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Endothelial Cell Costimulation of T Cell Activation Through CD58-CD2 Interactions Involves Lipid Raft Aggregation[1]

Javier Mestas and Christopher C. W. Hughes[2]

Human endothelial cells (EC) costimulate CD4$^+$ memory T cell activation through CD58-CD2 interactions. In this study we tested the hypothesis that EC activate distinct costimulatory pathways in T cells that target specific transcription factors. AP-1, composed of fos and jun proteins, is a critical effector of TCR signaling and binds several sites in the IL-2 promoter. EC augment c-*fos* promoter activity in T cells; however, deletion analysis reveals no transcription factor binding sites in the promoter uniquely responsive to EC costimulation. Overexpression of AP-1 proteins in T cells augments the activity of an AP-1-luciferase reporter gene equally in the absence or the presence of EC costimulation. Interestingly, EC stimulate a similar 2- to 3-fold up-regulation of AP-1, NF-AT, NF-κB, and NF-IL-2-luciferase reporters. CD2 mAbs completely block EC effects on all of these pathways, as well as costimulation of IL-2 secretion. We conclude that EC costimulation through CD2 does not trigger a single distinct costimulatory pathway in T cells, but rather, it amplifies several pathways downstream of the TCR. Indeed, we find that early EC costimulation acts "upstream" of the TCR by promoting lipid raft aggregation, thus amplifying TCR signaling. Soluble CD2 mAbs block EC-induced raft aggregation, whereas cross-linking CD2 promotes aggregation. These data are consistent with the critical role of CD2 in organizing the T cell-APC contact zone. *The Journal of Immunology,* **2001, 167: 4378–4385.**

Antigen-presenting cells initiate adaptive immune responses by presenting foreign peptide Ags to the TCR (1). However, this primary signal is not usually sufficient for full activation of the T cell. A second, costimulatory, signal is also required and consists of interactions between cell surface ligands on the APC and specific receptors on the T cell. Ligation of CD28 on T cells by Ab, or B7 molecules on APC, reduces the number of TCR that have to be triggered to induce T cell activation, and significantly up-regulates expression of cytokines such as IL-2 (2). In addition to CD28, several other molecules on the T cell have been shown to provide costimulatory signals early in the response, including CD2 (3, 4), CD5 (5), CD44 (6), and CD9 (7, 8). At late times, OX40-OX40 ligand interactions prolong IL-2 secretion and trigger the generation of memory T cells (9).

Costimulation, therefore, plays an important role in T cell activation by enhancing TCR-mediated signals and increasing cytokine synthesis, thereby allowing T cell proliferation in the presence of low Ag concentrations. Stimulation of a T cell by an APC initiates a signaling cascade from the TCR that results in the activation of members of the AP-1, NF-κB, octamer, and NF-AT families of transcription factors, all of which bind the IL-2 promoter and are involved in the regulation of T cell IL-2 synthesis (10–16).

Activated endothelial cells (EC)[3] express MHC class II molecules on their surface and provide costimulatory signals that allow them to fully activate resting T cells (17). EC costimulation of CD4$^+$ T cell activation is CD28 independent and augments T cell IL-2 mRNA levels by increasing IL-2 transcription (18), at least in part through AP-1 sites in the IL-2 promoter (19). We previously demonstrated that CD58 on human EC is a major costimulatory molecule and that blocking CD58 or its ligand, CD2, profoundly affects EC activation of T cells (17, 20). The surprisingly mild defect in CD2 knockout mice (21) likely reflects the fundamental differences in expression and affinity of the major mouse CD2 ligand, CD48, compared with the human ligand, CD58. Interestingly, the CD48 knockout mouse shows severe defects in T cell activation (22).

The role of costimulatory molecules in T cell activation is still poorly understood. The two-signal model of T cell activation maintains that costimulation provides a biochemical signal separate from those initiated by the TCR. An emerging model of T cell activation offers the paradigm that costimulation is a process by which TCR aggregation and signaling is enhanced as a result of ligation and segregation of costimulatory molecules at the T cell/APC interface, recently termed the immunological synapse (23, 24). At the focal point of APC/T cell contact there is a concentration of TCR molecules and signaling kinases surrounded by a ring of CD2, outside of which is an adhesion ring of LFA-1. These zones have been termed supramolecular activation clusters (23, 25).

In other systems it has been shown that engagement of the costimulatory molecule CD28 triggers an active accumulation of kinase-rich membrane microdomains (rafts) at the T cell/APC interface (26). T cell activation induced by CD3 cross-linking also involves the aggregation of rafts and the colocalization of T cell signaling molecules such as LCK, linker of activated T cells, and ζ-associated protein 70 (27). To date, the topological segregation model and the raft model have not been reconciled. Importantly, these models for early T cell activation events do not rule out a more traditional role for costimulatory molecules later in T cell activation. For example, the induction of cyclosporin A resistance appears to be a late (>8–12 h) event involving novel costimulatory pathways (28). Also, it has recently been shown that OX40/OX40 ligand interaction is a late event that sustains IL-2 synthesis and helps in the induction of memory T cells (9). In aggregate,

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[3] Abbreviations used in this paper: EC, endothelial cells; MβCD, methyl-β-cyclodextrin; FITC-CT-B, FITC-conjugated cholera toxin subunit B; CRE, cAMP response elements; Oct, NF-IL-2A; SAg, superantigen; β-gal, β-galactosidase.

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these results suggest that the initiation of T cell activation occurs through a mechanism in which TCR engagement promotes the formation of signaling complexes and possibly the aggregation of lipid rafts and lipid raft-associated T cell signaling molecules, and that costimulation serves to enhance this aggregation, thereby enhancing TCR-mediated signals.

In this study we report that EC costimulation of early T cell activation promotes CD2-dependent lipid raft aggregation and thus the amplification of TCR signaling. The data do not support a model involving independent costimulatory signaling pathways in the early stages of T cell activation by EC.

Materials and Methods

Reagents and Abs

PHA, methyl-β-cyclodextrin (MβCD), and FITC-conjugated cholera toxin subunit B (FITC-CT-B) were purchased from Sigma-Aldrich (St. Louis, MO). The superantigens (SAg) staphylococcal enterotoxin A and B and toxic shock syndrome toxin-1 were obtained from Toxin Technologies (Sarasota, FL). Abs to CD2 (TS2/18), CD58 (TS2/9), and a nonbinding IgG1 control (HB64) were purified from culture supernatants of cells purchased from American Type Culture Collection (Manassas, VA). Cy3-conjugated goat anti-mouse IgG was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). IFN-γ was purchased from BioSource International (Camarillo, CA), and fibronectin was obtained from Fisher Scientific (Pittsburgh, PA).

Plasmids

Mouse c-*fos* 5′ promoter deletion constructs p301–356, p301–151, p301–71, and p301–56 were gifts from Dr. M. Gilman (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY). Mouse c-*fos* promoter mutant construct −356 Mut1 *fos*-CAT (29) was a gift from Dr. N. C. Partridge (St. Louis University, School of Medicine, St. Louis, MO). The pGL2-enhancer luciferase reporter construct was purchased from Promega (Madison, WI). Luciferase reporter constructs containing tandem repeats of IL-2 promoter *cis*-regulatory elements upstream of the minimal TK promoter, AP-1-luciferase, NF-AT-luciferase, NF-κB-luciferase, NF-IL-2A (Oct)-luciferase, and minimal TK promoter-luciferase reporter plasmid pTK-luciferase were gifts from C. Zacharchuk (National Institutes of Health, Bethesda, MD). AP-1 protein expression vectors pBJ5-FOS, pBJ5-FRA1, and control plasmid pBJ5 were gifts from J. P. Northrop (Affymax Research Institute, Santa Clara, CA). AP-1 protein expression vectors pGEM1-JunB and pGEM1-FosB were gifts from Dr. R. Bravo (Bristol-Myers Squibb Pharmaceutical Research Institute, Princeton, NJ), and pBJ5-Ha-c-*jun* was a gift from Dr. G. Crabtree (Stanford University, Stanford, CA).

*Generation of c-*fos *promoter constructs*

All c-*fos* promoter constructs were cloned into the pGL2-enhancer luciferase reporter vector (Promega). Mouse c-*fos* promoter DNA from bp −356, −151, −71, and −56 to 109 relative to the transcriptional start site was isolated from plasmids p301–356, p301–151, p301–71, and p301–56, respectively, using restriction enzymes *Sal*I and *Xba*I. Blunt ends were generated using the Klenow fragment from *Escherichia coli* DNA polymerase I, and the resulting DNA was cloned into the *Sma*I site of pGL2-enhancer to generate pcfos-356, pcfos-151, pcfos-71, and pcfos-56. A mouse c-*fos* promoter construct with a single point mutation in the 3′ cAMP response element (CRE) site (previously described in Ref. 29) was subcloned from plasmid −356 Mut1 *fos*-CAT into pGL2-enhancer in the same manner as described above to generate pcfos-356 Mut1.

Preparation and transfection of T cells

PBMC were isolated from whole blood obtained from normal healthy donors by centrifugation over lymphocyte separation media according to the manufacturer's instructions (ICN Biomedicals, Aurora, OH). PBMC were transfected as previously described (19, 30) in the presence of 25 μg of reporter gene DNA. Where different mutant and deletion promoters were compared in a single experiment, pCMV-β-galactosidase (β-gal; 4 μg/ml) was added to allow for normalization of transfection efficiency. After electroporation, cells were washed, resuspended in fresh medium, and allowed to rest for 2 h at 37°C.

Coculture of EC and T cells

HUVECs were isolated and cultured as previously described (31, 32). In all experiments, cells were used at passage 2–6. For all transient transfection assays, 2×10^4 HUVEC were plated on gelatin-coated 96-well culture plates in 200 μl of full medium (M199 containing 20% heat-inactivated FBS, penicillin/streptomycin, EC growth supplement, and heparin) and were incubated at 37°C overnight to allow cells to adhere to the plate. Transfected PBMC were plated on a confluent monolayer of HUVEC or in an empty well at 3×10^5 cells in 200 μl of RPMI 1640/10% FBS with or without 5 μg/ml PHA. Where the involvement of CD2 was analyzed, mAb TS2/18 was added at 10 μg/ml. Cells were incubated at 37°C for 2 h (c-*fos* promoter constructs) or 8 h (IL-2 promoter element multimer constructs), and were then lysed for analysis of luciferase activity. In experiments where SAg was used as a primary stimulus, HUVEC were treated with 1000 U/ml IFN-γ (BioSource International) for 3–4 days to induce the expression of MHC class II (33). PBL were then plated on a confluent monolayer of MHC class II^+ HUVEC, or in an empty well, in 200 μl of RPMI 1640 medium with 0.5 ng/ml SAg and were then incubated at 37°C.

Overexpression of AP-1 proteins

PBMC were transfected with 2 μg of AP-1 protein expression vectors pBJ5-FOS, pGEM1-FosB, pBJ5-FRA1, pGEM1-JunB, pBJ5-Ha-c-*jun*, or pBJ5 along with 10 μg of luciferase reporter plasmid AP-1-luciferase and were cocultured with EC as described above. Cells were cultured for 8 h before harvest.

Reporter gene detection

Luciferase was detected using the luciferase assay system (Promega). One hundred-fifty microliters of supernatant was removed from each well of the transient transfection assays and 100 μl of 1.5× reporter lysis buffer was added to the remaining 50 μl. Cells underwent one freeze/thaw cycle and then 40 μl of cell lysate was added to 100 μl of luciferase assay reagent. Emitted light was measured in cuvettes on a Monolight 2010 luminometer (National Labnet, Woodbridge, NJ). β-Gal was detected using the β-gal enzyme assay system (Promega). Cells were lysed as above, and 40 μl of cell lysate was added to an equal volume of assay reagent. The lysates were incubated at 37°C for 30 min, and colorimetric analysis was performed on a 96-well Ceres 900C plate reader (Bio-Tek Instruments, Winooski, VT). All experiments were performed in triplicate and the results are expressed as mean ± SD.

IL-2 detection

IL-2 secreted into the media was measured as previously described (20). All experiments were performed in duplicate and at least two serial dilutions of each supernatant were tested. Results are expressed as mean ± SD. Where indicated, IL-2 was measured using the DuoSet ELISA Development System according to the manufacturer's instructions (R&D Systems, Minneapolis, MN). All experiments were performed in triplicate and are expressed as mean ± SD.

Blocking of lipid raft aggregation with MβCD

HUVEC were treated with 1000 U/ml IFN-γ (BioSource International) for 3–4 days to induce the expression of MHC class II. Cells were plated at 1×10^4 cells in 200 μl of medium on gelatin-coated 96-well culture plates and were incubated at 37°C overnight as above. PBL (a gift from Dr. A. Tenner, University of California, Irvine, CA) were treated with MβCD for 30 min at room temperature or left untreated. Cells were then washed and plated on a confluent monolayer of HUVEC, or in an empty well, at 3×10^5 cells in 200 μl of RPMI 1640 medium with 0.5 ng/ml SAg and were incubated at 37°C for 8 h. IL-2 secretion was assayed using the DuoSet ELISA Development System according to manufacturer's instructions (R&D Systems). All experiments were performed in triplicate and are expressed as mean ± SD.

Analysis of lipid rafts

PBL at 1×10^6 cells/ml were incubated with various concentrations of FITC-CT-B at room temperature for 30 or 60 min and were then fixed with 4% paraformaldehyde for 30 min. Alternatively, PBL were fixed before staining. Cells were then analyzed on a FACSCaliber (BD Biosciences, San Jose, CA). To analyze raft aggregation in individual cells, HUVEC were treated with 1000 U/ml IFN-γ (BioSource International) for 3–4 days to induce the expression of MHC class II, and were then plated at 1×10^4 cells in 200 μl of medium on glass chamber slides (Nalge Nunc International, Naperville, IL) and incubated overnight at 37°C. Chamber slides were pretreated with 1 μg/ml fibronectin in HBSS (Life Technologies, Grand Island, NY) for 1 h at 37°C. PBL were plated at 1×10^5 cells in 200 μl of RPMI 1640 medium on a monolayer of 75% confluent HUVEC with 0.5 ng/ml SAg either in the presence of control Ab (HB64) or anti-CD2 mAb (TS2/18), both at 10 μg/ml. Cells were incubated at 37°C for 4 h,

fixed with 4% paraformaldehyde in PBS for 30 min, stained with 20 μg/ml FITC-CT-B for 1 h, and mounted in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Cells were visualized by confocal microscopy on an Olympus IX70 Inverted Microscope (Olympus, Melville, NY). PBL were analyzed for the aggregation of lipid rafts as evidenced by polarization or "capping" of fluorescent label at the T cell/EC interface. Results are expressed as the percentage of T cells attached to EC and positive for lipid raft aggregation.

Analysis of CD2 receptor involvement in lipid raft aggregation

To analyze CD2 localization in cells exhibiting lipid raft aggregation, PBL were incubated with HUVEC in the presence of 0.5 ng/ml SAg at 37°C for 4 h, as above. Cells were then fixed with 4% paraformaldehyde in PBS for 30 min, stained with 10 μg/ml TS2/18, washed, and stained with 10 μg/ml FITC-CT-B and Cy3-conjugated goat anti-mouse IgG for 1 h.

To determine whether cross-linking CD2 can induce raft aggregation, PBL were incubated in CD2 mAb on ice, followed by Cy3-conjugated goat anti-mouse IgG and 20 μg/ml FITC-CT-B for 30 min. Cells were then fixed with 4% paraformaldehyde for 30 min and visualized by confocal microscopy.

Results

CD2-CD58 interactions are critical for the early stages of T cell activation by EC

We have previously shown that EC costimulation of T cells results in up-regulation of AP-1 activity, and we have speculated that AP-1 may represent the end-point of a distinct signaling pathway triggered in T cells by an EC costimulatory molecule (18). Moreover, there is mounting evidence that costimulation may be a fundamentally different process early in T cell activation compared with late (9, 28). We have shown that T cell CD2 is important in mediating signals that result in increased IL-2 transcription in response to EC costimulation and that anti-CD2 mAbs partially block EC costimulation late (24 h) in the activation process (28). To determine whether blocking CD2 signaling also inhibits IL-2 expression early during T cell activation, we stimulated T cells and over time measured IL-2 secretion in the presence or absence of blocking Abs to CD2 or CD58. EC costimulation augmented T cell IL-2 secretion as early as 6 h and this was blocked completely by preventing CD2-CD58 interactions (Fig. 1). In agreement with previous data, these Abs only blocked EC costimulation by 50–75% at later times. These results are consistent with the hypothesis that CD2-CD58 interaction is critical for the early stages of T cell activation by EC.

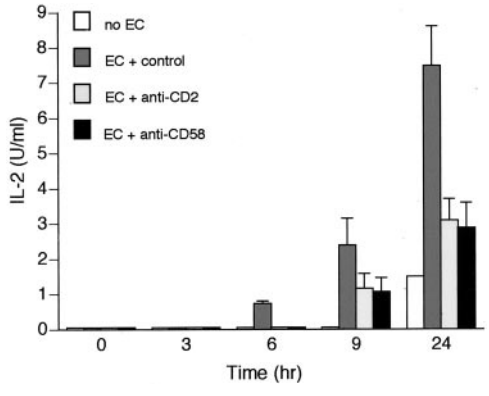

FIGURE 1. Inhibition of EC costimulation by blocking of CD2 interactions. T cells were incubated with EC and were stimulated with 5 μg/ml PHA either alone or in the presence of 10 μg/ml anti-CD2 mAb (TS2/18), 10 μg/ml anti-CD58 mAb (TS2/9), or 10 μg/ml isotype-matched control Ab. Supernatants were collected at 0, 3, 6, 9, and 24 h for measurement of IL-2 by HT-2 bioassay. Shown are means ± SD of triplicate samples. One of two similar experiments.

*EC costimulation targets the c-*fos *promoter*

Numerous stimuli can induce the expression of the c-*fos* gene, including EC costimulation (18, 34–36). We wished to test whether EC up-regulate c-*fos* mRNA levels by inducing c-*fos* transcription, and we wished to determine whether discrete EC-targeted pathways are responsible. We first transfected T cells with a c-*fos* promoter-luciferase construct to examine whether the reporter contains promoter regions that are responsive to EC signals. Transfected cells were stimulated with PHA in the presence or absence of EC and were harvested 2 h later. EC costimulation augmented transcription of the pcfos-356 construct by 2.2-fold (Fig. 2*A*), similar to the effect of EC on the endogenous *c-fos* gene (18). When EC costimulation was blocked using anti-CD2 mAb, transcription of the *c-fos* promoter decreased to levels seen in cells stimulated in the absence of EC (Fig. 2*A*), indicating that CD2-CD58 interaction is essential for augmentation of this immediate early gene.

We next attempted to localize a specific EC-responsive transcription factor binding site in the *c-fos* promoter by transfecting T cells with *c-fos* promoter deletion constructs (Fig. 2*B*). Cells were stimulated with PHA in the presence or absence of EC and were harvested for measurement of luciferase activity 2 h after plating. Deletion of the serum response element, *sis*-inducible element, glucocorticoid response element, and several nonconsensus CRE at the 5′ end had no effect on the transcriptional activity of the promoter relative to the full-length promoter (Fig. 2*B*). However, deletion of the 5′ CRE almost completely abolished promoter activity, reducing luciferase expression to near basal levels. Further deletions to include the 3′ CRE did not reduce transcriptional activity further. A single point mutation in the 3′ CRE, which has previously been shown to abolish protein binding (29), diminished the transcriptional activity of the promoter by ~3-fold. Interestingly, none of the deletions had any significant effect on the ability of EC to augment transcription from these promoters. For all constructs tested, EC augmented transcription by ~1.5- to 2.5-fold (Fig. 2*B*). Again, the addition of CD2 blocking mAb to the assay completely inhibited reporter gene expression in response to EC costimulation. These data indicate that there is a critical element between base pairs −71 and −151, most likely the 5′ CRE, that is important for the induction of *c-fos* transcription in this system. However, no single element appears to be uniquely responsive to EC signals.

EC costimulation of early T cell activation leads to the up-regulation of AP-1, NF-AT, NF-κB, and Oct activity

Our data analyzing EC effects on the c-*fos* promoter suggested that EC signals may act by amplifying pathways emanating from the TCR, rather than activating distinct pathways. As an alternative approach to test this hypothesis, we transfected T cells with luciferase reporters driven by multimerized transcription factor binding sites from the IL-2 promoter. These constructs "report" activity of AP-1, NF-AT, NF-κB, and Oct factors. T cells were then stimulated with PHA in the presence or absence of EC and were harvested for measurement of reporter gene expression 8 h after plating. As predicted, EC augmented reporter activity 2- to 3-fold regardless of the IL-2 promoter element used to drive luciferase expression (Fig. 3). Consistent with the important role of CD2 in early costimulation events, blocking this pathway completely blocked the ability of EC to augment reporter gene activity, again regardless of the promoter element used. Moreover, T cells cotransfected with an AP-1-luciferase reporter gene and expression vectors for several AP-1 proteins and then costimulated by EC exhibited a ~2-fold increase in AP-1-luciferase activity regardless

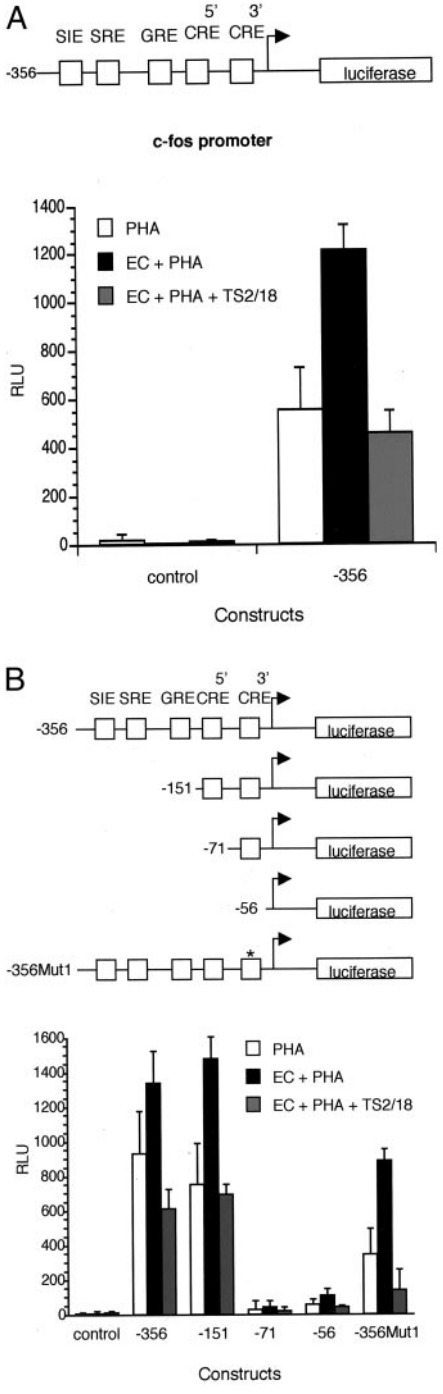

FIGURE 2. Targeting of the T cell c-*fos* promoter by EC costimulation. *A*, Diagram of c-*fos* promoter-luciferase construct. Base pair positions are relative to the transcriptional start site, which is indicated by an arrow. All promoter regions terminate at position 109 and are cloned into the luciferase reporter plasmid pGL2-enhancer (Promega). Normal T cells were transfected with the c-*fos* 5′ promoter-luciferase construct described above along with pCMV-β-gal as described under *Materials and Methods*. Experiments were performed in duplicate and were cultured for 2 h (luciferase) and 24 h (β-gal) before harvest. Transfected T cells were cultured with 5 μg/ml PHA either alone or in the presence of EC or in the presence of EC plus anti-CD2 mAb TS2/18 (10 μg/ml). β-gal expression was used to normalize data for transfection efficiency. Shown are means ± SD of triplicate samples. One of three similar experiments. *B*, Diagram of c-*fos* 5′ promoter deletion constructs. Base pair positions are relative to the transcriptional start site, which is indicated by an arrow. All promoter regions terminate at position 109 and are cloned into the luciferase reporter plasmid pGL2-enhancer (Promega). An asterisk indicates a point mutation in the major CRE. Normal T cells were transfected with the c-*fos* 5′ promoter deletion constructs described above, along with pCMV-β-gal as described under *Materials and Methods*. Experiments were performed in duplicate as in *A*. Shown are means ± SD of triplicate samples. One of three similar experiments.

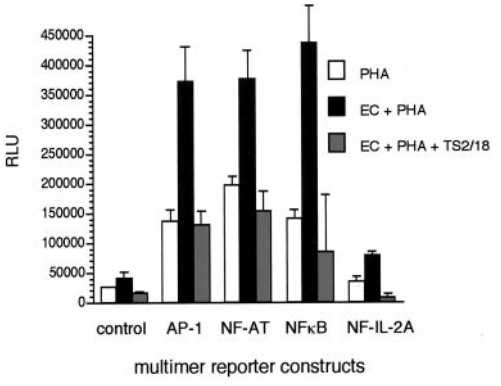

FIGURE 3. Effects of EC costimulation on IL-2 promoter *cis*-regulatory element-driven transcription in transfected normal T cells. Normal T cells were transfected with luciferase reporter plasmids, pAP-1-luciferase, pNF-AT-luciferase, pNF-κB-luciferase, pNF-IL-2-luciferase, or pTK-luciferase, as described under *Materials and Methods* and were cultured for 8 h before harvest. Transfected T cells were cultured with 5 μg/ml PHA either alone or in the presence of EC or in the presence of EC plus anti-CD2 mAb TS2/18 (10 μg/ml). Shown are means ± SD of triplicate samples. One of three similar experiments.

of the AP-1 protein overexpressed (data not shown), suggesting that EC costimulation does not target specific AP-1 proteins, but rather, amplifies the effect of TCR signaling on all downstream targets.

Taken together, our results show that EC costimulation of early T cell activation leads to the up-regulation of AP-1, as well as NF-AT, NF-κB, and Oct activity. The effects of EC signaling can be completely blocked at early times by anti-CD2 mAb, indicating the critical role CD2-CD58 interactions have in initiating the immediate early gene activation that precedes full T cell activation. Furthermore, we were not able to define a unique pathway that was responsive to EC signals. Rather, our data suggest that all signaling pathways that converge on the IL-2 promoter in response to EC signals, namely NF-AT, NF-κB, AP-1, and Oct, are equally targeted and that CD2 ligation is critical for this process. One interpretation of these data is that EC act upstream of the TCR to augment all pathways triggered by TCR ligation, and that CD2-CD58 interaction is essential for this activity.

Disrupting the structure of lipid rafts abolishes T cell activation by EC

One way that EC and other APC may act upstream of the TCR is by promoting lipid raft aggregation. Several reports have demonstrated that MβCD inhibits the aggregation of lipid rafts by extracting cholesterol from the plasma membrane (37, 38). We therefore used MβCD to determine whether lipid raft aggregation is necessary for T cell activation by EC. T cells were treated with 30 mM MβCD for 30 min and were then washed and plated on MHC class II$^+$ EC in the presence of 0.5 ng/ml SAg. SAg was used rather than PHA so that TCR engagement only occurred at the site of T cell/APC contact (33). Supernatants were collected 8 h after plating and were measured for IL-2 content by ELISA. MβCD treatment decreased IL-2 synthesis by over 60% in T cells costimulated by EC (Fig. 4). As a control for cell viability,

MβCD-treated cells were stimulated by PMA and ionomycin in the presence of EC. The combination of PMA and ionomycin bypasses the need for TCR ligation and should be resistant to the effects of MβCD. This indeed was the case; disrupting raft aggregation only minimally decreased PMA and ionomycin-induced IL-2 synthesis (Fig. 4). This experiment supports the hypothesis that lipid raft aggregation is an essential component of T cell activation by EC.

Analysis of lipid raft aggregation at the T cell/EC interface

To examine the aggregation of lipid rafts at the single cell level we used FITC-CT-B, which binds GM1 glycosphingolipids associated with lipid rafts. Optimization of the labeling procedure yielded the most intense staining when cells were fixed and labeled for 1 h with 20 μg/ml FITC-CT-B (data not shown).

To examine raft formation in T cells stimulated by EC, T cells were plated on MHC class II$^+$ EC and were cultured for 4 h in the presence of 0.5 ng/ml SAg. Cells were then fixed with 4% paraformaldehyde for 30 min and stained with 20 μg/ml CT-B. Lipid raft aggregation was visualized by confocal microscopy. T cells attached to EC and clearly not in contact with other T cells were counted and assessed for their ability to form a polarized "cap" of fluorescent label at the T cell/EC interface. Cells were counted as positive when there was clear evidence of a single polarized cap oriented toward the EC. In the absence of SAg, T cells do not become activated or produce IL-2 (data not shown), and consistent with this, no polarized caps are seen (Fig. 5*A*). In contrast, in the presence of SAg, T cells show polarized caps of fluorescent label oriented toward the presenting EC, indicating the aggregation of lipid rafts at the contact site (Fig. 5, *B–D*). These images are similar to those obtained when T cells are activated by Ab-coated beads (26) and they strongly suggest that EC are capable of driving T cell activation and raft aggregation. Interestingly, EC also label weakly with FITC-CT-B, suggesting that lipid rafts may also be present on these cells; however, we did not see strong evidence of aggregation of these rafts at the contact site with T cells. Concentration of MHC class II molecules in rafts has recently been demonstrated in B cells during Ag presentation to T cells (39). Because of the strong likelihood of rafts on the EC, we did not assay raft aggregation biochemically; it is not possible to purify T cells from the much larger EC with sufficient purity to make such an analysis reliable.

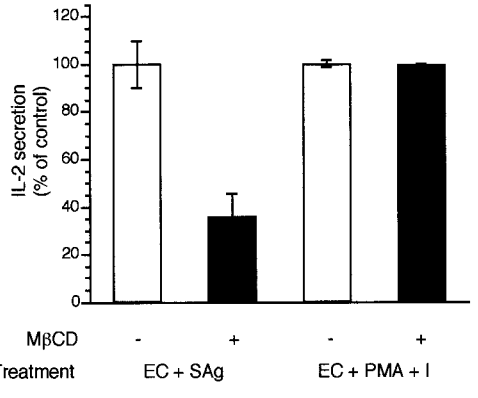

FIGURE 4. Effects of T cell membrane cholesterol depletion on IL-2 secretion in response to EC costimulation. T cells were treated with or without 30 mM MβCD for 30 min, washed, and then plated on EC pretreated with IFN-γ to induce MHC class II expression, in the presence of SAg (0.5 ng/ml) or PMA and ionomycin (25 ng/ml and 1 μg/ml, respectively). Supernatants were collected 24 h after plating, and IL-2 was measured by ELISA. Results are expressed as a percentage of the IL-2 secreted by untreated (control) cells. One of two similar experiments.

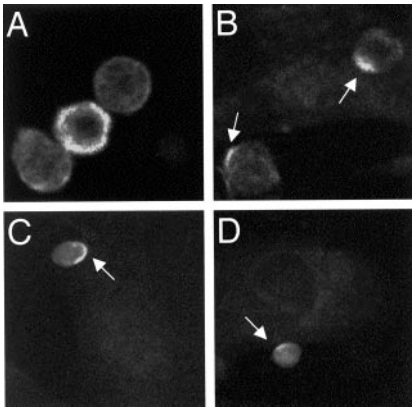

FIGURE 5. EC induce lipid raft aggregation at the T cell/EC interface. T cells were plated on EC pretreated with IFN-γ to induce MHC class II expression, in the presence of SAg (0.5 ng/ml) and were incubated for 4 h at 37°C. Cells were fixed with 4% paraformaldehyde for 30 min and stained with 20 μg/ml FITC-CT-B for 1 h. Cells were visualized by confocal microscopy. *A*, T cells not activated by EC exhibit a uniform labeling of the plasma membrane. *B–D*, T cells activated by EC form a fluorescent cap due to lipid raft aggregation at the T cell/EC interface. Arrows denote the T cell/EC interface.

Disrupting CD2-CD58 interaction prevents EC-induced lipid raft aggregation

To examine the role of EC engagement of T cell CD2 in promoting raft aggregation, we cultured class II$^+$ EC with T cells and SAg for 4 h in the presence of control (HB64) or CD2 (TS2/18) mAb, and we counted "capped" cells. In the presence of control Ab, 62–68% of T cells attached to EC exhibited capping, whereas in the presence of CD2 mAb, capping was inhibited by ~50% (Table I). Although the percentage of T cells attached to EC and exhibiting fluorescent capping decreased to 25–36% in the presence of CD2 mAb, the mAb did not reduce T cell binding to EC. These data suggest that CD2 is important for the aggregation of lipid rafts at the T cell/EC contact site, and are also consistent with the previously demonstrated association of CD2 with lipid rafts (38).

Association of CD2 with aggregated lipid rafts induced by EC costimulation

To provide further evidence for the involvement of CD2 in the rearrangement of lipid rafts at the T cell/EC interface, we examined the localization of CD2 receptors with respect to lipid raft aggregation. T cells were cultured with class II$^+$ EC and SAg for

Table I. *Disruption of CD2-CD58 interactions blocks lipid raft aggregation at the T cell/EC interface*[a]

	mAb	T Cells on EC	Capping	No Capping	% Capping
Expt. 1	Control	105	71	34	68
	CD2	101	25	76	25
Expt. 2	Control	180	111	69	62
	CD2	125	45	80	36

[a] EC were pretreated with IFN-γ for 3–4 days to induce MHC class II expression. T cells were added along with 0.5 ng/ml SAg and either 10 μg/ml anti-CD2 mAb (TS2/18) or 10 μg/ml isotype-matched control Ab (HB64) and incubated at 37°C for 4 h. Cells were fixed for 30 min with 4% paraformaldehyde and stained with 20 μg/ml FITC-cholera toxin subunit B for 1 hour. Cells were analyzed by fluorescence microscopy.

4 h and were stained for both CD2 and lipid rafts. Interestingly, we found that CD2 is localized throughout the T cell membrane (Fig. 6*B*) and is not found solely at the T cell/EC contact site. These findings are not surprising, as previous studies have shown that even in a highly controlled artificial bilayer system, only one quarter of the CD2 receptors on a T cell are engaged within the contact site (40). Importantly, CD2 was not excluded from the T cell/EC interface and is clearly associated with EC-induced lipid raft aggregation (Fig. 6*C*), which is consistent with previous findings of CD2 association with rafts (38). This is in marked contrast to the exclusion of CD45 receptors from lipid rafts upon the cross-linking of TCR (41).

To determine whether the effects of EC on T cell activation can be mimicked by the engagement of CD2 receptors, we cross-linked CD2 on T cells and stained for lipid raft aggregation. In resting T cells CD2 is homogenously distributed over the cell surface (Fig. 6*B*); however, on cross-linking, distinct caps are formed (Fig. 7*C*). In agreement with previous reports (38), cross-linking of CD2 aggregated lipid rafts, as shown by staining with FITC-CT-B (Fig. 7, *A* and *B*), and these caps colocalized with the CD2 caps (Fig. 7*D*). These data demonstrate that cross-linking of CD2 is sufficient to aggregate lipid rafts.

Collectively, our results show that EC costimulation is blocked by disrupting lipid raft aggregation, and that CD2 is crucial for the aggregation of lipid rafts at the T cell/EC interface. Furthermore, CD2 colocalizes with lipid rafts at the T cell/EC contact site and engagement of CD2 is sufficient for lipid raft aggregation. These data are consistent with the idea that engagement of costimulatory receptors such as CD2 promotes the rearrangement of lipid rafts and associated signaling molecules at the T cell/APC interface.

Discussion

Human EC costimulate and activate resting $CD4^+$ memory T cells, resulting in augmented levels of IL-2 secretion (17, 20, 33). In this study we demonstrate that this augmentation is due to the ability of EC to promote the aggregation of lipid rafts early in T cell activation and that the T cell surface receptor CD2 is critical for the initiation of T cell activation by EC.

Ag presentation to resting $CD4^+$ T cells is restricted to cells that express MHC class II molecules on their surface along with the costimulatory molecules necessary for inducing full activation. Traditionally, it has been thought that only dendritic cells, macrophages, and activated B cells perform this specialized role. However, over the last decade there has been increasing evidence that EC also present Ag to T cells (42–45). Numerous in vitro experiments have shown induction of proliferation of allogeneic $CD4^+$ T cells in response to MHC class II^+ EC, as well as augmentation of T cell IL-2 and IFN-γ synthesis (46). In vivo, allogeneic EC have been suggested to drive the T cell activation found in the walls of coronary arteries undergoing graft arteriosclerosis in transplanted hearts (42). Moreover, activated EC appear to represent the major target for immune-mediated damage in the HuPBL-SCID mouse model of skin transplantation (47).

We have proposed that human EC lie along a spectrum of Ag presenting ability (33). They differ from professional APC in that they lack the capacity to activate naive T cells due to the absence of B7.1 or B7.2 expression. However, they do efficiently activate resting memory T cells in culture, whereas fibroblasts and smooth muscle cells lack this ability (33). It is likely that this range in Ag presenting ability is determined by the ability of different lineages to provide costimulation. T cells exhibit thresholds with respect to the amount of signaling required for complete activation, and these thresholds appear to be set by a cell's response to costimulation. We and others (17, 20, 28, 48) have provided evidence that CD58-CD2 engagement is a critical component of the costimulatory signal provided by EC, although it is clear that EC also express other costimulatory molecules.

A recent model for the role of costimulation by APC early during T cell activation proposes that engagement of costimulatory receptors on the T cell promotes the organization of signaling complexes into an immunological synapse (24). Rearrangement of molecules at this synapse, based to a large extent on size, leads to the clustering of TCR, CD2, CD28, and various signaling kinases, and to the exclusion of the phosphatase CD45. Generation of the phosphatase-free zone is suggested to promote tyrosine phosphorylation of several signaling intermediates (49). An alternative model for costimulation suggests that TCR engagement promotes the aggregation of lipid rafts containing signaling molecules such as LCK, linker of activated T cells, and ζ-associated protein 70 (26, 27). The bringing together of these components with the TCR stabilizes the T cell/APC interface and promotes sustained signaling (50). These two models are clearly not mutually exclusive, though significant discrepancies still need to be resolved. Regardless, rearrangement of lipid domains and associated signaling molecules clearly has a role to play in Ag presentation.

The function of costimulatory molecules in raft aggregation and synapse formation is under intense scrutiny. We have shown that disruption of a major EC costimulatory ligand-receptor pairing, namely CD2-CD58, blocks formation of these aggregates at the contact point between the EC and the T cell. Viola et al. (26) recently demonstrated lipid raft aggregation in response to CD3 and CD28 mAbs, but not to either alone. Moran and Miceli (50) have demonstrated the importance of CD48-CD2 interaction in induction of lipid raft aggregation in mouse T cells. In this study

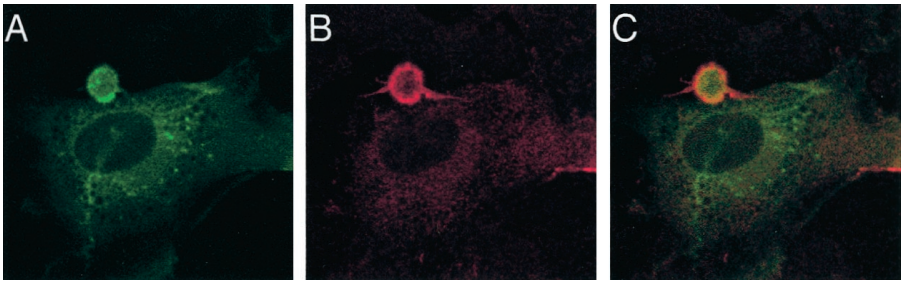

FIGURE 6. CD2 colocalizes with lipid rafts at the T cell/EC contact site. T cells were plated on EC pretreated with IFN-γ to induce MHC class II expression, in the presence of SAg (0.5 ng/ml) and were incubated for 4 h at 37°C. Cells were fixed with 4% paraformaldehyde and stained for CD2 and lipid rafts using TS2/18, Cy3-conjugated goat anti-mouse IgG, and FITC-CT-B, respectively. Cells were visualized by confocal microscopy. *A*, Lipid rafts aggregate at the T cell/EC contact site. *B*, CD2 receptors are homogenously distributed across the T cell surface. *C*, CD2 colocalizes with lipid rafts at the T cell/EC contact site.

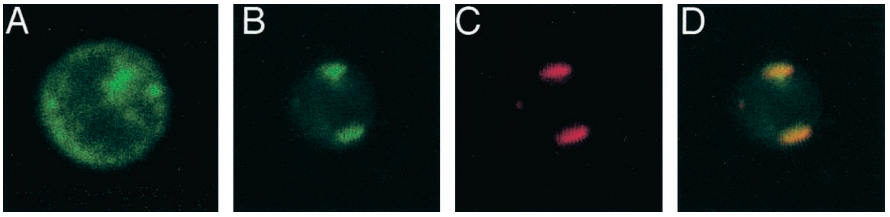

FIGURE 7. Cross-linking of CD2 induces lipid raft aggregation. T cell CD2 receptors were cross-linked using TS2/18 and Cy3-conjugated goat anti-mouse IgG and were stained for lipid rafts with FITC-CT-B before fixation with 4% paraformaldehyde. Cells were then visualized by confocal microscopy. *A*, Control cells show random distribution of lipid rafts over the cell surface. Cross-linking CD2 induces the aggregation of lipid rafts (*B*) as well as capping of CD2 (*C*). *D*, CD2 patches colocalize with aggregated lipid rafts.

we have shown that CD2 colocalizes with lipid rafts at the T cell/EC contact site and that cross-linking CD2 alone is sufficient to induce lipid raft aggregation. In addition, we have demonstrated that costimulation by EC is sensitive to MβCD, again indicating the importance of cholesterol-rich lipid rafts in the process. Whether EC play an active or a passive role in inducing raft aggregation is not clear yet; however, the presence of rafts on EC and the demonstration of raft-associated MHC class II molecules on APC (39) suggests that reciprocal signaling may occur.

Interestingly, CD2 mAb appeared to be more effective at blocking EC-induced IL-2 synthesis than in blocking raft aggregation. At early times, CD2 mAb completely blocked IL-2 synthesis in response to EC, but only reduced the number of capped T cells by 50%. This suggests that there may be a threshold of activation above which IL-2 synthesis occurs. Blocking CD2 may raise this activation threshold so that fewer cells are recruited into the activated T cell pool (33). This model suggests that in some situations, capping does not necessarily lead to T cell activation and IL-2 synthesis.

The data presented in this paper support the idea that the primary effect of EC costimulation early during T cell activation is to enhance the signals initiated at the TCR by colocalizing the receptors with kinase-rich signaling complexes. This is in contrast to a model whereby EC target specific and unique signaling pathways. We have demonstrated that EC costimulation targets several transcription factors known to be essential for IL-2 synthesis, including AP-1, NF-AT, NF-κB, and Oct, but it affects them equally. These transcription factors are downstream targets of numerous effectors and pathways, including Ca^{2+}, protein kinase C, PI3K, ras, and mitogen-activated protein kinase (51–53). None of these pathways appear to be uniquely responsive to EC signals, but are equally up-regulated by T cell contact with EC. We conclude that early costimulation by EC promotes lipid raft aggregation through a CD2-dependent mechanism, which leads to potentiation of signaling pathways emanating from the TCR. Our results do not rule out a more traditional role for costimulatory molecules later in the process of T cell activation.

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