immune cell activation (6, 9, 10, 31), and tissue morphogenesis (17, 32). CEACAM1-S isoforms lack these tyrosine-containing cytoplasmic sequences. We performed RT-PCR to ascertain which splice variants were expressed by the primary human monocytes. The products obtained correspond to CEACAM1 with 3 (207-bp product) and 4 (506-bp product) extracellular domains (Fig. 2C), a transmembrane domain, and a full-length cytoplasmic domain, indi-
FIGURE 2. Expression of CEACAM1 in T cells, B cells, and monocytes. A, histograms show the fluorescence intensity of CEACAM1 on the surface of CD3⁺ T cells, CD3⁻CD4⁺, and CD3⁻CD8⁺ T cell populations, CD19⁺ B cells, and freshly isolated CD14⁺ monocytes. Solid lines show the fluorescence intensity obtained with anti-human CEACAM1 mAb (D14HD11), and dashed lines indicate background fluorescence obtained with isotype-matched control mAb. Values listed represent the mean fluorescence intensity subtracted from the background fluorescence, with values of less than 5 being considered negative in this study. HeLa cells and a polyclonal population of HeLa cells transfected with human CEACAM1 cDNA were used as negative and positive FACS staining control, respectively. B, Western blot analysis of target protein of mAb D14HD11. Equal amounts of protein extracts from freshly isolated monocytes were subjected to Western blot analysis with mAbs of D14HD11 (cross-specific for human CEACAM1, CEACAM3, CEACAM5, and CEACAM6), 9A6 (specific for human CEACAM6), and Col-1 (cross-specific for human CEACAM3 and CEACAM5). C, RT-PCR detection of human CEACAM1. RNA isolated from FACS-sorted CD3⁺ T cells, CD4⁺, CD8⁺ T cells, CD19⁺ B cells, Epstein-Barr virus-transformed B cell line C1R, and freshly isolated CD14⁺ monocytes was subjected to RT-triple-PCR to detect RNA transcript encoding CEACAM1. The oligonucleotides FP49, BP60, and BP59 were used as primers in the PCR. No products corresponding to CEACAM1–4S or CEACAM1–3S were detected in any of these cell types. Only bands corresponding to CEACAM1–4L (506 bp) and CEACAM1–3L (207 bp) were evident. C⁻ lane indicates RT-PCR negative control using RNA extracted from untransfected HeLa cells. Amplification of a cDNA fragment of human housekeeping gene GAPDH indicated a comparable amount of RNA. Figure is representative of four similar experiments.
cating CEACAM1–3L and CEACAM1–4L, respectively. Consistent with previous work (7, 27), no products corresponding to CEACAM1 splice variants with a short cytoplasmic domain were detected in the monocytes.

**CEACAM1 Binding Suppresses the Activation of Caspase-3 and Up-regulates Bcl-2**—Caspase-3 represents an “executioner” protease that is central to both intrinsic and extrinsic apoptotic pathways (33). Synthesized as an inactive proenzyme, caspase 3 is proteolytically activated to generate a functional heterodimer. Given that CEACAM1 binding promoted survival of PBMCs and particularly monocytes, we predicted that caspase-3 activation should also be suppressed. To test this, we measured active caspase-3 by intracellular staining and flow cytometric analysis. CEACAM-specific mAb dramatically reduced caspase-3 activation compared with that evident in cultures incubated with an isotype-matched irrelevant mAb or

**FIGURE 3. Suppression of caspase-3 activation and up-regulation of Bcl-2 by anti-human CEACAM1 mAb.** PBMCs or freshly isolated monocytes were incubated with 1 μg/ml mAb D14HD11 or 1 μg/ml isotype-matched control mAb or left not stimulated for 48 h for caspase-3 analysis or 16 h for analysis of Bcl-2 expression. A, level of active caspase-3 was analyzed by intracellular staining using specific Abs for active caspase-3. The numbers show the percentage of active caspase-3 positive cells out of the total cells. B, elevation of intracellular expression of Bcl-2 protein by isolated monocytes was analyzed by intracellular staining using PE-conjugated Bcl-2 antibody reagent set, including Bcl-2PE and isotypePE antibodies. Values represent the mean fluorescence intensity obtained from Bcl-2-specific Ab staining subtracted from the value of matched isotype staining control (dotted histogram).
medium alone (Fig. 3A). Even when considering independent experiments together, activated caspase-3 expression in PBMC cultured for 48 h with medium alone or isotype-matched control mAb was 13.6 ± 3.1% (n = 5) and 12.8 ± 3.7% (n = 5) respectively. In contrast, active caspase-3 was detected in only 1.1 ± 0.4% of cells when 1 μg/ml of CEACAM1 mAb was added. The CEACAM-specific mAb inhibited caspase-3 activation in a dose-dependent manner, reaching a plateau at a concentration of 1 μg/ml of CEACAM1 mAb when 1 μg/ml of CEACAM1 mAb was added. The CEACAM-specific mAb inhibited caspase-3 activation in a dose-dependent manner, reaching a plateau at a concentration of 1 μg/ml (data not shown), which correlated with cell survival analysis by annexin V and PI staining (Fig. 1C). Also consistent with the viability staining, the effect of CEACAM-specific mAb on caspase-3 activation was most dramatic in monocytes (Fig. 3A), whereas an effect on caspase-3 in lymphocytes was not clearly evident (Fig. 3A and data not shown). The effect on monocyte caspase-3 activation was similar regardless of whether they were being cultured with other PBMCs or in isolation (Fig. 3A), again implying that it emanates from mAb D14HD11 binding directly to CEACAM1 on the monocytes.

To understand the molecular mechanism by which CEACAM binding promotes monocyte survival, we next measured the expression of Bcl-2. During intrinsic (i.e. mitochondrial) pathways of programmed cell death, this anti-apoptotic factor appears to function by protecting mitochondrial membrane integrity, blocking the release of cytochrome c and other factors that may ultimately lead to the activation of caspase-3. The CEACAM-specific mAb caused a marked increase in the expression of Bcl-2 by purified monocytes (Fig. 3B). A similar effect was apparent when monocytes were instead cultured together with other PBMCs; however, no similar increase in Bcl-2 staining was apparent in lymphocytes (data not shown).

Monocyte Survival Conferred by Soluble and Immobilized CEACAM1-specific Ligands—To ascertain both the influence of binding valency and the relevance of survival signals triggered by the CEACAM1-specific mAb, we tested the effect of other ligands specific for this receptor. Untreated cells (medium) and cells treated with bacteria-derived endotoxin...
(LPS), which promotes monocyte survival via a CD14-dependent pathway independent of CEACAM1 (34), were used as negative and positive controls, respectively. Caspase-3 activation and annexin staining of monocytes incubated with either soluble (Fig. 4, A and B) or immobilized (Fig. 4, C and D) CEACAM-specific mAbs were indistinguishable from that observed with endotoxin, indicating that CEACAM1-dependent survival signals are as potent as those in response to LPS. Immobilized isotype Ig, which does not bind CEACAM1, had no effect on monocyte survival, confirming that the effect of the CEACAM1-specific mAb was not because of monocyte Fc receptor binding to antibody bound on the surface of adjacent cells.

When a soluble chimera containing the four extracellular domains of CEACAM1 was applied to cells, a dramatic reduction of cell death was also apparent (CEACAM1-Fc; Fig. 4), indicating that the previously established homophilic (CEACAM1-CEACAM1) binding of this receptor is sufficient to trigger survival signals. Soluble CEACAM5 also reduced caspase-3 production (Fig. 4A, compare Medium and CEA); however, it did not significantly reduce annexin staining of the cells as compared with those incubated with medium alone (Fig. 4B). Immobilized CEACAM5 had no apparent effect on the monocytes (Fig. 4, C and D) under the conditions tested. Whether the different effects of CEACAM1 and CEACAM5 stem from a reduced affinity of
CEACAM1 for the latter and/or a steric difference in the binding interaction remains unclear.

Given the exquisite sensitivity of monocytes to endotoxin, we confirmed that antibody preparations were free of endotoxin by adding CD14-specific antibodies, which effectively blocked the survival-promoting effects of LPS without any effect on survival conferred by the CEACAM binding (Fig. 4E).

**CEACAM1-dependent Down-regulation of Caspase-3 Activity Requires PI3K**—Various growth factors (macrophage-colony-stimulating factor and granulocyte-macrophage colony-stimulating factor), cytokines (tumor necrosis factor-α, interleukin-1β, and interleukin-18), and other stimuli (LPS) promote the survival of human monocytes via a PI3K-dependent activation of the serine/threonine kinase Akt (35). Accordingly, we addressed whether this pathway was also involved in the CEACAM1-dependent monocyte survival. As illustrated in Fig. 5, culturing purified human monocytes with the CEACAM-specific mAb induced Akt phosphorylation, an effect that was not evident when the cells were instead cultured with the nonspecific isotype control or in medium alone. Moreover, the PI3K-specific inhibitor LY294002 blocked the CEACAM1-dependent Akt phosphorylation (Fig. 5) and removed the protective effect of CEACAM-specific antibodies, as evidenced by the increased annexin VFITC staining (Fig. 6A), dose-dependent reduction in monocyte viability (Fig. 6B), and dose-dependent increase in caspase-3 activation (Fig. 6, C and D) in the presence of LY294002. In each case, similar effects were not apparent when the cells were grown in the presence of ERK-specific inhibitor PD98059 or in the dimethyl sulfoxide solvent alone (Figs. 5 and 6).

**Effect of CEACAM1 Binding on FasL-Fas-mediated Apoptosis**—Spontaneous apoptosis of monocytes is mediated in part through CD95 L-CD95 interactions via an autocrine/paracrine mechanism (36). Thus, the effect of CEACAM1 antibody on Fas-mediated apoptosis of monocytes induced by exogenous soluble Fas-L protein was also tested. A dose-dependent enhancement of monocyte apoptosis was observed with exogenous Fas-L protein, and control Ig had no effect on this outcome (Fig. 7). The CEACAM-specific D14HD11 mAb effectively inhibited Fas-mediated cell death at all concentrations of FasL. The protective effect of anti-CEACAM mAb was partially overcome by high (500 ng/ml) concentrations of the ligand, suggesting that the relative level of these two stimuli determines cell survival.

**DISCUSSION**

Spontaneous apoptosis of monocytes occurs through two independent pathways, a death receptor (e.g. CD95) and a mitochondrial mediated pathway (34). CD95 ligand (FasL) expression on monocytes can signal through CD95 (Fas) in a paracrine/autocrine manner, resulting in activation of caspase 8 and caspase 3 downstream. In monocytes, this pathway has been shown previously to be inhibited by LPS or tumor necrosis factor-α (34). Stressful stimuli such as oxidative damage or the limitation of growth factors induce cellular Bcr homology domain 3-only proteins to associate with Bcl-2 on the mitochondrial membrane. Sequestration of Bcl-2 then allows the Bax and Bak protein-dependent release of cytochrome c and other effectors from the mitochondrion, leading to the activation of APAF-1 and caspase-9. Downstream activation of caspase-3 then ultimately leads to apoptotic cell death (33).

In this study, we explored the effect of CEACAM1 on the spontaneous apoptosis of primary monocytes cultured in vitro. CEACAM1-specific monoclonal antibodies had a dramatic effect on monocyte survival, effectively protecting them from apoptosis. Purified CEACAM1 reproduced these results, indicating that well described homophilic interactions between the IgV-like amino-terminal domain of the receptors was sufficient to confer this anti-apoptotic effect. CEACAM1 binding increased expression of the anti-apoptotic factor Bcl-2, reduced activation of the cysteine protease caspase-3, and prevented depolarization of the cytoplasmic membrane, as indicated by annexin V binding to surface-exposed phosphatidylserine. CEACAM1-specific antibodies also reduced monocyte death in response to soluble FasL, indicating that CEACAM1 binding protects against both intrinsic (mitochondrial)- and extrinsic (death receptor)-mediated pathways of apoptosis (33).

The anti-apoptotic effect of CEACAM1 requires active PI3K, which leads to phosphorylation of the serine/threonine kinase Akt (also called protein kinase B), a well established survival signal that stimulates Bcl-2 protein expression (33). Akt is activated upon translocation to the plasma membrane, which occurs by virtue of its pleckstrin homology domain binding to phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate generated by PI3K. We have previously observed the CEACAM1-dependent recruitment of class III PI3K to phagosomes containing...
FIGURE 6. PI3K activity is required for CEACAM1-dependent monocyte survival. Freshly isolated monocytes were preincubated with the PI3K inhibitor LY294002 or ERK inhibitor PD98059 for 30 min prior to incubation in 1 μg/ml of CEACAM1-specific mAb D14HD11, 1 μg/ml of isotype-matched control mAb (MOPC21), or medium alone for 48 h. A, cellular survival was monitored by flow cytometry, with live cells being defined as an absence of annexin V and PI staining. DMSO, dimethyl sulfoxide. B, the percentage of cells surviving after 48 h of incubation in medium supplemented with the indicated antibodies and/or dilutions of inhibitor was determined by flow cytometry, as outlined above. C, active caspase-3 was detected by intracellular staining with specific anti-human caspase-3 mAb conjugated with FITC. Dot plots depict sideward scatter versus caspase-3. D, the percentage of cells expressing active caspase-3 following culture in medium containing the indicated antibodies and/or dilutions of inhibitor was determined by flow cytometry. In each instance (A–D), a representative of at least three independent experiments is shown.
N. gonorrhoeae (37). However, the binding specificity of the Src homology 2 domain within this and other PI3K subclasses (38) suggests that their recruitment is likely via an adaptor protein rather than PI3K binding directly to CEACAM1.

In contrast to neutrophils (19), the activation of the ERK kinase was not apparent, and ERK-specific inhibitors did not abrogate the protective effect of CEACAM1 binding on monocytes. Although the ERK/MEK cascade is most intimately associ-
associated with control of cell cycle progression and differentiation and the PI3K/Akt cascade is considered central to cellular protection from apoptosis (39), these pathways are tightly interlinked. In this regard, it is interesting to consider that neutrophils are terminally differentiated, whereas monocytes can undergo further differentiation. Whether the lack of effect of ERK inhibitors in monocytes reflects a difference in cellular differentiation state and cell type or instead implies that PI3K/Akt activation overcomes an absence of ERK/MEK signaling awaits future work aimed at dissecting how these two pathways coordinate downstream of CEACAM1 in phagocytes. Given that tumors often down-regulate or lose CEACAM1 expression (12, 40–44), understanding the link between this receptor and cellular growth, differentiation, and apoptosis has obvious implications for the role of CEACAM1 in other systems.

In at least some cell types, CEACAM1 transcripts may be differentially spliced during progression through the cell cycle (6, 45). Recent studies suggest that the ratio of splice variants expressed may radically alter the effect of CEACAM1 binding. The full-length CEACAM1-L inhibits immune cell function (9, 10, 20), whereas a CEACAM1-S variant that lacks most of the cytoplasmic domain has no similar effect (13, 14). These differ-

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**FIGURE 7. CEACAM-specific mAb protects monocytes from FasL-mediated cell death.** Freshly isolated monocytes were incubated at 37 °C for 3 h with mAb D14HD11, isotype-matched control mAb, or medium alone in the presence of indicated concentrations (ng/ml) of soluble FasL. Caspase-3 expression was monitored by intracellular staining and flow cytometry. Values listed indicate the proportion of cells expressing active caspase-3.
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ences presumably result from the absence of an immunoreceptor tyrosine-based inhibitory motif (ITIM) in CEACAM1-S, leading to an inability to recruit Src homology 2 domain-containing signaling molecules such as the tyrosine phosphatases SHP-1 and/or SHP-2. It is, however, pertinent to note that the short cytoplasmic domain of CEACAM1-S is sufficient to engage some downstream effectors, including actin and tropomyosin (46), indicating an active role in other cellular processes.

CEACAM1-S promotes the normal apoptotic death of cells within the lumen of developing mammary alveoli, as cells lacking this receptor instead form masses within the extracellular matrix-containing Matrigel (17). When considered together with our observations, it appears that intercellular CEACAM1-L binding confers growth inhibitory signals that promote cell survival, whereas CEACAM1-S expression instead permits the normal apoptosis of cells in response to stresses such as a loss of integrin-mediated attachment to the extracellular matrix. Given the opposing roles of the two CEACAM1 splice variants in apoptosis, it is interesting that caspase-3 cleaves the cytoplasmic domain of full-length CEACAM1-L to generate a truncated version of the receptor reminiscent of CEACAM1-S (18). If the apoptotic stimuli is sufficient to overcome CEACAM1-L-mediated survival signals, the early induction of caspase-3 would effectively accelerate the cell death cascade by eliminating the cytoplasmic domain of the receptor.

Other immunoglobulin superfamily members have been shown to influence cell survival. The closely related carcinoembryonic antigen (CEA or CEACAM5) inhibits the normal apoptosis of cells that detach from the extracellular matrix-coated substratum, a process known as “anoikis” (Greek for “homelessness” (47)). Although the mechanism of this effect has yet to be described, CEA has no proteinaceous transmembrane or cytoplasmic domains, suggesting that processes distinct from those evident with CEACAM1 must mediate these effects. The more distantly related platelet endothelial cell adhesion molecule PECAM-1 (CD31), which is anchored via a proteinaceous ITIM-containing cytoplasmic domain (48), inhibits mitochondria-dependent apoptosis of endothelial cells and lymphocytes in a manner more reminiscent of that described with CEACAM1 in this study. PECAM-1 ligation with antibodies causes a PI3K-dependent activation of Akt and survival of CD14+CD34+ dendritic cell precursors (49), prompting the proposal that intercellular PECAM-1 binding promotes survival of these cells during their transendothelial migration. Importantly, another group reported no effect of PI3K inhibitors on the PECAM-1-dependent inhibition of apoptosis of HEK293T epithelial cells (50), suggesting that cell-specific differences may exist. Whether such differences account for the greater effect of CEACAM1 binding on monocyte versus lymphocyte survival in the present study remains to be assessed. However, it remains equally possible that the relatively low death rate of lymphocytes during the in vitro culture system used in our study merely masks the significance of the survival advantage of CEACAM1 binding in these cells.

This work represents the first to describe CEACAM1 function in monocytes. Moreover, although a number of studies suggest a link between CEACAM1 and cellular survival, our work is the first to describe a molecular link between CEACAM1, the PI3K/Akt pathway, and cellular survival. This relationship has important implications in vivo. Epithelial and endothelial cell expression of CEACAM1 increases in response to bacterial infection and cytokines (51, 52), and CEACAM1 expression by lymphocytes increases upon immune activation (7–9), causing sites of inflammation to be rich in CEACAM1. Although CEACAM1-dependent homophilic and heterophilic binding interactions may contribute to monocyte recruitment to sites of inflammation, CEACAM1-dependent survival signals will facilitate their persistence to allow their contribution to the development of effective immunity. Whether these interactions also protect other cells within the inflamed tissues from apoptosis remains an important topic for future study.

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