CD40 Ligand Binds to α5β1 Integrin and Triggers Cell Signaling*

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It was originally thought that the critical role of the CD40 ligand (CD40L) in normal and inflammatory immune responses was mainly mediated through its interaction with the classic receptor, CD40. However, data from CD40L−/− and CD40−/− mice suggest that the CD40L-induced inflammatory immune response involves at least one other receptor. This hypothesis is supported by the fact that CD40L stabilizes arterial thrombi through an α1β3-dependent mechanism. Here we provide evidence that soluble CD40L (sCD40L) binds to cells of the undifferentiated human monocytic U937 cell line in a CD40- and α1β3β3-independent manner. Binding of sCD40L to U937 cells was inhibited by anti-CD40L monoclonal antibody 5C8, anti-α5β1 monoclonal antibody P1D6, and soluble α5β1. The direct binding of sCD40L to purified α5β1 was confirmed in a solid phase binding assay. Binding of sCD40L to α5β1 was modulated by the form of α5β1 expressed on the cell surface as the activation of α5β1 by Mn2+ or dithiothreitol resulted in the loss of sCD40L binding. Moreover, sCD40L induced the translocation of α5β1 to the Triton X-100-insoluble fraction of U937 cells, the rapid activation of the MAPK pathways ERK1/2, and interleukin-8 gene expression. The binding of sCD40L to CD40 on BJAB cells, an α5β1-negative B cell line, and the resulting activation of ERK1/2 was not inhibited by soluble α5β1, suggesting that sCD40L can bind concomitantly to both receptors. These results document the existence of novel CD40L-dependent pathways of physiological relevance for cells expressing multiple receptors (CD40, α5β1, and α1β3) for CD40L.

The CD40 ligand (CD40L), also known as CD154 or gp39, is a type II transmembrane protein that belongs to the tumor necrosis factor (TNF) superfamily and is expressed on a variety of hematopoietic and non-hematopoietic cells. The main sources of CD154 are activated platelets and CD4+ T cells (1). CD154 is also variably expressed on activated CD8+ T cells, activated B cells, eosinophils, mast cells, basophils, natural killer cells, dendritic cells, monocytes, and macrophages as well as endothelial cells, epithelial cells, and smooth muscle cells (reviewed in Ref. 2). Expression of CD40L differs according to cell types and type of stimuli (2). The expression of CD154 is inducible, and its expression on T cells is triggered primarily by T cell receptor signaling and is regulated by CD28-dependent and independent pathways (3) CD40L is stored in platelets and a subpopulation of T cells and rapidly translocates to the cell membrane following T cell and platelet activation (4, 5). A soluble form of biologically active CD40L trimer (sCD40L) is present in the supernatant of activated T cells (6) and platelets (7) and results from the proteolytic cleavage of the homotrimeric CD40L by a metalloproteinase (7).

It was originally thought that CD40L had only one receptor, CD40, which is a type I transmembrane protein that is a member of the TNF receptor superfamily. CD40 is expressed on the surface of many immune and non-immune cells, including B lymphocytes, monocytes/macrophages, and dendritic cells, as well as platelets, epithelial, and endothelial cells (8). Most biological functions of CD40L have been attributed to its direct interaction with CD40. However, studies using CD40L−/− and CD40−/− mice have suggested that CD40L may also bind to one or more other receptors (9). In support of this hypothesis, it has been elegantly demonstrated that sCD40L interacts with α1β3 (GPⅡb/Ⅲa), an integrin expressed on platelets (10), triggering outside-in signaling and inducing platelet activation and spreading (11). CD40L−/− mice exhibit increased bleeding time (12) and reduced thrombus...
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stability (10), showing that the interaction between sCD40L and the integrin αIIbβ3 is physiologically relevant.

Based on the above observations, we hypothesized that CD40L may bind to another still unknown receptor on human monocytes and induce cellular activation. Our results show that sCD40L binds to CD40-negative human monocyctic U937 cells, an interaction that is inhibited by anti-α5β1 integrin (VLA-5) mAb as well as by soluble α5β1 integrin (sα5β1). The binding of sCD40L to immobilized purified α5β1 confirms the direct interaction of sCD40L with this integrin. sCD40L induces the translocation of α5β1 to the Triton X-100-insoluble fraction, the rapid activation of MAPKs ERK1/2 in U937 cells, and IL-8 gene expression, confirming the existence of a third functional receptor for CD40L on α5β1.

EXPERIMENTAL PROCEDURES

Cells—The myelomonocytic cell line U937 (ATCC, Manassas, VA) and the B cell lymphoma cell line BJAB (from Dr. J. Menezes, Sainte-Justine Hospital, Montréal, Quebec, Canada) were maintained in RPMI 1640 containing 10% heat-inactivated fetal bovine serum, 1-glutamine, penicillin, and streptomycin (Wisent, St-Bruno, Quebec, Canada).

Reagents and Antibodies—Recombinant trimeric soluble CD40L (rsCD40L) (13) was provided by Immunex Corp. (Seattle, OR). Avidin was procured from Sigma. Alexa Fluor-488 labeling of rsCD40L (rsCD40L-A) and avidin (avidin-A) was performed according to the manufacturer’s instructions (Molecular Probes, Eugene, OR). Anti-CD40L hybridoma 5C8 (IgG1) and anti-CD40 hybridoma G28.5 (IgG1) were obtained from the ATCC. The isotype controls anti-TSST-1 mAb 2H8 (IgG2b) and isotype control anti-SEB mAb 8C12 (IgG2a) and anti-CD40 hybridoma G28.5 (IgG1) were obtained from R & D Systems, Minneapolis, MN). Soluble recombinant sCD40L to immobilized purified α5β1 confir...
protease inhibitor mixture (Roche Applied Science), and 1% Triton X-100 (Fisher Scientific) for 30 min on ice. The cell lysates were centrifuged at 16,000 \( \times \) g for 15 min at 4 °C. Soluble and insoluble fractions were separated by SDS-PAGE under non-reducing conditions and analyzed by immunoblotting.

**Immunoblot Analysis**—Polyvinylidene difluoride membranes were blocked in BLOTTO (5% skim milk in Tris saline, pH 7.5, 0.15% Tween 20, Fisher Scientific) for 1 h at room temperature, and then incubated with rabbit anti-\( \alpha \)-\( \delta \)-antibody overnight at 4 °C followed by goat anti-rabbit IgG-HRP antibody or with mouse anti-CD40 antibody overnight at 4 °C followed by goat anti-mouse IgG-HRP antibody. The phosphorylation of ERK1/2 was assessed by immunoblotting using phospho-specific Abs according to the manufacturer’s instructions. Membranes were stripped (62 mM Tris-HCl, pH 6.8, 2% SDS/100 mM, 2-mercaptoethanol, 30 min, 50 °C) and reprobed with antibody recognizing total ERK1/2. Antigen-antibody complexes were revealed with ECL (GE Healthcare, Mississauga, Ontario, Canada).

**Analysis of IL-8 mRNA Expression**—U937 cells (2 \( \times \) 10^6 cells/100 l) were treated with 100 ng of rsCD40L in RPMI 5% fetal bovine serum at 37 °C for the indicated time points. Reactions were stopped by adding ice-cold RPMI, and cells were isolated by spinning at 14,000 rpm at 4 °C. Total RNA was prepared from each sample using the RNeasy total RNA isolation Kit (Qiagen Inc., Mississauga, Ontario, Canada). Single strand cDNA for a PCR template was synthesized from 1 \( \mu \)g of total RNA using a primer, oligo(dT)\(_{12-18}\) (Invitrogen), and superscript III reverse transcriptase (Invitrogen) under the conditions indicated by the manufacturer. Reverse transcription was inactivated at 95 °C for 5 min, and the products were kept on ice until needed for the PCR. Specific primers were designed from cDNA sequence for IL-8 and \( \beta \)-actin. Each cDNA was amplified by PCR using TaqDNA polymerase (Invitrogen). The sequences of the primers were as follows: IL-8F (5'-GCC-AAGGAGTGGCTAAAGGAC-3'), IL-8R (5'-CAGTGCTCACTGATTCTTG-3'), \( \beta \)-actin F (5'-AATCTGGCAACCACA-3') and cDNA were amplified by PCR using TaqDNA polymerase (Invitrogen).
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**A**

![Flow cytometric analysis of α5β1 integrin surface expression. Cells were incubated with anti-α5β1 mAb HA5 or isotype control mAb followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG Ab and analyzed by FACS.](image)

**B**

![Relative Fluorescence intensity](image)

**C**

![Relative Fluorescence intensity](image)

FIGURE 2. rsCD40L-A binding to α5β1-positive U937 cells but not to α5β1-negative BJAB cells was prevented by soluble α5β1 (sα5β1) and anti-α5 mAb P1D6. A, flow cytometric analysis of α5β1 integrin surface expression. Cells were incubated with anti-α5β1 mAb HA5 or isotype control mAb followed by fluorescein isothiocyanate-labeled goat anti-mouse IgG Ab and analyzed by FACS. B, soluble α5β1 prevented the binding of rsCD40L-A to U937 cells but not to BJAB cells. rsCD40L-A was preincubated with sα5β1 (10-fold molar excess) for 1 h at 37 °C prior to the addition of cells. Similar results were obtained with avidin-A and sα5β1 (avidin-A). C, preincubation of U937 cells with anti-α5β1 mAb P1D6 significantly prevented the binding of rsCD40L-A. Cells were incubated with mAb P1D6 or an isotype control (IgG3) for 30 min at 37 °C. Avidin-A or rsCD40L-A was then added, and the incubation continued for 1 h at 37 °C. This figure is representative of three independent experiments.

CTTCT-3′), and β-actin R (5′-TAATGTCACGACGATT-TCC-3′). Conditions for PCR were 35 cycles of 94 °C for 45 s, 55 °C for 45 s, and the 72 °C for 1 min. Additional 10 min of 72 °C was performed at the end of the PCR reaction. The products were analyzed on a 1% agarose gel containing ethidium bromide. The expected sizes of the PCR products for IL-8 and β-actin were 280 and 400 bp, respectively. We did not detect any band when we performed PCR without adding the cDNA template in this study. Genomic DNA contaminants were examined by performing PCR reaction on 1 μg of total RNA at similar conditions, and no contaminants were detected. Densitometric analyses were performed on each detected band using a Molecular Imager Gel Doc System and Quantity One analysis software from Bio-Rad. Results shown are normalized for two conditions, first based on β-actin levels at each time point and thereafter, based on the level of expression of each gene of samples indicated as time 0 (non-treated samples). The ratio was then blotted as fold increase of IL-8 mRNA after rsCD40L treatment versus time of the treatment.

**RESULTS**

**rsCD40L-A Bound to CD40-negative U937 Cells**—In an attempt to identify a novel receptor for CD40L, we first analyzed the expression of CD40 by flow cytometry and immunoblotting using various cell lines, and selected CD40-negative cell lines that could bind Alexa Fluor-labeled rsCD40L (rsCD40L-A). The CD40-positive human BIAB B cell line was used as a control. The undifferentiated monocytic U937 cell line did not express CD40, as assessed by flow cytometry (Fig. 1A) and immunoblotting (data not shown) but was able to bind rsCD40L-A at a level similar to that observed on BJAB cells (Fig. 1B). The specificity of this binding was confirmed by adding a 10-fold molar excess of unlabeled rsCD40L (Fig. 1B) or by preincubating rsCD40L with the anti-CD40 mAb 5C8 (Fig. 1C). Similar results were obtained with the CD40-negative erythroleukemic K562 and HEK 293 cell lines (data not shown). Based on these results, we looked for strong evidence of rsCD40L binding to U937 cells in a CD40-independent manner. Fig. 1D shows that preincubation of U937 cells with blocking anti-CD40 mAb 82102 did not interfere with the binding of rsCD40L-A, whereas the same treatment completely prevented the binding of rsCD40L-A to BJAB cells. These results indicated that U937 cells express at least one molecule that is distinct from CD40 and that acts as a receptor for sCD40L. They also confirmed that CD40 is the sole receptor for CD40L on BJAB cells.

**rsCD40L Bound to α5β1**—Based on the above results, and because sCD40L also binds to αIIbβ3 (10, 11), which is selectively expressed on platelets (18), hematopoietic progenitors (19), and mast cells (20) but not on U937 cells (data not shown), we hypothesized that binding of rsCD40L to U937 cells may also be mediated by other members of the integrin superfamily. We first looked for integrin superfamily members that are expressed on U937, K562, and HEK 293 cells but not on BJAB cells. Among others, we found that α5β1 was expressed constitutively on U937 cells (Fig. 2A), K562 cells (data not shown)
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Chemical Agents That Increase the Affinity of α5β1 for Fibronectin Negatively Affected the Binding of rsCD40L to U937 Cells—α5β1 is constitutively expressed on the cell surface in an inactive form that cannot bind fibronectin (reviewed in Ref. 23). Conformational changes triggered by outside-in or inside-out signaling result in the activation of the integrin (23) allowing it to bind to its natural ligand. Chemical agents such as Mn²⁺ and DTT can promote such changes (21). We wondered whether the activation of α5β1 integrin could also modulate the binding of rsCD40L to U937 cells. First, we confirmed that U937 cells did not constitutively bind to fibronectin and that Mn²⁺ and DTT strongly promoted their adhesion to fibronectin as evaluated by microscopy (Fig. 4A) and a colorimetric assay (Fig. 4B). In contrast, similar treatments of α5β1-negative BJAB cells did not promote their attachment to fibronectin (Fig. 4). Second, conformational changes induced by these chemical agents expose a β1 epitope, the mAb B44 epitope (21). Indeed, the results presented in Fig. 5A show that treatments with Mn²⁺ or DTT induced the expression of the B44 epitope on U937 cells but not on BJAB cells. We then assessed the binding of rsCD40L-A to U937 cells treated with Mn²⁺ or DTT. Interestingly, the treatment of U937 cells with Mn²⁺ reduced the binding of rsCD40L-A while the treatment with DTT almost completely inhibited the binding of rsCD40L-A to U937 cells (Fig. 5B). In contrast, similar treatments of BJAB cells had no effect on the binding of rsCD40L-A to CD40 (Fig. 5B). Thus, changes in the conformation of α5β1 that promote its binding to fibronectin prevent its interaction with sCD40L.

rsCD40L Induced α5β1 Recruitment to the Cytoskeleton in CD40-negative U937 Cells—One consequence of the binding of β1 integrins with their ligands is their association with the cell cytoskeleton (24). We thus looked at whether the interaction of rsCD40L with α5β1 would also result in its association with the cytoskeleton. To assess the recruitment of α5β1 to the cytoskeleton, cells were incubated with rsCD40L for 30 min at 37 °C and solubilized in Triton X-100 buffer. The soluble (Sol) and insoluble (Ins) fractions were separated by centrifugation and analyzed by immunoblotting with a rabbit polyclonal anti-α5 Ab (Fig. 6A). α5β1 was found exclusively in the Triton X-100-soluble fraction of unstimulated U937 cells, whereas a significant amount of α5β1 translocated into the Triton-X-100-insoluble fraction of rsCD40L-stimulated U937 cells. As expected, rsCD40L induced the translocation of CD40 to the detergent-insoluble fraction of BJAB B cells (25) and the formation of

(21), and HEK 293 cells (data not shown) (22) but not on BJAB cells (Fig. 2A). To determine whether rsCD40L-A could bind to α5β1, we performed a competitive binding assay using soluble α5β1 (sα5β1) as bait for rsCD40L-A. Fig. 2B shows that preincubation of rsCD40L-A with sα5β1 substantially inhibited the binding of rsCD40L-A to U937 cells. Preincubation of rsCD40L-A with sα5β1 did not affect rsCD40L-A binding to CD40 on B cells, suggesting that sCD40L could bind concomitantly to both CD40 and α5β1.

To provide further support for the notion that α5β1 is involved in the binding of rsCD40L to U937 cells, cells were preincubated with anti-α5 mAb P1D6 or isotype control mAb and then incubated with rsCD40L-A. Fig. 2C shows that mAb P1D6 inhibited the interaction of rsCD40L-A to U937 cells but, as expected, did not affect the binding of rsCD40L-A to α5β1-negative BJAB cells. Thus, the results presented here support our contention that α5β1 is the third receptor for sCD40L.

Lastly, to confirm that rsCD40L binds directly to α5β1, a solid phase binding assay was developed using immobilized soluble CD40-Fc as a control receptor (Fig. 3A). The results presented in Fig. 3B clearly demonstrate that rsCD40L binds directly to purified α5β1 in a dose-dependent manner. In contrast, rsCD40L did not bind to other purified integrins, αVβ3 (Fig. 3C) and αVβ5 (Fig. 3D), confirming the specificity of the rsCD40L-α5β1 interaction. Thus, α5β1, like CD40 and α1β2β3, is a receptor for sCD40L.
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**A**

| Medium | Mn²⁺ | DTT |
|--------|------|-----|
| BJAB   |      |     |
| U937   |      |     |

**B**

![Graph showing adherence of U937 cells to fibronectin](Image)

**FIGURE 4.** Mn²⁺ and DTT treatments induced the adherence of U937 cells but not BJAB cells to fibronectin. A, cells were treated with Mn²⁺ (1 mm) or DTT (10 mm) or not for 30 min at room temperature. Washed cells were then added to fibronectin-coated wells and allowed to adhere for 1 h at 37 °C. After removing unbound cells, adherence was assessed using a Zeiss microscope. B, colorimetric evaluation of cells adhering to fibronectin. Treated or untreated cells were tested for their adherence to immobilized fibronectin as described above. After removing unbound cells, a colorimetric assay was used as described under “Experimental Procedures” to evaluate the adherence of U937 and BJAB cells to fibronectin. These results are representative of four independent experiments.

CD40 homodimers (26) (Fig. 6B). Thus, like its natural ligand, fibronectin, the interaction of rsCD40L with α5β1 triggered its association with the cytoskeleton.

**rsCD40L Induced the Activation of the ERK1/2 Pathway in CD40-negative U937 Cells**—The above results prompted us to investigate signal-transducing events triggered by the engagement of α5β1 by rsCD40L. The stimulation of monocytes with membrane CD40L or sCD40L induces the activation of the MAPK pathways ERK1 and -2 (27–29). The binding of ligand to α5β1 also induces the activation of MAPK pathways (30). We thus investigated the phosphorylation of ERK1/2 in U937 cells following stimulation with rsCD40L and found, as shown in Fig. 7A, that ERK1/2 was rapidly phosphorylated in rsCD40L-activated U937 cells. As expected (31), rsCD40L induced the activation of ERK1/2 in BJAB cells (Fig. 7A). These results indicated that, like its classic ligand fibronectin, the interaction of sCD40L with α5β1 triggers the activation of signaling pathways, confirming the functional status of this interaction.

**Interaction between sa5β1 and rsCD40L Did Not Interfere with the CD40L-induced Activation of ERK1/2 via CD40**—We showed above by flow cytometry analysis that purified sa5β1 prevented the interaction of rsCD40L with U937 cells. As expected, the interaction of rsCD40L with sa5β1 also completely prevented the activation of the ERK1/2 in U937 cells (Fig. 7B). An interesting outcome of the binding experiments was the observation that rsCD40L may interact concomitantly with CD40 and α5β1 (Fig. 2B). This suggested that sCD40L bound to α5β1 can trigger signaling in CD40-positive cells. Indeed, our data in Fig. 7B show that rsCD40L bound to α5β1 induced the activation of ERK1/2 in BJAB cells. Thus, sCD40L may serve as a molecular bridge between CD40 and α5β1 expressed on two different cells and trigger signal transduction in both cells.

**rsCD40L Induced IL-8 Gene Expression in U937 Cells**—The interaction of monocytes with fibronectin leads to inflammatory cytokines expression (30, 32), cellular responses that could also be induced in these cells by sCD40L-triggered signaling. To assess the expression of biological mediators, we analyzed IL-8 gene expression in U937 cells stimulated with 100 ng of rsCD40L for 15 min to 4 h. In U937 cells, rsCD40L induced a weak but significant IL-8 gene expression (Fig. 8A), reaching more than a 2.5-fold increase at 2 h (Fig. 8B). Thus, the interaction of sCD40L with cells in a CD40-independent manner also promotes the expression of inflammatory mediators.

**DISCUSSION**

We designed this study to examine the ability of sCD40L to bind to cells that do not express its two known receptors, CD40 and α1β1β3, and, if such binding occurs, to identify the new CD40L receptor(s). The main findings of this study are that 1) sCD40L bound to α5β1, a widely distributed cell surface receptor, 2) the interaction of sCD40L with α5β1 was prevented by conformational changes of α5β1 that result in its activation and its binding to fibronectin, 3) binding of sCD40L to α5β1 induced signaling, translocation of α5β1 to the Triton X-100-insoluble fraction, and chemokine gene expression, and 4) sCD40L may simultaneously bind to CD40 and α5β1 on the cell surface. Thus, α5β1 is a functional receptor for sCD40L, the third described so far, two being members of the integrin family.

Extensive research over the last decade has documented the central role of CD40L in immune responses (reviewed in Refs. 33 and 34) and in the development of autoimmunity (reviewed in Refs. 35 and 36) and inflammatory disorders (reviewed in Refs. 2 and 37). It became evident that CD40L is not only expressed by activated T cells but also by other cell types following activation, especially in chronic inflammatory diseases and autoimmune, and that it plays a broader role than initially thought. Indeed, the expression of CD40L by immune cells is augmented and prolonged in many disease conditions, including systemic lupus erythematosus (38), rheumatoid arthritis (39, 40), inflammatory bowel disease (37), and cardiovascular disease (41), and is induced in non-immune cells such as fibroblasts (42) and endothelial cells (43). CD40L is also released as a soluble, trimeric, biologically functional molecule, and an increased level of circulating sCD40L is also a feature of many chronic inflammatory and autoimmune conditions (41, 44–46). The importance of CD40L in the development and maintenance of these inflammatory disorders has been demonstrated in mouse models and in a few human studies where an anti-CD40L treatment has been shown to be beneficial (35, 36, 47). CD40, the classic ligand for CD40L, is also expressed on many immune and non-immune cell types in inflammatory and
autoimmune diseases (2, 36, 37), which supports the contention that the CD154–CD40 axis plays a crucial role in autoimmunity and inflammatory disorders. However, the scope of the role of CD40L in cellular immunity and inflammation is not limited just to its interaction with CD40. André et al. (10, 11) have shown that sCD40L can bind to α5β1, triggering platelet activation, and is involved in thrombus stabilization (10, 11). We show here that α5β1 is also a functional receptor for sCD40L.

Like other members of the integrin superfamily, α5β1 is a cell surface receptor that binds to the extracellular matrix and, when doing so, provides cells with adhesive properties and a transmembrane link between the extracellular environment and the intracellular cytoskeleton. Ligand binding to integrins triggers signaling pathways such as MAPKs, leading to cytoskeleton organization and regulating cell fate and behavior (48–50). Like other integrins, α5β1 is not constitutively active and, in that form, cannot bind to fibronectin, its ligand (23). sCD40L, however, binds to inactive α5β1, and the activation of α5β1 by chemical agents that induce conformational changes in a manner similar to that observed in the ligand-occupied integrin (21) prevents the binding of sCD40L. Thus, although α5β1 acts as receptor for fibronectin and sCD40L, these two ligands cannot bind to α5β1 simultaneously.

The interaction of α5β1 with the ECM or ligation by anti-α5 antibodies induces the activation of ERK pathways in various cell types, including B cell progenitor cell line (51), fibroblasts (52), chondrocytes (53), and, as shown here, monocyte U937 cells (54). In many cell types such as chondrocytes (55), synovial cells (56), and monocyes (30, 54), signals transduced via α5β1 result in cell survival, proliferation, and cytokine and chemokine expression. We show herein that the engagement of α5β1 by sCD40L on U937 cells is also functionally relevant, because it induces the activation of ERK signaling pathways and the association of α5β1 with the Triton X-100-insoluble fraction. The trimeric nature of sCD40L (13) and native sCD40L (6, 57) may facilitate the formation of clusters of α5β1 that promotes the activation of signaling pathways and the association of α5β1 with the cytoskeleton in a manner similar to the ECM (24, 48).

A number of cell types such as monocytes, macrophages, and dendritic cells express both CD40 and α5β1. CD40L is important for macrophage activation as shown by the fact that CD40L knock-out mice have defective T cell-dependent macrophage-driven functions (58). To date, signaling and subsequent cellular responses induced by CD40L in CD40-positive cells have been attributed solely to its interaction with CD40, although in most cases the identity of the CD40L receptor has not been confirmed. The results presented herein point to the need for a reassessment of the contribution of CD40 to these cellular responses. Indeed, stimulation of CD40-positive/α5β1-positive

FIGURE 5. Treating U937 cells with Mn²⁺ or DTT exposed the B44 β1 epitope but decreased the binding of rsCD40L to α5β1. A, effects of Mn²⁺ and DTT treatments of U937 and BJAB cells on B44 β1 epitope expression. Cells were treated with Mn²⁺ and DTT as indicated above, and the expression of the B44 β1 epitope was assessed by flow cytometry. B, treatment of U937 cells with Mn²⁺ or DTT prevented the binding of rsCD40L-A. Cells were treated with Mn²⁺ or DTT as described above, and the binding of rsCD40L-A was assessed as described in Fig. 1. These results are representative of four independent experiments.

FIGURE 6. Stimulating U937 cells with rsCD40L led to the recruitment of α5β1 into the Triton X-100-insoluble fraction. Cells were stimulated for 30 min at 37 °C with rsCD40L and then lysed in 1% Triton X-100 buffer. Cell lysates were centrifuged at 16,000 × g for 20 min at 4 °C. Soluble (10⁶ cell equivalents) and insoluble (2 × 10⁶ cell equivalents) fractions were separated by SDS-PAGE under non-reducing conditions and analyzed by immunoblotting using anti-human α5 (A) and anti-human CD40 (B). This figure is representative of three independent experiments.
monocytes and macrophages with sCD40L or membrane-bound CD40L induces the activation of ERK (27, 28) and the synthesis of pro-inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α (27, 28, 59). These cellular responses are also induced in monocytes by fibronectin (30), mainly via its interaction with α5β1 (32). The signals induce in U937 cells by sCD40L lead to IL-8 gene expression, which suggests that the CD40L-α5β1 interaction in addition to the CD40L-CD40 interaction contributes to the pathogenesis of inflammatory conditions where cells such as neutrophils play a crucial role. Interestingly, vitamin D3-differentiated U937 cells have been reported to produce tissue factor in a CD40L-dependent but in a CD40-independent manner when incubated with CD40L+ T cells (60). It is thus tempting to hypothesize that tissue factor is induced in these cells by α5β1-mediated signals.

An interesting finding is that sCD40L can bind to α5β1 and CD40 simultaneously, as shown by cell binding and cell activation experiments in BJAB cells. The CD40 binding site has been mapped to the interface between adjacent CD40L monomers (61). The simultaneous binding of α5β1 and CD40 to sCD40L suggests that sCD40L interacts with α5β1 outside the CD40 binding site. These results raise the possibility that sCD40L trimers may serve as a molecular bridge between CD40 and α5β1 expressed on various cell types, triggering signal transduction in such cells, or may cross-link the two receptors expressed on the same cell, triggering signaling-related events via both the receptors.

Thus, the responses of monocytes as well as other CD40-positive and α5β1-positive cell types to sCD40L may be induced through both CD40 and α5β1 individually or in combination. The signaling pathways and subsequent cellular responses triggered by sCD40L would depend on the availability of the various receptors for interactions with sCD40L. For example, platelets express all three receptors for sCD40L that have been identified to date (CD40, α5β1, and αIIbβ3). Platelets are also the major source of circulating sCD40L (1) and, through their cell surface expression and release of CD40L, can play an important role in the immune response (62) and in inflammation (63). The roles played by each of these sCD40L receptors in platelet activation remain to be determined. Moreover, the expression of αIIbβ3 is not restricted to platelets and their progenitors as was recently shown with hematopoietic progenitors (64) and mast cells (20), which also expressed α5β1 (65). Like platelets, activated mast cells express both integrins α5β1 and αIIbβ3, and CD40L (66), and could be activated via α5β1 and αIIbβ3 following their interaction with CD40L. α5β1, through its interaction with CD40L, may play an important role in immune and inflammatory responses, because this integrin is broadly expressed (67). This possibility is currently under investigation in our laboratory. Given the important role of CD40L in inflammatory and autoimmune diseases, the identification of α5β1 as a new CD40L receptor may turn out to be crucial in understanding the development of
these diseases and may lead to the development of potential new strategies to counter them.

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