

Functional Interactions of T Cells with Endothelial Cells: The Role of CD40L–CD40-mediated Signals

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Summary

CD40 is expressed on a variety of cells, including B cells, monocytes, dendritic cells, and fibroblasts. CD40 interacts with CD40L, a 30–33-kD activation-induced CD4⁺ T cell surface molecule. CD40L–CD40 interactions are known to play key roles in B cell activation and differentiation in vitro and in vivo. We now report that normal human endothelial cells also express CD40 in situ, and CD40L–CD40 interactions induce endothelial cell activation in vitro. Frozen sections from normal spleen, thyroid, skin, muscle, kidney, lung, or umbilical cord were studied for CD40 expression by immunohistochemistry. Endothelial cells from all tissues studied express CD40 in situ. Moreover, human umbilical vein endothelial cells (HUVEC) express CD40 in vitro, and recombinant interferon γ induces HUVEC CD40 upregulation. CD40 expression on HUVEC is functionally significant because CD40L⁺ Jurkat T cells or CD40L⁺ 293 kidney cell transfectants, but not control cells, upregulate HUVEC CD54 (intercellular adhesion molecule-1), CD62E (E-selectin), and CD106 (vascular cell adhesion molecule-1) expression in vitro. Moreover, the kinetics of CD40L-, interleukin 1-, or tumor necrosis factor α -induced CD54, CD62E, and CD106 upregulation on HUVEC are similar. Finally, CD40L–CD40 interactions do not induce CD80, CD86, or major histocompatibility complex class II expression on HUVEC in vitro. These results demonstrate that CD40L–CD40 interactions induce endothelial cell activation in vitro. Moreover, they suggest a mechanism by which activated CD4⁺ T cells may augment inflammatory responses in vivo by upregulating the expression of endothelial cell surface adhesion molecules.

Endothelial cells express surface molecules, such as CD54 (intercellular adhesion molecule-1), CD62E (E-selectin), and CD106 (vascular cell adhesion molecule-1), that mediate adhesive interactions with leukocytes (1, 2). The expression of endothelial cell surface adhesion molecules orchestrates recruitment of leukocytes to sites of inflammation and therefore is subject to tight regulation (1, 2). Resting endothelial cells express low levels of CD54 and minimal or no CD62E or CD106. After activation with IL-1, TNF- α , or LPS, endothelial cells rapidly upregulate CD54, CD62E, and CD106 expression (1, 2). CD4⁺ T cells may contribute to upregulation of endothelial cell surface adhesion molecules by inducing endothelial cells or other target cells to secrete IL-1 or TNF- α (3). The molecular details involved in CD4⁺ T cell–endothelial cell interactions that induce endothelial cell activation, however, have not been completely delineated.

Interestingly, an immunohistologic study demonstrated that endothelial cells within rheumatoid arthritis synovial membrane express CD40 (4), a molecule whose counter-receptor is expressed on CD4⁺ T cells. CD40 is a 50-kD cell surface molecule also expressed on B cells (5, 6), mono-

cytes (7), dendritic cells (8), basophils (9), epithelial cells (10, 11), and fibroblasts (12). CD40 interacts with CD40L (T cell–B cell–activating molecule, gp39, TNF-related activation protein), a 30–33-kD cell surface molecule transiently expressed on activated CD4⁺ T cells (13–18). CD40L–CD40 interactions have been most extensively studied in the context of T–B cell interactions (19, 20), and they are essential for T cell–dependent B cell differentiation in vitro and in vivo. Although the functional significance of ligating CD40 on non-B cells in vivo is currently unknown, CD40 signaling induces activation, differentiation, or proliferation of monocytes (7), dendritic cells (21), epithelial cells (11), or fibroblasts (12) in vitro.

In this regard, CD40 ligation induces the secretion of various cytokines from monocytes (7), dendritic cells (21), epithelial cells (11), and fibroblasts (12). CD40L–CD40 interactions also upregulate CD54 and CD106 expression on fibroblasts (12), and they upregulate CD80, CD86, and MHC class II expression on dendritic cells (21). Moreover, CD40L-mediated signals induce morphologic changes in cultured dendritic cells (21), proliferation of fibroblasts (12), and tumoricidal activity by monocytes (7).

In this article, we demonstrate that endothelial cells in normal tissue express CD40. Moreover, we show that human umbilical vein endothelial cells (HUVEC)¹ express CD40 in vitro and that CD40L-CD40 interactions induce the up-regulation of endothelial cell surface adhesion molecules.

Materials and Methods

mAbs, Lectins, and Cell Lines. The IgG2a murine anti-CD40L mAb (5C8) was previously generated in our laboratory (13). Hybridomas W6/32 (anti-MHC class I), L243 (anti-MHC class II), 3C10 (anti-CD14), THB.5 (anti-CD21), G28.5 (anti-CD40), and GAP 8.3 (anti-CD45) were purchased from American Type Culture Collection (Rockville, MD). FITC conjugated anti-CD13, FITC conjugated anti-CD19, and PE conjugated anti-CD54 mAbs were purchased from Biosource International (Camarillo, CA), and anti-CD34 mAb was obtained from Biogenex (San Ramon, CA). An additional anti-CD54 mAb as well as anti-CD62E and anti-CD106 mAbs were kindly provided by Biogen, Inc. (Cambridge, MA). L243 and mAbs provided by Biogen, Inc., were biotinylated as previously described (22). PE-conjugated anti-CD80 and biotinylated anti-CD86 mAbs were purchased from Becton Dickinson & Co. (Mountain View, CA) and PharMingen (San Diego, CA), respectively. Isotype control mAbs used for FACS[®] analysis were purchased from Becton Dickinson & Co. or CALTAG Laboratories (South San Francisco, CA). P1.17 is an irrelevant control IgG2a murine mAb (Biogen, Inc.) used for functional studies. FITC-conjugated Ulex europaeus agglutinin-1 (UEA-1) was obtained from Sigma Chemical Co. (St. Louis, MO), and streptavidin-PE was purchased from Calbiochem (La Jolla, CA).

D1.1 is a Jurkat T cell subclone that constitutively expresses CD40L (13). B2.7 is a CD40L⁻ Jurkat T cell subclone (13). Stable transfected CD40L⁺ 293 kidney cells or CD8⁺ 293 kidney cells were generated as previously reported (22). Ramos 2G6 B cells respond to CD40L-mediated signals (23) and were obtained from American Type Culture Collection.

Endothelial Cell Cultures. HUVEC were cultured in M199 media (Gibco Laboratories, Grand Island, NY) supplemented with 25% FCS (Summit Biotechnology, Ft. Collins, CO), 5% human serum (Gemini, Calabasas, CA), 90 µg/ml heparin (Sigma Chemical Co.), 15 µg/ml endothelial cell growth factor (Collaborative Research Inc., Bedford, MA), and 1% penicillin-streptomycin (Sigma Chemical Co.) (M199 complete media). HUVEC were passaged by treatment for 3 min with 1% trypsin-EDTA (Sigma Chemical Co.). All HUVEC experiments were performed in M199 complete media after one to three passages.

Studies on the Effects of Cytokines on HUVEC CD40 Expression. HUVEC in six-well plates (Nunc, Roskilde, Denmark) were grown to near confluence and then incubated with 1,000 U/ml rIFN-γ (Biogen), 10 pg/ml rIL-1α (R&D Systems, Inc., Minneapolis, MN), or 200 U/ml rTNF-α (Upstate Biotechnology, Inc., Lake Placid, NY) in 3 ml of M199 complete medium. At the indicated times, medium was aspirated, cells were washed once with saline, and 1 ml of 1% trypsin-EDTA was added to the wells. Cold Isocove's modified Dulbecco's medium (Gibco Laboratories) containing 10% FCS (Summit Biotechnology) was added to the wells after 3 min, and the cells were collected for FACS[®] analysis.

¹Abbreviations used in this paper: HUVEC, human umbilical vein endothelial cells; MFI, mean fluorescence intensity; UEA, Ulex europaeus agglutinin-1.

Studies on Functional Consequences of HUVEC CD40 Ligation. HUVEC in six-well plates were cultured with 10⁶ CD40L⁺ Jurkat D1.1 cells, CD40L⁻ Jurkat B2.7 cells, CD40L⁺ 293 kidney cell transfectants or CD8⁺ kidney cell transfectants. Where indicated, CD40L⁺ cells were pretreated with anti-CD40L mAb 5C8 (10 µg/ml) or isotype control mAb P1.17 (10 µg/ml) before addition to HUVEC. After the indicated time in culture, the cells were collected by trypsinization, and two-color FACS[®] analyses were performed.

Functional Studies of CD40 Ligation on Ramos 2G6 Cells. Control experiments of CD40 ligation on Ramos 2G6 cells were performed by culturing 2 × 10⁵ Ramos 2G6 cells with 10⁵ D1.1 cells or control cells for 24 h in 96-well plates containing 200 µl of Isocove's Modified Dulbecco's Medium (Gibco Laboratories) supplemented with 10% FCS (Summit Biotechnology) and 1% penicillin-streptomycin (Sigma Chemical Co.).

Cytofluorographic Analysis. Single- or two-color cytofluorographic analyses were performed as previously described (13, 22). HUVEC were distinguished from Jurkat T cells in two-color FACS[®] analyses by positive staining with anti-CD13 mAb. CD13 (*N*-aminopeptidase) is expressed on monocytes, fibroblasts, and endothelial cells but is not expressed on T cells (24). Additionally, UEA-1, a lectin that selectively binds endothelial cells (25), was used to discriminate HUVEC from Jurkat cells or 293 kidney cells. Fluorescence intensity was measured on a FACSscan[®] cytofluorograph with Consort-30 software (Becton Dickinson & Co.). Mean fluorescence intensity (MFI) refers to values normalized to the log scale as calculated by Consort-30 software.

Characterization of Endothelial Cell CD40 Expression In Situ. Frozen sections of normal spleen, thyroid, skin, muscle, kidney, lung, or umbilical cord were studied for CD40 expression, as previously described (23). Immunohistologic analysis was performed with the indicated mAbs or control mouse IgG (Sigma Chemical Co.), and reactivity was detected using a Vector ABC Elite kit and 3-amino-9-ethylcarbazole (Vector Laboratories, Inc., Burlingame, CA) according to the manufacturer's instructions.

Results

In Situ and In Vitro Characterization of Endothelial Cell CD40 Expression. The first series of experiments was performed to determine if normal endothelial cells express CD40 in situ. Therefore, frozen sections obtained from normal spleen, thyroid, skin, muscle, kidney, lung, or umbilical cord were stained with anti-CD40 mAb or control mouse IgG, and endothelial cell reactivity was noted. Additional controls included staining with anti-CD34 mAb (reactive with hematopoietic stem cells and endothelial cells [26]) or anti-CD21 mAb (reactive with B cells and epithelial cells [10]). Endothelial cells from all tissues studied express CD40 in situ. Fig. 1 demonstrates representative CD40 staining of endothelial cells in normal skin or muscle. The pattern of endothelial reactivity was similar to that seen with anti-CD34 mAb (Fig. 1). In contrast, endothelial cells did not react with anti-CD21 mAb (Fig. 1) or mouse IgG (not shown).

To further explore endothelial cell CD40 expression and function in vitro we next asked if cultured HUVEC also express CD40 as determined by FACS[®] analysis. Morphologic and phenotypic analyses demonstrated that the cultures did not contain significant numbers of contaminating non-endothelial cells (not shown). HUVEC constitutively express

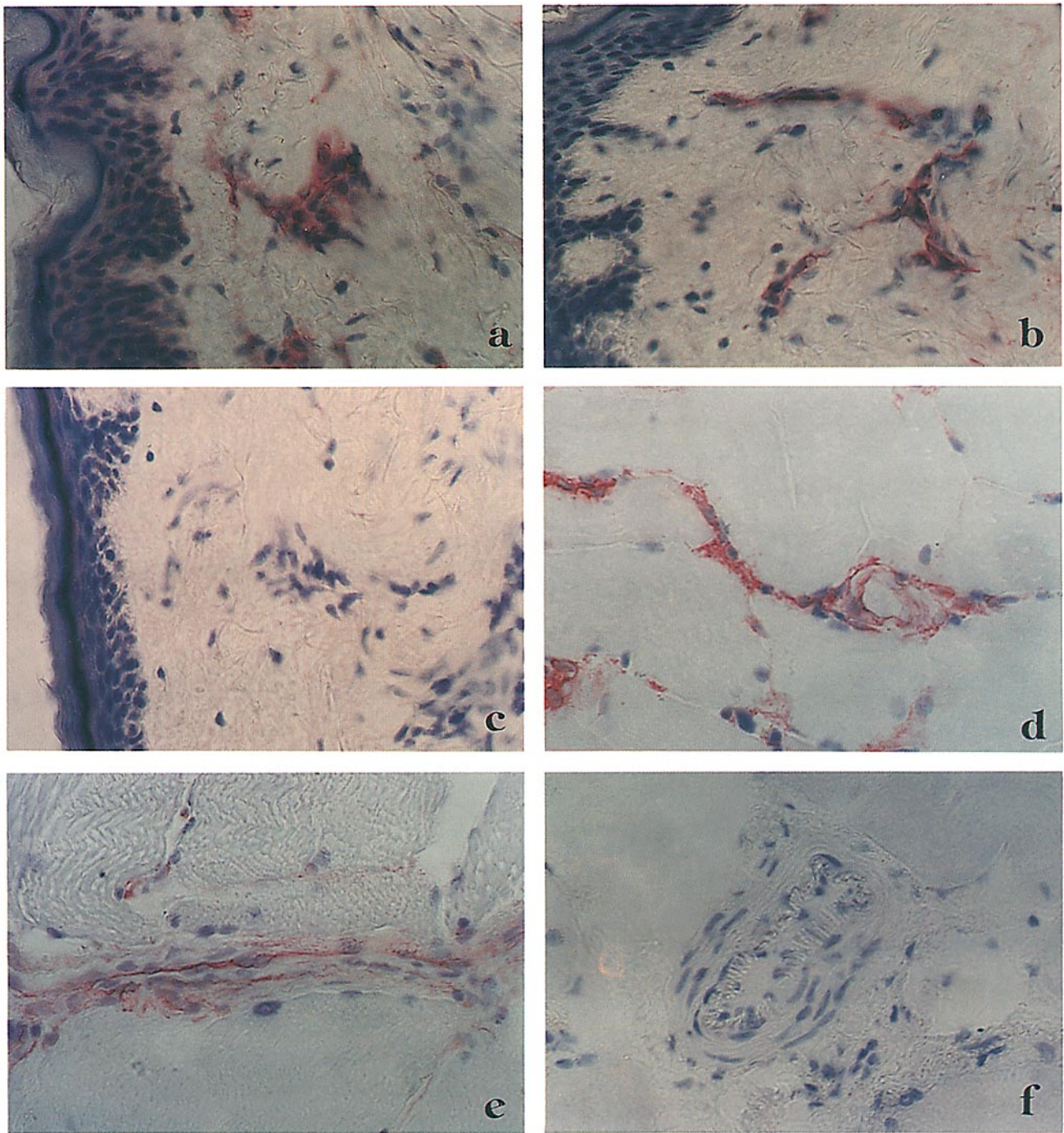


Figure 1. Expression of CD40 on endothelial cells in situ. Shown are immunohistologic studies of frozen sections demonstrating the expression of CD40 (*a* and *d*), CD34 (*b* and *e*), and CD21 (*c* and *f*) in normal skin (*a-c*) or muscle (*d-f*). $\times 40$.

CD40 in vitro (Fig. 2). Similar results were obtained from HUVEC isolated from 15 individuals. Moreover, rIFN- γ , in contrast to rIL-1 α or rTNF- α , induces a two- to three-fold increase in HUVEC CD40 expression (Table 1). Together, these studies demonstrate that endothelial cells from normal tissue express CD40 in situ and in vitro and that rIFN- γ upregulates endothelial cell CD40 expression in vitro.

Effect of CD40L-CD40 Interactions on HUVEC CD54, CD62E, and CD106 Expression. Activated endothelial cells rapidly upregulate expression of cell surface molecules, such as CD54, CD62E, and CD106, which play important roles in mediating intercellular adhesive interactions (1, 2). Interestingly, ligation of CD40 on B cells (27) or fibroblasts (12) induces the upregulation of adhesion molecules. Therefore, we next asked if CD40L-CD40 interactions affect the expression

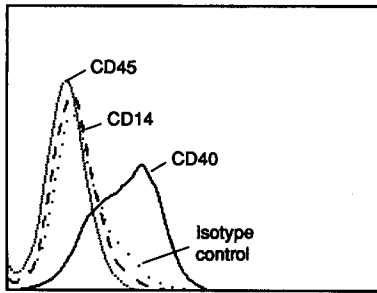


Figure 2. Expression of CD40 on HUVEC in vitro. Shown are overlapping FACS[®] analyses of CD14, CD40, CD45, or isotype control expression on HUVEC after the first passage. The mean fluorescence intensity of CD14, CD40, CD45, or isotype control expression is 7, 24, 5, and 9, respectively. The data shown is representative of CD40 expression on HUVEC isolated from 15 umbilical cords.

of CD54, CD62E, or CD106 on HUVEC in vitro as determined by two-color FACS[®] analysis. HUVEC were cultured with CD40L⁺ 293 kidney cell transfectants or CD8⁺ 293 kidney cell transfectants. As a positive control, HUVEC were also cultured with rIL-1 α . CD40L⁺ 293 cells, but not CD8⁺ 293 cells, induce CD54, CD62E, and CD106 upregulation on HUVEC (Fig. 3). Additionally, CD40L⁺ Jurkat D1.1 cells, in contrast to CD40L⁻ B2.7 cells, upregulate CD54, CD62E, and CD106 on HUVEC (Figs. 3 and 4) in a manner that is inhibited by anti-CD40L mAb 5C8, but not by control mAb (not shown). The kinetics of adhesion molecule upregulation induced by CD40L-CD40 interactions is similar to that reported for IL-1- and TNF- α -induced upregulation (1, 2) (Fig. 4). Together, these studies demonstrate that CD40L-CD40 interactions are sufficient to upregulate HUVEC CD54, CD62E, and CD106 expression in vitro.

Determining if CD40L-CD40 Interactions Upregulate CD80, CD86, or MHC Class II Expression on HUVEC. Activated endothelial cells are competent to express MHC class II molecules and deliver costimulatory signals to T cells (3, 28). Ligation of CD40 on B cells or dendritic cells upregu-

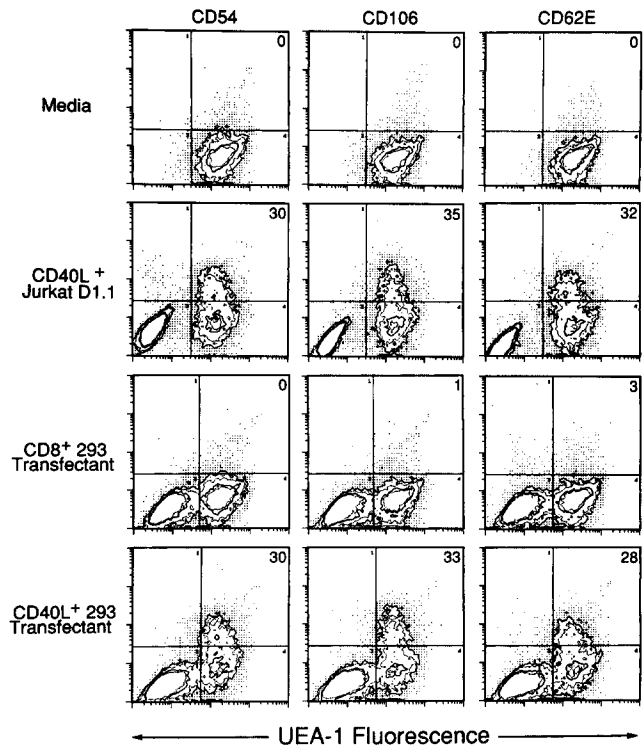


Figure 3. Effects of CD40L-CD40 interactions on HUVEC CD54, CD62E, and CD106 expression. Shown are two-color contour graphs demonstrating HUVEC CD54, CD62E, and CD106 expression after culture with medium, CD40L⁺ Jurkat D1.1 cells, CD8⁺ 293 kidney cell transfectants, or CD40L⁺ 293 kidney cell transfectants for 6 h. The x axis demonstrates UEA-1 expression and the y axis demonstrates CD54 (left), CD106 (middle), or CD62E (right) expression. The number in the upper right-hand corner of each graph indicates the percentage of UEA-1⁺ cells expressing CD54, CD106, or CD62E, as indicated (background staining of control mAb is subtracted for each value). The data shown is representative of three similar experiments with different HUVEC lines.

lates MHC class II expression, as well as the expression of the costimulatory molecules CD80 and CD86 (21, 22, 27, 29, 30). Therefore, the next series of experiments determined if CD40L-CD40 interactions similarly upregulate MHC class II, CD80, or CD86 expression on HUVEC. HUVEC were cultured with CD40L⁺ D1.1 cells, CD40L⁻ B2.7 cells, or rIFN- γ for 24 or 48 h, and CD54, CD80, CD86, and MHC class II expression was determined by two-color FACS[®] analysis. Preliminary experiments demonstrated that trypsinization did not affect CD80, CD86, or MHC class II expression on control B cell lines (not shown). As a positive control for CD40L-mediated CD80, CD86, and MHC class II upregulation, D1.1 cells were cultured with Ramos 2G6 B cells (23). In contrast to the effects of CD40 ligation on B cells or dendritic cells, CD40L-CD40 interactions do not upregulate MHC class II, CD80, or CD86 expression on HUVEC (Table 2).

Discussion

CD40 is a cell surface molecule constitutively expressed on a variety of cells, including B cells (5, 6), monocytes (7),

Table 1. Effect of Cytokines on HUVEC CD40 Expression

Stimuli	HUVEC expression	
	CD40 (MFI)	CD54 (MFI)
Media	17	22
rIFN- γ	42	44
rIL-1 α	24	51
rTNF- α	22	54

Shown is the MFI of CD40 or CD54 expression on HUVEC cultured in the presence or absence of rIFN- γ (1,000 U/ml), rIL-1 α (10 pg/ml), or rTNF- α (200 U/ml) for 48 h. CD40 or CD54 MFI was determined by FACS[®] analysis, and background staining of control mAb is subtracted for each value. Similar results were obtained in two additional experiments with different HUVEC lines.

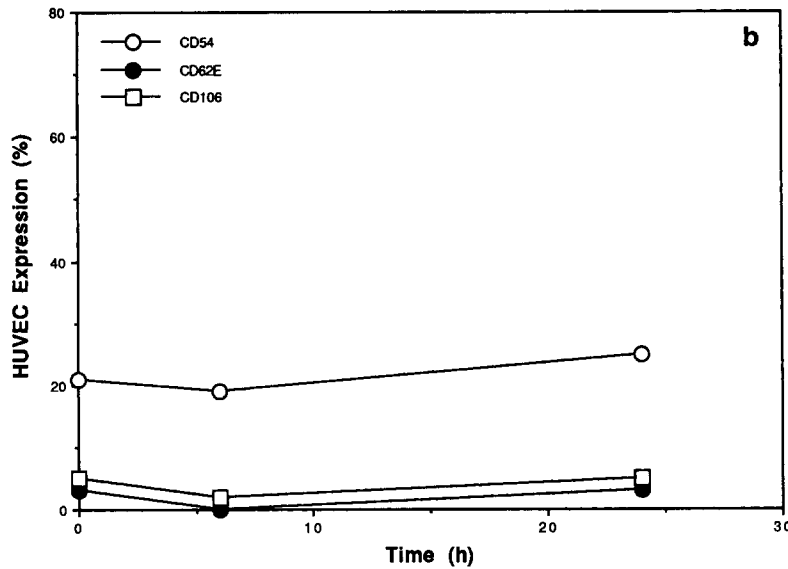
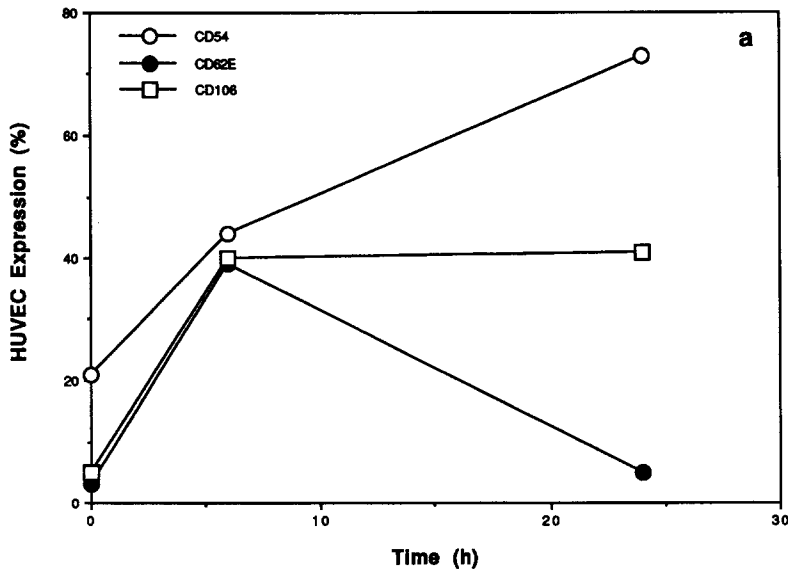


Figure 4. Kinetic analysis of CD40L-induced HUVEC CD54, CD62E, and CD106 upregulation. Shown are the percentages of HUVEC expressing CD54, CD62E, or CD106 after culture with CD40L⁺ Jurkat D1.1 cells (a) or CD40L⁻ Jurkat B2.7 cells (b) for 6 or 24 h. The percentages of HUVEC expressing CD54, CD62E, or CD106 were determined by two-color FACS[®] analysis (background staining of control mAb is subtracted for each value). The data shown is representative of three similar experiments with different HUVEC lines.

dendritic cells (8), epithelial cells (10, 11), basophils (9), and fibroblasts (12). The counterreceptor for CD40 is CD40L, a 30–33-kD activation-induced, transiently expressed CD4⁺ T cell surface molecule (13–18). In this article, we studied the expression and function of CD40 on normal endothelial cells. We show that endothelial cells in the spleen, thyroid gland, skin, muscle, kidney, lung, or umbilical cord express CD40 in situ. This finding is consistent with a previous report that endothelial cells in rheumatoid arthritis synovial membrane express CD40 (4). In addition, HUVEC express CD40 in vitro. Most importantly, CD40 expression on endothelial cells is functionally significant because CD40L⁺ Jurkat T cells or CD40L⁺ 293 kidney cell transfectants, but not control cells, upregulate the expression of intercellular adhesion molecules CD54 (intercellular adhesion molecule-1), CD62E (E-selectin) and CD106 (vascular cell

adhesion molecule-1) on HUVEC. Together, these studies demonstrate that endothelial cells express CD40, and CD40L–CD40 interactions induce endothelial cell activation in vitro.

While this article was in review, two papers were published demonstrating that dermal endothelial cells express CD40 in situ and rsCD40L induces upregulation of HUVEC adhesion molecules in vitro (31, 32). Our work confirms and significantly extends these studies by demonstrating that (a) endothelial cells in a variety of tissues express CD40; (b) the kinetics of CD40L-, IL-1-, and TNF- α -mediated upregulation of CD54, CD62E, or CD106 expression on HUVEC are similar; (c) in contrast to effects on B cells and dendritic cells, CD40L–CD40 interactions do not upregulate CD80, CD86, or MHC class II expression on HUVEC; and most importantly, (d) cell surface CD40L

Table 2. Effect of CD40L-CD40 Interactions on HUVEC MHC Class II, CD80, and CD86 Expression

Conditions	HUVEC expression (MFI)				Ramos expression (MFI)			
	CD54	CD80	CD86	MHC class II	CD54	CD80	CD86	MHC class II
Media	8	0	1	0	22	0	7	128
D1.1	78	0	0	0	71	8	13	223
B2.7	23	0	1	1	25	1	7	127
rIFN- γ	16	0	0	97	ND	ND	ND	ND

Shown is the mean fluorescence intensity of HUVEC CD54, CD80, CD86, or MHC class II expression after culture with medium, rIFN- γ (1,000 U/ml), CD40L⁺ Jurkat D1.1 cells, or CD40L⁻ B2.7 cells for 48 h. In a parallel experiment, the CD40L-responsive Ramos 2G6 B cell line (23) was cultured with medium, CD40L⁺ Jurkat D1.1 cells, or CD40L⁻ B2.7 cells for 24 h. HUVEC or Ramos 2G6 MHC class II, CD54, CD80, and CD86 expression was determined by two-color FACS[®] analysis. Background staining of control mAb is subtracted for each value. Shown is representative of three similar experiments with different HUVEC lines.

induces endothelial cell activation. This is not a trivial point because CD40L physiologically exists as a cell surface molecule and delivers contact-dependent signals to CD40⁺ cells. In this regard, CD40L is not known to normally function as a secreted cytokine. Interestingly, in contrast to our studies and those of Karmann et al. (31), Hollenbaugh and colleagues did not observe CD40L-mediated CD106 upregulation on HUVEC (32). This may be caused by methodological differences used to study upregulation of HUVEC cell surface molecules, or alternatively, by the rsCD40L molecule used by this group.

Together, these studies suggest that CD40L⁺CD4⁺ T cells play roles in augmenting the inflammatory process by interacting with CD40⁺ endothelial cells and upregulating CD54, CD62E, and CD106 expression. The functional consequences of this interaction would enable further adhesion and transmigration of immune cells at sites of inflammation. The fact that CD40 ligation regulates the expression of endothelial cell surface adhesion molecules is consistent with a general role for CD40 signaling in regulating the expression and/or function of adhesion molecules on a variety of cells. In this regard, we have recently shown that CD40L-mediated signals induce CD54 and CD106 upregulation on fibroblasts cultured from synovial membrane (12). CD40 ligation also upregulates CD54 expression on B cells (27) and induces CD54-dependent hom aggregation of B cells (33).

In contrast to effects on B cells and dendritic cells, CD40 ligation does not upregulate MHC class II, CD80, or CD86 expression on HUVEC *in vitro*. This finding is consistent with previous studies suggesting that human endo-

thelial cells do not express CD80 (28). CD80 and CD86 deliver important costimulatory signals to T cells necessary for IL-2 production and proliferation. Interestingly, endothelial cells are competent to present Ag to T cells *in vitro*, and they deliver appropriate costimulatory signals to T cells which are required for IL-2 production and proliferation (28). The costimulatory molecules expressed on endothelial cells are not precisely known. It will be of interest to determine if CD40L-mediated signals modulate the capacity of endothelial cells to activate T cells.

Finally, endothelial cells are activated in a variety of diseases mediated by CD4⁺ T cells. For example, endothelial cell surface adhesion molecules are upregulated in rheumatoid arthritis (34) and scleroderma (35). In addition, CD4⁺ T cells may play roles in atherosclerosis (36) and accelerated atherosclerosis associated with transplantation (37). The precise role of CD40L-mediated interactions with endothelial cells in these diseases is not known. It is of interest, however, that an antibody to CD40L, MR1, inhibits murine models of diseases mediated by CD4⁺ T cells and/or inflammatory cell infiltrates. For example, MR1 prevents the synovial lining cell hypertrophy and cellular infiltrate associated with collagen-induced arthritis, a murine model of rheumatoid arthritis (38). Moreover, MR1 inhibits a murine model of multiple sclerosis (experimental allergic encephalomyelitis) and inhibits allograft rejection (39). It is possible that blocking CD40L-dependent interactions with endothelial cells and/or fibroblasts mediates, in part, these effects of MR1. Together, these studies suggest that CD40L-CD40 interactions on the surface of endothelial cells may play immunopathogenic roles in inflammatory diseases.

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