Requirement of Oxidation-dependent CD40 Homodimers for CD154/CD40 Bidirectional Signaling*

Carlos Reyes-Moreno*1,2, Ehsan Sharif-Askari*1,2, Julie Girouard*1, Claire Léveillé4, Malek Jundi5, Ali Akoum**, Réjean Lapointe4, André Darveau5, and Walid Mourad*6

From the 1Centre de Recherche en Rhumatologie et Immunologie, Centre Hospitalier de l’Université Laval, Québec City, Québec G1V 4G2, Canada; the 2Laboratoire d’Immunologie Cellulaire et Moléculaire, Centre Hospitalier de l’Université de Montréal, Hôpital Saint-Luc, Montreal, Québec H2X 1P1, Canada; the 3Département de Médecine, Université de Montréal, Montréal, Québec H3T 1J7, Canada; the 4Département de Chimie-Biochimie, Université du Québec à Trois-Rivières, Québec G9A 5H7, Canada; the 5Département de Recherche en Endocrinologie de la Reproduction, Université Laval, Québec City, Québec G1V 4G2, Canada; 6Centre Hospitalier de l’Université de Montréal, Hôpital Notre-Dame, Montréal, Québec H2L 4M1, Canada, and the 7Département de Biochimie et Microbiologie, Université Laval, Québec City, Québec G1V 4G2, Canada

It is well established that the CD154/CD40 interaction is required for T cell-dependent B cell differentiation and maturation. However, the early molecular and structural mechanisms that orchestrate CD154 and CD40 signaling at the T cell/APC contact site are not well understood. We demonstrated that CD40 engagement induces the formation of disulfide-linked (dl) CD40 homodimers that predominantly associate with detergent-resistant membrane microdomains. Mutagenesis and biochemical analyses revealed that (a) the integrity of the detergent-resistant membranes is necessary for dl-CD40 homodimer formation, (b) the cytoplasmic Cys238 of CD40 is the target for the de novo disulfide oxidation induced by receptor oligomerization, and (c) dl-CD40 homodimer formation is required for CD40-induced interleukin-8 secretion. Stimulation of CD154-positive T cells with staphylococcal enterotoxin E superantigen that mimics nominal antigen in initiating cognate T cell/APC interaction revealed that dl-CD40 homodimer formation is required for interleukin-2 production by T cells. These findings indicate that dl-CD40 homodimer formation has a physiological role in regulating bidirectional signaling.

Primary immune responses are initiated by specific physical interactions between antigen-specific T cells and antigen-presenting cells (APCs), resulting in bidirectional signal transduction events that modulate cell functions (1). Various cytokine and co-stimulatory receptors provide the input to direct these processes. Elucidating the mechanisms underlying the regulation of cell/cell interactions is thus crucial for further understanding the immunological responses and improving therapeutic strategies aimed at treating cell/cell-interaction-mediated human diseases.

CD154 and CD40 molecules are pairs of ligand/receptor that belong to the tumor necrosis factor (TNF) and TNF receptor (TNFR) superfamily and that play a pivotal role in cell/cell interactions (1). In B cells, the CD154/CD40 interaction is responsible for clonal expansion, germinal center formation, isotype switching, affinity maturation, and rescue from surface Ig-induced apoptosis. In nonhematopoietic cells, ligation of CD40 with CD154 enhances the secretion of pro-inflammatory cytokines such as IL-6, IL-8, and TNF-α. The absence or disruption of the CD154/CD40 pathway leads to a severe perturbation of the immune system, for example, in X-linked immunodeficiency with hyper-IgM (1). This observation has been confirmed using CD154 and CD40 knock-out mice (2, 3). Like most ligand/receptor pairs, the CD154/CD40 interaction leads to a bidirectional signal that leads to proliferation and IL-2 production (3) as well as various cellular events that modulate T cell functions.

Because CD40 has no kinase domain, the transmission of its intracellular signals passes via the recruitment of several adapter proteins, such as Jak3 and TNFR-associated factor (TRAF) proteins, to specific domains in its cytoplasmic tail. This results in the activation of members of the Src kinase family (such as Lyn and Fyn) and other protein-tyrosine kinases (such as Syk and Btk), phosphatidylinositol 3-kinase, phospholipase C-β, and other signaling molecules, translocate and cluster into sphingolipid- and cholesterol-enriched microdomains and engage specific intracellular signaling pathways (5, 6). These discrete

- interleukin; TNF, tumor necrosis factor; TNFR; TNF receptor; TRAF, TNFR-associated factor; WT, wild type; ELISA, enzyme-linked immunosorbent assay; ROS, reactive oxygen species; TCR, T cell receptor(s); MHC, major histocompatibility complex.
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Detergent-resistant membrane (DRM) microdomains are very dynamic areas of the cell membrane that serve as platforms for cell signaling (7).

Although it is well established that CD154 is mainly present in a trimeric form at the cell surface and/or following release from the cell membrane (8), little research has been conducted on CD40 homodimers (9) and their functional importance during T cell/ APC interactions. We have previously reported that ligation of CD40 with soluble leucine zipper trimeric CD154 (stCD154) leads to the formation of the disulfide-linked CD40/CD40 (dl-CD40) homodimer (10, 11). A high degree of CD40 clustering and subsequent dl-CD40 homodimer formation are selectively required for certain CD40-triggered responses such as phosphatidylinositol 3-kinase-dependent expression of B7-2 on human B cells and IL-8 production (10, 11). We show here that the translocation of CD40 to DRM microdomains is a prerequisite for dl-CD40 homodimer formation and that a cysteine residue at position 238 is involved in dl-CD40 homodimer formation. We also provide evidence showing that CD154/CD40 bidirectional signaling triggered during cognate T cell/ APC interaction is critically dependent on CD40 homodimer formation.

EXPERIMENTAL PROCEDURES

Cell Cultures, Antibodies, and Reagents—All of the cells used in this study were obtained from the ATCC (Manassas, VA). BJAB and Jurkat D1.1 cells were maintained in RPMI 1640 containing 5% fetal bovine serum, l-glutamine, penicillin, and streptomycin (Wisent, St-Bruno, Canada). HEK 293 cells were maintained in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum, l-glutamine, penicillin, and streptomycin (Wisent). The hybridomas producing the mouse mAbs directed against human CD40 (G28-5; IgG1) were obtained from ATCC. The IgG1 isotype controls were produced in our laboratory. The horseradish peroxidase-conjugated anti-mouse IgG (Jackson ImmunoResearch, Cedarlane Laboratories Ltd., Burlington, Canada), and incubated for a further 30 min on ice. The cell lysates were diluted in an equal volume of 85% sucrose, overlaid with 2.4 ml of 35% sucrose and centrifuged at 35,000 rpm for 16 h at 4 °C. Eleven 380-μl fractions were harvested from the tube, starting at the top. The pellet was washed and resuspended in 380 μl of TNE buffer. Alternatively, the cells were lysed in ice-cold TNE buffer for 30 min on ice. The Triton-soluble fraction was separated from the Triton-insoluble fraction by centrifugation at 14,000 rpm for 30 min at 4 °C. The Triton-insoluble pellets were washed, resuspended in 380 μl of TNE buffer, and homogenized by sonication. To disrupt sphingolipid- and cholesterol-enriched microdomains, BJAB cells were pretreated with 10 mM MβCD for 10 min at 37 °C. For thiol alkylation and the antioxidant treatments, the cells were treated with cell-permeable NEM thiol alkylating agent or NAC antioxidant for 60 min at 37 °C. The concentrations were maintained throughout the CD40 stimulation treatments.

Immunoblotting—To study homodimer formation by intermolecular disulfide bond formation, the samples were diluted 1:5 in 5X Laemmli sample buffer under nonreducing conditions (without β-mercaptoethanol). The samples were boiled for 5 min and resolved by SDS-PAGE. The proteins were transferred onto polyvinylidene difluoride membranes (Millipore). After blocking with 5% skim milk and 0.1% Tween 20 in phosphate-buffered saline, the CD40 molecules were probed with mouse mAbs plus secondary Abs as described above. IL-8 release was detected from the cell membrane (8), which is recognized by staphylococcus protein A (Clontech, Mountain View, CA) and selected using 400 μg/ml hygromycin (Roche Applied Science). HEK 293 cells co-expressing CD40 and HLA-DR were generated by transfecting cells expressing CD40-WT or CD40-mutant expression vectors CD40-C6Q, CD40-C17A, CD40-C238A, and CD40-ΔCyto were generated by site-directed mutagenesis using the pCEP4/CD40 wild type (CD40-WT) as a template (10). HEK 293 cells were transfected by DNA-calcium phosphate precipitation (Clontech, Mountain View, CA) and selected using 400 μg/ml hygromycin (Roche Applied Science). HEK 293 cells co-expressing CD40 and HLA-DR were generated by transfecting cells expressing CD40-WT or CD40-C238A or cells mock transfected with the pBud-DR1-α/β expression vector (a generous gift from Dr. Jacques Thibodeau, Université de Montréal) and selected in 160 μg/ml and 200 μg/ml of Zeocin (InvivoGen, Cedarlane Laboratories, Burlington, Canada) and hygromycin, respectively.

Detection of IL-8 and IL-2—For the detection of IL-8 protein, HEK 293 transfectants (5 × 10⁶/well; in triplicate) were allowed to adhere in 96-well plates for 24 h before being stimulated with 50 ng/ml of phorbol 12-myristate 13-acetate or anti-CD40 mAbs plus secondary Abs as described above. IL-8 release was detected by ELISA as previously described (13). For IL-2, HEK 293 transfectants (5 × 10⁶/well; in triplicate) were plated in 96-well plates and co-cultured with the same number of Jurkat D1.1 T cells expressing Vβ8, which is recognized by staphylo-
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Localization—While seeking to determine the potential residue(s) involved in CD40-mediated disulfide bond formation, we previously demonstrated that a point mutation (C6Q) in the extracellular domain of the CD40 abolishes dl-CD40 homodimer formation (11). However, the basis of this inhibition was not investigated. As shown in Fig. 2B, treatment of HEK 293 cells stably expressing the CD40-C6Q mutant at levels comparable to that of CD40-WT with cross-linked anti-CD40 antibody resulted in impaired CD40 translocation to the Triton-insoluble fraction as well as dl-CD40 formation (data not shown). Together these results showed that a high affinity multivalent ligand/receptor interaction is required for CD40 to translocate to DRMs and that the relocation of CD40 within these microdomains may be necessary to form CD40/CD40 homodimers.

Prerequisite Requirement of DRM Integrity for dl-CD40 Homodimer Formation—To assess the role of DRM integrity in dl-CD40 homodimer formation, we treated cells with MβCD, a drug that disrupts the integrity of these microdomains by extracting cholesterol, prior to CD40 oligomerization. Because fractionation of cell lysates on a sucrose density gradient revealed no monomeric or dimeric CD40 in the pellets (Fig. 1, A and B, fraction 12), we performed straightforward ice-cold Triton-soluble (cytosolic) and Triton-insoluble (DRM and cytoskeleton) fractionation. Using this method, we found that treating cells with cross-linked anti-CD40 antibody resulted in the translocation of CD40 monomer from the Triton-soluble fraction to the Triton-insoluble fraction (DRM) and dl-CD40 homodimer formation (Fig. 1C). Disrupting DRMs prior to CD40 stimulation totally impaired CD40 translocation to the Triton-insoluble fraction as well as dl-CD40 formation (Fig. 1C). This inhibition was not due to lower surface CD40 expression, because MβCD had no effect on CD40 expression (Fig. 1D) or cell viability (data not shown), indicating that the integrity of DRMs is critical for dl-CD40 homodimer formation.

Coccal enterotoxin E (SEE) superantigen (a generous from Dr. Sekaly, Université de Montréal). The cells were incubated for 48 h in the absence or presence of 100 ng/ml of SEE, and IL-2 production was monitored using an ELISA kit (Mabtech Inc.). The ELISA results were analyzed using Statview 4.5 (Abacus Concept, Inc., Berkeley, CA). All of the values are expressed as the means of triplicates ± standard error of the mean.

RESULTS

Induction of dl-CD40 Homodimers in DRM Microdomains—There is a growing body of evidence to indicate that oligomerization of CD40 leads to a rapid increase in dl-CD40 homodimer formation (10, 11) and that some CD40 signaling requires DRMs integrity (14). These observations prompted us to investigate the role of DRM microdomains in dl-CD40 homodimer formation using cold 1% Triton X-100 solubilization over sucrose density gradient. To this end, BJAB B cells were left untreated or were treated with stCD154. They were then lysed, fractionated, and analyzed by Western blot under nonreducing conditions. As shown in Fig. 1, we observed three major phenomena in stCD154-treated cells compared with untreated cells: (a) there was a significant translocation of CD40 to DRM microdomains, (b) CD40 homodimer formation was mainly detected in DRMs, and (c) there was no detectable CD40 in the pellets (fraction 12). Triton-insoluble fractions (DRMs) were found exclusively in the low density fractions (3 and 4), in which raft-associated molecule ganglioside GM1 was detected, whereas the Triton-soluble proteins were present in the high density fractions (9–11), in which membrane phosphatase CD45 was detected (data not shown).

We previously demonstrated that the ligation of CD40 with anti-CD40 Abs is not efficient in inducing significant dl-CD40 homodimer formation. However, efficient homodimer formation occurs when the anti-CD40 Abs are cross-linked with a secondary antibody (11). We thus set out to assess the distribution of CD40 in cells treated with anti-CD40 Abs alone or with cross-linked anti-CD40 Abs. As can be seen in Fig. 1B, the anti-CD40 Abs treatment did not promote dl-CD40 homodimer formation. This failure to trigger dl-CD40 homodimer formation can be explained by the failure of anti-CD40 Abs to induce CD40 translocation to DRMs (Fig. 1B). In contrast, significant CD40 translocation to DRMs and homodimer formation in DRMs (Fig. 1B, fractions 3 and 4) were induced in cells incubated with cross-linked anti-CD40 Abs. CD40 translocation to and homodimer formation in DRM microdomains also occurred in Ramos, LG2, and primary human tonsillar B cells, indicating that these microdomains play a role in CD40 dimer formation (data not shown). Together these results showed that a high affinity multivalent ligand/receptor interaction is required for CD40 to translocate to DRMs and that the relocation of CD40 within these microdomains may be necessary to form CD40/CD40 homodimers.

FIGURE 1. Multivalent ligand-induced CD40 dimers are preferentially distributed in DRM microdomains. A, BJAB cells were treated with stCD154 for 30 min at 37 °C. B, BJAB cells were treated with anti-CD40 (αCD40) mAbs alone for 15 min on ice, or the anti-CD40 was cross-linked with goat anti-mouse Ab (αCD40/αlgG) for another 10 min at 37 °C. Untreated cells were used as a control. The cells were then lysed in 1% Triton X-100 and fractionated on a sucrose density gradient. DRM, soluble, and cytoskeleton fractions are marked. C, BJAB cells were treated with 10 μg MβCD for 15 min at 37 °C. The cells were then incubated in the presence or absence of cross-linked anti-CD40 mAbs as in B, then lysed, and fractionated as described under “Experimental Procedures.” Triton X-100-soluble (α) and -insoluble (β) fractions were resolved by SDS-PAGE in nonreducing condition. The blots were revealed using anti-CD40 mAb. The molecular masses are indicated in kilodaltons on the left side of each blot. The positions of the CD40 monomer (M) and CD40 dimer (D) are indicated on the right side of each blot. D, the cells were stained with anti-CD40 Alexa fluor 488-conjugated antibody and analyzed by flow cytometry. The results are representative of three independent experiments.
are required for inducing CD40 homodimer formation, these results suggest that it was the impairment of CD40 translocation to the DRMs that inhibited CD40-C6Q dimerization. Based on the disulfide structural model of members of the TNFR superfamily (15) and the CD40 structural model (16), Cys6 forms a disulfide intraloop with Cys17 on the extracellular domain of CD40. We thus hypothesized that a Cys17 mutation should also destabilize the intramolecular disulfide bond and prevent CD40 translocation to DRM and CD40 dimerization. Indeed, similar to CD40-C6Q, treatment of CD40-C17A-expressing cells (Fig. 3B) resulted in total inhibition of CD40 translocation to DRM and homodimer formation in DRMs (Fig. 2B). These results strongly suggest that the intramolecular disulfide bond between these two cysteines (Cys6 and Cys17) might play an important role in the localization of CD40 molecules in these microdomains following CD40 stimulation.

Involvement of Cytoplasmic Cysteine 238 in dI-CD40 Homodimer Formation—Having demonstrated that DRM microdomains integrity was required for CD40 homodimer formation and the indirect involvement of Cys6 and Cys17, we then generated HEK 293 cells expressing truncated CD40 (CD40-ΔCyto) (Fig. 3A) to study the possible requirement of intracellular signaling to induce dI-CD40 homodimer and to determine which residues mediate this phenomenon. We found that deleting the cytoplasmic domain of CD40 did not alter the extraction of CD40 from the Triton-insoluble fraction, but it did completely inhibit dI-CD40 homodimer formation (Fig. 3B). These results strongly imply that the formation of the CD40 homodimer requires a specific cytoplasmic-mediated intramolecular disulfide bond. We thus investigated the role of the sole cytoplasmic cysteine (Cys238) in the induction of CD40 homodimers. To this end, we generated a CD40-C238A mutant in which Cys238 was replaced by alanine. Stimulating CD40-C238A-expressing cells (Fig. 3A) revealed that there was a significant translocation of CD40 from the Triton-soluble to the Triton-insoluble fraction in the absence of dI-CD40 homodimer formation (Fig. 3B), strongly suggesting the crucial role for Cys238 in the induction of CD40 homodimers.

Role of Reactive Oxygen Species in dI-CD40 Homodimer Formation—It is well established that thiol disulfide exchange reactions are catalyzed by proteins with disulfide isomerase activity (17). The evidence gathered from the above experiments suggested that an intracellular disulfide isomerase activity is involved in the induction of intramolecular disulfide bond formation with Cys238. To confirm this hypothesis, we utilized a chemical approach to further illustrate this phenomenon. To this end, BJAB cells were preincubated with cell-permeable NEM thiol alkylating agent. The NEM concentrations used were selected based on preliminary experiments in which the treatment had no effect on cell viability (less than 5%) or CD40 surface expression levels (data not shown). As shown in Fig. 4A, cells preincubated with NEM showed a significant, dose-de-
that ROS play a role in the formation of dl-CD40 homodimers in DRM microdomains. As shown in Fig. 4, the formation of the dl-CD40 homodimer formation in BJAB cells was significantly reduced in the presence of NAC (50%) in a dose-dependent fashion (Fig. 4B). NAC acts as an antioxidant-scavenging ROS. The NAC concentrations used were selected based on preliminary experiments. To investigate the role of ROS in the formation of the dl-CD40 homodimer formation after CD40 cross-linking, BJAB cells were pretreated with the NAC, which is a source of sulfhydryl antioxidants on dl-CD40 homodimer formation.

To further investigate the role of ROS in the formation of the dl-CD40 homodimer formation, we cultured Jurkat D1.1 T cells that bear Vβ8, which is recognized by SEE (22) and that express high levels of CD154 with HEK 293 mock transfected cells or stable transfecants expressing CD40-WT, HLA-DR, CD40-WT/HLA-DR, or CD40-C238A/HLA-DR in the presence or absence of SEE. All transfectants with comparable levels of surface HLA-DR or CD40 expression were sorted out (Fig. 5C). As shown in Fig. 5B, IL-2 production was induced when SEE was presented by cells expressing HLA-DR alone (150 ± 20 pg/ml). Co-expression of CD40-WT with HLA-DR resulted in a significant enhancement of IL-2 production by D1.1 Jurkat cells (600 ± 115 pg/ml). Conversely, when SEE was presented by cells co-expressing the CD40-C238A mutant with HLA-DR, the level of IL-2 production decreased to levels very similar to that detected in Jurkat cells co-cultured with cells expressing HLA-DR alone (195 ± 30 pg/ml) (Fig. 5B).

DISCUSSION

Receptor oligomerization is vital for activating intracellular signaling, in part by initiating events that recruit effector and adaptor proteins to sites of active signaling. However, the mechanisms by which a cell uses its functional membrane to organize receptor oligomerization are poorly understood. Receptor clustering in DRM microdomains, where some signaling molecules reside and others are recruited where they may interact with the clustered receptors, has recently been identified as a central event in the initiation of signal transduction of many immune receptors, notably in signaling through B cell receptors (23, 24), MHC class II (12, 25), TCR (7, 26), and CD40 (6, 14) molecules. The data obtained in the present study demonstrated that DRM microdomains are also important for the homodimerization of cross-linked CD40. This relationship prompted us to investigate the physiological role of these microdomains in CD40 homodimer formation. We showed that the formation of dl-CD40 homodimers requires a dynamic association with an intact DRM structure. This result was supported by the fact that cholesterol depletion
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A comparison of the transmembrane domain of CD40 and the transmembrane domain of the tyrosine phosphatase CD45, which is excluded from DRMs, suggests that the association of CD40 in these microdomains is mediated by the complementary charges of specific transmembrane domain amino acid residues of CD40 and structural components of DRMs (30). However, such models fail to explain the specific affinity of CD40 homodimers for DRMs, unless they are formed from cross-linked monomers that are translocated into these domains. This hypothesis is consistent with the fact that the association of CD40 with DRMs occurs even in the absence of CD40 dimer formation, as observed in HEK 293 transfectants expressing mutated Cys238 and cytoplasmic tail-truncated CD40 (Fig. 3B).

The crucial role of DRM microdomains in the formation of CD40 homodimers was further extended through abolishing the putative intrachain disulfide bond between Cys6 and Cys17 in the first cysteine-rich extracellular domain of CD40 that prevented CD40 homodimerization and completely eliminated the association of CD40 with DRMs. This implies that CD40 oligomerization is required but not sufficient to ensure the association of CD40 with DRMs. It further suggests that other mechanisms are involved in DRMs translocation upon CD40 oligomerization. For example, CD40 homodimers might undergo a structural change mediated by the Cys6–Cys17 intraloop disulfide bond that might facilitate or force the integration of ligand-clustered receptors into DRMs. Like other TNF receptor family members, homotypic interactions between CD40 molecules at the cell surface occur via the preligand-binding assembly domain, which is important for the assembly of functional receptors (31). Oligomerization of CD40 may thus be stabilized via this domain, which is the first cysteine-rich domain. Although CD154 and anti-CD40 mAb G28-5 bind to CD40 via the second and third cysteine-rich domains (15, 16), but not via preligand-binding assembly domain, this domain may be important in the outcome of CD40 engagement, although it is not essential for all CD40-mediated signals (32). In fact, rapid dl-dimerization and partitioning into

Our finding concerning dl-CD40 homodimer formation is also consistent with the observation that immune receptors such as B cell receptors and TCR associate with DRMs only upon multivalent ligand-mediated receptor aggregation (23, 24, 26), possibly through a oligomerization-dependent change that allows a stable association with DRMs. On the other hand, the association of CD40 with DRMs does not require a specific CD40-induced signal because cytoplasmic tail-truncated CD40 translocates into these microdomains. Whereas a mechanistic reason cannot yet be proposed, translocation to DRM is independent of cell signaling events, as has also been reported for B cell receptors (24) and TCR (29). Furthermore, a biochemical

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FIGURE 5. Cys238-mediated dl-CD40 is required for CD40-mediated responses. A, dl-CD40 is required for CD40-induced IL-8 expression. Mock transfected HEK 293 cells and cells stably transfected with CD40-WT or CD40-C238A expression vector were stimulated in the presence of cross-linked anti-CD40 mAbs as described under “Experimental Procedures.” Phorbol 12-myristate 13-acetate (PMA, 50 ng/ml) was used as positive control. B, dl-CD40 is required for superantigen-induced IL-2 production. Mock transfected HEK 293 cells and cells stably transfected with CD40-WT, HLA-DR, CD40-WT/HLA-DR, or CD40-C238A/HLA-DR expression vector were incubated with CD154-expressing D1.1 Jurkat T cells in the presence or absence of SEE superantigen (100 ng/ml) as described under “Experimental Procedures.” IL-8 and IL-2 production was assessed by ELISA. The graphs show the means of IL-8 and IL-2 expression levels ± S.D. (p < 0.05). C, cells with comparable levels of CD40 receptor and HLA-DR co-expression were selected. The results are representative of three independent experiments.
CD40 homodimers are a feature shared with other members of the TNFR superfamily such as TNFR-I (33) and TNFR-II (34), respectively, following stimulation with TNF. Based on these results and on recent observations showing that the transmembrane domain of CD40 is involved in the association with DRM (30), we hypothesized that a mutation in the first loop of the extracellular domain of CD40 might decrease the affinity of anti-CD40 by altering the structural conformation of the receptor. This hypothesis was further confirmed using another anti-CD40 mAb (S2C6) directed against an epitope that is distinct from the one recognized by G28,5, as well as by analyzing the binding of recombinant CD154 to CD40-WT and CD40-mutant expressing HEK 239 cells. Data revealed that single mutation in CD40-C6Q or CD40-C17A induced conformational changes that prevented the translocation of CD40 monomer into DRMs, which is a crucial step in homodimer formation (data not shown). Furthermore, our findings indicated that the Cys238 plays a direct role in dl-CD40 homodimer formation following multivalent ligand stimulation and that the association of CD40 with DRM microdomains occurred prior to CD40 dimerization, providing evidence that ligand-mediated CD40 receptor aggregation is a multi-step process. Because the intracellular domain Cys238 residue is only a few residues away from a well-characterized TRAF-binding site threonine at position 234 (Thr234), one might argue that the inhibition of CD40 homodimer formation in Cys238 mutant resulted from inhibition of the association of this intracellular domain with TRAF signaling adapter protein. However, we have clearly demonstrated in our previous work (11) that dl-CD40 homodimer formation was totally independent of TRAF1/2/3/5 associations with the Thr234 in the cytoplasmic tail of the CD40 molecules. Ligation of CD40-T234A mutant or CD40-WT proteins with anti-CD40 mAbs induced the formation of dl-CD40 homodimers at similar kinetics. Furthermore, utilizing a cell-permeable chemical approach, we have shown previously that dl-CD40 homodimer formation is mediated by a disulfide bond that was completely prevented when cells were pretreated with the irreversible thiol-alkylating agent iodoacetamide (10). However, the effect of Cys238 mutation on TRAF association and signaling is currently under investigation. It is worth noting that we did not observe any effect on TRAF2/3/and 6 localization after anti-CD40 cross-linking and sucrose density gradient of cells expressing CD40-WT or CD40-C238A mutant (data not shown). We are currently dissecting the effect of Cys238 mutation on TRAFs association and subsequent signaling.

Most of the disulfide bonds are mediated by two cysteines upon oxidation by ROS (18, 35). Signaling via CD40 triggers the generation of ROS in various cell types (data not shown). It has been reported that ROS are produced in murine B cells following CD40 ligation and that these ROS are important for CD40-mediated signaling and gene expression (19). Furthermore, previous reports clearly demonstrate that CD40-mediated proximal events, which include protein serine phosphorylation, protein translocation between membranes and cytosol, as well as receptor complex formation, were inhibited after the pretreatment of cells with the NAC (19). We showed here that the antioxidant NAC significantly prevents the formation of dl-CD40 homodimers (50% at highest concentration used) without interfering with the translocation of CD40 into DRMs. Thus, CD40-mediated signals leading to ROS production precede the formation of homodimer. It remains to be confirmed whether dl-CD40 homodimers assemble into DRM microdomains under the action of a common accessory molecule such as thioredoxin or a protein-disulfide isomerase. These enzymes are present at the plasma membrane, particularly in DRM fractions, and in the cytoplasmic and nuclear compartments of various cells (17), including human B cells (36, 37). Further study is undertaken to assess whether any specific disulfide isomerase is required during dl-CD40 homodimer formation.

Using SEE as a model of T cell activation, we showed that (a) CD154 is a co-stimulatory molecule for IL-2 production from T cells and (b) dl-CD40 dimerization is a prerequisite for reverse signaling via CD154 for optimal activation of T cells in response to TCR engagement by SEE. Functionally, two signals are required to activate the T cell-TCR engagement by nominal antigen presented in the context of self-MHC class II and costimulation (CD28-CD80/CD86 and/or CD154-CD40 interactions). As reported above, receptor clustering in lipid rafts at the synaptic interface is a central event in the initiation of signal transduction of MHC class II, TCR, CD28, and CD40 (7, 12, 14, 25–28) molecules. Recently, Grassme et al. (5) provided evidence for the novel concept that CD40 clustering depends on reciprocal clustering of the CD40 ligand, CD154. In this context, we showed that positioning CD40 monomers in DRMs is not sufficient for CD154-induced IL-2 expression in T cells and CD40-induced IL-8 secretion. In fact, a dynamic association with DRMs is required for both the formation and functioning of dl-CD40 homodimers and optimal CD154-induced activation of human T cells. Our studies confirmed that efficient T cell differentiation and effector function requires reciprocal feedback that must be provided to the antigen-presenting cell (dendritic cell, macrophage, B cell) through MHC class II, CD40, and B7 engagements by TCR, CD154, and CD28, respec-
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tively, and to T cells through the binding of TCR, CD154, and CD28 by their respective ligands.

In summary, based on data presented here and previously published observations, we propose a model (Fig. 6) describing the interrelationship between DRM microdomains and CD40 homodimer formation as well as the involved signals. This model shows that oligomerization of CD40 triggers CD40 translocation to DRM microdomains and CD40 homodimer formation. CD40 homodimer formation that is mediated by Cys238 and dependent on oxidation is an absolute requirement for CD40-induced IL-8 production and IL-2 production by T cells during cognate T cell/APC interaction. In light of our studies, it is crucial to investigate the role of dl-CD40 homodimers in an in vivo system in conjunction with autoimmune and auto-inflammatory diseases known to have altered CD154/CD40 interactions.

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