

# Ligation of CD28 by Its Natural Ligand CD86 in the Absence of TCR Stimulation Induces Lipid Raft Polarization in Human CD4 T Cells<sup>1</sup>

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Stimulation of resting CD4 T cells with anti-CD3/CD28-coated beads leads to rapid polarization of lipid rafts (LRs). It has been postulated that a major role of costimulation is to facilitate LR aggregation. CD86 is up-regulated or expressed aberrantly on immune cells in a wide array of autoimmune and infectious diseases. Using an Ig fusion with the extracellular domain of CD86 (CD86Ig) bound to a magnetic bead or K562 cells expressing CD86, we demonstrated that ligation of CD28 by its natural ligand, but not by Ab, induced polarization of LR at the cell-bead interface of fresh human CD4 T cells in the absence of TCR ligation. This correlated with activation of Vav-1, increase of the intracellular calcium concentration, and nuclear translocation of NF- $\kappa$ B p65, but did not result in T cell proliferation or cytokine production. These studies show, for the first time, that LR polarization can occur in the absence of TCR triggering, driven solely by the CD28/CD86 interaction. This result has implications for mechanisms of T cell activation. Abnormalities in this process may alter T and B cell tolerance and susceptibility to infection. *The Journal of Immunology*, 2005, 175: 7848–7854.

One of the most important paradigms of T cell activation is the requirement of two distinct signals for the effective activation of Ag-specific T cells: signal 1, which represents the Ag-specific component via the TCR, and signal 2, which is noncognate and is also called the “costimulatory” signal (1). The CD28/B7 pathway represents a prominent costimulatory pathway for T cells. However, the issue of whether CD28/B7 signaling is completely dependent on signal 1 (or TCR signaling) or whether ligation of CD28 also results in activation of TCR-independent signaling pathways has not been fully resolved. Independent early signaling events through CD28 have been described, among them the activation of PI3K (2) and an increase in intracellular calcium concentration (3). Recently, it has been reported that cross-linking of CD28 leads to phosphorylation of Vav-1 (4) and GSK-3 (2).

However, CD28-cross-linking alone does not lead to proliferation of T cells or release of cytokines (5) and it is still poorly understood which of the signaling pathways initiated through CD28 complement TCR activation and which ones are independent.

Lipid rafts (LRs)<sup>4</sup> are distinct T cell membrane compartments involved in T cell signaling. They serve as platforms for recruiting and concentrating signaling components (6) and thus enable the close interaction of essential molecules during the cell activation process. Movement of the rafts accompanies changes in LR composition: in the early stages of T cell/APC interaction, LR are enriched with signal transduction molecules and cluster in the region of the immune synapse (7). Viola et al. (8) showed, using a cell/bead system, that in the presence of TCR ligation, binding of CD28 is also required for the recruitment of LR to the cell/bead contact region. In this study, we show that ligation of CD28 by its natural ligand CD86 alone can induce LR polarization. This suggests that LR polarization in primary human CD4 T cells is not only driven by a costimulatory signal, it can also be independent of a TCR signal. We also show that important T cellular signaling pathways involved in T cell activation (e.g., increase of intracellular calcium concentration, translocation of NF- $\kappa$ B to the nucleus, and gene transcription) can be initiated through CD28 alone. Considering that CD28/B7 interactions between an APC and T cell will likely occur before much lower density and lower affinity MHC Ag/TCR interactions, our data suggest that the signal through CD28 may play an important role in the proper initiation of T cell activation. These studies may also advance the understanding of the pathogenesis of autoimmune or infectious diseases, specifically systemic lupus erythematosus (SLE) and HIV-1 infection, which are noted for having aberrant CD86 expression.

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Received for publication May 27, 2004. Accepted for publication September 29, 2005.

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<sup>1</sup> T.H.F. was supported by National Institutes of Health Grants (NIH) RO1 AI35513 and AI30575, the Kahn Foundation, and the Joseph Hollander Chair of Pediatric Rheumatology. B.K. was supported by a Howard Hughes Medical Institute Postdoctoral Fellowship and an Amgen Rheumatology Fellowship Award. A.K.T. was supported by a grant from the Dr. Mildred-Scheel-Stiftung Deutsche Krebshilfe. J.L.R. was supported by NIH Grants AI057838 and AI060477. We also thank Chip Nickoloff for his financial support.

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<sup>4</sup> Abbreviations used in this paper: LR, lipid raft; SLE, systemic lupus erythematosus; aAPC, artificial APC; CD86Ig, Ig fusion with the extracellular domain of CD86; LAT, linker for activation of T cells; RRI, relative recruitment index; MFI, mean fluorescence intensity; CTB, cholera toxin B; EGR, early growth response.

## Materials and Methods

### Cell purification, generation of artificial APCs (aAPCs), and activation conditions

PBLs were obtained from normal donors by leukopheresis and elutriation, and CD4 T cells were purified by negative selection as described (9). Initial T cell preparations were >94% CD3<sup>+</sup>CD4<sup>+</sup>.

Two different systems of aAPCs were used to stimulate CD4 T cells. To prepare Ig fusion with the extracellular domain of CD86 (CD86Ig)-coated beads, CD86Ig (a gift from B. Carreno, Inflammation, Wyeth Research, Cambridge, MA) was coated on tosyl-activated M-450 magnetic beads (DynaL Biotech) as per the manufacturer's instructions. Beads containing only anti-CD3 (OKT3), anti-CD28 (9.3, mouse IgG2a), anti-CD28 (5D10, mouse IgG1 $\kappa$ ), or an equal mixture of anti-CD3 and anti-CD28 (CD3/28), were prepared as described (10). In some experiments, K562 cells (American Type Culture Collection) transfected with CD32 or both CD32 and CD86 were used as aAPCs (11).

### Generation and purification of linker for activation of T cells (LAT)-GFP transfected human CD4<sup>+</sup> T cells

A total of  $5 \times 10^6$  purified human CD4<sup>+</sup> T cells were washed and resuspended in 100  $\mu$ l of the Nucleofector solution (Amaxa VPA-1002; T Cell Solution) added with 2  $\mu$ g of LAT-GFP plasmid (12) and transfected with Amaxa electroporation. The transfected cells were incubated in a 12-well plate containing 2 ml of RPMI 1640 complete media at 37°C for 24 h. The cells were suspended in 2% PBS/FBS and sorted for GFP-positive CD4 T cells by flow cytometry.

### Immunofluorescence staining and microscopy

CD4 T cells were incubated with aAPCs at a 1:3 APC:T cell ratio for various times at 37°C, settled onto poly-L-lysine-coated coverslips, fixed, and labeled for GM1, as previously described (13). When K562 cells were used as aAPCs, T cells were first labeled with biotinylated cholera toxin B (CTB; Sigma-Aldrich), then incubated with K562 cells, fixed and labeled with streptavidin-Cy3 (Sigma-Aldrich). For quantification of LRs, 20 cell bead conjugates and 30 cell/K cell conjugates were identified in each experiment and the percentage of conjugates with LR polarization was calculated. For the experiments with the LAT-GFP transfected human CD4<sup>+</sup> T cells, GFP-positive cells were spun together with K86 or K32 cells at a 1:1 ratio at  $300 \times g$  for 1 min and incubated at 37°C for 5 min for conjugate formation. The cells were then gently resuspended in media and incubated on coverslips coated with poly-L-lysine for 5 min at 37°C before fixation with 3% formaldehyde/PBS for 15 min at room temperature.

Staining for NF- $\kappa$ B p65 and the cell nucleus was performed using a polyclonal anti-p65 antiserum (Santa Cruz Biotechnology) and 4',6'-diamidino-2-phenylindole (Sigma-Aldrich), followed by donkey-anti goat Cy3 (The Jackson Laboratory). In some experiments, the T cells were preincubated with anti-CD28 Fab (prepared using the ImmunoPure Fab Preparation kit; Pierce), prior to stimulation of the cells with CD86Ig beads.

Immunofluorescence and corresponding Nomarski images of T cell/aAPC conjugates were recorded as previously described (13). Within each experiment, all images were normalized to the same range of intensity. To quantitate the polarization of GM1 to the cell-aAPC contact area, data masks of equal size were drawn around the cell-aAPC contact area, the regions of the T cell not in contact with the aAPC and a background area outside of the conjugate. The relative recruitment index (RRI) of LRs was calculated as follows: (mean fluorescence intensity (MFI) at the contact area - background)/(MFI at regions not in contact with aAPC - background). For the analysis of translocation of p65 to the nucleus, data masks were created to include the 4',6'-diamidino-2-phenylindole staining (=the nucleus) for each cell-bead conjugate. Within each mask the MFI for Cy3 (=p65) was measured.

Statistical significance was calculated using the Student *t* test.

### Western blotting

A total of 10 million T cells was stimulated with magnetic beads loaded with either anti-CD28 and anti-MHC class I, anti-MHC class I alone, anti-CD28 or CD86Ig for 1, 5, or 15 min. Cells were lysed as previously described (14). Vav-1 was coupled with 1  $\mu$ g of anti-human Vav-1 Ab (Upstate Biotechnology) and immunoprecipitated with protein G-Sepharose beads. Immunoprecipitates were resolved by SDS-PAGE and transferred to nitrocellulose. Blots were probed with anti-phospho-Vav-1 (BioSource International), stripped, and reprobed with anti-human Vav-1 Ab. Blots were visualized as described previously (14). Densitometry was performed using the Bio-Rad Gel Doc EQ Gel Documentation System.

### Recording of calcium flux

Jurkat T cells (American Type Culture Collection) were labeled with the calcium-sensitive dye fura-2 (Molecular Probes), settled onto cell culture dishes at 37°C for 20 min and transferred to a Zeiss Axioplan microscope equipped with a heated stage. Fluorescence excitations at 340 and 380 nm, image recording, and analysis were performed as previously described (12) using Slidebook software (Intelligent Imaging Innovations). For cell activation, K562 cells transfected with CD32 and CD86 or CD32 alone were added to the culture dish at  $t = 0$  s and pictures were recorded at regular intervals. At the end of each experiment, different focal planes were scanned to ensure that the recorded cells had formed proper conjugates with the K562 cells. All picture sequences were saved as movies using QuickTime (Apple) software and still pictures were taken from these movies to create Fig 5.

### Proliferation assay

A total of 100,000 CD4 T cells were stimulated with aAPC at a 3:1 aAPC:T cell ratio in 96-well plates. A total of 18 h prior to harvest, cultures were pulsed with 1  $\mu$ Ci/well [<sup>3</sup>H]thymidine (DuPont NEN). Cell harvesting and [<sup>3</sup>H]thymidine incorporation was performed as previously described (11).

### Quantitative RT-PCR

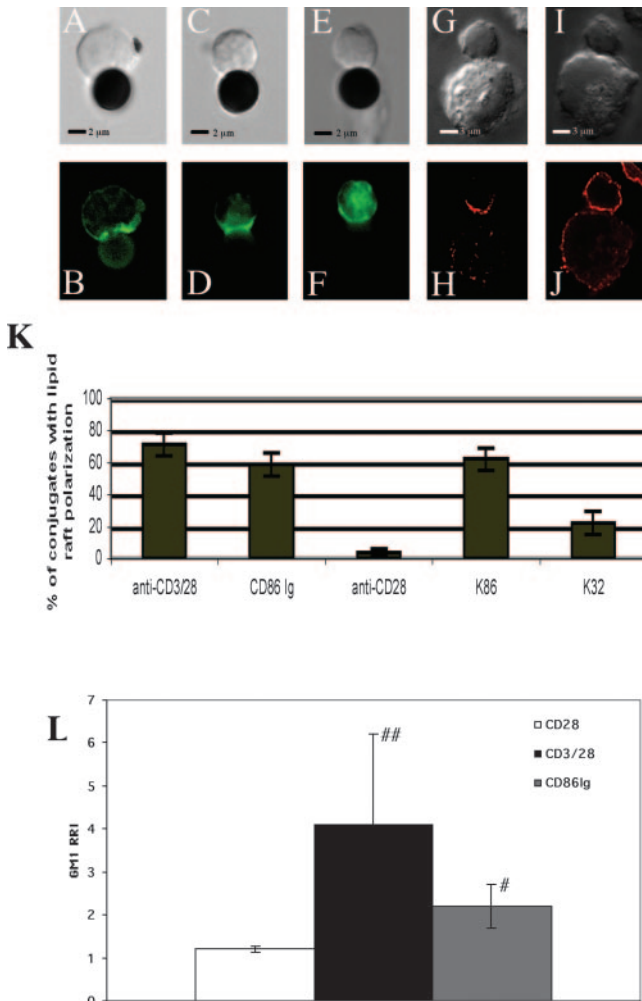
Primers and probes to detect early growth response (EGR) 1, IL-2, and 28S rRNA were designed using Primer Express software (Applied Biosystems) and are available upon request. Purification of cytosolic RNA, reverse transcription, and real-time PCR amplification and product detection were performed as previously described (14).

## Results

### Ligation of CD28 by CD86Ig can polarize LRs to the aAPC/CD4 T cell contact region

Previous work (11) has shown that the accumulation of LRs at the T cell/APC contact site, in resting CD4 T cells, depends on the simultaneous ligation of CD3 and CD28. In those experiments, Viola et al. (8) used Abs bound to beads to ligate the T cell surface receptors. Because there are affinity differences between Ab and natural ligand, we postulated that ligation of CD28 by its natural ligand, CD86, triggers T cells in a sufficiently different manner to induce LR polarization in the absence of TCR ligation. Accordingly, beads coated with CD86Ig alone led to polarization of LRs (Fig. 1, *D* and *K*). There was no difference in the frequency of LR polarization in response to CD86Ig when primary mixed CD4 T cells were compared to purified naive or memory T cells (data not shown). We did not observe LR polarization in T cells stimulated with either anti-CD3 (data not shown) or anti-CD28 (CD28) 9.3 coated beads (Fig. 1*F*), but did observe LR polarization upon stimulation with anti-CD3/28 (CD3/28)-coated beads (Fig. 1*B*). Beads coated with an anti-CD28 Ab (5D10) that can induce proliferation in the absence of TCR stimulation did not induce LR polarization (Fig. 2). Thus, LR polarization could be achieved using a natural ligand of CD28 and without engagement of the TCR.

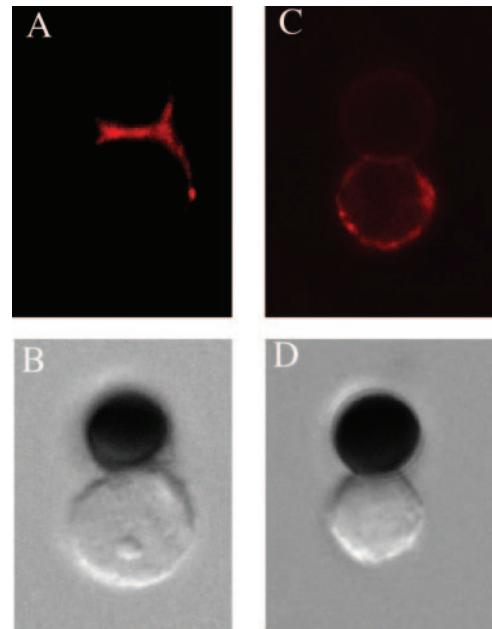
Although magnetic beads are an excellent tool for the study of signaling properties of molecules in isolation and have been used previously as artificial APCs, unlike cell-based APCs, they lack membrane fluidity and the ability to "cross-talk" with T cells. Recently, we have shown that K562 cells transfected with CD32 (K32 cells) permit the binding of anti-CD3 and anti-CD28 Abs. Ab-coated K32 cells rapidly expand CD4 T cells (11). K562 cells lack MHC class II and CD86 expression. Thus, K562 that express CD86 (K86 cells) are an ideal platform to test whether CD86 can induce LR polarization in T cells in the absence of TCR engagement. CD86 expression on K86 cells is comparable to that observed on mature DCs (11). Incubation of CD4 T cells with K32 or K86 cells resulted in similar numbers of APC/T cell conjugates, presumably mediated by the many integrins (e.g., CD54 and CD58) that K562 cells express (11). However, only K86 cells were able to induce LR polarization in the T cells in a majority of these



**FIGURE 1.** Ligation of CD28 by CD86 induces the polarization of LRs in primary CD4 T cells. Primary human CD4 T cells were incubated with CD3/28 beads (*A* and *B*), CD86Ig beads (*C* and *D*), or CD28 beads (*E* and *F*) for 30 min and stained with CTB-FITC. In *H* and *J*, cells were pre-labeled with CTB-Bio and then incubated with K86 (*G* and *H*) or K32 (*I* and *J*) cells for 30 min prior to staining with streptavidin-Cy3. Data are representative of four experiments (*A–F*) and two experiments (*G–J*), respectively. *K*, Quantification of LR polarization from the experiments with beads/T cells (average  $\pm$  SEM of four experiments) and K cells/T cells (average  $\pm$  SD of two experiments). In *L*, the RRI of GM1 to the cell/aAPC contact area was calculated as described in *Materials and Methods*. It represents the mean  $\pm$  SD of 20 cells from two different experiments. ##,  $p = 0.06$  and #,  $p = 0.04$  vs CD28 beads.

conjugates (Fig 1, *H* vs *J* and *K*), indicating the importance of CD28 signaling in this process.

Quantification of our experiments showed that ~60% of CD86Ig bead/T cell conjugates, 70% of CD3/28 bead/T cell conjugates, 62% of K86/T cell conjugates, 22% of K32/T cell conjugates, but only 4% of CD28 bead/T cell conjugates, showed LR polarization (Fig. 1*K*). The fact that LR polarization can be seen to a certain extent in K32 cells (which do not express CD86) is probably due to binding and signaling of other molecules (e.g., adhesion factors). There was no difference in the amount of LR polarization when primary mixed CD4 T cells were compared to purified naive or memory cells (data not shown). Fig. 1*L* shows the relative recruitment index of LRs to the cell-aAPC contact site, as previously calculated by Tavano et al. (15). Cells incubated with CD3/28 beads showed the greatest extent of polarization (mean



**FIGURE 2.** Ligation of CD28 by CD86Ig induces the polarization of LRs in primary CD4 T cells, while ligation of CD28 by the stimulating anti-CD28 Ab 5D10 does not. Primary human CD4 T cells were incubated with CD86Ig beads (*A* and *B*) or anti-CD28 5D10 beads (*C* and *D*) for 30 min and stained with anti-GM1. Data are representative of two experiments.

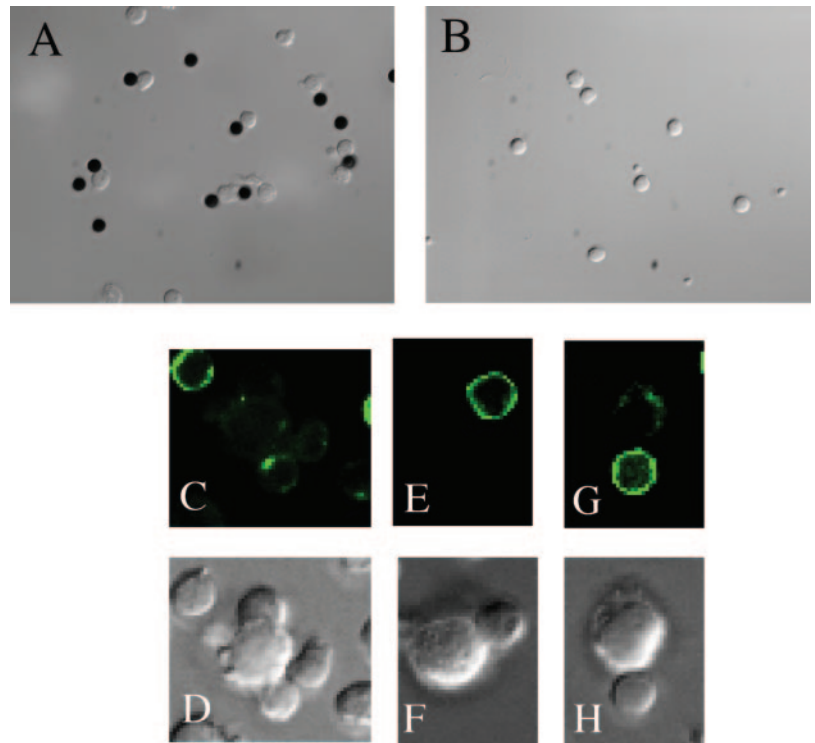
index 4.1,  $p = 0.06$ , vs CD28 beads), followed by CD86Ig beads (mean index 2.2,  $p = 0.04$ , vs CD28 beads).

It was shown recently that B7-dependent costimulation can function in mice that lack CD28 and CTLA-4 (16), indicating that there might be an additional receptor for B7 on T cells. It is possible that the LR polarization we observe in response to CD86Ig is mediated by a receptor other than CD28 on the T cells. To explore this, we preincubated the CD4 T cells with an anti-CD28 Fab Ab prior to stimulation of the cells with CD86Ig beads. This completely inhibited bead-cell conjugate formation (Fig. 3*B*), indicating that CD86Ig on the beads primarily binds CD28, and that CD28 is required to promote a stable interaction between the bead and the T cell. Similarly, preincubation of T cells with anti-CD28 Fab prior to stimulation with K86 cells inhibited the polarization of LRs (Fig. 3*G*).

#### *Ligation of CD28 by CD86Ig can activate early signal transduction pathways in CD4 T cells*

Other studies have addressed CD28 signaling after Ab engagement in human T cells (17) and Kaga et al. (18) showed that B7.2 expressed on CHO cells can induce actin polymerization in mouse lymph node T cells. However, there is limited information about early signal transduction events in resting human T cells when CD28 is stimulated by its natural ligand. We investigated whether binding of CD28 by CD86Ig can induce phosphorylation of Vav-1. We chose Vav-1 because it cooperates with CD28 to induce NF- $\kappa$ B activation (19, 20) and is essential for LR clustering in T cells (21). Furthermore, in a Vav-1 overexpression system using Jurkat T cells, Michel et al. (22) showed that ligation of CD28 by B7.1 could induce phosphorylation of Vav. Incubation of resting CD4 T cells with CD86Ig beads induced a marked increase in Vav-1 phosphorylation, whereas the response following incubation of CD4 T cells with Ab-coated beads was much less pronounced (Fig 4). The CD28-induced phosphorylation of Vav-1

**FIGURE 3.** Interaction of B7 and CD28 is required for LR polarization in resting CD4 T cells. *A* and *B*, CD4 T cells were incubated with CD86Ig beads for 30 min and Nomarski images were obtained. *B*, cells were preincubated with an anti-CD28 Fab Ab, which blocked cell/bead conjugate formation. The data are representative of two experiments with different donors. *C–H*, CD4 T cells were transfected with a GFP-LAT construct and incubated with K86 cells (*C*, *D*, *G*, and *H*) or K32 cells (*E* and *F*) for 5 min prior to fixation. *G* and *H*, T cells were incubated with anti-CD28 Fab prior to incubation with K86 cells, which inhibited LR polarization.



was rapid in onset, seen prominently after 1 min of stimulation and was sustained after 5 min, returning to baseline by 15 min after stimulation (data not shown). Resting CD4 T cells alone and CD4 T cells incubated with MHC I beads (anti-MHC I, negative control) had a similar amount of phosphorylated Vav-1 (data not shown).

Another early signal transduction event in T cells is the increase in intracellular calcium. We loaded Jurkat T cells with fura-2, and K86-induced calcium flux was measured using real-time imaging. Representative images from an experiment are shown in Fig. 5. Jurkat cells that bound to the K86 cells showed an increased intracellular calcium concentration (indicated by the red color), whereas Jurkat cells not in contact with K86 cells remained in their resting state (indicated by blue or green). Incubation of K32 cells with Jurkat cells did not induce calcium flux (data not shown). Thus, cross-linking of CD28 by its natural ligand, CD86, leads to the initiation of early signal transduction pathways.

#### *Stimulation of CD28 in the absence of TCR ligation induces translocation of NF- $\kappa$ B p65 to the nucleus and initiates gene transcription of EGR1*

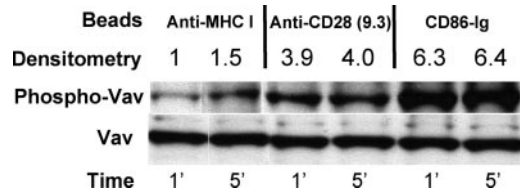
Activation of the NF- $\kappa$ B pathway is an important step required for gene transcription. Although activation of NF- $\kappa$ B is thought to be a result of CD28 activation in the presence of TCR ligation (i.e., costimulation; Ref. 23), it is unclear whether NF- $\kappa$ B can be activated through stimulation of CD28 alone. We performed experiments using CD3/CD86Ig and CD86Ig beads, respectively, to stimulate freshly isolated human CD4 T cells, and stained for NF- $\kappa$ B p65 (Fig. 6). As expected, p65 moved to the nucleus after 25 min of activation with CD3/CD86Ig beads (Fig. 6*D*). Interestingly, translocation of p65 to the nucleus was also observed following activation of the cells with CD86Ig beads alone (Fig. 6*D*). The extent of translocation of p65 to the nucleus is shown in Fig. 6*M*. Less p65 was observed in the nuclei of cells stimulated with CD86Ig beads alone than in cells stimulated with CD3/CD86Ig beads. Almost no translocation of p65 to the nucleus was observed

in the nuclei of cells bound to MHC I beads (Fig. 6, *L* and *M*, negative control). These data are consistent with previously published data showing that the magnitude of gene regulation is higher after CD3 and CD28 coligation, compared to CD28 ligation alone (9). Of importance, no translocation of p65 to the nucleus was visible in CD4 T cells that did not form conjugates with beads. Another intriguing observation was the accumulation of p65 in the cell/bead contact area after 5 min of activation with CD3/CD86Ig beads (Fig. 6*B*) or CD86Ig beads (Fig. 6*F*). This indicates that, prior to p65 translocation to the nucleus, p65 is recruited to the immune synapse, in response to CD86 triggering. This surprising result implies the functional integration of transcription factors in the immune synapse after ligation of CD28 by its natural ligand.

To date, only stimuli leading to full T cell activation have been shown to induce LR polarization. We showed that CD86Ig stimulation was unable to induce cytokine expression (Fig. 7*A*) or T cell proliferation (Fig. 7*B*). The ability of T cells to induce EGR1 expression upon CD86 stimulation was previously demonstrated (9) and served as a control to demonstrate intact CD86Ig stimulation (Fig. 7*C*). These results demonstrate that CD86 ligation promotes a variety of signal transduction pathways that may lead to the regulation of a small subset of genes (24), of which EGR1 is a representative member. The fact that these signals do not result in T cell proliferation or cytokine production severs the link between LR polarization and full T cell activation.

## Discussion

It has been established that the optimal activation of resting T cells requires both signal 1, which represents the Ag-specific component via the TCR, and signal 2, which is noncognate and is also called the "costimulatory" signal (1). Signaling through the TCR must clearly be considered "signal 1" in terms of importance, because without TCR ligation on resting CD4 T cells, cell expansion and induction of effector functions do not occur. However, in an APC/T cell conjugate, the density of the receptor/ligand pair, CD28/B7, is much higher than the density of peptide-MHC/TCR.



**FIGURE 4.** Cross-linking of CD28 by CD86 Ig regulates phosphorylation of Vav-1 in human T cells. CD4 T cells were incubated with anti-MHC class I, anti-CD28, or CD86Ig beads for 1 or 5 min. Vav-1 activation was detected by Western blot for Tyr<sup>160</sup>-phosphorylated Vav-1 (top panels, phospho-Vav). The blot was then stripped and reprobed for total Vav-1 (bottom panels, Vav). Densitometry results are shown above the blots.

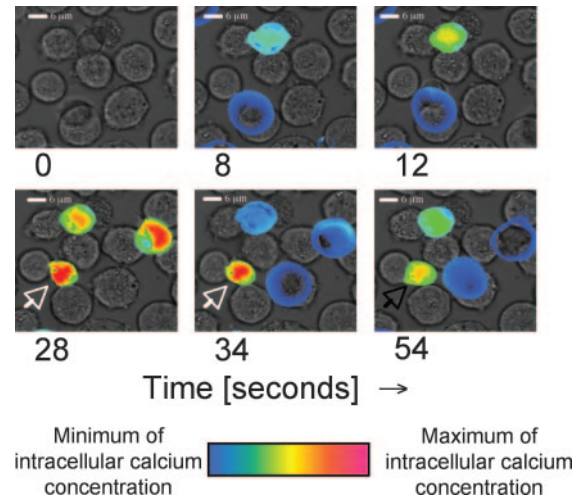
Moreover, the binding affinity of CD86 to CD28 is several orders of magnitude higher than that of the TCR to Ag/MHC complexes. Thus, signaling through CD28 (“signal n”) might be essential for preparing the T cell for TCR signaling (“signal n + 1”) and hence could be a necessary step in terms of sequential activation of T cell signaling pathways.

Our experiments show for the first time that ligation of CD28 by natural ligand alone (without triggering of the TCR) can induce the polarization of LRs and suggest that signaling via CD28 plays a more immediate role in the T cell activation process than previously thought. The CD28/B7 ligand pair facilitates and enhances conjugate formation of APCs and T cells in the absence of TCR stimulation. Therefore, CD28 might participate in the dynamic organization of the T cell/APC contact area by enhancing conjugate formation, and by facilitating TCR-mediated signaling through concentration of essential signaling molecules in the immune synapse. CD28 would thus enhance scanning of the APC for Ag recognition. Of note, recent studies suggest that CD86 is recruited to LRs on dendritic cells, leading to effective priming of T cells (25). In contrast to our findings, however, the recruitment of CD86 to LRs on dendritic cells is Ag dependent.

We have also seen that, in contrast to CD86 beads, beads coated with an anti-CD28 Ab alone cannot induce polarization of LRs. Ebert et al. (26) showed that immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes do not polarize LRs in response to TCR-mediated signals and used anti-CD28 mAb-coated beads in their experiments. However, a recent study by Hare et al. (27) suggests that when CD80 is introduced on thymic epithelium, CD4<sup>+</sup>CD8<sup>+</sup> thymocytes show marked polarization of LRs. These data support our conclusion that in terms of LR polarization, the signal provided by the B7 family is superior to that of an anti-CD28 mAb.

It remains to be determined which intermediary signaling components are involved in the LR polarization induced by CD86Ig ligation of CD28. In our experiments, we observed increased phosphorylation of Vav-1 after incubation of resting T cells with CD86Ig-coated beads. Villalba et al. (21) reported that Vav-1 is required for recruitment of LRs to the immune synapse, a mechanism that is dependent on intact actin cytoskeleton reorganization. We observed less Vav-1 phosphorylation after incubation of T cells with anti-CD28-coated beads. Because CD28 beads also cannot induce LR polarization in the absence of TCR ligation, it is possible that the additional Vav-1 activation induced by CD86Ig facilitates the magnitude of signaling needed to induce LR polarization. Recently, Tavano et al. (15) showed that in human resting T cells, TCR and CD28 coengagement induces Lck recruitment to the immune synapse and that Vav-1 is necessary for this process. Interestingly, an intact CD28 molecule is necessary for the recruitment of Lck (15).

Our results are in contrast to those of Burack et al. (28) who reported that LR accumulation at the immune synapse is indepen-

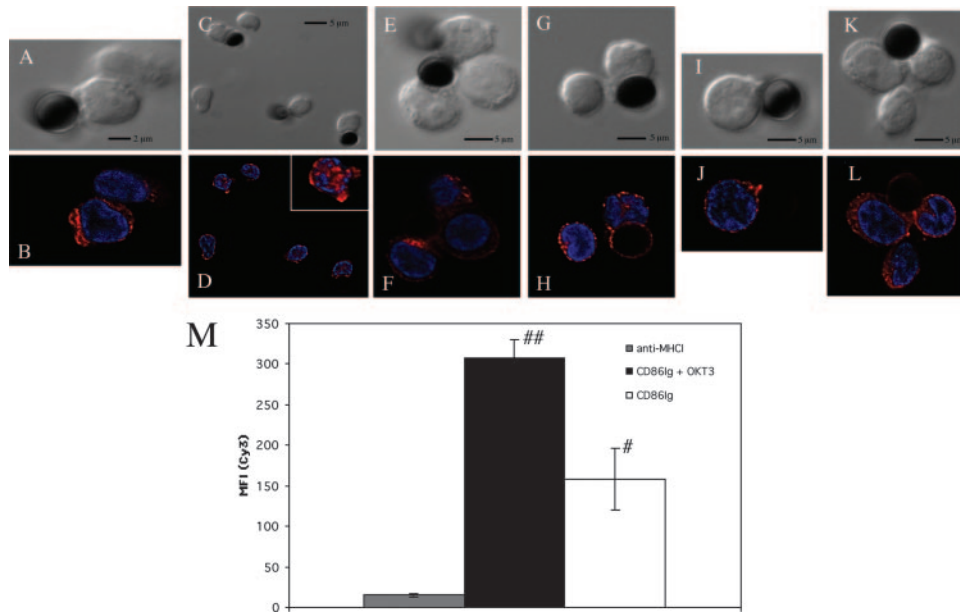


**FIGURE 5.** Jurkat T cells show intracellular calcium flux upon stimulation with K86 cells. Each picture shows the composition of a bright field image overlaid with a transparent color scale of the ratio of fluorescence emissions at 340 and 380 nm of fura-2. The ratio correlates with intracellular calcium concentration: green color indicates resting (low), red color indicates high intracellular calcium concentration. The first picture shows K86 cells alone; in the following pictures, fura-2-loaded CD4 T cells are interacting with the APCs. These data are representative of three experiments.

dent of the CD28/B7 system. These results are not directly comparable to our experiments, because they were performed using activated mouse T cells. In the LR polarization experiments shown here, primary human CD4 T cells were used exclusively. We have performed experiments in PHA-activated human CD4 T cells, as well as in Jurkat cells, and have observed that, in contrast to resting human cells, activated cells do not require costimulation through CD28 for LR polarization (data not shown), in agreement with previous results. Tuosto et al. (29) published similar findings.

It has been known for a long time that ligation of CD28 can initiate T cell signaling pathways independent of TCR ligation. In the experiments presented here, we show that ligation of CD28 by its natural ligand, CD86, can induce intracellular calcium mobilization and translocation of NF- $\kappa$ B p65 to the nucleus of primary human CD4 T cells. These signaling events are also important sequelae of activation through the TCR. Raab et al. (30) have shown that CD28 drives NFAT into the nucleus of COS cells and Marinari et al. (19) showed that CD28 engagement by CD80 induced NF- $\kappa$ B transcriptional activity in Jurkat cells. Using DNA microarrays, a small number of transcripts (including members of the EGR family) were specifically up-regulated by anti-CD28 stimulation and, to an even greater extent, by CD86Ig stimulation (9). The ability of CD86 ligation to induce LR polarization and p65 translocation to the nucleus may explain the enhanced gene regulation induced by CD86Ig stimulation. Furthermore, these results further disassociate T cell proliferation from LR polarization. Previously, we demonstrated that CD8 T cells can be activated and expanded in the absence of LR polarization (13). Here, we show that LR polarization can be induced in CD4 T cells without subsequent T cell proliferation.

What might be the physiologic significance of these results? Individuals with SLE aberrantly express CD86 on resting B cells, whereas resting B cells from healthy people express minute levels of CD86 (31, 32). The use of anti-CD86 blocking Ab in a SLE mouse model prevents onset of disease (33), and findings that B7 expression on B cells is sufficient to break tolerance (34) suggest



**FIGURE 6.** Ligation of CD28 by CD86 induces translocation of NF- $\kappa$ B p65 to the nucleus. CD4 T cells were incubated for 5 (A and B, E F, I and J) or 25 min (C and D, G and H, K and L) with CD3/CD86lg beads (A and B, C and D), CD86lg beads (E and F, G and H), or MHC I beads as control (I and J, K and L), and then stained for NF- $\kappa$ B p65 (red). The nucleus is identified in blue. M, The extent of p65 translocation to the nucleus at 25 min. ##,  $p < 0.005$  and #,  $p = 0.01$  vs MHC I beads. All T cells bound to either CD86lg or CD3/CD86lg beads showed translocation of p65 to the nucleus after 25 min. Data are representative of three experiments.

a central role of CD86 in either initiating or exacerbating autoimmune disease. Perhaps these CD86 expressing, resting B cells induce LR on T cells, helping autoreactive T cells to break tolerance. The relationship between aberrant B7 expression and autoimmune disease is not limited to SLE and in fact is found in a wide array of autoimmune diseases (31). Whether the ability of CD86 to induce LR on CD4 T cells explains the relationship between CD86 expression and autoimmune disease remains to be seen, but it is a tempting speculation. Equally interesting, are the implications of CD86-mediated LR polarization as they relate to HIV-1 disease. HIV-1 virions preferentially incorporate CD86 into their membranes (35) and LR facilitates HIV-1 entry. These virions were shown to trigger NF- $\kappa$ B activation in a CD86-dependent manner similar to that shown in our studies (36). The heightened immune

activation observed in HIV-infected individuals enhances CD86 expression, which in turn could induce LR polarization between activated APC and resting T cells, permitting HIV-1 entry into noncycling T cells. The ability of CD86 to induce LR polarization may in part explain the in vivo susceptibility of resting T cells to HIV-1 infection (37).

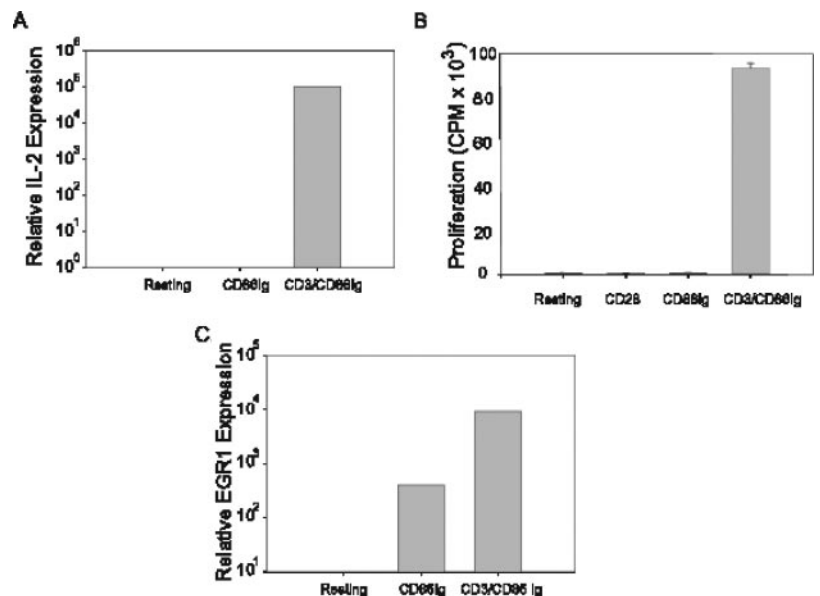
**Acknowledgments**

We thank Drs. Carl June and Gary Koretzky for helpful discussions and support, Dr. Beatriz Carreno for providing the CD86lg, and Dr. Larry Samelson for providing the LAT-GFP construct.

**Disclosures**

The authors have no financial conflict of interest.

**FIGURE 7.** LR polarization induced by CD86lg alone does not lead to CD4 T cell cytokine secretion or proliferation. A and C, CD86lg ligation induces EGR1 transcription but no IL-2 production. CD4 T cells were either left unstimulated (resting) or stimulated with CD86lg or anti-CD3 and CD86lg beads for 2 h. EGR1 and IL-2 expression were quantified by RT-PCR, as expressed in comparison to values obtained from unstimulated cells. Data are representative of three independent experiments. B, LR polarization induced by CD86lg alone does not lead to proliferation of human CD4 T cells. Purified CD4 T cells were cultured for 72 h with anti-CD28 (mAb 9.3), CD86lg, or anti-CD3 and CD86lg beads. Cultures were pulsed with [<sup>3</sup>H]thymidine for 18 h, harvested, and analyzed for [<sup>3</sup>H] incorporation. The error bars indicate the SD of one experiment and the data are representative of three independent experiments. The use of CD3/CD86lg beads gave equivalent proliferation compared to CD3/CD28 beads (data not shown).



## References

- Schwartz, R. H. 1990. A cell culture model for T lymphocyte clonal anergy. *Science* 248: 1349–1356.
- Appleman, L. J., A. A. van Puijenbroek, K. M. Shu, L. M. Nadler, and V. A. Boussiotis. 2002. CD28 costimulation mediates down-regulation of p27<sup>kip1</sup> and cell cycle progression by activation of the PI3K/PKB signaling pathway in primary human T cells. *J. Immunol.* 168: 2729–2736.
- Ledbetter, J. A., J. B. Imboden, G. L. Schieven, L. S. Grosmaire, P. S. Rabinovitch, T. Lindsten, C. B. Thompson, and C. H. June. 1990. CD28 ligation in T-cell activation: evidence for two signal transduction pathways. *Blood* 75: 1531–1539.
- Hehner, S. P., T. G. Hofmann, O. Dienz, W. Droge, and M. L. Schmitz. 2000. Tyrosine-phosphorylated Vav1 as a point of integration for T-cell receptor- and CD28-mediated activation of JNK, p38, and interleukin-2 transcription. *J. Biol. Chem.* 275: 18160–18171.
- Zaru, R., C. P. Berrie, C. Iurisci, D. Corda, and S. Valitutti. 2001. CD28 costimulates TCR/CD3-induced phosphoinositide turnover in human T lymphocytes. *Eur. J. Immunol.* 31: 2438–2447.
- Horejsi, V. 2003. The roles of membrane microdomains (rafts) in T-cell activation. *Immunol. Rev.* 191: 148–164.
- Bi, K., Y. Tanaka, N. Coudronniere, K. Sugie, S. Hong, M. J. van Stipdonk, and A. Altman. 2001. Antigen-induced translocation of PKC- $\theta$  to membrane rafts is required for T-cell activation. *Nat. Immunol.* 2: 556–563.
- Viola, A., S. Schroeder, Y. Sakakibara, and A. Lanzavecchia. 1999. T lymphocyte costimulation mediated by reorganization of membrane microdomains. *Science* 283: 680–682.
- Riley, J. L., M. Mao, S. Kobayashi, M. Biery, J. Burchard, G. Cavet, B. P. Gregson, C. H. June, and P. S. Linsley. 2002. Modulation of TCR-induced transcriptional profiles by ligation of CD28, ICOS, and CTLA-4 receptors. *Proc. Natl. Acad. Sci. USA* 99: 11790–11795.
- Levine, B. L., J. D. Mosca, J. L. Riley, R. G. Carroll, M. T. Vahey, L. L. Jagodzinski, K. F. Wagner, D. L. Mayers, D. S. Burke, O. S. Weislow, et al. 1996. Antiviral effect and ex vivo CD4<sup>+</sup> T-cell proliferation in HIV-positive patients as a result of CD28 costimulation. *Science* 272: 1939–1943.
- Thomas, A. K., M. V. Maus, W. S. Shalaby, C. H. June, and J. L. Riley. 2002. A cell-based artificial antigen-presenting cell coated with anti-CD3 and CD28 antibodies enables rapid expansion and long-term growth of CD4 T lymphocytes. *Clin. Immunol.* 105: 259–272.
- Barda-Saad, M., A. Braiman, R. Titerence, S. C. Bunnell, V. A. Barr, and L. E. Samelson. 2005. Dynamic molecular interactions linking the T cell antigen receptor to the actin cytoskeleton. *Nat. Immunol.* 6: 80–89.
- Kovacs, B., M. V. Maus, J. L. Riley, G. S. Derimanov, G. A. Koretzky, C. H. June, and T. H. Finkel. 2002. Human CD8<sup>+</sup> T-cells do not require the polarization of lipid rafts for activation and proliferation. *Proc. Natl. Acad. Sci. USA* 99: 15006–15011.
- Parry, R. V., C. A. Rumbley, L. H. Vandenberghe, C. H. June, and J. L. Riley. 2003. CD28 and inducible costimulatory protein Src homology 2 binding domains show distinct regulation of phosphatidylinositol 3-kinase, Bcl-x<sub>L</sub>, and IL-2 expression in primary human CD4 T lymphocytes. *J. Immunol.* 171: 166–174.
- Tavano, R., G. Gri, B. Molon, B. Marinari, C. E. Rudd, L. Tuosto, and A. Viola. 2004. CD28 and lipid rafts coordinate recruitment of Lck to the immunological synapse of human T lymphocytes. *J. Immunol.* 173: 5392–5397.
- Mandelbrot, D. A., M. A. Oosterwegel, K. Shimizu, A. Yamada, G. J. Freeman, R. N. Mitchell, M. H. Sayegh, and A. H. Sharpe. 2001. B7-dependent T-cell costimulation in mice lacking CD28 and CTLA4. *J. Clin. Invest.* 107: 881–887.
- Siefken, R., S. Klein-Hessling, E. Serfling, R. Kurrle, and R. Schwinzer. 1998. A CD28-associated signaling pathway leading to cytokine gene transcription and T-cell proliferation without TCR engagement. *J. Immunol.* 161: 1645–1651.
- Kaga, S., S. Ragg, K. A. Rogers, and A. Ochi. 1998. Cutting edge: stimulation of CD28 with B7-2 promotes focal adhesion-like cell contacts where Rho family small G proteins accumulate in T cells. *J. Immunol.* 160: 24–27.
- Marinari, B., A. Costanzo, A. Viola, F. Michel, G. Mangino, O. Acuto, M. Levrero, E. Piccolella, and L. Tuosto. 2002. Vav cooperates with CD28 to induce NF- $\kappa$ B activation via a pathway involving Rac-1 and mitogen-activated kinase kinase 1. *Eur. J. Immunol.* 32: 447–456.
- Piccolella, E., F. Spadaro, C. Ramoni, B. Marinari, A. Costanzo, M. Levrero, L. Thomson, R. T. Abraham, and L. Tuosto. 2003. Vav-1 and the IKK $\alpha$  subunit of I $\kappa$ B kinase functionally associate to induce NF- $\kappa$ B activation in response to CD28 engagement. *J. Immunol.* 170: 2895–2903.
- Villalba, M., K. Bi, F. Rodriguez, Y. Tanaka, S. Schoenberger, and A. Altman. 2001. Vav1/Rac-dependent actin cytoskeleton reorganization is required for lipid raft clustering in T-cells. *J. Cell Biol.* 155: 331–338.
- Michel, F., G. Mangino, G. Attal-Bonnefoy, L. Tuosto, A. Alcover, A. Roumier, D. Olive, and O. Acuto. 2000. CD28 utilizes Vav-1 to enhance TCR-proximal signaling and NF-AT activation. *J. Immunol.* 165: 3820–3829.
- Kane, L. P., J. Lin, and A. Weiss. 2002. It's all Rel-ative: NF- $\kappa$ B and CD28 costimulation of T-cell activation. *Trends Immunol.* 23: 413–420.
- Diehn, M., A. A. Alizadeh, O. J. Rando, C. L. Liu, K. Stankunas, D. Botstein, G. R. Crabtree, and P. O. Brown. 2002. Genomic expression programs and the integration of the CD28 costimulatory signal in T-cell activation. *Proc. Natl. Acad. Sci. USA* 99: 11796–11801.
- Meyer zum Buschenfelde, C., J. Unternaehrer, I. Mellman, and K. Bottomly. 2004. Regulated recruitment of MHC class II and costimulatory molecules to lipid rafts in dendritic cells. *J. Immunol.* 173: 6119–6124.
- Ebert, J. R., J. F. Baker, and J. A. Punt. 2000. Immature CD4<sup>+</sup>CD8<sup>+</sup> thymocytes do not polarize lipid rafts in response to TCR-mediated signals. *J. Immunol.* 165: 5435–5442.
- Hare, K. J., J. Pongracz, E. J. Jenkinson, and G. Anderson. 2003. Modeling TCR signaling complex formation in positive selection. *J. Immunol.* 171: 2825–2831.
- Burack, W. R., K. H. Lee, A. D. Holdorf, M. L. Dustin, and A. S. Shaw. 2002. Cutting edge: quantitative imaging of raft accumulation in the immunological synapse. *J. Immunol.* 169: 2837–2841.
- Tuosto, L., I. Parolini, S. Schroder, M. Sargiacomo, A. Lanzavecchia, and A. Viola. 2001. Organization of plasma membrane functional rafts upon T-cell activation. *Eur. J. Immunol.* 31: 345–349.
- Raab, M., S. Pfister, and C. E. Rudd. 2001. CD28 signaling via VAV/SLP-76 adaptors: regulation of cytokine transcription independent of TCR ligation. *Immunity* 15: 921–933.
- Salomon, B., and J. A. Bluestone. 2001. Complexities of CD28/B7: CTLA-4 costimulatory pathways in autoimmunity and transplantation. *Annu. Rev. Immunol.* 19: 225–252.
- Folzenlogen, D., M. F. Hofer, D. Y. Leung, J. H. Freed, and M. K. Newell. 1997. Analysis of CD80 and CD86 expression on peripheral blood B lymphocytes reveals increased expression of CD86 in lupus patients. *Clin. Immunol. Immunopathol.* 83: 199–204.
- Nakajima, A., M. Azuma, S. Kodera, S. Nuriya, A. Terashi, M. Abe, S. Hirose, T. Shirai, H. Yagita, and K. Okumura. 1995. Preferential dependence of autoantibody production in murine lupus on CD86 costimulatory molecule. *Eur. J. Immunol.* 25: 3060–3069.
- Roth, R., T. Nakamura, and M. J. Mamula. 1996. B7 costimulation and autoantigen specificity enable B cells to activate autoreactive T-cells. *J. Immunol.* 157: 2924–2931.
- Esser, M. T., D. R. Graham, L. V. Coren, C. M. Trubey, J. W. Bess, Jr., L. O. Arthur, D. E. Ott, and J. D. Lifson. 2001. Differential incorporation of CD45, CD80 (B7-1), CD86 (B7-2), and major histocompatibility complex class I and II molecules into human immunodeficiency virus type 1 virions and microvesicles: implications for viral pathogenesis and immune regulation. *J. Virol.* 75: 6173–6182.
- Bounou, S., N. Dumais, and M. J. Tremblay. 2001. Attachment of human immunodeficiency virus-1 (HIV-1) particles bearing host-encoded B7-2 proteins leads to nuclear factor- $\kappa$ B- and nuclear factor of activated T-cells-dependent activation of HIV-1 long terminal repeat transcription. *J. Biol. Chem.* 276: 6359–6369.
- Zhang, Z., T. Schuler, M. Zupancic, S. Wietgrefe, K. A. Staskus, K. A. Reimann, T. A. Reinhart, M. Rogan, W. Cavert, C. J. Miller, et al. 1999. Sexual transmission and propagation of SIV and HIV in resting and activated CD4<sup>+</sup> T cells. *Science* 28: 1353–1357.