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Internalization of CD40 regulates its signal transduction in vascular endothelial cells

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Abstract

The CD40 ligand (CD40L)-CD40 dyad can ignite proinflammatory and procoagulatory activities of the vascular endothelium in the pathogenesis and progression of atherosclerosis. Besides being expressed on the activated $CD4^+$ T cell surface (mCD40L), the majority of circulating CD40L reservoir (sCD40L) in plasma is released from stimulated platelets. It remains debatable which form of CD40L triggers endothelial inflammation. Here, we demonstrate that the agonistic antibody of CD40 (G28.5), which mimics the action of sCD40L, induces rapid endocytosis of CD40 independent of TRAF2/3/6 binding while CD40L expressed on the surface of HEK293A cells captures CD40 at the cell conjunction. Forced internalization of CD40 by constitutively active mutant of Rab5 preemptively activates NF-κB pathway, suggesting that CD40 was able to form an intracellular signal complex in the early endosomes. Internalized CD40 exhibits different patterns of TRAF2/3/6 recruitment and Akt phosphorylation from the membrane anchored CD40 complex. Finally, mCD40L but not sCD40L induces the upregulation of proinflammatory cytokines and cell adhesion factors in the primary human vascular endothelial cells in vitro, although both forms of CD40L activate NF-κB pathway. These results therefore may help understand the molecular mechanism of CD40L signaling that contributes to the pathophysiology of atherosclerosis.

Keywords: CD40; CD40L; TRAFs; PI3K/Akt; NF-κB; Endothelial cells; Endocytosis

CD40 is a member of the tumor necrosis factor receptor (TNFR) superfamily that constitutively expresses on professional antigen presenting cells (APCs), such as B cells, macrophages/monocytes, and dendritic cells. Bi-directional signaling between CD40 and its ligand CD40L (also known as CD154 or gp39), which primarily expresses on the surface of activated $CD4^+$ T cells (mCD40L), is essential to development of the adaptive immunity [1,2]. CD40 lacks intrinsic catalytic activity, but it can use the cytoplasmic tail to recruit TNF receptor-associated factors (TRAFs) therefore couple CD40 to the activation of the Jun N-terminal protein kinase (JNK), phosphoinositide 3-kinase (PI3K)/Akt, p38 mitogen-activated protein kinase (MAPK), and NF-κB signaling pathways in a cell type-dependent fashion [3,4]. CD40 is also present on nonprofessional APCs [4] such as endothelial cells (EC), vascular smooth muscle cells, and fibroblasts, and activation of CD40 signaling in these cells leads to elevated expression of proinflammatory genes and adhesion molecules in chronic inflammatory diseases, such as rheumatoid arthritis, graft-vs-host disease, and atherosclerosis [2,5,6]. Antibody blockade of CD40L–CD40 interaction can therefore effectively reduce the lesion development and progression of atherosclerosis in murine models [5].

Besides cell surface expression, CD40L can also exist in a soluble, biologically active form (sCD40L) that is shred from activated T cells [7] or stimulated platelets [8,9], the latter accounting for 95% of circulating sCD40L [10].

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Therefore, it is generally speculated that the increased levels of plasma sCD40L due to abnormal platelet activation most likely play a role in vascular endothelial inflammation and atherogenesis [11]. This notion, however, is contradicted by the in vitro finding that only mCD40L on the surface of platelet [12] but not sCD40L [9] activates EC with characteristic upregulation of adhesion molecules, proinflammatory cytokines and chemokines. The atherogenic role of sCD40L in circulation is also challenged by a recent large-scale survey that fails to associate plasma sCD40L levels with the atherosclerosis risk [13]. Therefore, it is of particular interest to delineate the molecular mechanisms of how mCD40L and sCD40L transduce EC inflammatory signaling.

The specificity of TNFR superfamily signaling, like other receptor tyrosine kinase mediated signal transduction, depends partly on whether the activated receptors remain at the cell membrane or become internalized [14]. The endocytic pathway could either switch off or activate receptor signaling [15], depending on which cytoplasmic complex that the receptor recruits and intracellular compartments it targets. Previous studies suggest that CD40 can induce internalization of CD40L and thereby downregulation of CD40L signaling in T cells [16], and mCD40L on the surface of fibroblast cells can induce endocytosis of CD40 in B cells [17]. However, whether CD40 endocytosis is involved in regulation of proinflammatory activation of EC remains unknown. We hypothesized that different forms of CD40L, either sCD40L or mCD40L, might trigger differential CD40 signal transduction via distinct intracellular traffic pathway. We tested the hypothesis by comparison of CD40 internalization and trafficking, Akt phosphorylation, and NF-κB activation in response to sCD40L or mCD40L. We further determined the disparity in their ability to induce inflammatory response in primary vascular endothelial cells in vitro.

Materials and methods

Cell lines and reagents. Porcine aortic endothelial (PAE) cells were routinely maintained as previously described [18]. EYFP fusion vectors expressing wild type CD40 or various mutant derivatives were transfected in PAE cells and stable lines (PAE-EYFP-CD40 and variants) were selected using G418. HEK293A cells stably expressing ECFP-CD40L (designated 293A/CD40L) were selected and cell surface expression of CD40L was verified by immunofluorescence microscopy. 293A/CD40L cells were usually paraformaldehyde fixed before added to PAE or HeLa cells unless specified. Primary HUVEC cells were obtained from Cascade Biologics and routinely maintained in Medium 200 (Portland, USA).

Soluble recombinant human CD40L trimer was purchased from Alexis Biochemicals (San Diego, CA). The agonistic antibody to human CD40 was either purchased from Pharmingen (5C3, San Diego, CA) or purified (G28.5) from the supernatants of hybridoma cells (HB-9110, ATCC) free of endotoxin. The following antibodies were used: CD40 and CD40L from Santa Cruz Biotech (Santa Cruz, CA), phosphor-Akt and total Akt from Cell Signaling (Beverly, MA), and human VDAC1 from Calbiochem. TRIzol Reagent was from Gibco-BRL (Carlsbad, CA), M-MLV reverse transcriptase, random primers, oligo$(dT)_{15}$ primer, bicistronic control plasmid pCMV-hRL, and dual-luciferase reporter assay system from Promega (Madison, WI), and Effectene transfection reagent from Qiagen (Valencia, CA). LPS and all fine chemicals were from Sigma (St. Louis, MO).

Plasmid constructs. The cDNA encoding full length human CD40 or CD40L was subcloned in between *Bgl*II/*Kpn*I or *Xho*I/*Bam*HI restriction sites of pEYFP-N1 or pECFP-C1, respectively, (Clontech, Mountain View, CA) by add-on PCR amplification of a human fetal liver cDNA library (designated as pEYFP-CD40 or pECFP-CD40L). The stop codon was introduced into CD40 at amino acid 193, 208, 212 or 230 by site-directed mutagenesis (QuikChange, Stratagene, La Jolla, CA) to yield a series of C-terminal truncation mutants (designated as pEYFP-CD40-Δ193–277, pEYFP-CD40-Δ208–277, pEYFP-CD40-Δ212–277 or pEYFP-CD40-Δ230–277, respectively). The wild type Akt, kinase-dead mutant Akt-K179A (kindly provided by M. Birnbaum, Univ Penn Sch Med) [19] was subcloned into pECFP-N1 between *Eco*RI and *Bam*HI sites to yield pECFP-Akt and pECFP-Akt-K179A, respectively. Plasmids expressing wild-type Rab5 (pECFP-Rab5), its dominant negative mutant (pECFP-Rab5-S34N), and GTPase deficient mutant (pECFP-Rab5-Q79L) and Rab7 (pEGFP-Rab7) were kindly provided by Sorkin [20]. Dominant negative (T22N) and positive (Q67L) mutants of Rab7 were generated by site-directed mutagenesis. All constructs were verified correct by DNA sequencing analyses. Primer sequences are available upon request. The cDNA plasmids encoding human TRAF2, TRAF3, and TRAF6 were gifted by Dr. Genhong Cheng (UCLA) and subsequently cloned into pECFP-C1 accordingly.

NF-κB reporter assay. Transient transfection using Effectene was performed according to manufacturer's manual (Qiagen). In brief, 400 ng IFN-β promoter derived 3× κB-Luc reporter plasmid (kindly provided by G. Cheng, UCLA) and various Rab5 (200 ng) or Akt (400 ng) constructs were transfected into 1×10^6 PAE or derivative cells, and grown in a 35-mm plate for 36–48 h before assays. pCMV-hRL (1 ng) was co-transfected as the internal control of transfection efficiencies. Transfectants were then split into 24-well plates for anti-CD40 crosslinking or sCD40L ligation under conditions where indicated. Dual luciferase activities were measured in a luminometer (TD-20, Turner Designs Instruments) according to manufacturer's manual (Promega). Data were presented as means ± SD and statistical analysis was performed by the paired Student's *t*-test and $P < 0.05$ was considered significant.

Western and nuclear Western blotting. Phosphorylation of Akt was detected by phosphor-Akt specific antibody according to manufacturer's instruction.

Real-time RT-PCR. HEK293A/CD40L cells were briefly trypsinized followed by incubation with 1% paraformaldehyde at 25 °C for 10 min. After washing with PBS for three times, cells were resuspended in 0.5 mL medium 200 (5×10^6/mL) and mixed with HUVEC cells (4×10^5) in 60-mm plates for 4 h. For G28.5 treatment, the antibody was added directly into HUVEC cell culture (24 μg/mL) and incubated for 4 h. Total RNA was extracted with TRIzol and 2 μg was used in the reverse transcription according to manufacturer's manual (Promega). Real-time PCR were carried out in an iCycler (Bio-Rad) with SYBR Green I (Molecular Probes) as the fluorescent dye. The amplification of 18S rRNA was used as the internal control. The primers used for ICAM-1, E-selectin, P-selectin, IL-8, and 18S rRNA are available on request.

Internalization by fluorescence microscopy. PAE-EYFP-CD40 or HeLa cells grown on coverslips were serum starved in F12 medium for 16 h before addition of sCD40L trimer (100 ng/mL), G28.5 (5 μg/mL) or trypsinized 293A-CD40L cells (293A:PAE = 10:1 or 293A:HeLa = 3:1) at 37 °C for the indicated time. To study the colocalization of CD40 and Rab5 or its mutants, PAE-EYFP-CD40 cells (1×10^5) were transiently transfected with 0.2 μg pECFP-Rab5, pECFP-Rab5-S34N, or pECFP-Rab5-Q79L. Transfectants were treated with G28.5 as mentioned above for the indicated time. The live cells were mounted onto a fluorescence microscope (Nikon TE2000-E) and visualized with FITC/YFP and CFP filter channels as described previously [21]. Images were analyzed with Slidebook 4.1.0 imaging workstation (3I, Denver, CO). Typically, 3-D deconvolution was performed from more than ten two-dimensional images acquired from bottom to top of a cell to visualize intracellular vesicles in a large flat area.

Results

sCD40L triggered internalization of CD40 independent of TRAFs

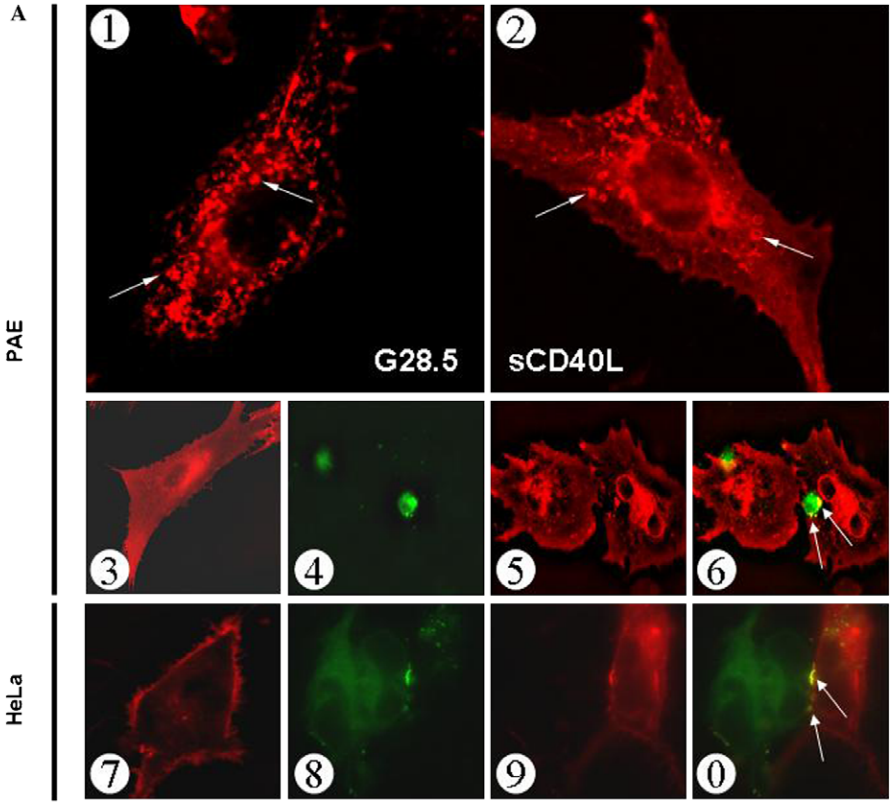
We chose porcine aortic endothelial cell line (PAE) to study EC signaling because PAE cells are much easier to maintain in tissue culture than the human primary umbilical vein endothelial cells (HUVEC) or the cell line, and PAE cells possess the intrinsic CD40 signaling pathway that can be activated by human mCD40L to express E-selectin, vascular adhesion molecule-1 (VCAM-1) [22]. In order to assess the potential difference between sCD40L and mCD40L in activation of CD40 signaling, we first treated PAE cells that stably expressed EYFP-CD40 (PAE-EYFP-CD40) with either G28.5 or the recombinant CD40L trimer, both can mimic the action of sCD40L shred from activated platelets. Each caused rapid internalization of CD40-EYFP in less than 30 min, with the formation of punctuate vesicles reminiscent of early endosomes (Fig. 1A, panels 1 and 2). Another agonistic antibody to CD40 (5C3) could not do so (data not shown), indicating the specific agonistic effect by G28.5 on CD40 endocytosis. We then co-cultured PAE-EYFP-CD40 cells (Fig. 1A, panel 5) with HEK293A cells that expressed membrane bound CD40L (293A/CD40L, Fig. 1A, panel 4). This form of mCD40L induced CD40 to cluster at the site of contact (Fig. 1A, panel 6), despite prolonged incubation (data not shown). To avoid the size difference between HEK293A and PAE cells that might cause imaging artifacts, we also co-cultured 293A/CD40L cells with HeLa cells that transiently over-expressed CD40-EYFP (HeLa-EYFP-CD40), and fluorescent microscopy analysis depicted the similar patches of CD40L/CD40 complex in between the cell junction (Fig. 1A, panels 7–0). The similar CD40L-CD40 clustering structure in lipid rafts is also observed within the immunological synapse between T cells and APCs [23], and CHO cell-surface expressed CD40L and B cells [24].

The cytoplasmic domain of human CD40 has a proximal TRAF6 binding site and a more distal TRAF2/3/5 binding site, both possessing distinct functions for CD40 signaling in B lymphocytes [25]. In an attempt to identify the specific requirement of TRAF binding for CD40 internalization, we then used G28.5 to activate PAE cells that transiently expressed EYFP-CD40 or its various truncation mutants (Fig. 1B). Fluorescent microscopy showed that, while the full length CD40 became rapidly internalized, deletion of all known TRAFs binding sites in CD40 (CD40-Δ212–277, CD40-Δ230–277) did not affect its endocytosis unless the transmembrane domain was abrogated (CD40-Δ193–277 or CD40-Δ208–277). These CD40 mutants without the transmembrane domain were actually entrapped in the cytoplasm. It is intriguing to note that the three amino acids (209–211) of CD40 contain a di-leucine sequence that can function as a receptor endocytosis and lysosome targeting signal [26]. Addition of the di-leucine sequence to CD40-Δ208–277 enabled CD40-Δ212–277 to be internalized (Fig. 1B). Stimulation of cells with the recombinant CD40L trimer yielded the similar intracellular localization for CD40 and its mutant derivatives (data not shown). Therefore, TRAF2/3/6 binding was dispensable for CD40 internalization in PAE cells, contrary to the previous finding where the shared binding site for TRAF2 and TRAF3 is required for CD40 internalization in B cells [27].

Constitutive activation of Akt causes endothelial cell senescence within atherosclerotic plaques that secrete a large number of proinflammatory cytokines responsible for vascular pathophysiology [28,29]. We found that G28.5 induced Akt phosphorylation in a time- and dose-dependent fashion (Fig. 2A). At the fixed concentration of G28.5, Akt was quickly phosphorylated with the peak reached around 30 min then gradually dephosphorylated after 12 h (further discussion in Fig. 4C). Unlike that for CD40 internalization, TRAF recruitment to CD40 was required for Akt phosphorylation because overexpression of EYFP-CD40-Δ212–277 significantly reduced Akt phosphorylation (Fig. 2B) as compared to wild type EYFP-CD40. NF-κB activation by CD40 was downstream of Akt phosphorylation in PAE cells, because when we transiently transfected PAE-EYFP-CD40 cells with the dominant negative mutant, Akt-K179A, NF-κB reporter activities were effectively inhibited (Fig. 2C). In contrast, inhibition of either MAPKp42/44 by PD98059 or MAPKp38 by SB203580 did not apparently block NF-κB activation by G28.5 in these cells (data not shown). The requirement of Akt phosphorylation for NF-κB activation in PAE cells seemed cell-type specific, because the PI3K-Akt pathway is not required for CD40 activation of NF-κB in vascular smooth muscle cells [30] or B cell receptor signaling [31]. Therefore, using agonistic antibody of CD40, our results supported previous findings that Akt can be activated by various atherogenic stimuli including CD40L [32,33] through direct interaction of CD40 with various TRAFs [4]. Our results further indicated that endocytosis of CD40 per se might not require the function of TRAFs, but Akt phosphorylation and NF-κB activation depended on appropriate TRAFs binding to the trafficking CD40.

Rab5 GTPase activity modulated CD40 endocytosis and NF-κB activation

Rab5 involves in early endosomal fusion events and is a key regulator of receptor transport from plasma membrane to the early endosomes [34]. Indeed, Rab5 GTPase activity is the rate-limiting component of the endocytic pathways [35]. On the other hand, Rab7 regulates traffic from the early to late endosomes, and ablation of Rab7 function inhibits endosomal transport and therefore lysosomal degradation of sorted receptors [36,37]. Internalized EYFP-CD40 was able to traffic to the early/enlarged endosomes as clearly demonstrated by its co-localization with ECFP-Rab5 (Fig. 3A). The involvement of Rab5 GTPase in CD40 internalization was specific, because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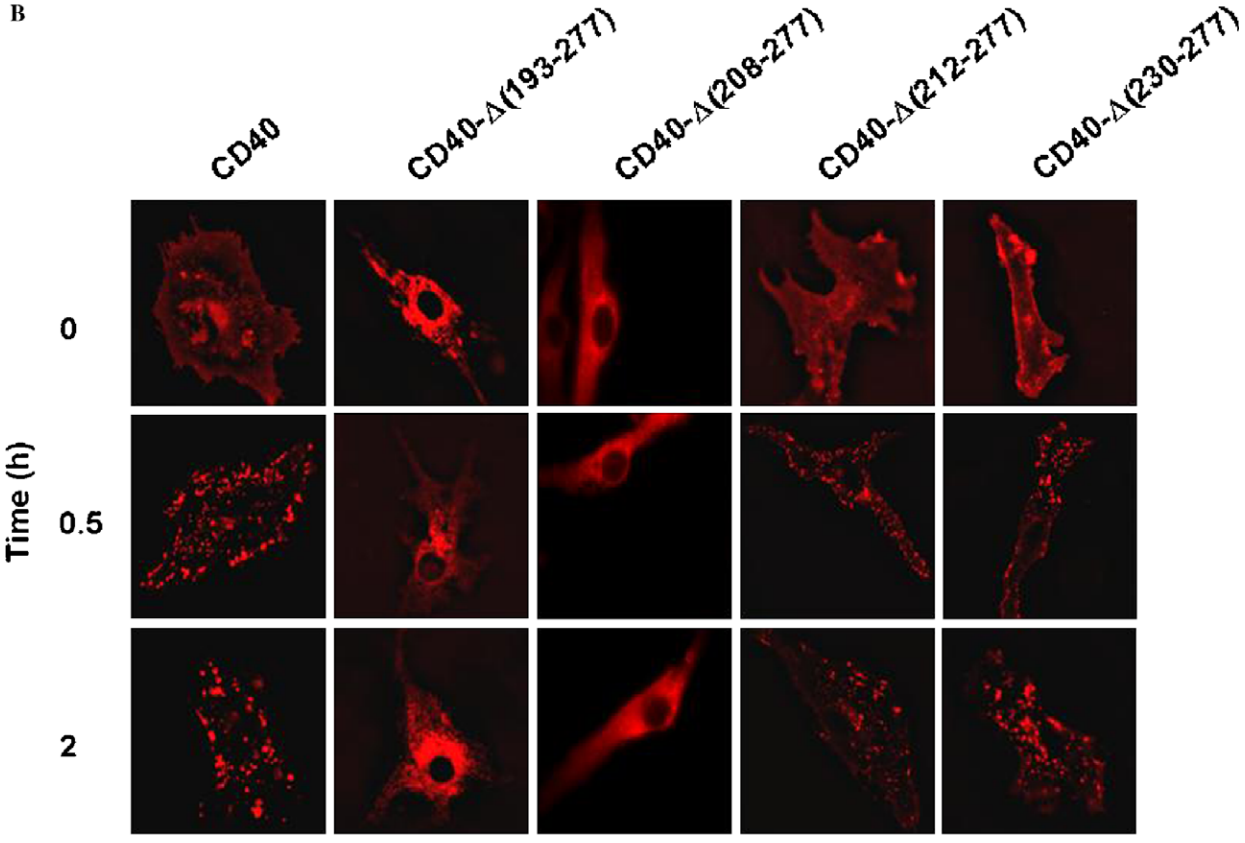
B
CD40
CD40-Δ(193-277)
CD40-Δ(208-277)
CD40-Δ(212-277)
CD40-Δ(230-277)
Time (h)
0
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2

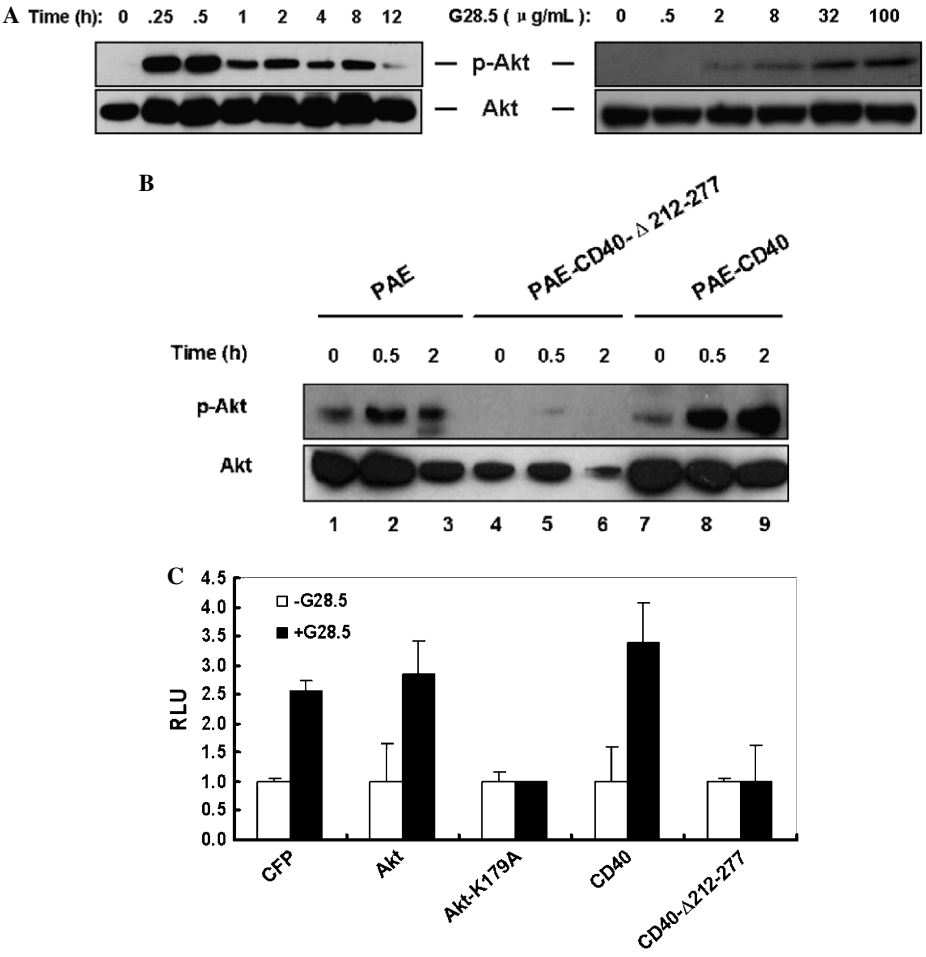

Fig. 2. CD40L activated NF-κB and Akt phosphorylation in PAE-CD40 cells. (A) Phosphorylation of Akt in PAE-EYFP-CD40 stable cell line. PAE-EYFP-CD40 cells (2×10^6) were serum-starved and stimulated with 24 μg/mL G28.5 for indicated time (left panel) or at different doses for 0.5 h (right panel). Cells were then lysed and immunoblotted with the indicated antibodies. (B) PAE-EYFP-CD40 or PAE cells stably expressing the C-terminus deleted CD40 mutant (PAE-CD40-Δ212–277) were stimulated with 24 μg/mL G28.5 for the indicated time. Phosphorylation of Akt was measured as in (A). (C) PAE cells were transiently co-transfected with 3× κB luciferase reporter plasmid and each of the following plasmids (400 ng): pECFP-Akt, pECFP-Akt K179A, pEYFP-CD40 or pEYFP-CD40-Δ212–277. Parental vector pECFP-N1 was used as the control transfection. Transfectants were stimulated with 5 μg/mL G28.5 for 12 h. The luciferase activities from three independent experiments were averaged and the relative luciferase activities (RLU) were normalized against the unstimulated controls. Data presented as means ± SD and statistical analysis was performed by the paired Student's *t*-test and $P < 0.05$ was considered significant.

overexpression of the dominant active mutant, Rab5-Q79L, in PAE-EYFP-CD40 cells induced CD40 endocytosis even without CD40 activation (Fig. 3B, arrows, time 0). In contrast, overexpression of the inhibitory Rab5 derivative, Rab5-S34N, effectively attenuated endocytosis of CD40 before or after G28.5 crosslinking (Fig. 3B). Internalization of CD40 promoted by Rab5-Q79L led to preemptive NF-κB activation by ~4-fold in the absence of G28.5 (Fig. 3C). Excessive CD40 internalization by Rab5-Q79L did not allow any further increase of NF-κB reporter activities after G28.5 crosslinking, maybe due to unavailability of CD40 present on the cell surface. As expected, overexpression of Rab5-S34N, which restricted CD40 on the cell surface, responded to G28.5 crosslinking which increased the NF-κB reporter activity (Fig. 3C). On the other hand,

Fig. 1. Soluble CD40L triggered endocytosis of CD40. (A) CD40 internalization (red) was shown after PAE-EYFP-CD40 cells were treated with 5 μg/mL G28.5 (1) or recombinant sCD40L trimer (2) using the YFP channel. Arrows indicate punctuate endosomal structures. 293A/CD40L cells (green, 4) were co-cultured with PAE-EYFP-CD40 (red, 5) for 3 h and colocalized CD40L and CD40 at the site of contact (yellow, 6) are indicated by arrows after merging CFP and YFP channels. In parallel, co-culturing of 293A/CD40L cells (green, 8) with HeLa-EYFP-CD40 (red, 9) also showed membrane patches of CD40L and CD40 (yellow, 0). PAE-EYFP-CD40 or HeLa-EYFP-CD40 before addition of 293A/CD40L is indicated in (3) and (7), respectively. (B) PAE cells were transiently transfected with pEYFP-CD40 and the various C-terminal truncation mutants as indicated, and incubated with G28.5 for the indicated time. Localization of YFP fluorescence is depicted. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

endocytosis of receptors usually attenuates signal transduction due to receptor traffic to late endosomes and degradation in lysosomes [15]. However, internalized CD40 did not traffic to late endosomes because neither down-regulation of CD40 protein levels (Fig. 3D), nor apparent changes in NF-κB activities were observed in the presence of active (Rab7-T22N) or inactive (Rab7-Q67L) mutant forms of Rab7 after cells were treated with G28.5 (Fig. 3E). It further supported our hypothesis that CD40 could form a signaling complex that activates Akt and NF-κB in early endosomes, as effective as that membrane associated CD40 signaling complex (also see Fig. 4D).

mCD40L, but not G28.5, up-regulated inflammatory cytokines in endothelial cells independent of NF-κB activation

Because both internalized and plasma membrane-sequestered CD40 by Rab5-Q79L and Rab5-S34N could activate NF-κB, we then sought to determine the specific regulatory effects by sCD40L and mCD40L. We decided to assay whether CD40 might differentially recruit various TRAFs in response to sCD40L or mCD40L. Fluorescent colocalization analyses showed that G28.5 stimulation caused membrane bound EYFP-CD40 and ECFP-TRAF2 or ECFP-TRAF3 transiently expressed

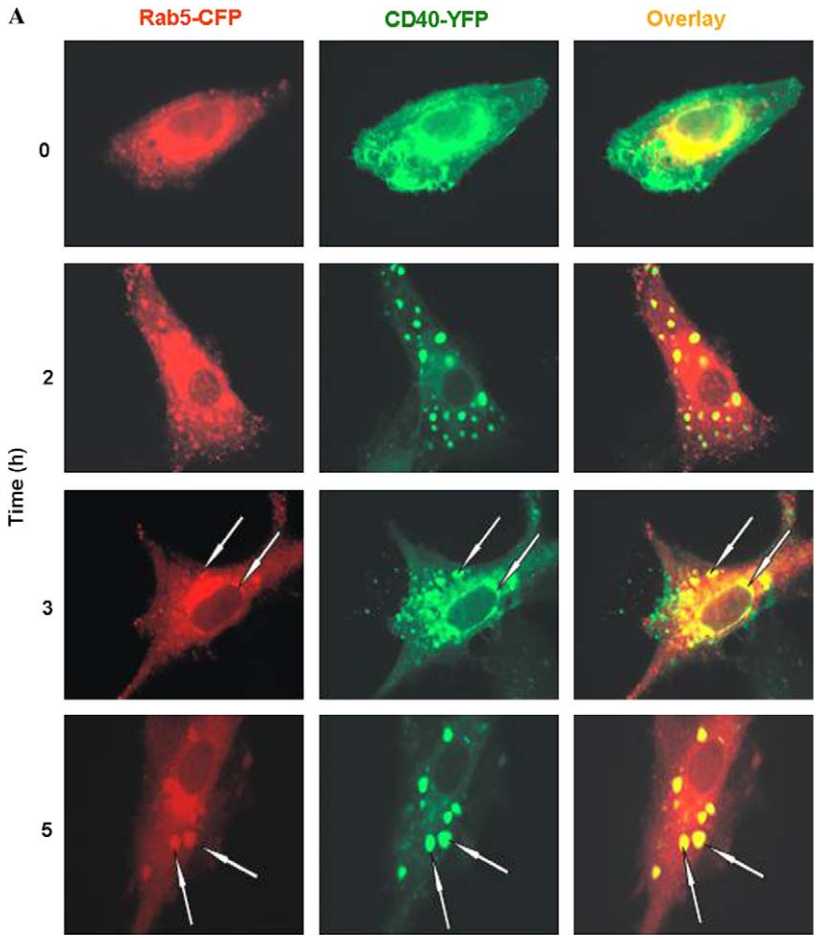

Fig. 3. Rab5 activities modulated endocytosis of CD40. (A) CD40 colocalized with Rab5 after CD40 crosslinking. PAE cells were transiently co-transfected with pECFP-Rab5 and pEYFP-CD40 (200 ng each). Cells were treated with G28.5 (5 μg/mL) for the indicated time. The coverslips were directly mounted to the microscopy chamber and visualized through YFP and CFP filter channels, respectively. The localization of Rab5 (red, left) and CD40 (green, middle) is merged to show their colocalization (yellow, right). Arrows indicate the typical enlarged endosomes. The data represented the general features of several repeated experiments. (B) PAE-EYFP-CD40 cells on a coverslip were transiently transfected with 400 ng pECFP-Rab5-Q79L or pECFP-Rab5-S34N and were treated with G28.5 for 30 min. The co-localization of Rab5-Q79L or Rab5-S34N (red) with CD40 (green) is shown in the overlay (yellow). Arrows indicate the typical enlarged endosomes. The data represented the general features of several repeated experiments. (C) Forced endocytosis of CD40 preemptively activates NF-κB pathway. PAE-EYFP-CD40 cells were co-transfected with 3× κB reporter and pECFP-Rab5, pECFP-Rab5-Q79L, pECFP-Rab5-S34N, or pECFP-N1 as the control. Transfectants were then treated with G28.5 (black bars) or not with G28.5 (white bars) for 12 h and dual luciferase activities were assayed. NF-κB activation was plotted after normalized to *Renilla* luciferase activities in each group. Data represented the average of three independent experiments and statistical analysis was performed as in Fig. 1C. (D) Internalized CD40 did not degrade. PAE-EYFP-CD40 cells were stimulated with G28.5 for indicated time. Cell lysates were resolved and immunoblotted with anti-CD40 antibody. The mitochondrial outer membrane protein, VDAC1, was detected to normalize the protein loading. (E) Internalized CD40 did not go through the late endosomes. PAE-EYFP-CD40 cells were transiently co-transfected with 3× κB luciferase reporter plasmid and wild type pECFP-Rab7, pECFP-Rab7-Q67L or pECFP-Rab7-T22N. NF-κB reporter activities were assessed as in (C). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

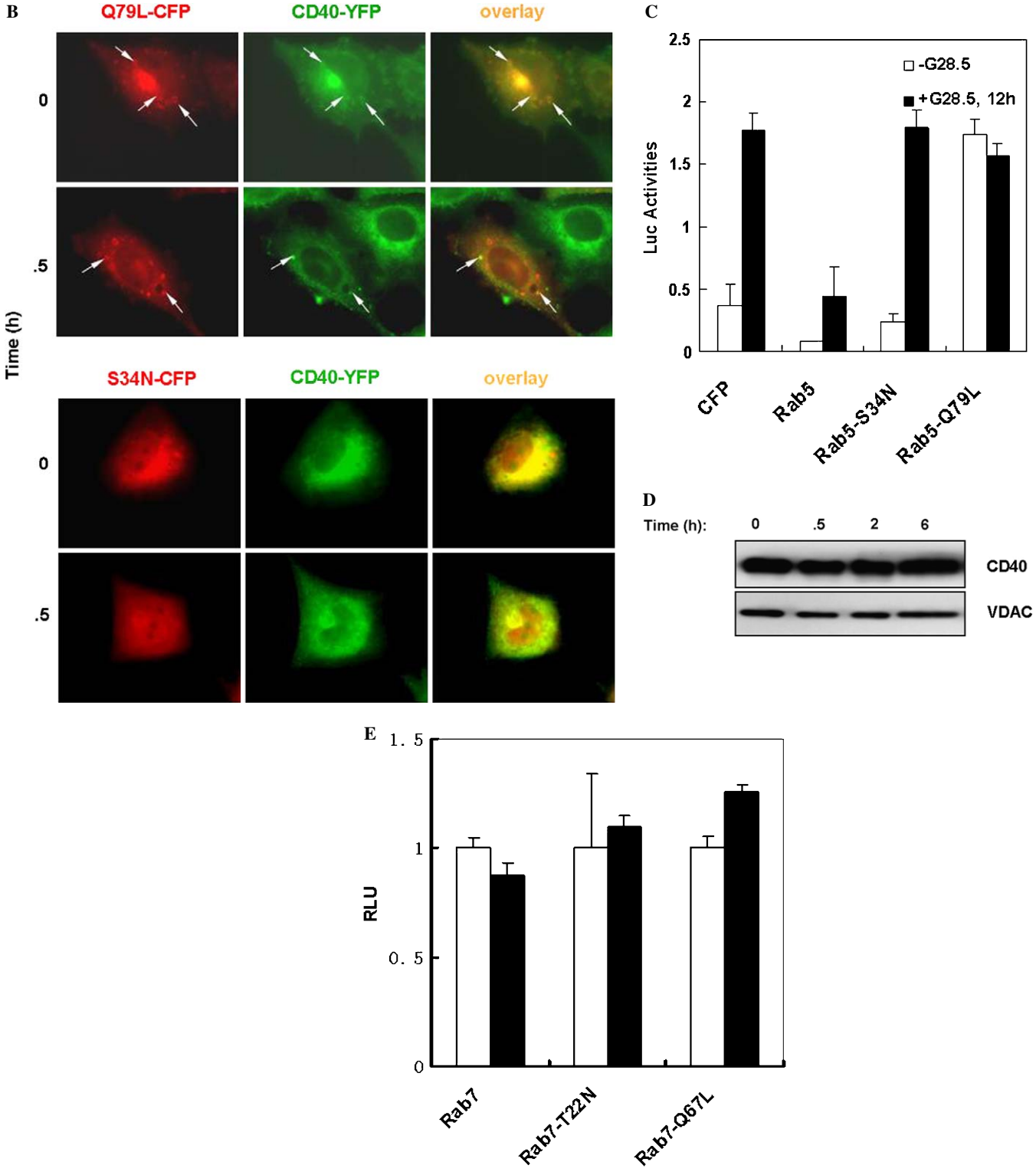

Fig. 3. (*continued*)

in HeLa cells to completely internalize and co-migrate into the enlarged endosomes, while otherwise cytosolic ECFP-TRAF6 became partially residing with the EYFP-CD40 endoplasmic vesicles (Fig. 4A). The recombinant trimeric sCD40L induced the similar CD40 trafficking and TRAF recruitment (data not shown). Like that in PAE-EYFP-CD40 cells, incubation of 293A/CD40L with HeLa-EYFP-CD40 triggered CD40 to cluster on cell membrane, with complete colocalization for ECFP-TRAF3 and ECFP-TRAF6, and partial colocalization for ECFP-TRAF2, with EYFP-CD40 (Fig. 4B). Therefore, recruitment of TRAF2 and TRAF6 to CD40 behaved differently in response to G28.5 or mCD40L.

Differential recruitment pattern of TRAFs may play a role in the proinflammatory activation of EC by CD40. In regard to the specific contribution of different TRAFs to inflammation responses, recent data show that TRAF6 is an essential mediator of sCD40L-activated proinflammatory pathways in monocytes and macrophages [38], and overexpression of TRAF3 can block sCD40L mediated endothelial activation [39] or TRAF2/5 mediated non-canonical NF-κB activation by mCD40L [40]. This could be partially reflected in the kinetic difference in Akt phosphorylation along the time course of G28.5 or mCD40L treatment. Akt became hyperphosphorylated in less than 30 min upon G28.5 crosslinking of PAE-EFYP-CD40 cells and waned at ~12 h (Fig. 4C, left panel). However, 293A/CD40L slowly activated Akt phosphorylation, peaked at 6 h but lasted beyond 12 h (Fig. 4C, right panel). In the control experiments, MAPKp38, MAPKp42/44, and JNK were activated by either form of stimuli without any significant difference (data not shown). Under this condition, both G28.5 and 293A/CD40L activated the NF-κB reporter to the similar degree and with similar kinetics (Fig. 4D), supporting the notion that NF-κB activation is a critical but not the only downstream effector of CD40 signaling [25].

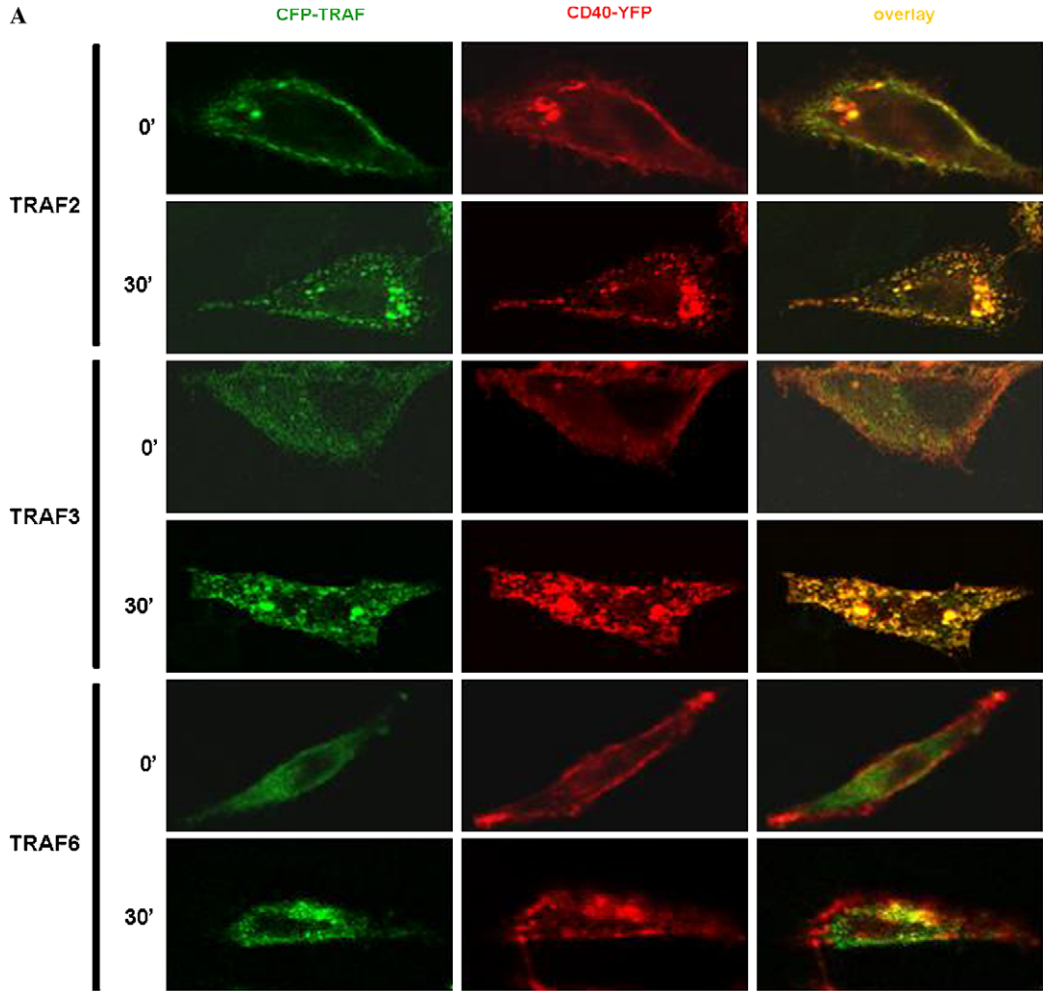

Fig. 4. Activation of endothelial inflammation by sCD40L or mCD40L differed. HeLa cells were seeded on glass coverslips in six-well plates and transfected by Effectene method with pEYFP-CD40 (1 μg) and pCFP-TRAF2/3/6 (3 μg each). About 24 h after transfection, the cells were stimulated with (A) G28.5 (10 μg/mL) or (B) 293A/CD40L for 30 min. Localization and traffic of TRAFs (green) and CD40 (red) is depicted with CFP and YFP channels, respectively, and overlaid (yellow). (C) The tendency of Akt phosphorylation varied in response to different stimuli. PAE-EYFP-CD40 cells were serum-starved before addition of 8μg/mL G28.5, or paraformaldehyde fixed 293A/CD40L (5×10^6/mL), for the indicated time. Cells were then lysed and immunoblotted with indicated antibodies. (D) PAE-EYFP-CD40 cells in 24-well plates were transfected with 3 × κB-luc reporter, and 24 h later, cells were serum starved before unfixed 293A/CD40L cells or G28.5 were added and incubated for the indicated time. LPS was used as a positive control. Luciferase activities were calculated as mentioned above and RLU was derived by normalization against the untreated controls in each group. The data represented the typical results of at least three independent experiments and statistically analyzed as in Fig. 2C. (E) mCD40L but not sCD40L induced endothelial inflammation. Primary HUVEC cells were treated with G28.5 (black bars), 293A/CD40L (grid bars), or LPS (grey bars) for 4 h. Real-time RT-PCR was performed to measure the expression levels of IL-8, ICAM, E-selectin, and P-selectin genes. The fold change after normalized to the mock treatment (white bars) is plotted. The data represented the typical results of at least three independent experiments and statistically analyzed as in Fig. 2C. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this paper.)

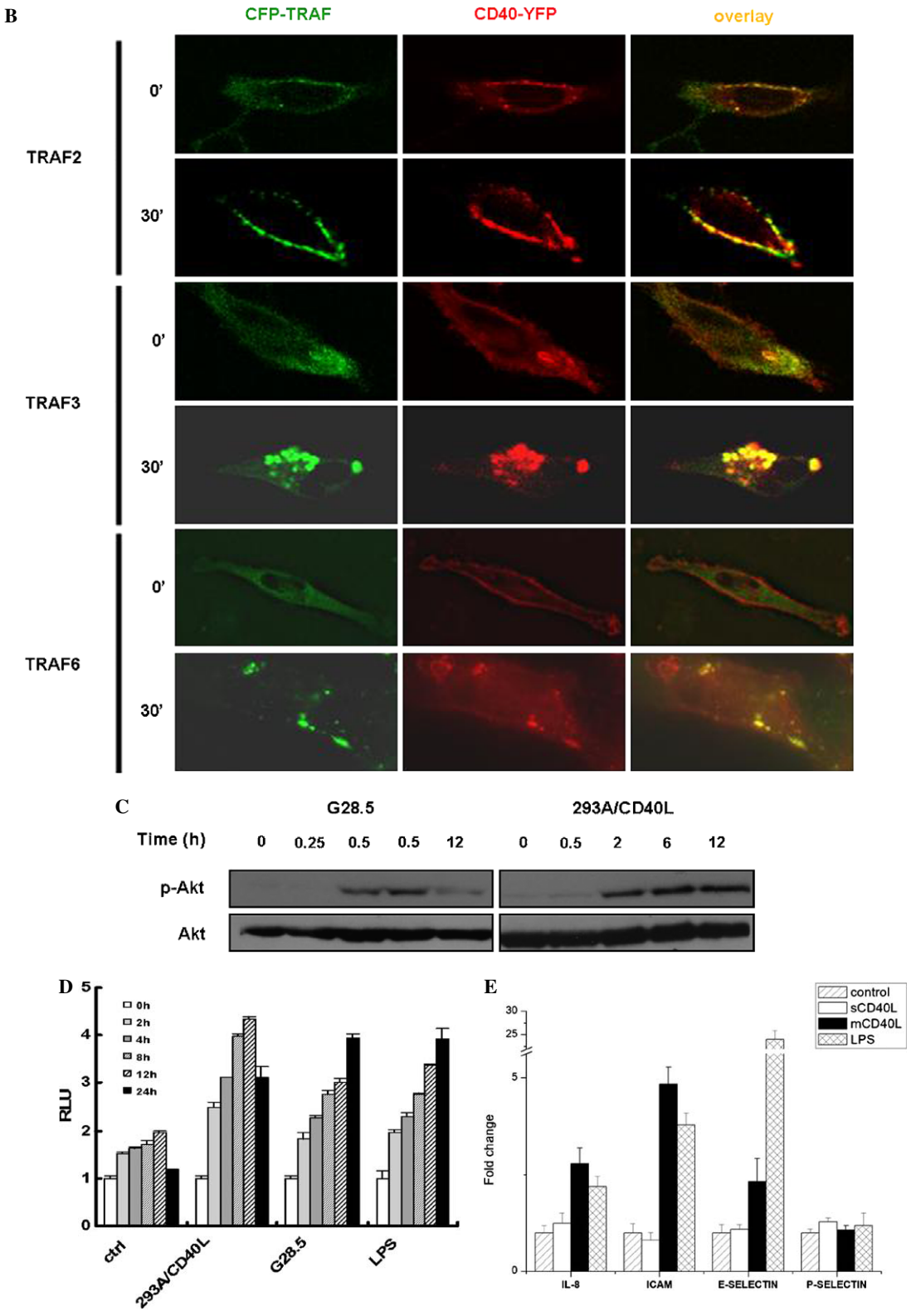

Fig. 4. (*continued*)

Because different traffic routes of the signaling complex should yield the divergent target gene activation, we then analyzed whether sCD40L and mCD40L might differentially regulate the inflammatory responses of cardiovascular endothelial cells. To do that, we treated the primary HUVEC cells with G28.5 or 293A/CD40L and real-time RT-PCR analysis showed that 293A/CD40L upregulated expression of atherogenic cytokines, IL-8, ICAM-1

(CD54), E-selectin (CD62E), while G28.5 resulted in no significant changes at all (Fig. 4E). Very weak or no induction of P-selectin was observed for either mCD40L or LPS, which was not surprising because they promote endothelial inflammation only by enhancing P-selectin secretion. This result was reminiscent of the previous finding that only platelet surface CD40L can induce the similar spectrum of cell adhesion molecules and proinflammatory factors in co-cultured HUVEC cells [12]. This result also agreed with the observation that mCD40L but not sCD40L upregulates IL-6 expression in B cells, although both can activate NF-κB [41]. Our in vitro experiments also indicated that NF-κB activation might be dispensable for mCD40L-induce vascular endothelial cell inflammation, because G28.5 (as well as sCD40L trimer, data not shown) exerted null proinflammatory activation of EC cells even though NF-κB was activated. Whether it is mCD40L rather than sCD40L that serves as an atherosclerotic risk factor in vivo remains to be thoroughly investigated.

Discussion

Despite the significant contribution of CD40 signal transduction to inflammatory diseases, relatively limited has been characterized of its role in EC activation. Abnormal activation of Akt leads to endothelial cell senescence in atherosclerotic plaques that secrete a large number of proinflammatory cytokines [28,29]. We demonstrated that, although dispensable for CD40 endocytosis, TRAFs binding to CD40 was essential for Akt phosphorylation thereby proinflammatory activation of EC. This dependence on TRAFs binding can rule out direct association of CD40 with PI3K in activation of Akt [42], but we cannot exclude the possibility of indirect association of Ras with PI3K [33] that may contribute to Akt phosphorylation upon CD40 engagement. Nevertheless, it is very tempting to speculate that the appropriate Akt phosphorylation might require a specific order and spatial arrangement of TRAFs recruitment for CD40L proinflammatory signaling. Recent data show that TRAF6 is an essential mediator of sCD40L-activated proinflammatory pathways in monocytes and macrophages [38], whether perfect colocalization of TRAF6 with CD40 triggered by mCD40L in our experiments was prerequisite for EC activation remains to be studied. This suggestion also raises the issue of cell-type specific regulation of CD40 signaling by TRAFs. For example, G28.5 triggered TRAF2/3 to localize with CD40 in endosomes in PAE cells, but the earlier experiments showed that TRAF2/3 colocalized with CD40 in lipid rafts in B cells [24]. Our observation that TRAF3 was recruited to cell surface CD40 by mCD40L was reminiscent of the previous findings [24], but rather different from the results that only TRAF2 and TRAF6 formed a cell surface complex with CD40 for constitutive activation of NF-κB pathway in B cell lymphoma [43].

Our results complemented the previous finding in which CD40 induces mCD40L internalization in T cells [16] and further showed that CD40 was internalized upon G28.5 or sCD40L treatment. This result is contradictory to the previous findings [17,24], because we showed that it was sCD40L but not mCD40L that triggered CD40 endocytosis in both PAE and HeLa cells. Our data further suggested that internalized CD40 could form intracellular signaling vesicles fully capable of Akt phosphorylation and NF-κB activation. Previous results indicate that CD40 forms a signalosome complex within the lipid rafts macrodomain on the surface of lymphoma cells, and antagonist antibodies to CD40L or CD40 can disrupt such a complex thereby inhibiting constitutive CD40 activation of NF-κB [43]. Because internalized CD40 by both Rab5-Q79L and sCD40L stimulation readily transduced the signals (e.g., Akt phosphorylation and NF-κB activation) with altered its specificity (e.g., inability to induce proinflammatory cytokines), we therefore speculate that the composition and functionality of the potential endosomal CD40 complex would differ from the signalosome on endoplasmic membrane. Indeed, only a small percentage of TRAF6 associated with the endosomal CD40 while it completely colocalized with the cell surface CD40.

Our data also showed that mCD40L but not sCD40L induced overexpression of proinflammatory cytokines and adhesion molecules in NF-κB independent manner. This finding is particularly intriguing given the contradictory reports on the function of CD40L in vascular endothelial inflammation and atherogenesis [9,11–13,44]. Our results and the recent risk assessment [13] favor the model that sCD40L level may not be a valid prognosis for chronic inflammation of vascular endothelial cells or the acute coronary dysfunction. There are several lines of evidence support this hypothesis and the notion that sCD40L may be a secondary risk factor of atherosclerosis, for example, in endothelial regeneration after injured by plaque erosion [45]. First, the release of sCD40L from alpha granules upon platelet activation is much slower than its presentation on cell surface [9], and the concentration of sCD40L in the plaque is much lower than it is in circulation, arguing whether plasma sCD40L is functionally relevant to atherogenesis [46]. Second, reduction of plasma sCD40L by statins intervention fails to revert abnormal platelet activation, inflammation, and hypercoagulation in patients with overt cardiovascular diseases [47]. G28.5 is a dimeric agonistic antibody that has been widely used as a functional ligand substitute in studies of CD40 signaling. Although G28.5 was functionally equivalent to the soluble trimeric sCD40L in many aspects of CD40 signaling, including the sufficient inductivity of CD40 endocytosis, Akt phosphorylation, and NF-κB activation, it should still be cautious to interpret the physiological role of G28.5 on endothelial cells in vivo. Nevertheless, our results suggest an important insight into pathophysiological function of membrane bound CD40L in atherosclerosis. Further characterization of the signaling network

exerted by different forms of CD40L might facilitate to pin down the precise mechanisms of vascular endothelial inflammation.

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