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► **To cite this version:**

Christiane Barbat, Maylis Trucy, Maurizio Sorice, Tina Garofalo, Valeria Manganelli, et al.. p56 lck, LFA-1 and PI3K but not SHP-2 interact with GM1- or GM3-enriched microdomains in a CD4-p56 lck association-dependent manner. *Biochemical Journal*, 2006, 402 (3), pp.471-481. 10.1042/BJ20061061 . hal-00478630

HAL Id: hal-00478630

<https://hal.science/hal-00478630>

Submitted on 30 Apr 2010

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**P56LCK, LFA-1 AND PI3K BUT NOT SHP-2 INTERACT WITH GM1
OR GM3-ENRICHED MICRODOMAINS IN A CD4/P56LCK
ASSOCIATION-DEPENDENT MANNER**

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Keywords:

CD4 T cells, adhesion molecules, rafts, gangliosides, signal transduction, kinases.

short title :

CD4/p56^{lck}-dependent interaction of specific proteins in DRM

SYNOPSIS

We previously showed that the association of CD4 and GM3 ganglioside induced by CD4 ligand binding was required for the down-regulation of adhesion and that aggregation of ganglioside-enriched domains was accompanied by transient colocalization of LFA-1, PI3K and CD4. We also showed that these proteins co-localized with the GM1 ganglioside that partially co-localized with GM3 in these domains. In this study, we show that CD4/p56^{lck} association in CD4 signaling is required for the redistribution of p56^{lck}, PI3K, LFA-1 in ganglioside-enriched domains, since ganglioside aggregation and recruitment of these proteins were not observed in a T cell line (A201) expressing the mutant form of CD4 that does not bind p56^{lck}. In addition, we show that although these proteins associated in different ways with GM1 and GM3, all of the associations were dependent on CD4/p56^{lck} association. Gangliosides could associate with these proteins that differ in affinity binding and could be modified following CD4 signaling. Our data suggest that through these associations, gangliosides transiently sequester these proteins and consequently inhibit LFA-1-dependent adhesion. Furthermore, while structural diversity of gangliosides may allow association with distinct proteins, we show that the tyrosine phosphatase SHP-2 also required for the down-regulation of LFA-1-dependent adhesion transiently and partially co-localized with PI3K and p56^{lck} in detergent-insoluble membranes without association with GM1 or GM3. We propose that CD4 ligation and binding with p56^{lck} and their interaction with GM3 and/or GM1 gangliosides induce recruitment of distinct proteins important for CD4 signaling to form a multimolecular signaling complex.

INTRODUCTION

CD4 signaling, in the absence of T cell receptor (TCR) engagement, is a critical event in the down-regulation of leukocyte function-associated antigen 1 (LFA-1)-dependent adhesion between T and B cells. We have previously reported that CD4-induced down-regulation requires intermediary proteins between CD4 and LFA-1 such as CD4-associated tyrosine kinase p56^{lck}, the phosphatidylinositol-3 kinase (PI3K), and the Src homology 2 domain-containing phosphatase SHP-2 [1]. In addition, given the rapid and transient complex of signaling proteins induced by CD4 signaling and signal inhibition by cholesterol removal, we suggested that these proteins were recruited into different membrane compartments [2]. Lateral heterogeneity in the classic fluid-mosaic model of cell membranes indicates that the plasma membrane contains distinct microdomains that are enriched in cholesterol, glycosylphosphatidylinositol (GPI) linked glycoproteins [3], and glycosphingolipids such as GM1 and GM3 which are involved in modulating signal transduction [4]. Asymmetric distribution of T cell rafts containing GM1 and GM3 glycosphingolipids indicates that rafts have distinct functions and play a role in several signaling processes involving receptors and integrins. Isolation of detergent-resistant membranes (DRM) has proved to be a valuable tool for the analysis of lipid rafts and a useful starting point for defining membrane subdomains and their composition [5, 6], even though DRM do not necessarily represent all the rafts in living cells. However, although detergent extraction disrupts lipid-lipid interactions, and lipid-protein interaction, a minor fraction of cell membrane is preserved. Only those proteins that are strongly interacting with highly ordered domain retain their association with lipids and are recovered in DRM. These DRM or rafts allow lateral segregation of proteins and provide a mechanism for the compartmentalization of signaling components by concentrating or excluding certain components such as CD45 that inhibit T cell signaling [7] [8]. Thus, lipid rafts may function as platforms to control the localisation and function of proteins for the formation of multicomponent transduction complexes. The role of lipid rafts in T cell signaling has been also emphasized given that disruption and/or displacement of signaling molecules abolishes TCR-mediated signaling events [9]. In lymphocytes, GM3 and

cholesterol, along with the src-family kinase p56^{lck} and a fraction of the CD4 pool are acylated by saturated fatty acids and are selectively recovered in the rafts where they associate [10, 11]. Mutant versions of p56^{lck} and CD4 that are not palmitoylated and consequently not localized within rafts, do not function in T cell activation [12, 13]. Polarization and lipid raft recruitment to the receptor engagement site in immune cells following solid-phase engagement of the CD4 molecule alone and subsequent redistribution of different proteins such as integrins requires p56^{lck} signaling [14]. Raft localization of integrins appears to be correlated with integrin activity, since inactive integrins such as LFA-1 are tethered away from lipid rafts by cytoskeletal restraints [15]. Other proteins such as SHP-2 and PI3K can affect both within raft-integrin localization and function. SHP-2 associates with adhesion molecules [16] and SHP-2 partitioning in raft domains triggers integrin-mediated signaling [17]. SHP-2 also associates with F-actin [18] and plays a role in cytoskeletal organization, cell adhesion and migration [19]. PI3K can also be recruited in raft domains following activation, and disruption of these domains inhibits the PI3K pathway [20]. PI3K, through its different associations with PKC ζ , RhoA or cytohesin-1, is also important for integrin regulation [21, 22] as well as for the polar redistribution of LFA-1 induced by chemokines [23].

We have previously reported that lipid raft integrity is required for the down-regulation of Ag-independent LFA-1-dependent adhesion induced by CD4 ligands, and more precisely by an anti-CD4 Ab (13B8.2) that specifically binds the NH₂-terminal-CD4 domain (D1) [24]. Since CD4 triggering induces aggregation of GM1- and GM3-enriched raft domains that are partially co-localized, suggests that GM1 and GM3 may also be localized in distinct domains and may associate with distinct proteins. Raft involvement in CD4-triggered events has been strengthened by the results showing that the CD4/GM3 association induced by CD4 ligand binding was required for the down-regulation of LFA-1-mediated adhesion [24]. Raft aggregation was accompanied by transient colocalization of LFA-1, PI3K and CD4 in these domains. We investigate here whether LFA-1, PI3K and SHP-2 colocalize and associate with gangliosides after CD4 ligation and whether gangliosides act as intermediary partners for the sequential association of these different proteins. In addition, since p56^{lck} associated with CD4 was also required for the down-regulation of Ag-independent LFA-1-

dependent adhesion induced by CD4 ligands [25], we also investigate whether CD4/p56^{lck} association is also required for the LFA-1, PI3K and SHP-2 colocalization and association with gangliosides.

MATERIALS AND METHODS

Antibodies and reagents

The following antibodies (Ab) were used: 13B8.2, 25.3 (IgG1, anti-CD4 and anti-LFA1 α mAbs respectively), anti-HLA class I Ab from Immunotech, Marseille, France, anti-p85-PI3-kinase polyclonal Ab (Upstate Biotechnology, Inc, Lake Placid, NY), anti-p56^{lck} polyclonal Ab, anti-SHP-2 monoclonal Ab (Santa Cruz, TEBU). F(ab')₂ goat anti-mouse IgG (GAMiG; Jackson Immunoresearch Laboratories, Inc) was used for cross-linking experiments. For confocal immunofluorescence experiments, the following Abs were used: TRITC-conjugated GAMiG and FITC-conjugated sheep anti-rabbit IgG (Jackson Immunoresearch Laboratories, Inc) and anti-GM1 and anti-GM3 monoclonal Abs (Seikagaku Corp. Japan; Coger).

Cell cultures

The A201 T cell line was a CEM-derived T cell line, transfected with wild type CD4 cDNAs (A201-CD4), or with a mutated form of CD4 cDNAs (A201-2C>A) in which the two cysteines required for association with p56^{lck} were replaced by two alanines [25]. These T cell lines normally express CD4 and equivalent amounts of active p56^{lck} at the cell surface [25]. No association between the mutated form of CD4 and p56^{lck} was found in A201-2C>A. Cells were cultured in RPMI 1640 medium (Life Technologies, Biocult, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine and 0.5mg/ml of G418 (Life Technologies).

Isolation of DRM by flotation experiments

Optiprep gradient analysis was performed according to a previously described method with some modifications [26]. Briefly, as previously reported [24], A201-CD4 T cell lines were starved overnight in serum-free medium; 25x10⁶ cells were then incubated with anti-CD4 antibody for 20 minutes at 4°C, washed and incubated for the indicated time at 37°C with a GAMiG. Cells were then lysed on ice for 15 minutes in 900 μ l of lysate buffer A

(50mM Tris-HCL pH7.4, 110mM NaCl, 10mM EGTA, leupeptin and pepstatin, 1µg/ml, aprotinin 2µg/ml and 1mM PMSF) without detergent and sonicated gently (5s bursts, 5W; Branson sonifier 250). After centrifugation for 5 minutes at 800 x g at 4° C, the post-nuclear supernatant (PNS) was incubated with 1% Brij 58 at 4°C for 1 hour. Detergent-resistant membranes were isolated by ultracentrifugation (4h at 28000rpm at 4°C) in a SW41 rotor (Beckman Instruments Inc), in a 40%-30%-5% Optiprep density gradient (Sigma). Seven fractions were collected from the top of the tube. F1 corresponds to the top of the gradient. The low-density fractions 2-3-4 contained detergent insoluble raft fractions enriched in gangliosides. As expected, transferrin receptor that does not reside in rafts, was only detected in fractions 6 and 7 that contained detergent soluble membrane. Normalized protein amounts for each fraction were determined using the Bio-Rad kit (Bio-Rad, Richmond, CA) with bovine serum albumin as standard. Each fraction was then immunoprecipitated with specific antibodies and analyzed by 8% SDS-PAGE and Western blotting as previously described [24]. Proteins were visualized using a chemiluminescence detection system (ECL-Amersham, Arlington Heights, IL) with an anti-rabbit or anti-mouse Ig coupled to horseradish peroxidase as secondary Ab (Amersham). GM1 and GM3 gangliosides were detected using high performance thin layer chromatography (HPTLC) [1], as reported below. The ganglioside extract was split into two aliquots. The first one was run on silica gel 60 HPTLC plates (Merck) and stained with resorcinol to detect GM3. The second one was run on HPTLC aluminium-backed silica gel 60 (20x20) plates (Merck). The plates were immunostained with 0.5µg/ml of CT-HRP for 1h to detect GM1 at room temperature. Immunoreactivity was assessed by chemiluminescence.

Ganglioside detection by high performance thin layer chromatography (HPTLC)

A201-CD4 or A201-2C>A T cell lines were incubated overnight in serum-free medium before incubation with anti-CD4 antibody. 40×10^6 cells were incubated for 20 minutes at 4°C with anti-CD4 antibody then washed and incubated for the indicated time at 37°C with a GAMIg. Cells were then lysed on ice for 20 minutes in 900 µl of lysate buffer A supplemented with 1%NP40, and clarified by centrifugation at 12,000 x g for 15 minutes. The

same amount of each PNS (4mg) was immunoprecipitated with specific antibodies and analyzed for the presence of the gangliosides by HPTLC. Gangliosides were extracted twice in chloroform:methanol:water (4:8:3) (v:v:v) and subjected to Folch partition by the addition of water resulting in a final chloroform:methanol:water ratio of 1:2:1.4. The upper phase, containing polar glycosphingolipids, was purified of salts and low molecular weight contaminants using Bond Elut C18 columns (Superchrom, Milan, Italy). The eluted glycosphingolipids were dried and separated by HPTLC using silica gel 60 HPTLC plates (Merck, Darmstadt, Germany). Chromatography was performed in chloroform:methanol:0.25% aqueous KCl (5:4:1) (v:v:v). The plates were stained with resorcinol (ganglioside-specific stain) or with cholera toxin for GM1, and anti-GM3 Ab [1]. Quantification was carried out by densitometric scanning analysis using a Mac OS 9.0 (Apple Computer International) and NIH Image 1.62 software. The amount of immunoprecipitated protein was checked by control Western blotting with the antibodies used for specific immunoprecipitation.

Colocalization experiments using scanning confocal microscopy

As previously described [24], after activation with cross-linked anti-CD4 Ab and saturation of the free sites with GAMIg incubation at 4°C, cell suspensions containing 8×10^4 cells/slide were layered onto poly-L-lysine-coated coverslips for 45 minutes at room temperature. Immunofluorescence staining of cell surface molecules was performed with the appropriate mAbs in the absence of permeabilizing agent, followed by FITC or TRITC-anti-mouse IgG1 or anti-rabbit secondary Abs. Intracellular proteins were stained for 1 minute in 0.05% saponin-permeabilized cells using the appropriate mAbs. We have previously checked that labeled-GAMIg does not reveal anti-CD4 Ab [24]. Confocal microscopy was performed on a Zeiss LSM-510 confocal microscope. Images were acquired using the maximum signal detection setting below the saturation limit of the detector.

RESULTS

The CD4/p56^{lck} association is required for the aggregation of GM1(+) and GM3(+) DRM induced by anti-CD4 Ab

We have previously shown that CD4 ligand binding specifically induces CD4 aggregation and partial co-localization of GM1- and GM3- enriched domains [24]. Integrity of raft domains and the CD4/p56^{lck} association were required for the down-regulation of LFA-1-dependent adhesion induced by CD4 ligand binding [24]. We have therefore investigated whether the CD4 ligand induces ganglioside-enriched domain aggregation in a CD4/p56^{lck} dependent manner. We observed that GM1- and GM3- enriched domains aggregation, detected following CD4 ligation (Fig 1A) was dependent upon CD4/p56^{lck} association, since it was not observed in A201-2C>A, an A201-T cell line transfected with a mutated form of CD4 that does not bind p56^{lck} (Fig 1B). Patching was not observed with a control Ab specific to transferrin receptor (Fig1A and B, Ct). HPTLC showed that both T cell lines expressed the same amount of ganglioside in the tested conditions (Fig 1C).

CD4 ligation induces the redistribution of LFA-1 and PI3K with GM1 and GM3 in a CD4/p56^{lck} association-dependent manner

We have previously reported that PI3K and LFA-1 partially co-localized with GM1 and GM3 in DRM following CD4 triggering [24]. In this study, we investigated whether CD4/p56^{lck} association was also required for these colocalizations. Distribution of LFA-1 and PI3K, that is uneven and punctuated over the plasma membrane following CD4-cross-linking, appeared distributed all over the membrane without detectable clustering in the A201-2C>A T cell line; this was also observed in both T cell lines in the absence of CD4 triggering (NA) and in the control Ab-treated cells (Ct) (Figs 2A and 3A). These immunofluorescence experiments also showed that the co-localization of GM1, LFA1 and PI3K respectively, and of GM3 and PI3K were partial but stable over a 20-minute period. Colocalization of LFA-1 with GM3 was more dominant after a short period of incubation with anti-CD4 Ab (2 minutes) than following longer incubation (Fig 2A, left panel).

To determine whether LFA-1 and PI3K interact with GM1 and GM3 gangliosides following CD4 cross-linking, cell-free lysates from anti-CD4-treated cells were immunoprecipitated with an anti-LFA-1 mAb (Fig 2B and Fig S1A of Supplemental Data available with this article online) or an anti-PI3K Ab (Fig 3B and Fig S1B), and gangliosides were detected by HPTLC as previously reported [1]. Immunoprecipitation with an anti-LFA-1 mAb (Fig 2B and Fig S1A of Supplemental Data available with this article online) and gangliosides detected by HPTLC showed a faint GM3 band but no GM1 band following control incubation with GAMlg, (Fig 2B). In contrast, after 2 minutes of CD4 cross-linking, two main resorcinol-positive bands co-migrating with GM3 (AU=82±10, FigS1A) and, to a lesser extent, with GM1 (AU=57±5, FigS1A) were detected. Coprecipitation of GM3 with LFA-1 rapidly decreased (AU=30±5; 24±2 after 10 and 20 minutes respectively; FigS1A). In contrast, coprecipitation of GM1 with LFA-1 was lower but stable and decreased more slowly (FigS1A). Confocal microscopy analysis also showed that the LFA-1/GM1 interaction was more stable than the LFA-1/GM3 interaction. Colocalization in patches (Fig 2A) and coprecipitation of LFA-1 and gangliosides (Fig 2B) both required CD4/p56^{lck} association since neither were detected in the A201-2C>A T cell line. The same amount of LFA-1 was immunoprecipitated in the different conditions used in A201-CD4 and A201-2C>A T cell lines as shown with the anti-LFA-1 immunoblot (Fig 2B).

In the PI3K immunoprecipitates (Fig 3B and Fig S1B), resorcinol-positive bands were not detected following control activation (Ct), whereas GM1 and, to a lesser extent, GM3 were detectable following activation with the anti-CD4 Ab. Although coprecipitation was stable for 20 minutes, GM3 coprecipitation with PI3K was always lower than with GM1 (Fig 3B and FigS1B). No ganglioside bands were detected in the extracts from the PI3K immunoprecipitates at the surface of the A201-2C>A T cell line (Fig 3B), indicating that PI3K interaction with lipid rafts also requires CD4/p56^{lck} association. The same amount of PI3K was immunoprecipitated in the different conditions used in A201-CD4 and A201-2C>A T cell lines as shown with the anti-PI3K immunoblot (Fig 3B).

Anti-CD4 Ab induces redistribution of p56^{lck} and SHP-2 with GM1 and GM3 in a CD4/p56^{lck} association dependent manner.

The above results indicated that CD4-associated-p56^{lck} is required for the aggregation of GM1, GM3, and the colocalization of PI3K and LFA-1 after CD4 signaling, and that p56^{lck} may also be redistributed with these gangliosides. Scanning confocal microscopy revealed p56^{lck} clustered in patches costained with anti-GM1 and anti-GM3 Abs (Fig 4A). All of the GM3-containing domains but only some of the GM1-containing domains colocalized with p56^{lck} (Fig 4A). Aggregation of p56^{lck} induced by CD4 ligand binding was not detected in the A201-2C>A T cell line, indicating that aggregation was dependent on CD4/p56^{lck} association (Fig 4A). Patching was not detected in either of the T cell lines following cross-linking with a control Ab (Fig 4A, Ct-2). However, CD4 cross-linking led to a transient increase in coprecipitation of GM3 with p56^{lck}, which was also dependent upon CD4/p56^{lck} association since it was not detected in the A201-2C>A T cell line (Fig 4B). TLC immunostaining analysis (a more sensitive method than resorcinol staining) using cholera toxin (CTxB) that specifically binds GM1 and anti-GM3Ab for GM3 detection (Fig 4C) revealed coprecipitation between p56^{lck} and GM1. Ganglioside association was not detected following incubation with a control Ab (Fig 4B, Ct). The same amount of p56^{lck} was immunoprecipitated in the HPTLC (Fig4B) and TLC (Fig4C) analysis in both T cell lines.

SHP-2 tyrosine phosphatase was previously reported to be required for the formation of a multi-protein complex with PI3K induced by CD4 ligand binding [2]. In this study, CD4 triggering induced very rapid redistribution of SHP-2 with both GM1 and GM3 (Fig 5A). After CD4 cross-linking for 20 minutes, SHP-2 was distributed predominantly in the cytoplasm, and was less aggregated in patches on plasma membrane, indicating that SHP-2 only transiently localized in these ganglioside-enriched domains. In contrast, GM1 and GM3 aggregation was maintained, indicating that the delocalization of SHP-2 from these domains was not due to disruption of the ganglioside-enriched domains. The rapid and transient localization of SHP-2 in GM1-and GM3-enriched domains was also dependent on CD4/p56^{lck} association, since SHP-2 aggregation was not detected in the A201-2C>A T cell line (Fig

5A). Patching was not detected following incubation with a control Ab (Fig 5A, Ct-2). However, coprecipitation between SHP-2 and GM1 and GM3 was not detected, suggesting that either SHP-2 does not interact directly with these gangliosides or alternatively, the affinity of these interactions was too low to be detected in immunoprecipitation conditions (Fig 5B).

Anti-CD4 Ab induces distinct interactions between p56^{lck}, SHP-2 and PI3K in DRM

We then investigated whether p56^{lck} co-localized and interacted with SHP-2 and PI3K in DRM. Immunofluorescence analysis showed that p56^{lck} did not colocalize with SHP-2 and PI3K in the same manner. Indeed, CD4 cross-linking induced a transient colocalization of p56^{lck} with SHP-2 (Fig 6A), whereas the colocalization of p56^{lck} with PI3K appeared stable up to 20 minutes (Fig 7A). These observations were consistent with those described above, showing the transient localization of SHP-2 in raft domains (Fig 5A), in contrast to PI3K (Fig3A) and p56^{lck} (Fig4A). Partial colocalization between SHP-2 and PI3K was observed (Fig 6B). Patching was not observed following incubation with a control Ab (Fig 6A-B, 7A).

The GM1- and GM3-enriched DRM containing the isolates from activated and unstimulated T cells obtained by Optiprep density gradient and ultracentrifugation were mainly detected in fractions F2-F4 (Fig 6C, top panel). F1 corresponds to the top of the gradient, and the detergent-soluble-membrane was detected in fractions F6 and F7. Neither GM1 nor GM3 were detected in F1, F6 and F7 (Fig 6C). P56^{lck} (Fig 6C, bottom panel and Fig S2A) was detected in the immunoprecipitates from DRM fractions 2 to 4 and one of the detergent-soluble fractions (F6) in the absence of, or following, CD4 cross-linking. In contrast, SHP-2 was mainly detected in detergent soluble fraction (F6) and DRM fraction 4 in the absence of CD4 cross-linking (Fig 6D and Fig S2B). Following CD4 cross-linking for 2 minutes, SHP-2 was predominantly detected in F3 (RN=4, Fig 6C and Fig S2B) and F4 DRM-containing fractions (RN=2.5, Fig 6C RN=5, Fig S2B). Following longer CD4 ligation, SHP-2 was detected in all of the tested fractions (Fig S2B). However, SHP-2 did not coprecipitate with p56^{lck} in any of the DRM fractions with the exception of the detergent-soluble fraction 6 (Fig 6C and FigS3A). Similarly, SHP-2 only coprecipitated with PI3K (Fig

6D and Fig S3B) in detergent-soluble membrane fraction (F6), as previously reported in other conditions [2]. Coprecipitation between SHP-2 and PI3K was not detected in any of the DRM fractions (Fig 6D and Fig S3B). In contrast, PI3K was detected in all of the fractions following CD4 ligation (Fig S2C and S3B) as expected, and was also co-precipitated with p56^{lck} in all of the fractions (Fig 7B and Fig S3C).

DISCUSSION

Overall, these results indicate that CD4 ligand binding induces colocalization and aggregation of several proteins involved in CD4 signaling in detergent-insoluble membrane domains. Lateral compartmentalization of the plasma membrane into raft domains is a key feature of immune cell activation and subsequent functions [27]. In previous studies, we have shown that CD4 ligation induced both aggregation of GM1 and GM3 gangliosides and colocalization of LFA-1 and PI3K required for the down-regulation of LFA-1-mediated T-B cell adhesion [24]. In the present study, we show that CD4 signaling also induces a strong protein-ganglioside association within lipid rafts and we provide evidence that the CD4-associated tyrosine kinase p56^{lck} required for the down-regulation of LFA-1-dependent adhesion induced by CD4 ligation [25] is also localized in ganglioside-enriched domains. In addition, given the absence of aggregation and protein recruitment when a mutant T cell line expressing CD4 unable to bind p56^{lck} was tested, we propose that p56^{lck} is required for the aggregation of GM1 and GM3, as well as for the recruitment and association of LFA-1 and PI3K with these gangliosides. These results appear to contrast with those reported by Fragoso *et al* showing that association between CD4 and p56^{lck} is not essential for CD4-induced lipid raft aggregation [12]. The most likely explanation for this difference could be the use of an anti-CD4 Ab (OKT4) that binds to the region of CD4 (D4) proximal to the transmembrane domain by Fragoso's team, whereas we used the 13B8.2 which recognizes an epitope located within the first NH₂-terminal domain (D1). The signal induced by these two Abs differs because only anti-CD4 Abs binding to the D1 CD4 domain (such as Leu3a, OKT4a and 13B8.2) inhibit Ag-independent LFA-1-dependent T cell adhesion to B cells [28]. In our study, inhibition was not detected with other anti-CD4 Abs, such as OKT4 (F. Mazerolles, unpublished observation). In addition, the pattern of tyrosine phosphorylated proteins following binding of either OKT4 or D1 domain specific ligands is distinct [29, 30]. Fragoso *et al* observed enhanced tyrosine phosphorylation of several proteins induced by OKT4 binding in the absence of association between CD4 and p56^{lck} in these mutant lines, showing that this setting

differs from ours. Moreover, as OKT4a and Leu3a epitopes are required for GM1 signaling in contrast to OKT4 [31], it appears that cross-linking of distinct CD4 epitopes induces different signaling and aggregation of raft domains in a CD4/p56^{lck} association dependent or independent manner. Since 13B8.2 binds to the HLA class II interactor domain of CD4, it may be a more appropriate physiological model for inducing CD4 aggregation than OKT4. Furthermore, our results are consistent with reports showing that in the absence of p56^{lck}, lipid rafts do not form caps following CD4 engagement so CD4 ligation may provide a sufficient signal to induce aggregation of lipid rafts in p56^{lck} (+) Jurkat cells in the absence of TCR engagement [14].

We also show that p56^{lck} totally colocalized with GM3-containing domains in a CD4/p56^{lck} association-dependent manner, while interaction between p56^{lck} and GM1 was less intense. Since CD4 associates with GM3 [32], CD4 appears to be the main intermediary between p56^{lck} and GM3. On the contrary, as CD4 does not associate with GM1 in a similar fashion, additional partners are likely to be inserted between GM1 and the CD4/p56^{lck}-complex (as proposed in the model depicted in Fig 8). Interestingly, the association between p56^{lck} and GM1 and GM3 gangliosides did not persist after prolonged CD4 cross-linking (20 minutes) whereas p56^{lck} remained localized in the DRM domains, indicating that p56^{lck} can persist in rafts without association with gangliosides. The localization of p56^{lck} in lipid rafts in the absence of TCR activation is controversial. In unstimulated cells, most of the p56^{lck} colocalizes with CD4 outside of lipid rafts and translocates into raft domains upon antigen stimulation [12]. Raft integrity has been shown to be important for p56^{lck} activity [33]. On the other hand, others have reported that p56^{lck} localizes mainly in lipid rafts [32, 34]. Our results support the view that p56^{lck} can localize within rafts in the absence of antigen presentation, but is further recruited and clustered in these domains following CD4 engagement. They also confirm that CD4 ligation plays a critical role in the recruitment of signaling proteins to rafts and may facilitate cellular reorganization by recruiting adhesion and signaling molecules [14].

We also provide evidence that CD4/p56^{lck} association is required for the recruitment of LFA-1, PI3K, and also SHP-2 which is another partner involved in the down-regulation of

LFA-1-dependent adhesion induced by CD4 ligand binding [2]. This result supports the hypothesis that CD4 associated p56^{lck} could induce the recruitment of adhesion and signaling molecules in DRM likely to concentrate these proteins in signaling platforms [12]. Our finding that LFA-1 and PI3K were not always coprecipitated with the same gangliosides during CD4/p56^{lck} induced signaling suggests that these proteins move to different domains containing GM1 and/or GM3 gangliosides which are not always colocalized. However, spatial resolution would be necessary to differentiate GM1- or GM3-containing domains. It is possible that prolonged CD4 ligation induced a modification of affinities between proteins and gangliosides and cannot be excluded. Indeed, PI3K did not appear to move from the DRM during CD4 ligation. It is likely that PI3K indirectly associates with GM3 via the p56^{lck}/CD4 complex because CD4 cross-linking also induced association between PI3K and p56^{lck} in the GM3-enriched fractions. The fact that PI3K was less associated with GM3 than with GM1 suggests that the PI3K isoforms (α and β , indistinguishable with the antibody we used) have a distinct affinity for these gangliosides. On the other hand, LFA-1 partially and stably colocalized with GM1, without notable modification after longer CD4 ligation, while the coprecipitation of LFA-1 with GM1 only slowly decreased. In addition, LFA-1 totally colocalized with GM3 after a short period of CD4 ligation, followed by a decrease in colocalization. This decrease was correlated with a rapid decrease in LFA-1/GM3 coprecipitation, suggesting a possible modification of affinity between these molecules. This modification can also induce a modification of LFA-1 localization in distinct ganglioside-enriched-domains. A similar raft segregation of membrane-receptor redistribution has also been described in migrating cells [35, 36]. Integrins colocalized with GM1-enriched rafts redistributed to the uropod, whereas PI3K colocalized with GM3-enriched rafts redistributed to the leading edge. As the GM3/CD4 association is required for CD4 signaling [32] [37] leading to the down-regulation of the LFA-1-dependent adhesion induced by CD4 ligation [24], GM3 could be a transducer of transmembrane signaling and play a role in cell adhesion regulation by triggering partners in a multi-protein complex formation [38]. The molecular basis of these ganglioside/protein interactions remains to be determined. Indeed, isoforms of

PI3K and LFA-1 are not acylated by saturated fatty acids such as p56^{lck} or CD4 to partition into rafts. Thus, we suggest, that in addition to p56^{lck}, other unidentified proteins moving in lipid rafts (X and Y in the model) are also involved in these interactions [15]. LFA-1 may bind cytohesin-1, which binds D3 phospholipids that are enriched in rafts, and other partners participating in the down-regulation of LFA-1 adhesion [2]. This localisation in rafts would allow LFA-1 to have a stronger affinity for its ligand and enhance its stability, and increase the activity of PI3K. SHP-2, which associates with PI3K outside of rafts following CD4 binding [2], may be another intermediate regulator of src kinase activation due to its localization in raft domains [39]. However, although CD4 ligation induces rapid and transient SHP-2 localization in a CD4/p56^{lck} association-dependent manner, direct interaction between SHP-2 and gangliosides or with p56^{lck} and PI3K was not detected. Nevertheless, SHP-2 was coprecipitated with PI3K and p56^{lck} after CD4 ligation in detergent-soluble membrane. The absence of coprecipitation in rafts suggests a possible alternative role for SHP-2 and other partners. We recently observed that outside of raft domains, SHP-2 can associate with the serine kinase PDK1, a specific effector of PI3K which also binds D3 phospholipids (Mazerolles et al unpublished data). Thus, it is possible that PDK1 also migrates to rafts with SHP-2 and could be the intermediary between PI3K and SHP-2. This hypothesis is under investigation.

In conclusion, these results further support our previous findings of the key role of CD4/p56^{lck} association in CD4 signaling. Indeed, this association, which is important for p56^{lck} and PI3K activities induced by CD4 ligation [25, 29], appears to be required for the redistribution of these kinases in ganglioside-enriched domains and colocalized with LFA-1 and SHP-2 that is involved in the regulation of adhesion. Our results also suggest that gangliosides associate with distinct proteins that probably have different affinity binding, and can be modified following CD4 signaling. Through these associations, gangliosides could transiently sequester these proteins and consequently inhibit LFA-1-dependent adhesion.

Acknowledgements

We thank J.Lipecka for invaluable help with image acquisition with the confocal microscope.

Grant support:

M. Trucy was supported by a doctoral fellowship from La Ligue Nationale Contre le Cancer.

This work was supported by INSERM

Abbreviations

AU: Arbitrary units

GAMIg: Goat anti-mouse Ig

SHP-2: Src homology 2 domain containing phosphatase

PDK1: Phosphoinositide-dependent-protein kinase-1

FIGURE LEGENDS

Figure 1. Role of CD4/p56^{lck} association on GM1 and GM3 distribution following CD4 ligation

A201-CD4 (panels A and C) and A201-2C>A T cell lines (panels B and C) were incubated at 4°C for 15 minutes with either anti-CD4 Ab (A) or an anti-transferrin receptor Ab used as control (Ct) then either cross-linked with GAMIg during the time indicated or incubated with GAMIg alone (NA or G-) at 37°C for 10 minutes. GM1 and GM3 were detected with appropriate Abs-and colocalizations are shown in the merged panels. A threefold enlargement (3X) of single cells is shown in the merged panels (marked with an arrow). Data depict one representative experiment out of ten. In panel C, following CD4 cross-linking, cells were washed, lysed and the presence of the gangliosides was detected by HPTLC. The standard gangliosides are indicated (St).

Figure 2. Role of CD4/p56^{lck} association on colocalization and association of LFA-1 with GM1 and GM3 gangliosides following CD4 ligation.

A201-CD4 and A201-2C>A T cell lines (5×10^4 in panel A, 40×10^6 in panel B) were incubated at 4°C for 15 min with either an anti-CD4 Ab (A-) or an anti-transferrin receptor Ab (panel A; Ct-2) or an anti-HLA-I Ab (panel B; Ct), used as control, then cross-linked with GAMIg during the indicated time or treated with GAMIg alone at 37°C for 20 min (NA in panel A, G in panel B). In panel A, LFA-1, GM1 and GM3 are shown with the appropriate Abs; colocalizations are shown in merged panels. The right column shows a threefold enlargement (3X) of single cells marked with an arrow in merged panels. Data depict one representative experiment out of eight. In panel B, proteins were immunoprecipitated with an anti-LFA-1 Ab and analyzed for the presence of the gangliosides by HPTLC. Standard gangliosides are indicated (St). The amount of LFA-1 immunoprecipitated was verified with an anti-LFA-1 immunoblot.

Figure 3. Role of CD4/p56^{lck} association on colocalization and association of PI3K with GM1 and GM3 gangliosides following CD4 ligation.

A201-CD4 and A201-2C>A T cell lines (5×10^4 in panel A, 40×10^6 in panel B) were incubated at 4°C for 15 min either with an anti-CD4 Ab (A-) or with an anti-transferrin receptor Ab (panel A) and an anti-HLA-I Ab (panel B) used as control (Ct) then cross-linked with GAMIg for the indicated time or treated with GAMIg alone at 37°C for 20 min (NA in panel A, G in panel B). In panel A, PI3K, GM1 and GM3 were detected with appropriate Abs and colocalizations are shown in merged panels. The right column depicts a threefold enlargement (3X) of single cells stained in the merge. Data depict one representative experiment out of six. In panel B, proteins were immunoprecipitated with an anti-PI3K Ab and analyzed for the presence of the gangliosides by HPTLC. Standard gangliosides are indicated (St). The amount of PI3K immunoprecipitated was verified with an anti-PI3K immunoblot.

Figure 4. Colocalization and association of p56^{lck} with GM1 and GM3 induced by CD4 ligation is dependent on CD4/p56^{lck} association

A201-CD4 and A201-2C>A T cell lines (5×10^4 in panel A, 40×10^6 in panel B) were incubated at 4°C for 15 min either with an anti-CD4 Ab (A-) or an anti-transferrin receptor Ab (panel A) and an anti-HLA-I Ab (panel B) used as control (Ct) then cross-linked with GAMIg for the indicated time, or treated with GAMIg alone at 37°C for 20 min (NA in panel A, G in panel B). In panel A, localization of p56^{lck} with GM1 and GM3 was detected with appropriate Abs. Colocalizations are shown in the merge panels. Data depict one representative experiment out of six. In panel B, proteins were immunoprecipitated with an anti-p56^{lck} Ab and analyzed for the presence of the gangliosides by HPTLC staining analysis by resorcinol (panel B) or by cholera toxin (CTxB) for GM1 detection, and anti-GM3 Ab (panel C). Standard gangliosides are indicated (St). Densitometric analysis of GM1 and GM3 co-precipitated with p56^{lck} was indicated in arbitrary units (AU). The amount of p56^{lck} immunoprecipitated was verified with an anti-p56^{lck} immunoblot.

Figure 5. Colocalization without association of GM1 and GM3 with SHP-2 in a CD4/p56^{lck} association dependent manner.

A201-CD4 T cell lines (5×10^4 in panel A, 40×10^6 in panel B) and A201-2C>A T cell lines (5×10^4 in panel A, 40×10^6 in panel B) were activated as described in previous figures. In panel A, localization of SHP-2 with GM1 and GM3 was detected with appropriate Abs and colocalizations were shown in merged panels. A threefold enlargement (3X) of single cells marked with an arrow in merged panels, was shown. Data depict one representative experiment out of six. In panel B, proteins were immunoprecipitated with an anti-SHP-2 Ab and analyzed for the presence of the gangliosides by HPTLC as described in Figure 2. Standard gangliosides are indicated (St). Densitometric analysis of GM1 and GM3 coprecipitated with SHP-2 was indicated in Arbitrary Units (AU). The amount of SHP-2 immunoprecipitated was verified with an anti-SHP-2 immunoblot.

Figure 6: Distribution and association of SHP-2 with p56^{lck} and PI3K in GM1- and GM3- containing microdomains following anti-CD4 Ab incubation.

A201-CD4 T cell lines (5×10^4 in panels A and B, 25×10^6 in panels C and D,) were incubated for 15 min at 4°C either with anti-CD4 Ab or an anti-transferrin receptor Ab used as control (Ct), then cross-linked with F(ab')₂ GAMIg during the indicated time at 37°C, or treated with GAMIg alone (NA and G-20min). In panels A and B, p56^{lck}, SHP-2 and PI3K are detected with appropriate Abs and colocalizations are shown in merged panels. The threefold enlargement (3X) of single cells is marked with an arrow in merged panels. In panels C and D, raft containing fractions were collected from cells that were activated, lysed and subjected to Optiprep density gradient (F1: top of the gradient; F2-F4: raft fractions; F6 and F7: fractions containing soluble-detergent membrane). GM1 was detected by HPTLC immunostaining using a CT-HRP conjugate and GM3 by HPTLC followed by resorcinol staining. Proteins isolated from the different fractions were immunoprecipitated with an anti-p56^{lck} Ab (panel C) or an anti-SHP-2 Ab (panel D) then detected by immunoblotting with an anti-p56^{lck} Ab (panel C) or an anti-SHP-2 (panels C and D) or an anti-PI3K (panel D).

Control Ab (ip Rb) was used for the non-specific immunoprecipitation. Data depict one representative experiment out of six. Proteins are indicated in each panel. Quantification of the gangliosides detected in each fraction (indicated in arbitrary units (AU)), and the intensity of each band detected by anti-SHP-2, anti-p56^{lck} or anti-PI3K-specific antibodies were calculated using NIH software. Relative number (RN) from each fraction (Panels C and D), represents the increased factor of the different immunoprecipitated proteins (p56^{lck} or SHP-2) and co-precipitated proteins (SHP-2 or PI3K) in activated cells over unstimulated cells as compared to the respective GAMig control (G-20min).

Figure 7: Distribution and association of p56^{lck} with PI3K in GM1- and GM3-containing microdomains following anti-CD4 Ab incubation.

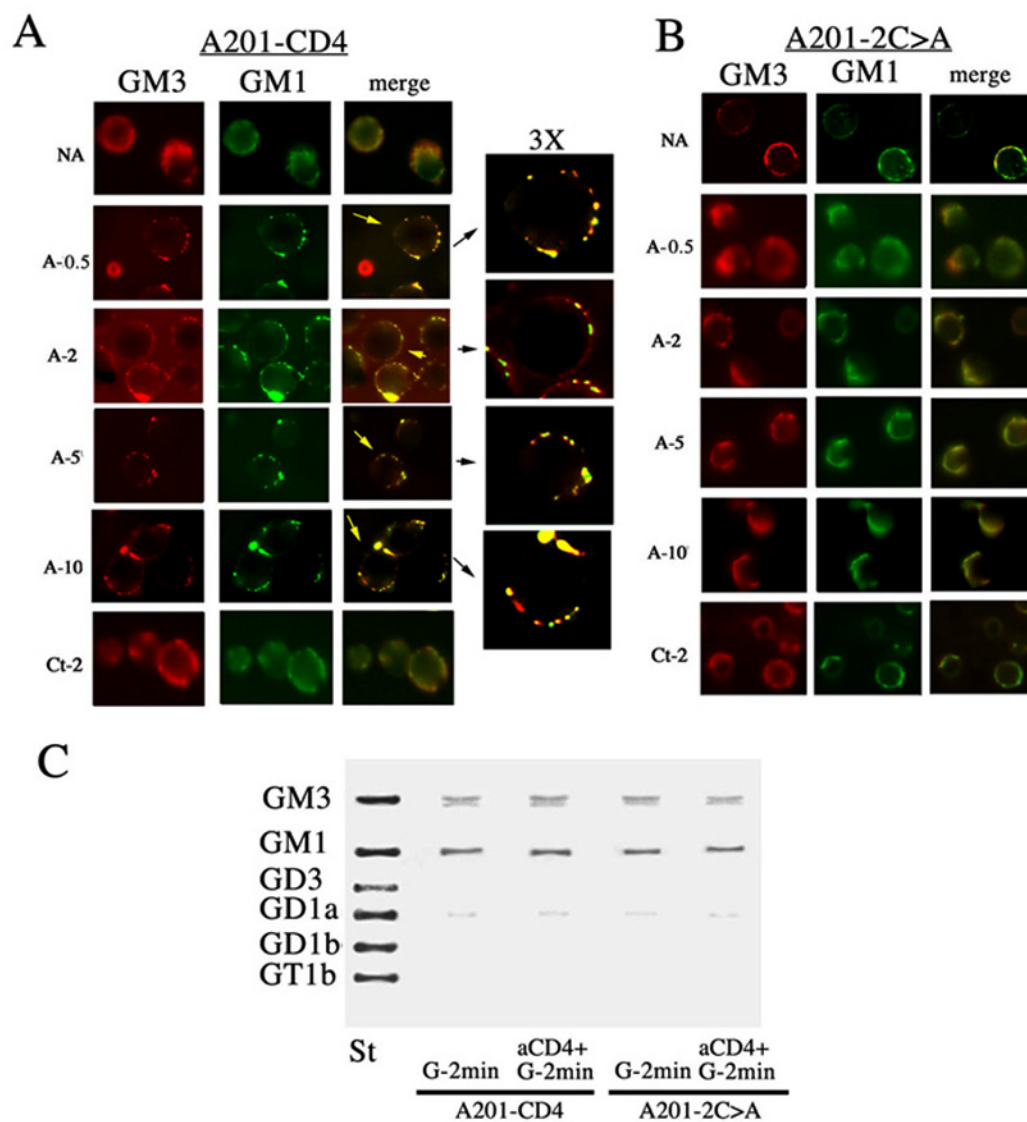
A201-CD4 T cell lines were similarly treated as described in Fig 6. In panel A, p56^{lck} and PI3K are detected with appropriated Abs and colocalizations are shown in merged panels. A threefold enlargement (3X) of single cells marked with an arrow in merged panels is shown. In panel B, as described in Fig 6, proteins were isolated from the seven fractions collected by Optiprep density gradient and ultracentrifugation and immunoprecipitated with an anti-p56^{lck} Ab then detected with an anti-p56^{lck} Ab or with an anti-PI3K. Control Ab (ip Rb) was used as a non-specific immunoprecipitation control. Data depict one representative experiment out of six. Proteins are indicated in each panel. Relative number (RN) in each fraction represents the ratio of p56^{lck} and PI3K immunoprecipitated in activated cells over unstimulated cells as compared to the respective GAMIg control (G-20min).

Figure 8: A model for the distinct membrane associations between LFA-1, PI3K, p56^{lck} and SHP-2 induced by CD4 ligation, in a CD4/p56^{lck} dependent manner.

In absence of CD4 ligation (0), LFA-1 is mainly localized in detergent-soluble domains of the membrane (Mb) and PI3K is associated with p56^{lck} in gangliosides-enriched microdomains or DRM, without association with GM1, nor with GM3, in contrast to p56^{lck}. After CD4 cross-linking for 2 minutes (aCD4+G-2min), LFA-1 has migrated into raft domains and is mainly associated with GM3 (directly or via the hypothetical intermediate protein Y) and with GM1

but to a lesser extent (directly or via the hypothetical intermediate protein X). In contrast, PI3K is mainly associated with GM1 (directly or via the hypothetical intermediate protein X), and its association with p56^{lck} increases. After CD4 cross-linking for 20 minutes (aCD4+G-20min), LFA-1 has moved and is mainly associated with GM1 and PI3K (directly or via X) that binds with GM1, and GM3 to a lesser extent. SHP-2 is detected in detergent soluble microdomains where it only associates with PI3K. It is also detected in DRM without association with gangliosides, p56^{lck} or PI3K.

FIGURE 1



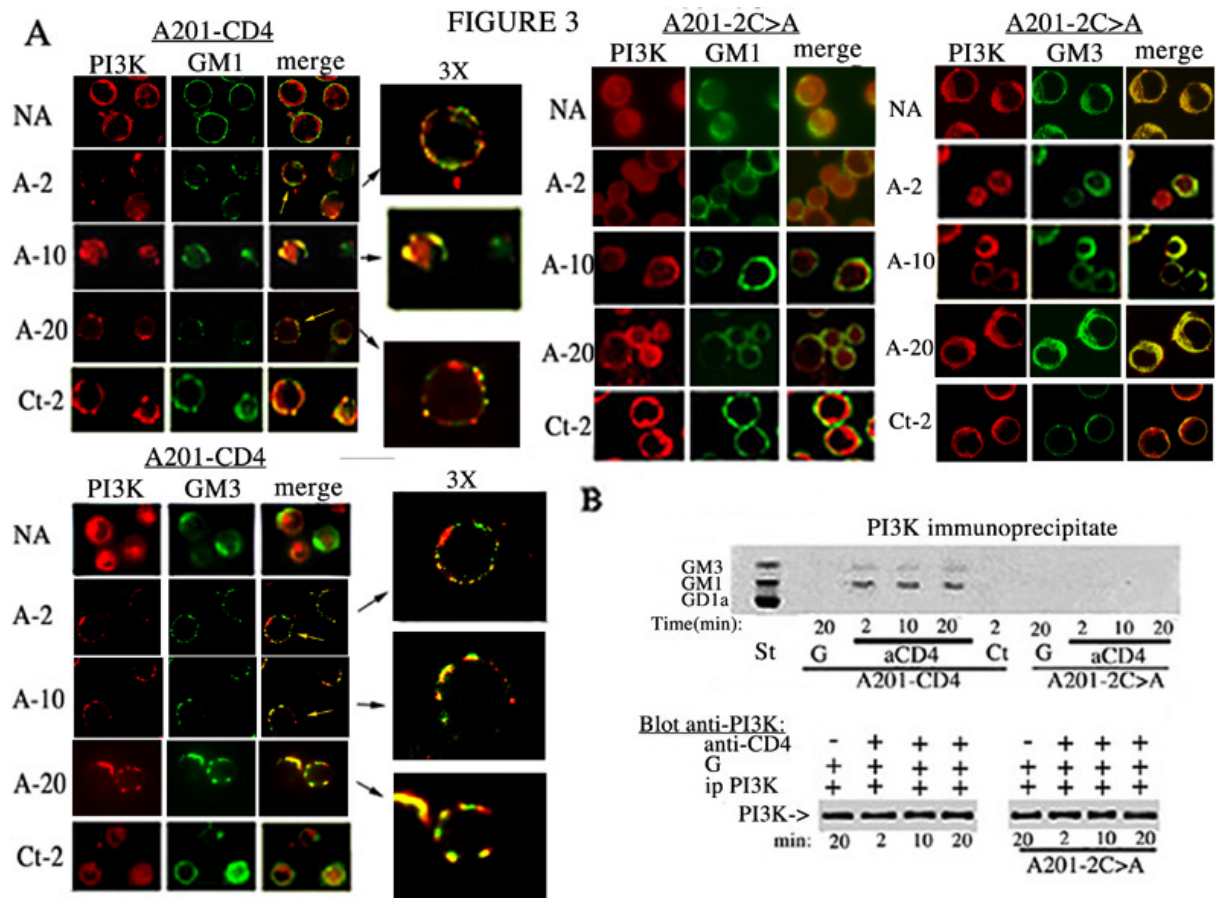
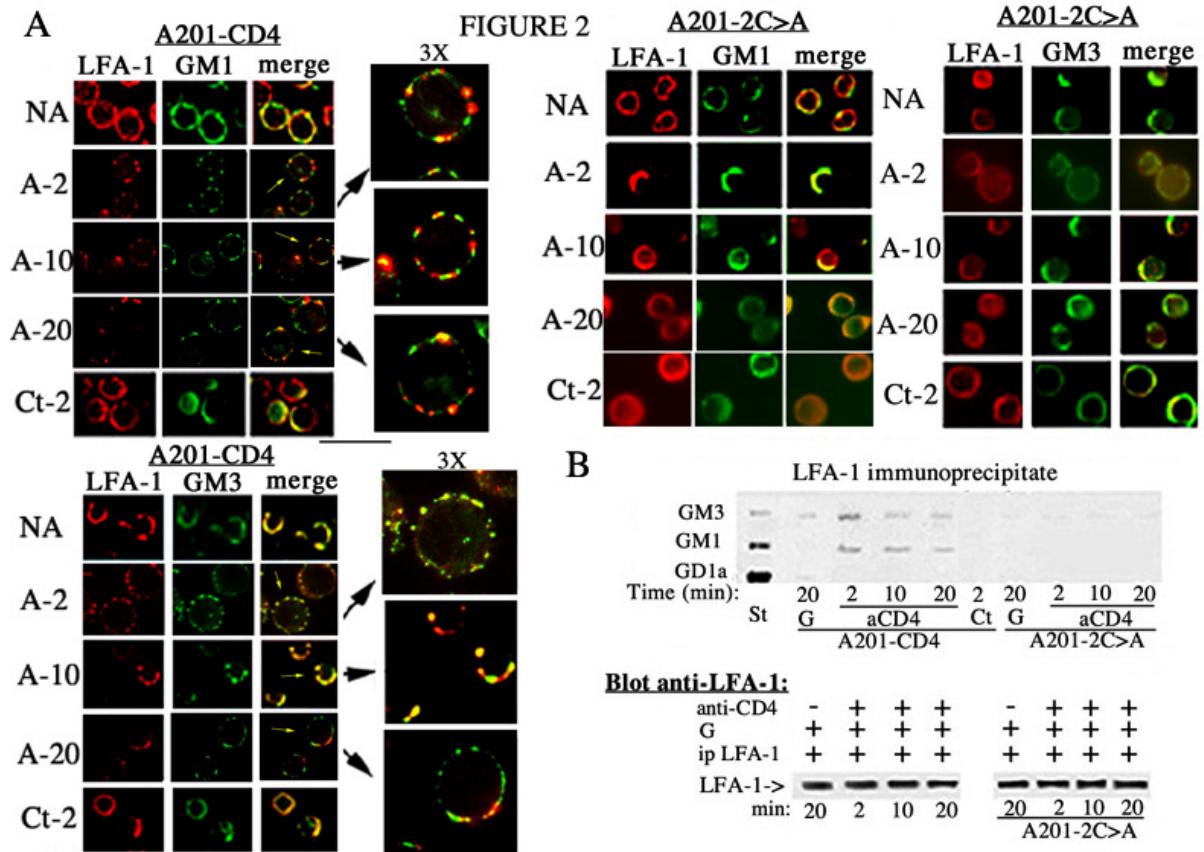


FIGURE 4

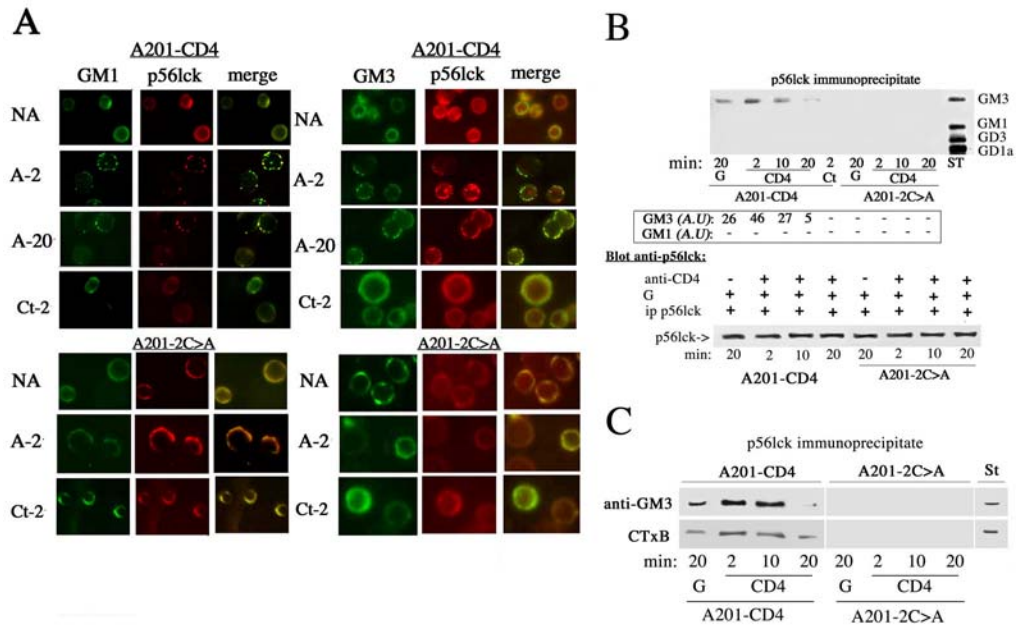


FIGURE 5

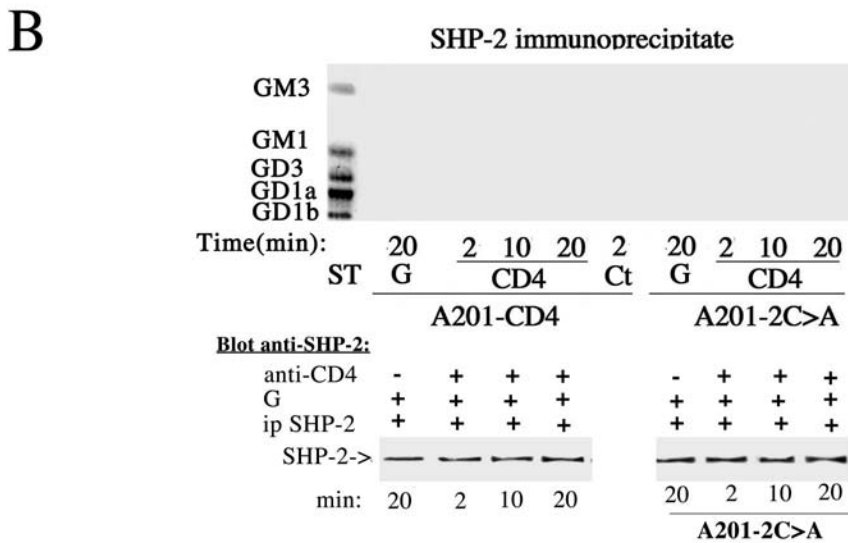
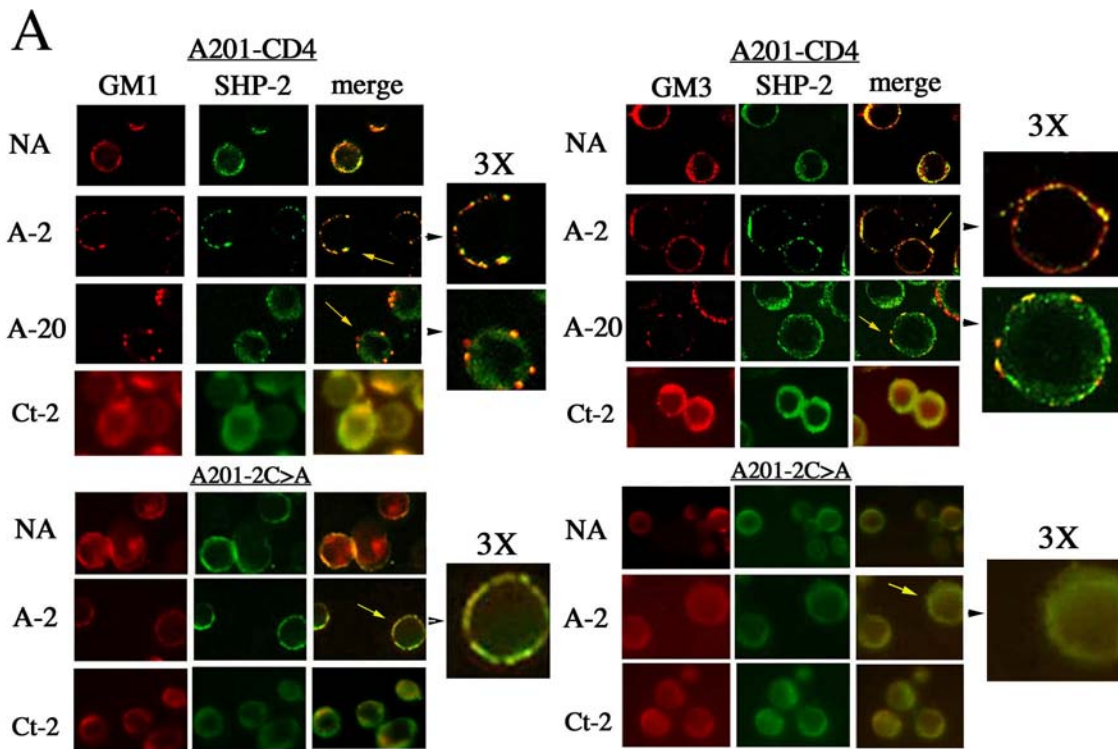


FIGURE 6

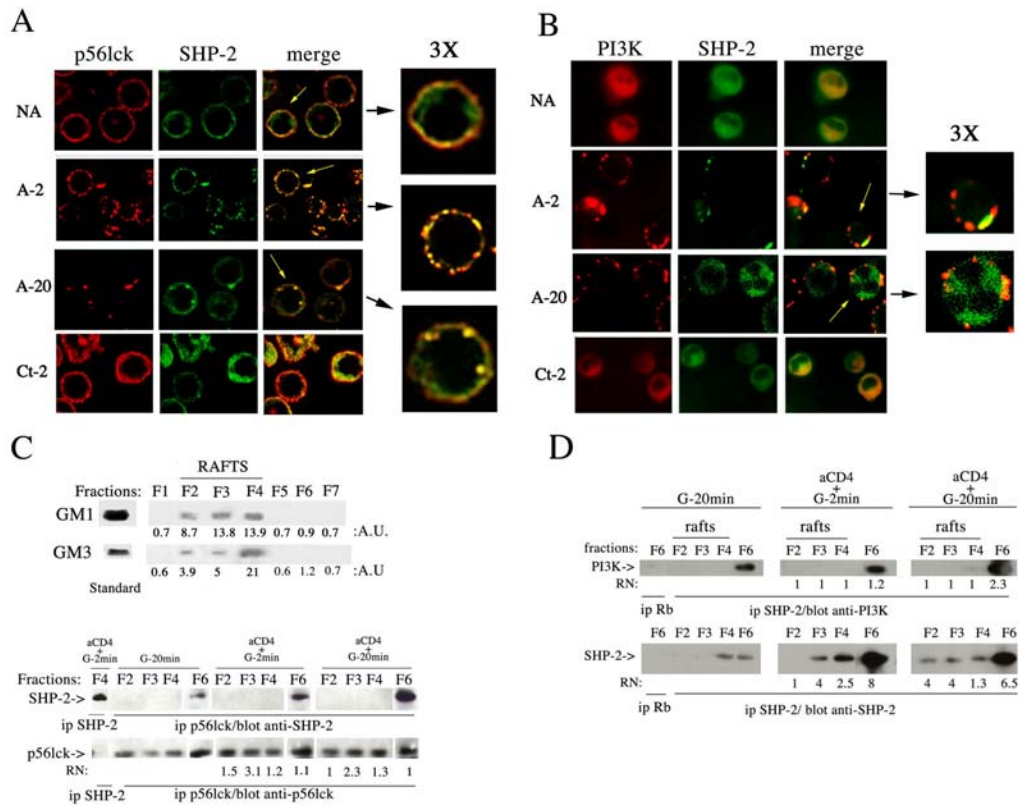


FIGURE 7

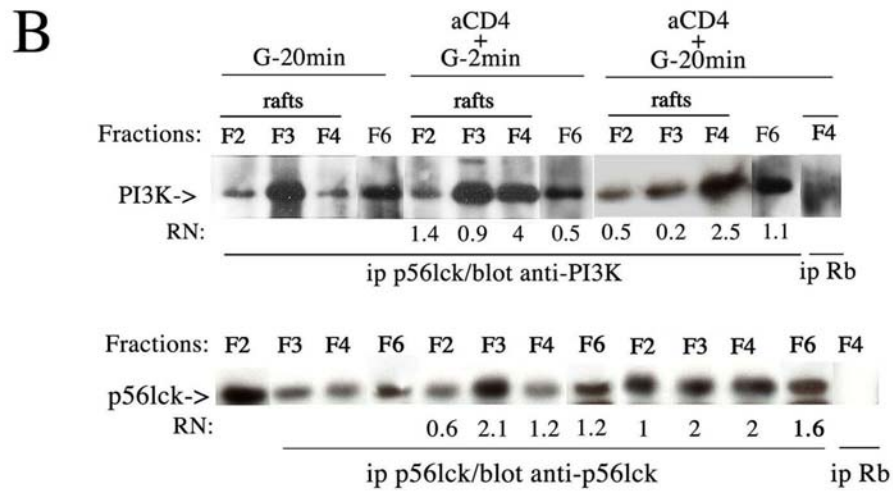
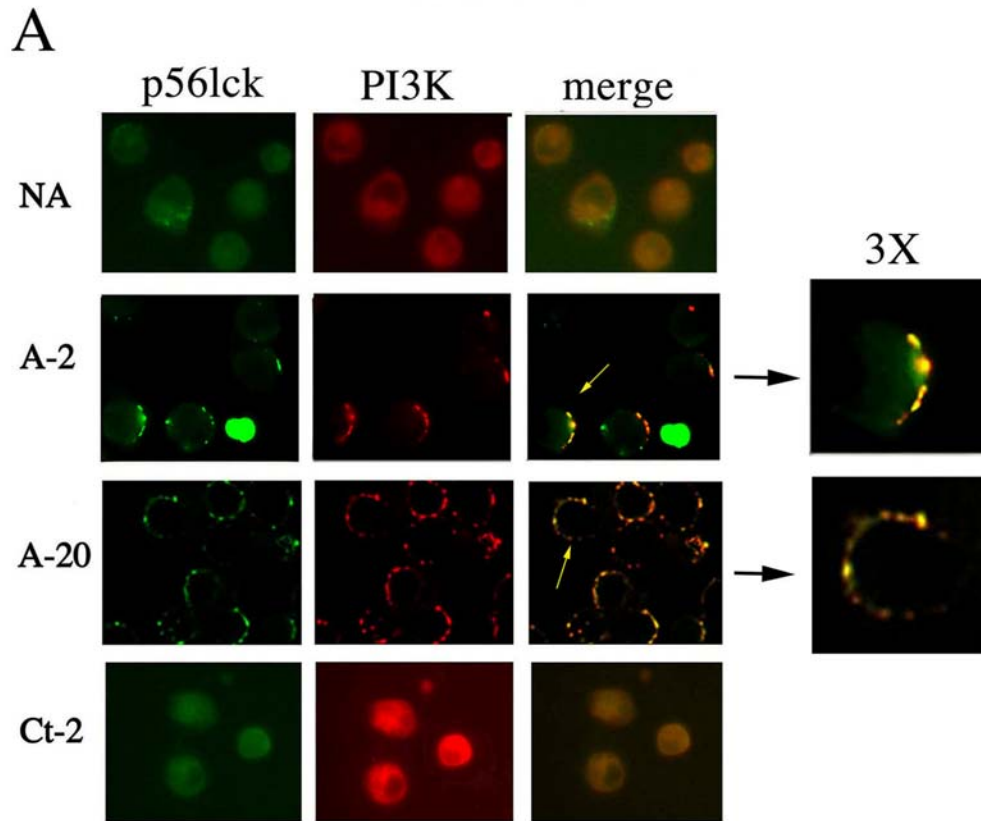
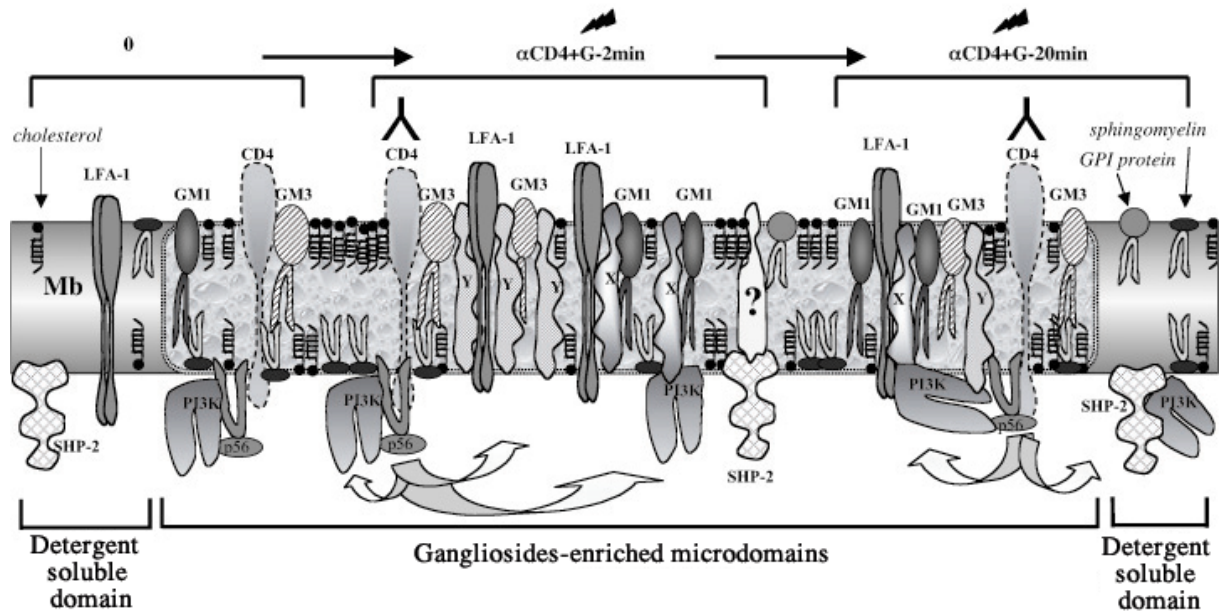


FIGURE 8

DISTINCT ASSOCIATIONS INDUCED BY CD4CROSS-LINKING IN DRM
IN A CD4/P56LCK DEPENDENT MANNER



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