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Different states of integrin LFA-1 aggregation are controlled through its association with tetraspanin CD9

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Running title: CD9 functionally associates with LFA-1 integrin

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Abstract:

The tetraspanin CD9 has been shown to interact with different members of the β 1 and β 3 subfamilies of integrins, regulating through these interactions cell adhesion, migration and signaling. Based on confocal microscopy co-localization and on co-immunoprecipitation results, we report here that CD9 associates with the β 2 integrin LFA-1 in different types of leukocytes including T, B and monocytic cells. This association is resistant to stringent solubilisation conditions which, together with data from chemical crosslinking, *in situ* Proximity Ligation Assays and pull-down experiments, suggests a primary/direct type of interaction mediated by the Large Extracellular Loop of the tetraspanin. CD9 exerts inhibitory effects on the adhesive function of LFA-1 and on LFA-1-dependent leukocyte cytotoxic activity. The mechanism responsible for this negative regulation exerted by CD9 on LFA-1 adhesion does not involve changes in the affinity state of this integrin but seems to be related to alterations in its state of aggregation.

1. Introduction

The $\beta 2$ subfamily of integrins comprises four distinct members, $\alpha L\beta 2$, $\alpha M\beta 2$, $\alpha X\beta 2$, and $\alpha D\beta 2$, that are selectively expressed on leukocytes (for review (Gahmberg et al., 1999; Harris et al., 2000; Cabanas et al., 2008)). The integrin $\alpha L\beta 2$, also termed LFA-1 (Lymphocyte Function-associated Antigen-1) or CD11a/CD18 antigen, is expressed on most types of leukocytes and primarily on lymphocytes, whereas expression of the rest of members of this subfamily is rather restricted to myeloid cells. LFA-1 interacts with intercellular adhesion molecules (ICAM-1, -2, or -3), playing a pivotal role in many crucial leukocyte functions that require intercellular adhesion, such as extravasation into tissues, organization of the immune synapse and antigen presentation, inter-lymphocyte collaboration and killing of target cells by CTL or NK cells (Springer et al., 1987; Sims and Dustin, 2002; Hogg et al., 2003; Anikeeva et al., 2005).

LFA-1 can exist in different states of activation regarding its ability to bind ligands. Resting T lymphocytes express LFA-1 with low affinity and avidity for ligands, necessary for their normal circulation in the blood as individual cells. However, activation by the TCR-CD3 complex or receptors for different cytokines and chemokines, results in rapid activation of LFA-1 enabling T cells to adhere to other cells (Dustin and Springer, 1989; van Kooyk et al., 1989; Cabanas et al., 2008). Activation of LFA-1 can be induced through changes in the affinity of individual integrin molecules, reflecting conformational alterations, or through changes in the valency of interactions with multivalent ligands (Carman and Springer, 2003). In terms of ligand binding affinity, at least three different conformational states of LFA-1 have been identified: a bent conformation of low affinity, an extended conformation with closed headpiece displaying intermediate affinity, and a high affinity extended conformation with open headpiece and separated intracellular tails (Carman and Springer, 2003; Hogg et al., 2011). PMA induces the intermediate affinity state but also increases the diffusion of LFA-1 molecules on the cell surface, which in the presence of multivalent ligand, leads to aggregation/clustering of this integrin (Carman and Springer, 2003; Kim et al., 2004). On the other hand, extracellular Mn^{2+} induces the high affinity conformation of LFA-1 (Dransfield et al., 1992b; Carman and Springer, 2003) but also induces changes in the local density (i.e. the clustering) of integrin

molecules, which is reflected by changes in the number of effective adhesive bonds (i.e. the valency) with ligand (Rodriguez-Fernandez et al., 2001; Cluzel et al., 2005).

CD9 is a member of the tetraspanin family of integral membrane proteins (Charrin et al., 2009; Yanez-Mo et al., 2009) abundantly expressed on the surface of endothelial cells, some leukocytes and many types of tumour cells (Sincock et al., 1997; Charrin et al., 2001; Hemler, 2013). CD9 was initially characterized as a lympho-hematopoietic marker (Boucheix et al., 1985) and received the name “Motility-Regulatory Protein” (MRP) (Miyake et al., 1991). CD9 has been also implicated in the formation and maintenance of muscular myotubes (Tachibana and Hemler, 1999), in nervous cell neurite outgrowth (Schmidt et al., 1996), and in sperm-oocyte fusion (Chen et al., 1999). Like other tetraspanins, CD9 associates on the cell surface with different integrins and many of the functional effects that have been attributed to CD9 may be indeed related to its ability to associate to integrin molecules (Berditchevski, 2001; Bassani and Cingolani, 2012).

Functional interactions of CD9 with several members of the $\beta 1$ and $\beta 3$ subfamilies of integrins have been reported, usually based on the effects exerted by CD9-specific mAbs on the associated integrin-dependent adhesion, migration and signalling (reviewed in (Berditchevski, 2001; Bassani and Cingolani, 2012). However, to our knowledge, only one published report includes some indication –merely based on co-immunoprecipitation evidence- suggestive of a possible interaction between CD9 and the $\beta 2$ integrin subunit (Takeda et al., 2003), although this study did neither address the type or functional aspects of this interaction. In addition, functional association of LFA-1 with tetraspanins CD81 (the most closely related to CD9) and CD82, has been described on T lymphocytes (Shibagaki et al., 1999; VanCompennolle et al., 2001), which prompted us to investigate in more detail whether CD9 is also functionally associated with the $\beta 2$ integrin LFA-1 on leukocytes.

We report here that CD9 associates directly with LFA-1 in different types of leukocytes and exerts inhibitory effects on its adhesive capacity and on leukocyte LFA-1-dependent cytotoxic activity.

2. Materials and Methods

Cells and cell cultures. Primary T lymphoblasts were obtained from peripheral blood mononuclear cells from healthy donors treated with 5 µg/ml phytohemagglutinin (Amersham Biosciences) for 48 h, as described previously (Dransfield et al., 1992a; Luque et al., 1996). Cells were then cultured for 7-10 days in RPMI-1640 containing 10% FBS and 50 U/ml IL-2 (Eurocetus). HSB-2 and Jurkat (T cell lines), JY and Daudi (B cell lines) and THP-1 and U937 cells (monocytic cell lines) were cultured in RPMI-1640 supplemented with 10% FBS, antibiotics and glutamine. THP-1 and U937 differentiation into macrophage-like cells was induced with PMA (100 ng/ml) for 24 h.

Expression Constructs and RNA silencing transfection. For stable transfection experiments, HSB-2 and U937 cells were electroporated with 20 µg pcDNA3-CD9 plasmid at 200 V (2 x 10 ms pulses in a 0.4 cm electroporation cuvette) using an ECM830 BTX electroporation system and selected with 1 mg/ml G418. For CD9 silencing, Jurkat cells were retrovirally transduced (OriGene Technologies) with the shRNA-coding plasmids TI356235 (the plasmid with the CD9 shRNA cassette insert) and TR20003 (“TR2” control plasmid without shRNA insert), according to manufacturer’s indications and selected with 1 µg/ml puromycin.

Antibodies and reagents. ICAM-1-Fc chimeric protein consisting of the five domains of ICAM-1 fused to the Fc region of human IgG1 was prepared as described (Berendt et al., 1992). Anti-β-tubulin antibody was purchased from Sigma and the anti-CD18 biotin-conjugated antibody (MEM-48) from ImmunoTools. The antibodies anti-β2 integrin Lia3/2 (Campanero et al., 1993) and TS1/18 (Sanchez-Madrid et al., 1983), anti-αL TP1/40 (Campanero et al., 1993) and TS1/11 (Sanchez-Madrid et al., 1983), anti-αM Bear-1 (Keizer et al., 1985) and anti-αX HC1/1 (Cabanas et al., 1988), anti-CD9 VJ1/20 (Yanez-Mo et al., 1998), PAINS-10 and PAINS-13 (Gutierrez-Lopez et al., 2003), anti-CD147 VJ1/9 and anti-CD59 VJ1/12 (Yanez-Mo et al., 1998) and anti-HSPA8 PAINS-18 (Gutierrez-Lopez et al., 2003; Gilsanz et al., 2013) were purified by protein A or protein-G affinity chromatography. The anti-CD81 5A6 mAb was kindly provided by Dr. Shoshana Levy (Stanford University School of Medicine, USA), the β2 stimulatory mAb KIM185 by Dr. Martyn K. Robinson (UCB-Celltech., Slough, UK),

the anti-CD105 mAb P4A4 by Dr. Carmelo Bernabeu (CIB-CSIC, Madrid, Spain) and the anti- β 2 mAb m24 by Dr. Nancy Hogg (Cancer Research UK, London, UK).

Flow Cytometry Analysis. For protein surface expression analysis cells were washed twice in RPMI-1640, incubated with primary antibodies at 4 °C for 30 min, followed by FITC-conjugated anti-mouse IgG (Sigma) and fixed in 2% formaldehyde in PBS. For flow cytometric analysis of m24 epitope expression, HSB-2 and JK cells were washed in cation-free PBS and incubated for 15 min at 37 °C with Mn^{2+} (10, 20, 40, 100 and 400 μ M) or with Ca^{2+}/Mg^{2+} (0.5 mM and 1 mM respectively) in the presence of mAb 24 (5 μ g/ml), washed and stained with secondary FITC-anti-mouse IgG. Fluorescence was measured using a FACScan™ flow cytometer (Beckton-Dickinson).

Immunofluorescence, Confocal and TIRF Microscopy. For immunofluorescence studies cells, treated or not with 0.4 mM Mn^{2+} for 20 min at 37 °C, were seeded on 12-mm glass coverslips coated with poly-L-lysine (50 μ g/ml). Cells were fixed in 2% paraformaldehyde, blocked in 1% BSA in TBS and incubated for 1 h with TS1/11 or TS1/18 mAbs (10 μ g/ml), followed by secondary antibody Alexa Fluor™-594 anti-mouse IgG (Invitrogen), rabbit polyclonal anti-CD9 antibody H-110 (Santa Cruz Biotechnology) and Alexa Fluor™-488 anti-rabbit IgG (Invitrogen). For THP-1 cells, Fc receptors were saturated with human gammaglobulin for 30 min, prior to fixation. Samples were mounted with Mowiol reagent (Calbiochem) and images were obtained with a Zeiss LSM510 Meta inverted microscope. Fluorescence co-localization histograms and Pearson coefficient values were obtained using the Fiji plug-in “Intensity correlation analysis” (Li et al., 2004; Schindelin et al., 2012). Fiji software was also used for setting the threshold and for detection and quantitation of fluorescent objects.

For TIRF (total internal reflection fluorescence) microscopy, JK TR2 and JK shCD9 cells were first activated with PMA (200 ng/ml for 2 hours), then plated onto ICAM-1-Fc-coated (14 μ g/ml) 35 mm petri dishes with glass bottom (2.5×10^5 cells/plate), and incubated for 90 min at 37 °C to allow adhesion. After washing non adhered cells with PBS, adhered cells were fixed in 2% paraformaldehyde (10 min, room temperature) and then permeabilized with 0.3% Triton-X100 in TBS buffer. Immunofluorescence staining of beta-2 integrin with mAb TS1/18 was performed as described above, and

images were obtained with an inverted Olympus Xcellence IX83P2ZF TIRF microscopy system. Fiji software was used for setting the threshold and for detection and quantitation of fluorescence in clusters.

Co-immunoprecipitations. Co-immunoprecipitation experiments were performed using intact cells, in order to detect only surface protein-protein interactions. Cells were incubated for 15 min at 37 °C (or for 60 min at 4 °C in parallel control experiments) with the specific TS1/18 (anti-β2) or VJ1/20 (anti-CD9) or control anti-CD105 and anti-CD59 antibodies in the presence of 0.5 mM Ca²⁺/1 mM Mg²⁺, followed by washing the antibody excess. Cells were then lysed for 15 min at 4 °C in TBS containing 1% Brij-97 or 1% Triton-X100 in the presence of corresponding extracellular cations and protease inhibitors and, after removal of insoluble material, incubated overnight at 4 °C with protein G-Sepharose. Beads were then washed with 1:5 diluted lysis buffer, boiled in nonreducing Laemmli buffer, resolved by 8% (for β2 detection) or 12% (for CD9) SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were then blocked with 3% BSA and developed with the β2 (MEM-48) or CD9 (VJ1/20) biotin-conjugated antibodies followed by streptavidin HRP (Thermo scientific) and ECL-chemiluminescence.

Covalent chemical cross-linking. THP-1/PMA and JY cells were extracted in 1% Brij97 lysis buffer (containing 20 mM Hepes, 150 mM NaCl, 0.5 mM CaCl₂, 3 mM MgCl₂ and protease inhibitors), pH 7.4. After removal of insoluble material, lysates were treated with 0.25 mM thiol-cleavable cross-linker 3,3'-dithiobis(sulfosuccinimidylpropionate) (DTSSP) for 30 min at 4°C. The cross-linking reaction was quenched for 15 min at room temperature with 10 mM glycine, pH 7.4, and then Triton X-100 was added to a final concentration of 1% (v/v) to cross-linked lysates and to parallel non-cross-linked lysates used as controls. Samples were immunoprecipitated with anti-β2 integrin mAb TS1/18, with anti-CD9 mAb VJ1/20 or with anti-CD9 pAb (H110), as indicated, resolved by SDS-PAGE either under reducing conditions (to break the thiol bond in DTSSP-crosslinked protein complexes) or under non-reducing conditions and subsequent immunoblotting with biotinylated anti-CD9 (VJ1/20) or anti-β2 integrin (MEM-48) mAbs, followed by streptavidin-HRP (Thermo Scientific) and ECL-chemiluminescence.

Pull-down assays. GST-fusion proteins containing the LEL region from human wt CD9, wt CD81 and wt CD63 were prepared as previously described (Higginbottom et al., 2003; Barreiro et al., 2005; Gutierrez-Lopez et al., 2011). JY and THP-1 cells were washed three times in PBS and either left untreated or their surface proteins biotinylated with 1 mM EZ-Link^R Sulfo-NHS-LC-Biotin (Thermo Scientific) in PBS with 1mM CaCl₂, 1mM MgCl₂ for 30 minutes at 4°C. Cells were then washed twice in PBS+100 mM glycine to quench and remove excess biotin. Biotinylated and non-biotinylated cells were lysed in 1% Brij-97 or in 1% Triton-X100 buffer and incubated overnight at 4 °C with equal amounts of GST-fusion proteins, pulled down with glutathione-agarose 3 h at 4 °C, washed in 1:10 diluted lysis buffer and boiled in non-reducing Laemmli buffer. For non-biotinylated cells, the presence of β2 integrin in the pulled-down complexes was revealed by immunoblotting with biotinylated mAb MEM-48 and detection with streptavidin-HRP and ECL-chemiluminescence. The levels of pulled-down GST-fusion proteins were assessed by western-blot using an anti-GST rabbit polyclonal antibody (Santa Cruz Biotechnology). For surface-biotinylated cells, all lysate proteins pulled-down by GST or by LEL-GST-CD9 were isolated with glutathione-sepharose beads and detected with streptavidin-HRP and ECL-chemiluminescence.

Cell Adhesion Assays. Static cell adhesion to ICAM-1-coated dishes was performed as described elsewhere (Rodriguez-Fernandez et al., 1999; Gilsanz et al., 2013). 96-well flat-bottom plates were pre-coated with 6 µg/ml (for THP-1/PMA and T-Lymphoblast cells) or 12 µg/ml (for JY, HSB2 and JK cells) of ICAM-1-Fc and blocked with 1% BSA. For PMA-stimulated cell adhesion, cells were incubated with 50 or 200 ng/ml of PMA in RPMI-1640 for 2 hours at 37 °C. Cells were loaded with the fluorescent probe BCECF-AM (Sigma) and added (2×10^5 cells/well) in adhesion medium (Hepes 20 mM, NaCl 149 mM, 2 mg/ml glucose), stimulated with 0.5 mM Ca²⁺/1 mM Mg²⁺ or 20-400 µM Mn²⁺ and incubated for 20-60 min at 37 °C. When indicated, 20 µg/ml of anti-CD9 (VJ1/20, PAINS-10 and PAINS-13), anti-β2 (Lia3/2 and KIM185) or the control anti-HASP8 (PAINS-18) mAbs were pre-incubated with cells for 15 min at 4 °C before transferring the plates to 37 °C. The plates were then washed by gravity with warm PBS for 20 min at 37 °C. The percentage of adherent cells was calculated by determining their fluorescence in a microplate reader (TecanGENios), considering as 100% the total

fluorescence of cells before washing. For determining cell adhesion under flow conditions, JK TR2 and JK shCD9 cells were first activated with PMA (100 ng/ml for 1 h) and labeled with CFSE or CMAC fluorescent probes and allowed to adhere for 15 min at 37°C to immobilized ICAM-1 (5 µg/ml). Shear stress was started at 0.5 dyn/cm² and increased up to 20 dyn/cm² at 1 min intervals. Cell detachment was calculated by normalizing the number of adhered cells relative to the number of cells observed at the minimal flow rate of 0.5 dyn/cm².

Lymphokine-activated Killer Cell Assay. The LAK cell assay was performed essentially as described (Groscurth et al., 1990; Robertson et al., 1990) with 4 x 10⁴ Daudi cells/well as targets and 4 x 10⁵ T lymphoblasts as effector cells (Dransfield et al., 1992a) in triplicates in 96-well U-bottom plates in a final volume of 200 µl. For antibody inhibition studies, purified mAbs were used at 50 µg/ml. Then the plates were incubated at 37 °C for 5 h and the percentage of specific target cytolysis is determined from the amount of LDH activity, measured as INT reduction, in the culture supernatant. 20 µl of lactate solution (Sigma, 36 mg/ml in 10 mM Tris buffer, pH 8.5) were added to the cell-free supernatant, followed by addition of 20 µl INT solution (Sigma, 2 mg/ml in PBS) and 20 µl of a solution containing NAD⁺/diaphorase (NAD⁺: Sigma, 3 mg/ml; diaphorase: Boehringer, 13.5 U/ml; BSA: 0.03%; sucrose: 1.2%; in PBS) and incubated for 20 min. The reaction was terminated with 20 µl of the LDH inhibitor oxamate (Sigma, 16.6 mg/ml in PBS) and the absorbance at 492 nm was determined in a microplate reader.

In situ Proximity Ligation Assays. In situ proximity ligation assays (PLAs) (Duolink kit, Olink Bioscience, Uppsala, Sweden) allows detection of direct or closely proximal protein–protein interactions in cell samples by fluorescence microscopy (Soderberg et al., 2006; Weibrecht et al., 2010). THP-1/PMA cells were seeded, fixed and blocked as described above. Next samples were incubated simultaneously with mouse mAbs anti-β2 TS1/18, anti-αL TS1/11, anti-CD147 VJ1/9 or anti-CD81 5A6 mAbs, and with the anti-CD9 H-110 rabbit polyclonal antibody (sc-9148, Santa Cruz Biotechnology), followed by specific oligonucleotide-labeled secondary antibodies (anti-mouse-plus probe and anti-rabbit minus probe). Only if the two different target proteins are in close proximity (≤ 40 nm), the oligonucleotides of the two probes will hybridize and after a

rolling-circle amplification reaction and detection with a different fluorescently labeled oligonucleotide a fluorescent dot signal can be visualized and analyzed by microscopy.

Statistical analysis. One-factor ANOVA analysis was performed using the statistics software SPSS (IBM). The data distribution was tested for normality by Bonferroni test.

3. Results

3.1. CD9 associates with $\beta 2$ integrin on the leukocyte surface

To investigate the possible association between CD9 and the $\beta 2$ integrin LFA-1, we first studied the co-localization of these molecules by double immunofluorescence staining with specific mAbs and confocal microscopy analysis. We employed monocytic THP-1 cells because they constitutively display detectable surface expression of the three major $\beta 2$ integrins ($\alpha_L\beta_2$, $\alpha_M\beta_2$, $\alpha_X\beta_2$) and CD9 (**Fig. 1A, upper histograms**). Confirming previous reports, we observed that the surface expression of both CD9 (Ouchi et al., 1997; Gutierrez-Lopez et al., 2011) and $\beta 2$ integrins (Aller et al., 1992) was increased during PMA-induced differentiation of THP-1 cells into macrophage-like cells (**Fig. 1A, lower histograms**). As shown in **Fig. 1B**, CD9 and $\beta 2$ integrin subunit were found to co-localize partially on the surface of undifferentiated monocytic THP-1 cells and this co-localization became much more evident on PMA-differentiated macrophage-like THP-1 (THP-1/PMA) cells, particularly at the cell-cell contact regions in the cellular aggregates that form during this differentiation process, as indicated by the increase in the Pearson co-localization coefficient. As we reported previously (Rodriguez-Fernandez et al., 2001), when activation of $\beta 2$ integrin was induced with extracellular divalent cation Mn^{2+} the localization of $\beta 2$ integrin molecules changed from an evenly-distributed pattern to a patched/clustered distribution and interestingly CD9 was clearly found co-localizing with clustered $\beta 2$ integrin. Since the $\beta 2$ -specific mAb stained all the $\beta 2$ -containing integrins we also performed double immunofluorescence using an α_L -specific mAb, confirming the pattern of co-localization between CD9 and LFA-1 (**Fig. 1 C**). The specificity of the co-localization between CD9 and LFA-1 was evidenced by the almost complete lack of co-localization observed between CD9 and CD147 (**Supp. Fig. 1**), which is another abundantly expressed surface protein on these cells (**Fig. 1A**).

Similar co-localization results were also observed in other leukocytes, including the B lymphoblastic JY and T leukemic Jurkat cells, which only express LFA-1, but not $\alpha_M\beta_2$ or $\alpha_X\beta_2$ (**Supp. Fig. 2**), and collectively suggest that CD9 associates with $\beta 2$ -integrins, and particularly with LFA-1 ($\alpha_L\beta_2$), on the surface of different types of leukocytic cells.

To confirm the association of CD9 with LFA-1, co-immunoprecipitation experiments were performed using JY and THP-1/PMA cells. All immunoprecipitations were

performed using intact cells that were incubated with the immunoprecipitating mAbs at 37°C prior to their lysis, thus ensuring that only interactions between cell surface molecules were detected. Immunoprecipitation using the β 2-specific mAb TS1/18, from Brij-97 lysates of THP-1/PMA and JY cells, followed by immunoblotting with anti-CD9 mAb VJ1/20 clearly showed that this tetraspanin was co-precipitated with integrin LFA-1 (**Fig. 2A**). Co-immunoprecipitation was also detected in the reverse order, i.e. immunoprecipitating CD9 followed by immunodetection of β 2 integrin (**Figure 2B**). Moreover, the association of LFA-1 with CD9 observed in Brij97-based lysates of THP-1/PMA and JY cells persisted under more stringent solubilisation conditions such as the use of detergent Triton-X100 (1.0 %) (**Fig. 3A**), thus pointing to a strong/direct type of interaction taking place between these molecules on the leukocyte surface. Similar results were also obtained using Triton-X100 (1.0 %) lysates of Jurkat cells (not shown). Parallel immunoprecipitations were also carried out with cells incubated with immunoprecipitating antibodies at 4°C and subsequently lysed in Triton-X100, to rule out that the observed association between CD9 and β 2 integrin is caused by pre-incubation with antibodies at 37°C (**Supp. Fig. 3**), and also with cells lysed at 4°C with this detergent prior to the addition of precipitating antibodies, yielding essentially the same results (data not shown).

When chemical crosslinking of solubilized proteins from JY cells was performed with thiol-cleavable DTSSP an important increase in the amount of CD9 co-immunoprecipitated with β 2 integrin could be detected after cleavage of the cross-linked protein complexes in reducing conditions (**Figure 3B, left panel**). Essentially the same results were also obtained with THP-1/PMA cells (data not shown). Furthermore, immunoprecipitation with anti- β 2 and anti-CD9 mAbs of non-cleaved cross-linked protein complexes resolved under non-reducing conditions clearly showed high molecular weight complexes of around 300 kDa which were immunodetected both with anti- β 2 and anti-CD9 antibodies (**Figure 3B, right panel**), indicating that these molecules had been directly cross-linked. It is worth indicating that these ~300 kDa bands are compatible with covalently cross-linked complexes containing both LFA-1 subunits (α L = 185 kDa; β 2 = 95 kDa) plus a molecule of CD9 (24 kDa). **Definitive proof that these ~300 kDa bands correspond to complexes including CD9 and β 2**

integrin, was provided by the fact that they were immunoprecipitated both with anti- $\beta 2$ and anti-CD9 antibodies, and immunodetected in both cases with an anti- $\beta 2$ antibody (MEM-48) (**Supp. Fig. 4**). Taken together, these crosslinking experiments support a direct-type of interactions occurring between endogenous LFA-1 and CD9 in leukocytic cells.

Further support for the direct nature of CD9-LFA-1 interaction on the leukocyte surface was provided by *In situ* Proximity Ligation Assays (PLAs) on non-permeabilized THP1/PMA cells. PLA signal is only detected when the secondary probes directed against the two different molecules whose interaction is suspected are within a short range distance (<40 nm) compatible with direct or closely proximal molecular interactions. As shown in **Fig. 3C**, PLA signal between CD9 and both subunits of LFA-1 (α_L and β_2) was clearly revealed on the surface of THP-1/PMA cells, being the PLA signal particularly evident at cell-cell contact regions. The same procedure with the abundantly expressed membrane molecule CD147/EMMPRIN (**Fig 1A**) did not provide any detectable PLA signal, revealing the specificity of PLA signal. As a positive control for primary/direct interactions, we assayed the association of CD9 with CD81, as these two tetraspanins are known to interact forming heterodimers and higher order oligomers on the cell surface (Kovalenko et al., 2004; Le Naour et al., 2006; Gutierrez-Lopez et al., 2011).

Most reported lateral interactions of tetraspanins with other proteins, and particularly with integrins, occur through the variable region of their Large Extracellular Loop (LEL) domain (Hemler, 2003; Stipp et al., 2003; Levy and Shoham, 2005). To assess whether CD9-LFA-1 interaction is mediated through this domain, we carried out pull-down assays employing a GST-fusion protein corresponding to the LEL domain of CD9 (CD9-LEL-GST) (Ho et al., 2006; Gilsanz et al., 2013). The $\beta 2$ subunit of endogenous LFA-1 was pulled down by CD9-LEL-GST from Brij-97 (not shown) and Triton-X100 (**Fig. 3D, left panel**) lysates of JY cells (and THP-1/PMA cells, not shown). As a positive control, another GST construct corresponding to the LEL domain of CD81, a tetraspanin closely related to CD9, also pulled-down the $\beta 2$ integrin subunit, as previously reported (Takeda et al., 2003). In contrast, the GST-LEL construct of another tetraspanin, CD63, (CD63-LEL-GST) did not pull-down the $\beta 2$ integrin subunit,

reflecting the specificity of these LFA-1/tetraspanin interactions. Furthermore, the CD9-GST-LEL fusion protein selectively recovered both the $\beta 2$ and the αL subunits of LFA-1 from a lysate of biotin-labelled JY cells, indicating that on these cells LFA-1 is a major surface protein that is selectively engaged in specific interactions with the LEL domain of CD9 (**Fig. 3D, right panel**).

3.2. CD9 regulates the adhesive function of integrin LFA-1

We decided to explore whether CD9 could regulate the adhesive function of LFA-1. For this goal, we first assessed the effects of several CD9-specific mAbs (VJ1/20, PAINS-10 and PAINS-13), which exert an agonist-like action on CD9 (Gutierrez-Lopez et al., 2003; Ovalle et al., 2007; Gutierrez-Lopez et al., 2011; Gilsanz et al., 2013), on LFA-1-mediated leukocyte adhesion. Treatment of THP-1/PMA cells with the three different anti-CD9 mAbs inhibited significantly their adhesion to immobilized ligand ICAM-1 (**Fig. 4A, left panel**). The LFA-1-dependence of this assay was confirmed by the nearly complete inhibition of adhesion with the blocking anti- $\beta 2$ mAb Lia3/2 and by the important stimulation of adhesion with the $\beta 2$ -activating mAb Kim185. Similar results were also obtained with B lymphoblastic JY cells, which do not express $\beta 1$ integrin (**Fig. 4B**).

In contrast to freshly isolated resting human lymphocytes, which express very little CD9 on their surface (Tohami et al., 2004; Barrena et al., 2005), PHA/IL-2-activated lymphocytes (“lymphoblasts”) express abundantly the integrin LFA-1 as well as variable (from donor to donor) though consistently detectable levels of CD9 on their surface, together with modest $\alpha_M\beta 2$ and negligible $\alpha_X\beta 2$ integrin expression (**Fig. 4C**). Using these primary human lymphoblasts in static adhesion assays on ligand ICAM-1, we observed essentially the same regulatory effects of CD9-specific mAbs on the adhesive activity of LFA-1 (**Fig. 4D**), indicating that CD9 exerts inhibitory effects on LFA-1-mediated adhesion.

Interaction of integrin LFA-1 with specific ligands is a crucial step in many leukocyte intercellular interactions and particularly in the cytotoxic lymphocyte-mediated killing

of target cells (Blanchard et al., 1987; de Vries et al., 1989; Dransfield et al., 1992a; Hogg et al., 2003; Anikeeva et al., 2005). Interestingly, the observed inhibitory effect exerted by anti-CD9 mAbs (VJ1/20, PAINS-10 and PAINS-13) on LFA-1 was also reflected in LAK (lymphokine-activated killer cells)-mediated killing of target Daudi cells (**Fig. 4E**). The allosterically inhibiting anti-CD18 mAb TS1/18 (Salas et al., 2004) was used as a blocking control. Taken together, these results confirm that mAbs with agonist-like effect on CD9 regulate negatively the adhesive function of LFA-1.

We next wanted to address whether changes in the expression levels of CD9 could also regulate LFA-1 adhesiveness, either by ectopically expressing this tetraspanin in the CD9-deficient HSB-2 T lymphoblastic cell line or, conversely, by suppressing CD9 expression using shRNA interference in Jurkat T cells (**Fig. 5A and B**). Ectopic expression of CD9 in HSB-2 cells reduced significantly LFA-1-mediated adhesion to immobilized ligand ICAM-1 (**Fig. 5C**), whereas silencing CD9 in Jurkat cells enhanced LFA-1-mediated adhesion to ICAM-1 (**Fig. 5D**). Interestingly, incubation of HSB-2/CD9 and Jurkat T cells with the anti-CD9 mAbs VJ1/20, PAINS-10 and PAINS-13, further reduced LFA-1-mediated adhesion to ICAM-1, confirming the agonist-like effect of these mAbs on CD9 function.

3.3. CD9-mediated regulation of LFA-1 adhesion does not involve changes in integrin affinity but alters its clustering

Integrin adhesive capacity is mainly regulated by two different mechanisms involving either, alterations in the conformation of individual integrin molecules that are reflected by affinity changes, or modifications in the aggregation of integrin molecules which affect the valency of their interactions with ligand. To investigate which of these mechanisms is involved in the observed CD9-mediated inhibition of LFA-1 adhesive function, we first analysed the induction of expression of the m24 epitope by divalent cation Mn^{2+} , which reports the high affinity conformation of LFA-1. As shown in **Fig. 6A**, expression of m24 epitope was similarly induced by Mn^{2+} (over the 10-400 μM concentration range) on cells expressing CD9 (HSB-2/CD9 and JK TR2 cells) and on their respective counterparts lacking CD9 (HSB-2 and JK shCD9), clearly indicating that the presence of this tetraspanin does not interfere with the affinity state of LFA-1. As expected, Mn^{2+} induced LFA-1-mediated adhesion of JK (**Fig. 6B**) and HSB-2 (**Fig.**

6C) T cells in a concentration-dependent manner over a 20-400 μM range. Interestingly, the differences in static cell adhesion between cells lacking CD9 and their CD9-expressing counterparts disappeared at the highest (400 μM) Mn^{2+} concentration. The inhibitory effect of anti-CD9 mAb VJ1/20 was also abrogated at 400 μM Mn^{2+} . These results show that CD9 does not affect LFA-1 adhesive function when cell adhesion is mediated by integrin molecules in the high affinity state (i.e. at high Mn^{2+} concentration).

However, when T cell adhesion was promoted with phorbol ester PMA (at 50 and 200 ng/ml), which induces the intermediate affinity state of LFA-1 as well as ligand-dependent clustering of this integrin, CD9-caused inhibition of static adhesion was clearly observed even at the highest PMA dose (200 ng/ml) (**Fig. 7A and B**), suggesting that CD9 effect was somehow related to the aggregation/clustering of LFA-1. It is worth indicating that these CD9-caused inhibitory effects on adhesion are still maintained at higher concentrations of PMA (400 ng/ml) (data not shown), although cell viability under these conditions begins to be compromised. Importantly, these differences in PMA-induced LFA-mediated cell adhesion to ICAM-1 between T cells expressing or lacking CD9 were also consistently observed under flow conditions, therefore highlighting the relevance of CD9-mediated regulation of LFA-1 function under conditions that resemble a more physiological setting (**Fig. 7C**).

To analyse in more detail the implication of CD9 in the organization of LFA-1 molecules, we first quantitated the number and size of LFA-1 clusters on the adhesive surface of PMA-stimulated T cells either expressing (JK TR2) or lacking CD9 (JK shCD9). As shown in **Fig. 8A (upper panel)**, the number of PMA-induced LFA-1 clusters detected by confocal microscopy on the cellular adhesive surface in contact with immobilized ligand ICAM-1 was significantly higher on Jurkat T cells expressing CD9 than on their CD9-silenced counterparts. Interestingly, although fewer LFA-1 clusters were observed on cells lacking CD9 expression, their size was bigger than on CD9-expressing cells. Likewise, the presence of CD9 induced the organization of LFA-1 molecules into an increased number of smaller clusters in PMA-stimulated monocytic U937 cells adhering onto ICAM-1 (**Fig. 8A, lower panel**). These results were corroborated by TIRF microscopy, which recognizes with high resolution and high signal-to-noise ratio the organization of cell surface molecules located specifically in

the area of contact with the substrate (ICAM-1). Quantitation of fluorescence from TIRF microscopy images shows that CD9-expressing T cells (JK TR2) display an increased number of clusters but of a smaller size as well as a significant proportion of LFA-1 molecules with a dispersed/unclustered appearance, compared to their CD9-lacking (JK shCD9) cell counterparts (**Fig. 8B**).

As a complementary biochemical approach to get further insight on how CD9 affects the organization of LFA-1 molecules on the leukocyte surface, the differential resistance of LFA-1 molecules to extraction with increasing concentrations of detergent Triton X-100 (ranging from 0.02 to 1%) in Jurkat cells expressing (JK TR2) or lacking CD9 (JK shCD9) was analysed. As shown in **Fig. 8C**, LFA-1 molecules were much more easily extracted when CD9 is expressed on the cell surface, as would be expected from the increased proportion of LFA-1 found in a dispersed/unclustered form and organized in smaller clusters.

Collectively, the confocal and TIRF microscopy data together with the biochemical extraction results show that CD9 affects the organization of LFA-1 molecules into clusters, as evidenced by the differences in the number and size of LFA-1 aggregates on the cell surface as well as by their resistance to detergent extraction.

4. Discussion

We report here that the $\beta 2$ integrin LFA-1 associates with CD9 in different types of leukocytes, including T (Jurkat) and B (JY) lymphocytic cell lines, and PMA-differentiated THP-1 macrophage-like cells.

The CD9/LFA-1 association was evidenced by co-localization, *in situ* Proximity Ligation Assays (PLA), as well as biochemical studies based on co-immunoprecipitation, chemical cross-linking and pull-down assays. These interactions resist stringent cell solubilization conditions (i.e. 1% Triton X-100) in co-immunoprecipitation and pull-down experiments which, together with chemical cross-linking and PLA data, collectively support the direct nature of the interaction between CD9 and LFA-1. Both co-localization and PLA signal were particularly evident at the cell-cell contact regions of THP-1 cell aggregates formed during their PMA-induced differentiation process, which are areas enriched in LFA-1 molecules actively engaged in interactions with ICAM-1 ligand expressed on opposing cells, suggesting that the association of CD9 with LFA-1 might be important in the regulation of LFA-1 adhesive function. Tetraspanin-integrin interactions seem to be consistently mediated through the variable region of the LEL domain of tetraspanins (Yauch and Hemler, 2000; Berditchevski et al., 2001; Hemler, 2005; Charrin et al., 2009); in this regard our pull-down experiments with a recombinant construct corresponding to the CD9 LEL-domain indicate that the interaction between $\beta 2$ integrin and CD9 is also mediated by this domain.

After demonstrating the existence of CD9-LFA-1 complexes in different types of leukocytes we next explored whether CD9 exerted any functional effects on the adhesive capacity of LFA-1. For this purpose, we have made use of three different anti-CD9 mAbs (VJ1/20, PAINS-10 and PAINS-13) considered to exert, at the cellular level, an agonist-like action on the basis that in different cellular systems the functional effects caused by treating CD9⁺ cells with these antibodies are similar to those derived from the neo- or over-expression of CD9, but opposite to those observed after silencing this tetraspanin (Ovalle et al., 2007; Gutierrez-Lopez et al., 2011; Gilsanz et al., 2013). The use of these three anti-CD9 mAbs, as well as ectopic expression of CD9 in HSB-2 T cells or silencing the endogenous CD9 expression in Jurkat T cells using specific

shRNAs, collectively show that CD9 exerts a negative regulatory role on LFA-1-mediated leukocyte adhesion. Consistently with the CD9 inhibitory effect on adhesion, LAK-cellular cytotoxicity against target cells, which is largely dependent on LFA-1-mediated intercellular adhesion, was also inhibited by these anti-CD9 mAbs. Engagement of tetraspanins CD81 or CD82 with specific mAbs or overexpression of these molecules on T cells have been reported to up-regulate the adhesive and signalling capacities of the integrin LFA-1 (Shibagaki et al., 1999; VanCompernelle et al., 2001), whereas for the tetraspanin CD9 we report here the opposite: a clear inhibitory effect on LFA-1-mediated cellular adhesion and *in vitro* cellular cytotoxicity. One possibility is that the different functional effects of distinct tetraspanins on LFA-1 adhesive function might depend on the specific cellular system under study or the specific ligand employed (ICAM-3 employed in some of these previous reports *versus* ICAM-1 employed here), or alternatively CD9 may exert specific functional effects on LFA-1.

Integrin adhesive capacity can be regulated essentially by mechanisms involving either alterations in the affinity of individual integrin molecules or changes in their aggregation/clustering on the cell surface which regulate the valency of their interactions with ligand. As reported by the expression of the m24 epitope, the high affinity conformation of LFA-1 induced by Mn^{2+} was not altered by ectopic expression or silencing of CD9; accordingly, no differences were observed in LFA-1-mediated adhesion stimulated by 400 μM Mn^{2+} between cells expressing or lacking CD9. In contrast, it is interesting that at lower Mn^{2+} concentrations (20 and 40 μM), at which presumably only a proportion of total LFA-1 molecules are in the high affinity conformation, differences in adhesion between cells expressing or lacking CD9 could be observed. Moreover, the differences between cells expressing and lacking CD9 were also consistently observed when cell adhesion was promoted with PMA, which induces the intermediate affinity state of LFA-1 as well as ligand-dependent clustering of this integrin. All these results clearly indicated that the effect of CD9 on LFA-1-mediated adhesion was mainly related to the aggregation/clustering state of this integrin and not to changes in its affinity. Indeed, confocal and TIRF microscopy analyses of PMA-induced LFA-1 clustering specifically at the adhesive cellular surface in contact with immobilized ligand ICAM-1 showed that in leukocytic cells expressing CD9 an increased number of clusters but with a smaller size could be detected in comparison

with their CD9-lacking cell counterparts. TIRF microscopy images also revealed that in T cells expressing CD9 a significant proportion of LFA-1 molecules showed a dispersed/unclustered appearance, which might correspond to the nanoclusters of LFA-1 that have been characterized by other higher resolution microscopy techniques. In this regard, through the use of NSOM (near-field scanning optical microscopy) and SDT (single dye tracking) super-resolution optical techniques, it has been recently reported that LFA-1 is preorganized in nanoclusters in “hotspot” regions of the leukocyte membrane. Ligand binding favors the lateral mobility and growth of LFA-1 clusters through coalescence of individual nanoclusters to form microclusters, in a process that also critically depends on transient cytoskeleton anchorage, which in turn mediate efficient stable leukocyte adhesion under shear flow (van Zanten et al., 2009; Bakker et al., 2012). Our findings are therefore compatible with these observations, and we can speculate that CD9 might have an important role in regulating these transitions among the different states of LFA-1 organization into distinct types of differently-sized clusters as well as the anchorage to the actin cytoskeleton.

Interestingly, we have observed that the $\beta 1$ integrin-mediated adhesion of leukocytes to the extracellular matrix protein fibronectin was enhanced by anti-CD9 mAbs or following ectopic expression of this tetraspanin (data not shown), which concurs with our previous data with colorectal carcinoma cells (Ovalle et al., 2007). Therefore, CD9 seems to have a dual functional regulatory role on leukocyte adhesion mechanisms by increasing $\beta 1$ adhesion to fibronectin but down-regulating LFA-1-mediated adhesion. Reciprocal control of the activity of members of distinct subfamilies of integrins co-expressed on the same cell has been previously described. For instance, in some leukemic T cell lines functionally active integrin $\alpha 4\beta 1$ occurs only when LFA-1 is either not expressed or inactive (van Kooyk et al., 1993), whereas in human erythroleukemic K562 cells transfected with integrin $\alpha v\beta 3$, ligation of the $\beta 3$ integrin subunit inhibits the phagocytic function of endogenously expressed $\alpha 5\beta 1$ integrin (Blystone et al., 1994; Blystone et al., 1995). Similarly, in human T lymphoblasts induction of activation of LFA-1 resulted in decreased adhesion through $\alpha 4\beta 1$ and $\alpha 5\beta 1$ integrins, rendering cells with a less adhesive and more migratory phenotype (Porter and Hogg, 1997). However, the underlying mechanisms for this type of regulation are still unclear. Association with cytoskeleton is essential for integrin activation and

adhesion. Interestingly, actin cytoskeleton seems to play a differential role in the control of $\beta 1$ and $\beta 2$ integrin function. In this regard, in resting leukocytes LFA-1 molecules are maintained in an inactive low-avidity state through association with actin microfilaments and release from these cytoskeletal constraints, caused by drugs such as cytochalasin D or latrunculin B, increases their lateral diffusion which is accompanied by enhanced clustering and avidity and augmented LFA-1-mediated leukocyte adhesion to ICAM-expressing cells (Kucik et al., 1996; van Kooyk and Figdor, 2000). In contrast, most $\beta 1$ integrins (with the notable exception of $\alpha 4\beta 1$ interaction with VCAM-1) mediate a more stable type of cell adhesion to extracellular matrix proteins such as fibronectin, collagen and laminin, and for this purpose seem to require firmer links with cytoskeletal components (Sanchez-Mateos et al., 1996; Berrier and Yamada, 2007; Chen et al., 2013). Several tetraspanins, including CD81 and CD9, are linked to actin microfilaments through ERM (Ezrin-Radixin-Moesin) proteins, either directly or through tetraspanin-associated partners EWI-2 and EWI-F (Sala-Valdes et al., 2006). The fact that CD9 may modulate in an opposite manner the adhesive function of $\beta 2$ and $\beta 1$ integrins points to CD9-centered TEMs as crucial players in this balanced regulation. An attractive possibility is that, by reinforcing the integrin-cytoskeletal links, CD9 may restrict the membrane lateral diffusion of LFA-1 molecules resulting in inhibition of its adhesive function, while at the same time, stabilizing $\beta 1$ integrins with concomitant adhesion enhancement to matrix components. Further research will be required to properly address this attractive hypothesis.

The model depicted in Figure 9 summarizes the main findings of this report. **State “1”** in the model is characterized by expression of dispersed and inactive/bent form of LFA-1 (**Low-valency/Low-affinity**) and by the absence/very low expression of CD9, as occurs physiologically in most resting/non-stimulated lymphocytes (Tohami et al., 2004; Barrena et al., 2005). Conversion from **state “1”** into a **High-valency/Intermediate-affinity state (“2”)** is characterized by aggregation of integrin molecules into large clusters and the acquisition of intermediate affinity conformation, both induced by phorbol ester PMA. Within the context of TEMs, augmented CD9 function induced by its ectopic neoexpression (or by the use of agonist-like mAbs to this tetraspanin, not shown in the model), alters the organization of LFA-1 molecules at the cell adhesion surface, as evidenced by an increase in “dispersed” LFA-1 molecules

and in the number of clusters with a reduced size, defining an **Intermediate-valency/Intermediate-affinity state** (“3”) characterized by diminished adhesive efficiency to ligand ICAM-1 relative to state “2”. On the other hand, **state “4”** is induced by high concentration of Mn^{2+} and is characterized by clustering of LFA-1 molecules in a high affinity conformation (**High-valency/High-affinity state**); in this case, transition from state “4” into “5”, caused by the ectopic expression of CD9 (or by the use of agonist-like mAbs to this tetraspanin, not shown in the model), is not accompanied by a down-modulation of the adhesive efficiency because, although probably some reduction in the ligand-interaction valency occurs, this effect is not potent enough to decrease cell adhesion when mediated by a majority of LFA-1 molecules in the high affinity conformation. The inhibitory effects on LFA-1-mediated cell adhesion (observed in transitions “2”→”3”) could place CD9 as a novel target for therapeutic intervention aimed at reducing the activity of LFA-1, which would be potentially beneficial in a number of inflammatory disorders.

5. Conclusions

Our data demonstrate that the tetraspanin CD9 associates with integrin LFA-1 in different types of leukocytes, and through these interactions, CD9 exerts inhibitory effects on LFA-1 adhesive function and leukocyte cytotoxic activity. The mechanism responsible for this negative modulation exerted by CD9 does not involve changes in the affinity state of LFA-1 but relates to alterations in its state of aggregation. These data contribute to our understanding of the regulation of adhesive activity of LFA-1, an integrin that plays a pivotal role in many crucial leukocyte functions that require intercellular adhesion.

List of abbreviations

BSA (Bovine serum albumin); FBS (Fetal bovine serum); FITC (Fluorescein isothiocyanate); GST (Glutathione S-transferase); ICAM-1 (Intercellular adhesion molecule-1); IL-2 (Interleukin 2); INT (2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium chloride); LDH (Lactate dehydrogenase); LAK (Lymphokine-activated killer); LEL (Large extracellular loop); mAb (monoclonal antibody); M.F.I. (Mean fluorescence intensity); PBLs (Peripheral blood leukocytes); PBS (Phosphate buffered saline); PLAs (Proximity ligation assays); PMA (Phorbol myristate acetate); SDS (Sodium dodecyl sulfate); SDS-PAGE (Sodium dodecyl sulfate polyacrylamide gel electrophoresis); SEM (Standard error of the mean); TBS (Tris buffered saline); TEM (Tetraspanin-enriched microdomain); TIRF (Total internal reflection fluorescence).

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Authors' contributions

RR carried out the confocal and TIRF microscopy, biochemical studies, flow cytometry, gene transfection, silencing and cell adhesion experiments. She also participated in the design of the study and performed the statistical analysis. **AM** performed confocal microscopy and some biochemical studies and adhesion experiments. She also participated in the design of the study. **MYM** carried out confocal and TIRF microscopy and biochemical experiments and participated in the interpretation of data and helped to draft the manuscript. **BC** performed some biochemical experiments. **GM** performed cell adhesion experiments. **AG** performed some adhesion experiments. **YM** participated in the flow cytometry and cell adhesion

experiments. **EL** participated in the transfection, silencing and cell adhesion experiments and helped to draft the manuscript. **PM** participated in the pull-down experiments and in the interpretation of data. **FSM** participated in the conception and design and interpretation of data, and helped to draft the manuscript. **CC** conceived the study, and took responsibility for its design and coordination and wrote the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figure Legends:

FIGURE 1. CD9 co-localizes with LFA-1 on THP-1 cell surface. A) Flow cytometric detection of CD9 (mAb VJ1/20), $\beta 2$ (mAb TS1/18), αL (mAb TP1/40), αM (mAb Bear-1), αX (mAb HC1/1) and CD147 (mAb VJ1/9) proteins on the surface of THP-1 monocytic cells or THP-1/PMA macrophage-like cells. Gray filled histograms correspond to negative controls; empty histograms correspond to the expression of the indicated molecules. The numbers in boxes represent M.F.I. values. **B)** and **C)** Confocal microscopy of THP-1, THP-1 in the presence of 0.4 mM Mn^{2+} and THP-1/PMA differentiated cells showing in panel **B** co-localization of CD9 (green) and $\beta 2$ (red) and in panel **C** co-localization of CD9 (green) and αL (red) at the cell surface. Representative images of confocal sections for each channel (green and red) and merged channels are shown together with co-localization histograms (right panels) showing Pearson co-localization values. Scale bars = 5 μm .

FIGURE 2. Co-immunoprecipitation analysis of the association between LFA-1 and CD9. THP-1/PMA (left panels) and JY (right panels) cells were incubated with the immunoprecipitating mAbs TS1/18 (anti- $\beta 2$) in panel **A**, and VJ1/20 (anti-CD9) in panel **B**, prior to their lysis in 1% Brij-97-containing lysis buffer. Protein immunocomplexes were precipitated with Protein-G-sepharose, then resolved by 8% (for detection of $\beta 2$) or 12% (for detection of CD9) SDS-PAGE under non-reducing conditions, and immunoblotted with anti- $\beta 2$ (MEM-48) (upper panels) or anti-CD9 (VJ1/20) (middle panels) biotin-conjugated mAbs or with anti-CD105 or anti-CD59 control proteins (lower panels). Blots are representative of three different experiments. In some cell lysates, several $\beta 2$ bands (indicated by grey arrowheads), probably corresponding to differences in glycosylation or partial degradation, are immunodetected. WL: whole cell lysate. pG: Protein-G-sepharose without precipitating antibody.

FIGURE 3. Direct interaction of CD9 and LFA-1 at the cell surface is mediated by the Large Extracellular Loop of CD9. A) THP-1/PMA or JY cells were lysed with 1% Triton-X100-based lysis buffer after incubation with immunoprecipitating mAbs TS1/18 (anti- $\beta 2$) or VJ1/20 (anti-CD9). Immunoblotting of co-immunoprecipitated proteins was performed as described in Materials and Methods. WL: whole cell lysate. pG: Protein-G-sepharose without precipitating antibody. **B)** 1% Brij-97 extracts of JY cells were treated with DTSSP cross-linker and Triton X-100 was then added to a final concentration of 1% (v/v) to cross-linked lysates and to parallel non-cross-linked lysates used as controls, as indicated. Samples were immunoprecipitated with anti- $\beta 2$ integrin

mAb TS1/18 (as indicated in the left panel and lanes 1 and 2 in the right panel) or with anti-CD9 mAb VJ1/20 (lanes 3 and 4 in the right panel), resolved by SDS-PAGE either under reducing conditions to break the thiol bond in DTSSP-crosslinked protein complexes (**left panel**) or under non-reducing conditions (**right panel**) and subsequently immunodetected with biotinylated anti-CD9 (VJ1/20) or anti- β 2 integrin (MEM-48) mAbs. **C**) *In situ* proximity ligation assays (PLAs) were performed on THP-1/PMA macrophage-like cells as described in Materials and Methods. Red fluorescent dots reveal molecular interactions between CD9 and β 2 or α L integrin subunits of LFA-1. The interactions between CD147/CD9 and CD81/CD9 are also shown as negative and positive controls, respectively (left panels). Maximal projections of representative confocal stacks are shown. Scale bars = 5 μ m. The right panel shows the quantitation of total fluorescence per cell. *** $p < 0.001$. **D**) Non biotinylated (left panel) or surface-biotinylated (right panel) JY cells were lysed in Triton-X100-based buffer and incubated with the LEL-GST constructs of human CD81 or CD9, as indicated. The GST and CD63-LEL-GST constructs were used as negative controls for specificity of binding. Formed complexes were pulled-down with glutathione-sepharose beads. For non-biotinylated cells, the presence of β 2 integrin in the pulled-down complexes was revealed by immunoblotting with mAb MEM-48 (upper left-panel) and loading controls of GST fusion proteins stained with anti-GST polyclonal antibody are also shown (lower left-panel). For surface-biotinylated cells, all lysate proteins pulled-down by GST, CD63-LEL-GST or by CD9-LEL-GST were isolated with glutathione-sepharose beads and detected with streptavidin-HRP. As a control, β 2 integrin was also immunoprecipitated with mAb TS1/18 from 1% Triton-X100 lysates of either surface-biotinylated or non biotinylated (NB) JY cells and detected with streptavidin-HRP (which also clearly reveals the accompanying biotinylated 185 kDa band corresponding to the α L subunit of LFA-1) or by immunodetection with anti- β 2 mAb MEM48, respectively.

FIGURE 4. Anti-CD9 mAbs inhibit LFA-1 adhesion and LAK cytotoxicity. A) THP-1/PMA cells were loaded with the fluorescent probe BCECF-AM (Sigma) and then allowed to adhere to ICAM-1-Fc-coated wells (6 μ g/ml) for 20 min at 37 °C in the presence of the indicated mAbs (20 μ g/ml). Data represent the percentage of adherent cells (mean \pm SEM of four experiments, each performed in triplicates) that remains in the wells after washing non-adherent cells. **B)** JY B lymphocytic cells, loaded with the fluorescent BCECF-AM probe, were seeded in 96-well plates pre-coated with ICAM-1-Fc (12 μ g/ml) and incubated for 20 min a 37 °C with the corresponding mAbs (20 μ g/ml) specified. The bars-graph represents the percentage of adhesion (mean \pm SEM) of 3 different experiments, performed in triplicates. **C)** Flow cytometric analysis of CD9

(mAb VJ1/20), $\beta 2$ (mAb TS1/18), αL (mAb TP1/40), αM (mAb Bear-1) and αX (mAb HC1/1) surface molecules on human T-lymphoblasts from two different donors. **D**) Adhesion of T-lymphoblasts to plastic-immobilized ICAM-1-Fc (6 $\mu\text{g/ml}$). Cells were allowed to adhere for 20 min in the presence of the specified mAbs (20 $\mu\text{g/ml}$). Data shown correspond to the percentages of adherent cells (mean \pm SEM of four experiments) relative to 100% cell adhesion (dotted line) considered in the absence of antibody treatment. **E**) LAK cytotoxic cells were pre-treated with control anti-HSPA8 mAb (PAINS-18), anti-CD9 antibodies (VJ1/20, PAINS-10 and PAINS-13) or the inhibitory anti- $\beta 2$ antibody TS1/18 and their cytotoxicity was analysed by incubating them with Daudi target cells at a 10:1 (effector:target) ratio. Cytotoxicity was determined from the amount of LDH released to the medium. The data show the percentage of cytotoxicity (mean \pm SEM) of three different experiments performed in triplicates. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

FIGURE 5. Ectopic expression or silencing of CD9 regulates LFA-1 mediated adhesion. A) and B) Ectopic neoexpression of CD9 in HSB-2 cells was achieved by stable transfection with the pcDNA3-CD9 (HSB-2/CD9) plasmid and CD9 knock-down in Jurkat cells was achieved by retroviral transduction with CD9-specific shRNA (JK shCD9). Jurkat cells transduced with an empty shRNA vector (JK TR2) were used as control. The neoexpression or silencing of CD9 in these cells was confirmed by flow cytometric analysis (**A**) and Western Blotting (**B**). **C**) After loading cells with BCECF-AM, HSB-2 (left panel) or Jurkat (right panel) cells were allowed to adhere for 60 min to immobilized ICAM-1-Fc (12 $\mu\text{g/ml}$), in the presence or absence of the indicated mAbs (20 $\mu\text{g/ml}$). The graphic shows the percentage of cell adhesion (mean \pm SEM) of three independent experiments, each performed in triplicates. * $p < 0.05$ and ** $p < 0.01$.

FIGURE 6. CD9 regulation of LFA-1-mediated adhesion does not involve alteration of integrin affinity. A) Flow cytometric analysis of epitope m24 expression induced by Mn^{2+} (at 10, 20, 40, 100 and 400 μM) relative to its basal expression in $\text{Ca}^{2+}/\text{Mg}^{2+}$ (0.5 mM and 1 mM, respectively) on the surface of HSB-2 (dark blue solid line), HSB-2/CD9 (light blue dotted line), JK TR2 (red dotted line) and JK shCD9 (brown solid line) cells. **B) and C)** JK TR2 (red bars) and JK shCD9 (brown bars) (**B**) or HSB-2 (dark blue bars) and HSB-2/CD9 (light blue bars) (**C**) cells were loaded with BCECF-AM. Then cells were treated with different Mn^{2+} concentrations and allowed to adhere for 45 min to immobilized ICAM-1-Fc (12 $\mu\text{g/ml}$), in the presence or absence of the indicated mAbs (20 $\mu\text{g/ml}$). Each bars panel represents the percentages of adherent cells (mean \pm SEM of three experiments) for each Mn^{2+} concentration, and data corresponding to

these different Mn^{2+} concentrations are plotted in the right panel. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$.

FIGURE 7. CD9 regulates PMA-induced LFA-1 adhesion through an increment in integrin clustering. **A) and B)** Prior to adhesion, JK TR2 and JK shCD9 (red and brown bars, respectively) **(A)** or HSB-2 and HSB-2/CD9 (dark and light blue bars, respectively) **(B)** cells were treated 2 hours at 37 °C with different concentrations of PMA (0, 50 or 200 ng/ml). Then cells were loaded with the fluorescent probe BCECF-AM and allowed to adhere to plastic-immobilized ICAM-1-Fc (12 μ g/ml) for 45 min at 37 °C in the presence or absence of the indicated mAbs (20 μ g/ml). Data represent the percentages of adherent cells (mean \pm SEM of three experiments), and data corresponding to these different PMA concentrations are plotted in the right panel. **C)** Adhesion of PMA-stimulated JK TR2 and JK shCD9 cells to ICAM-1 under shear flow conditions. Left panel contains a representative image showing the cells that remain adhered for each different flow rate, and the right panel graph shows the calculated ratios of adherent cells from 9 different microscopic fields for each flow rate condition. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Scale bars = 30 μ m.

FIGURE 8. CD9 regulates the distribution of LFA-1 molecules into clusters. **A)** Confocal microscopy images of immunofluorescence-stained β 2 integrin clustering at the adhesive surface in contact with ligand ICAM-1-Fc of PMA-stimulated JK TR2, JK shCD9, U937/pcDNA3 and U937/CD9 cells. Representative confocal images are shown on the left panels and quantitation of the number and size (mean area) of clusters/cell corresponding to ten individual cells are shown on the right panels. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$. Scale bars = 5 μ m. **B)** TIRF microscopy images of β 2 integrin clustering at the adhesive surface in contact with ligand ICAM-1-Fc of PMA-stimulated JK TR2, JK shCD9. Five representative images are shown for each cell type on the left panel and quantitation of the number and size of clusters/cell corresponding to 15 individual cells are shown on the right panels. *** $p < 0.001$. Scale bars = 5 μ m. **C)** Differential resistance of LFA-1 molecules to extraction with increasing concentrations of detergent Triton X-100 (0.02 / 0.05 / 0.1 / 0.5 %) from PMA-stimulated Jurkat cells either expressing (JK TR2) or lacking CD9 (JK shCD9), adhered to ICAM-1-Fc. Extracted LFA-1 was detected by immunoblotting with the anti- β 2 mAb MEM48, and quantitated relative to β -actin content.

FIGURE 9. Hypothetical model which summarizes the regulation by CD9 of the different LFA-1 functional states, as described in the Discussion.

Figure 1
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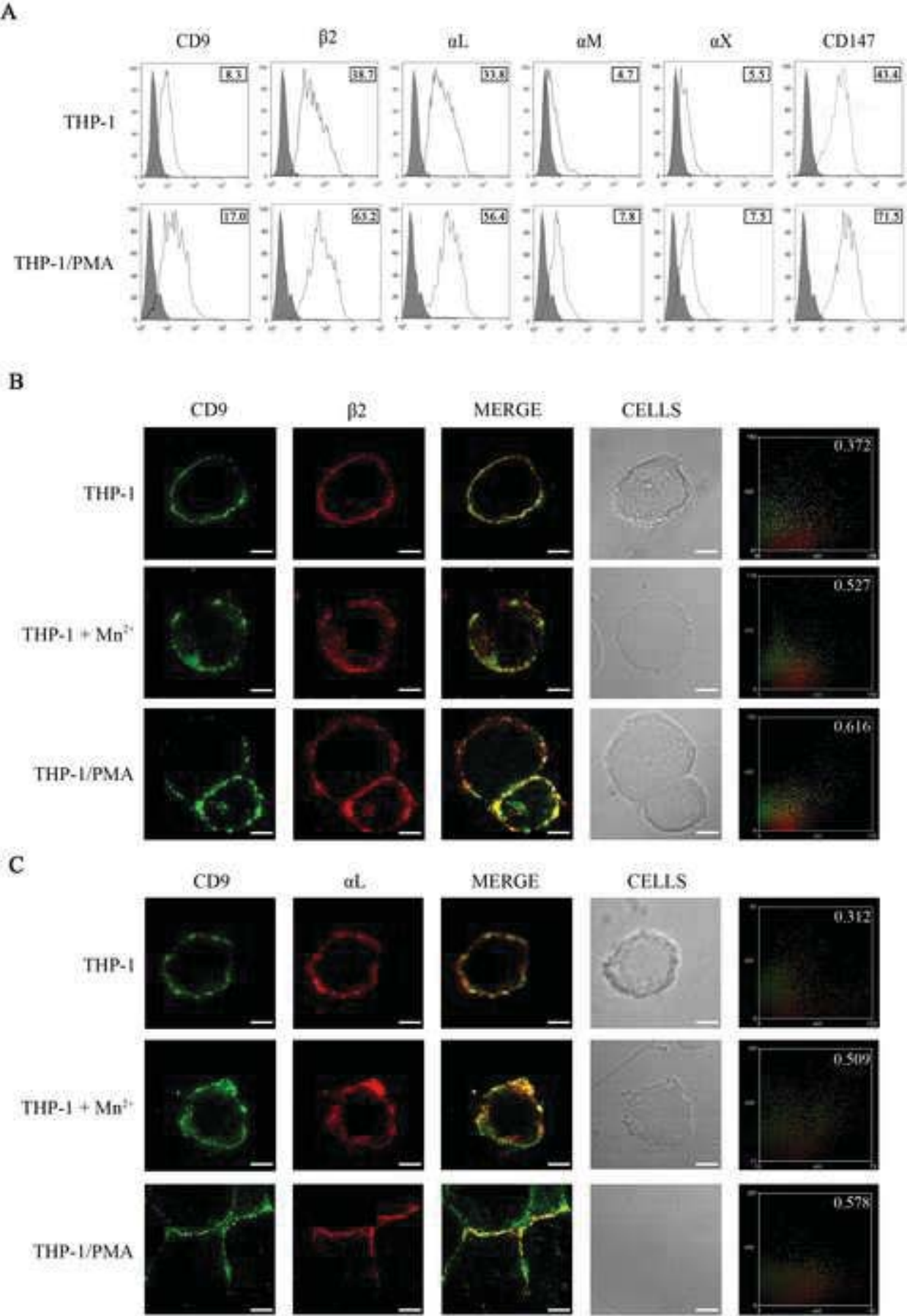
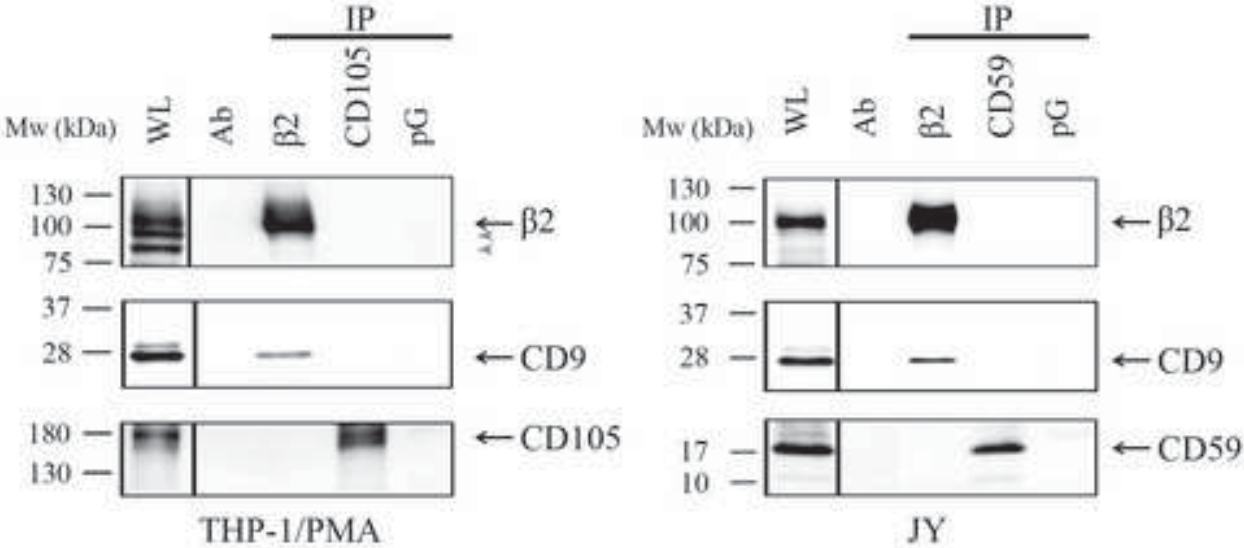


FIGURE 1

Figure 2
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A



B

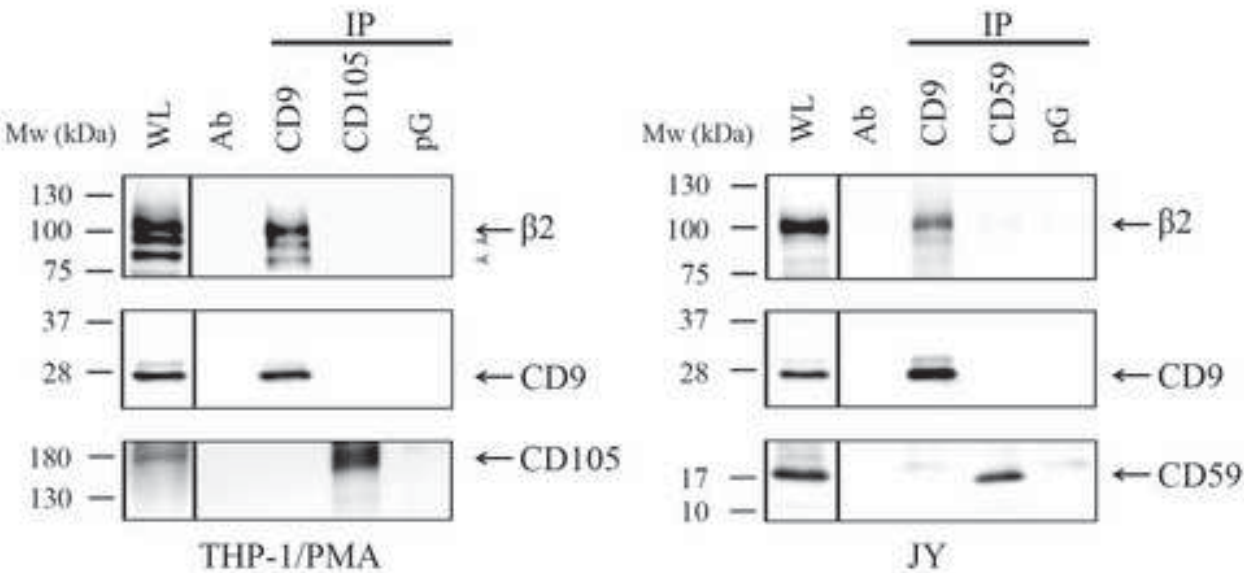


FIGURE 2

Figure 3
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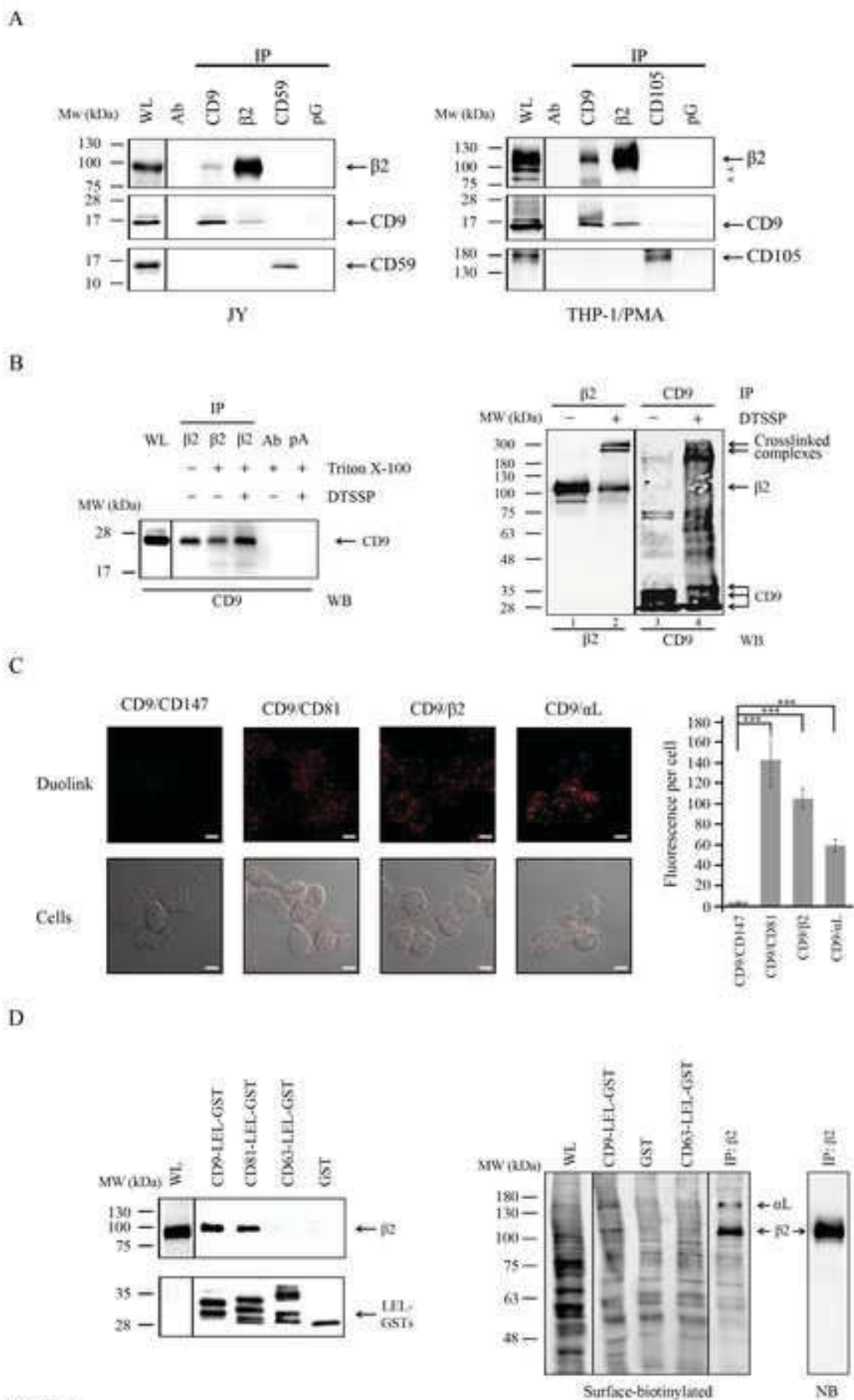


FIGURE 3

Figure 4
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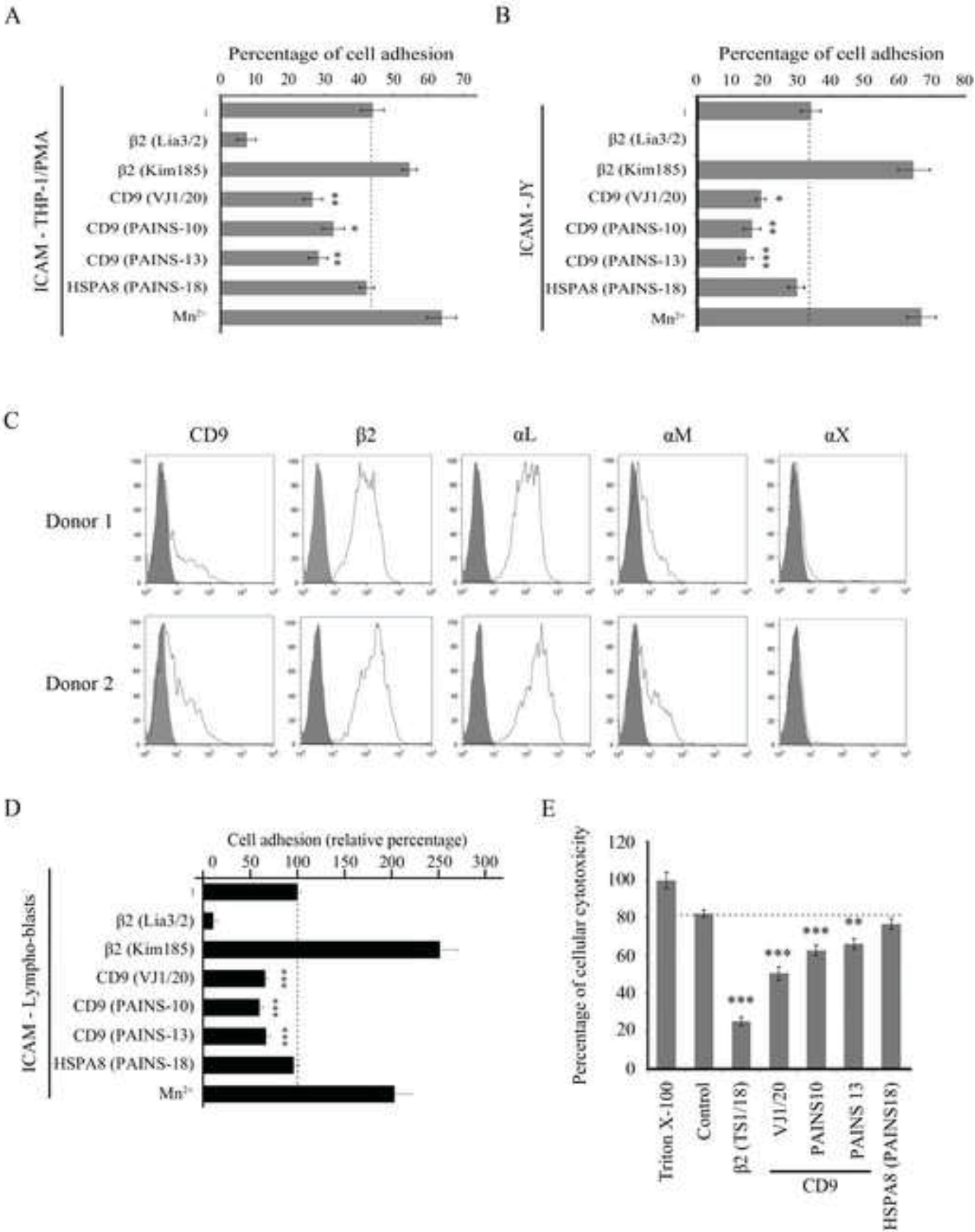


FIGURE 4

Figure 5
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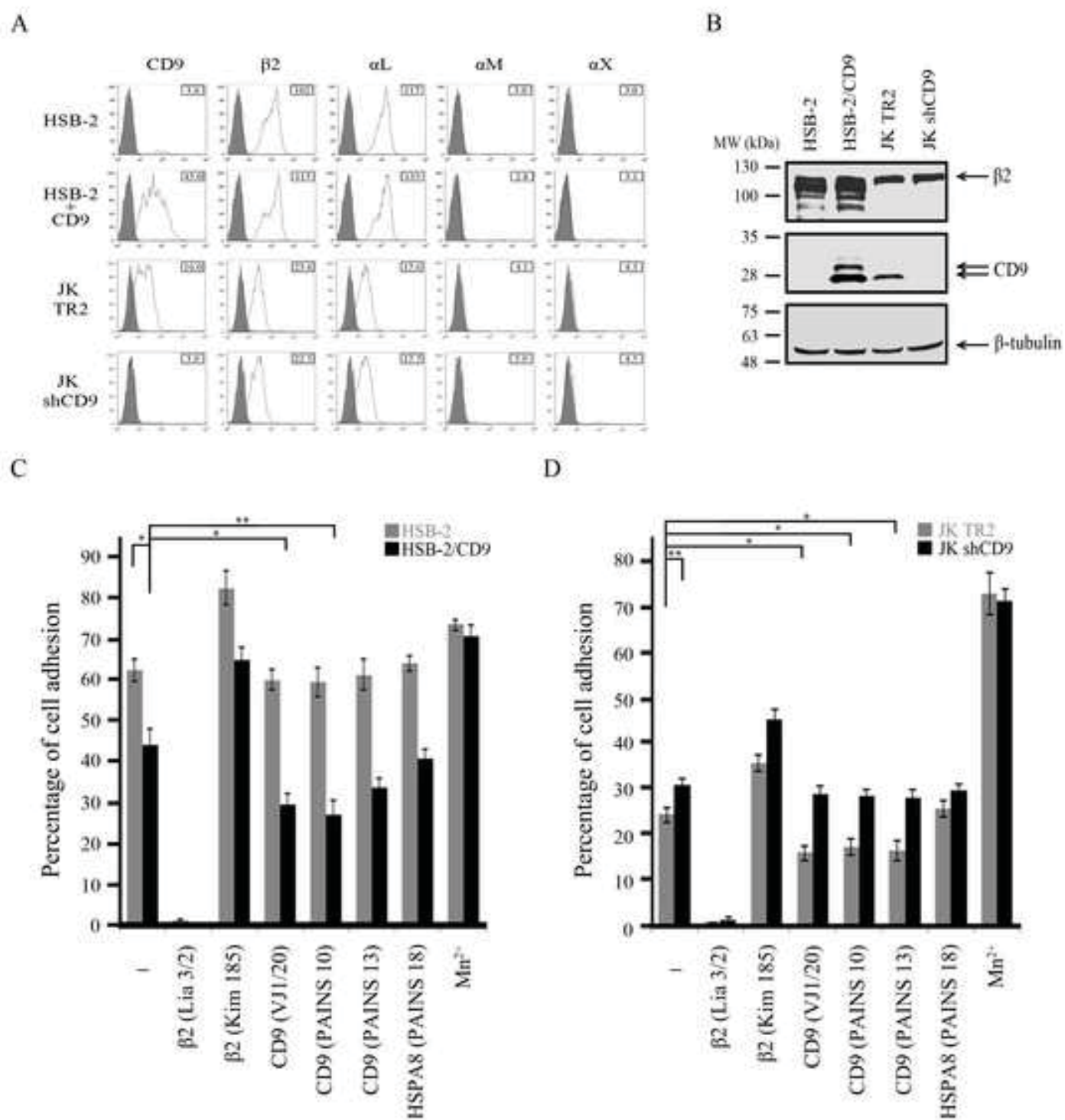
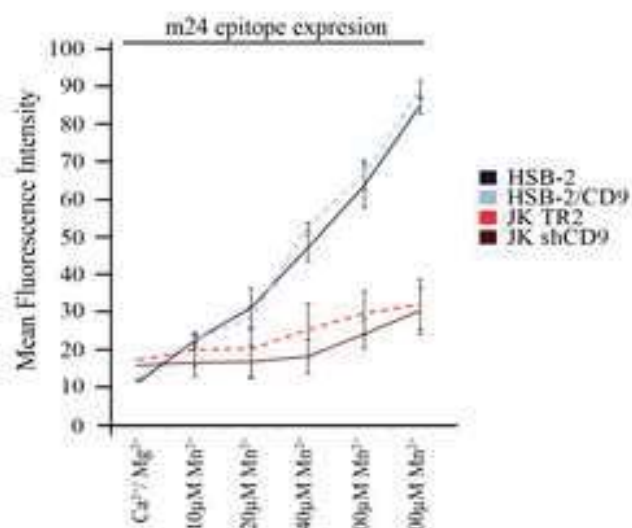


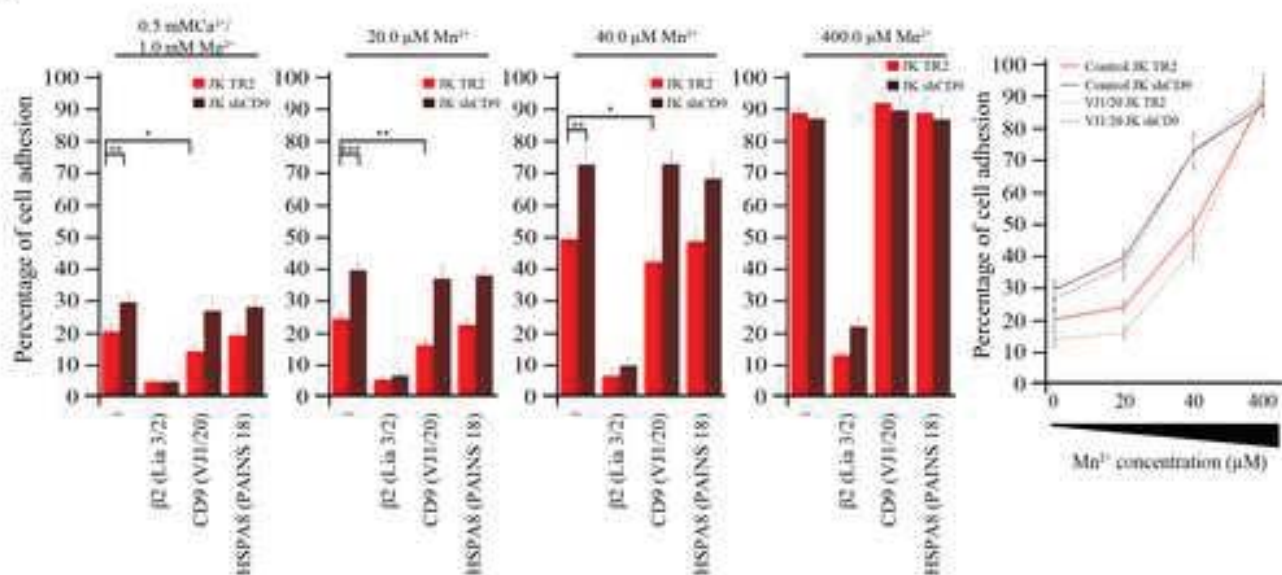
FIGURE 5

Figure 6
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A



B



C

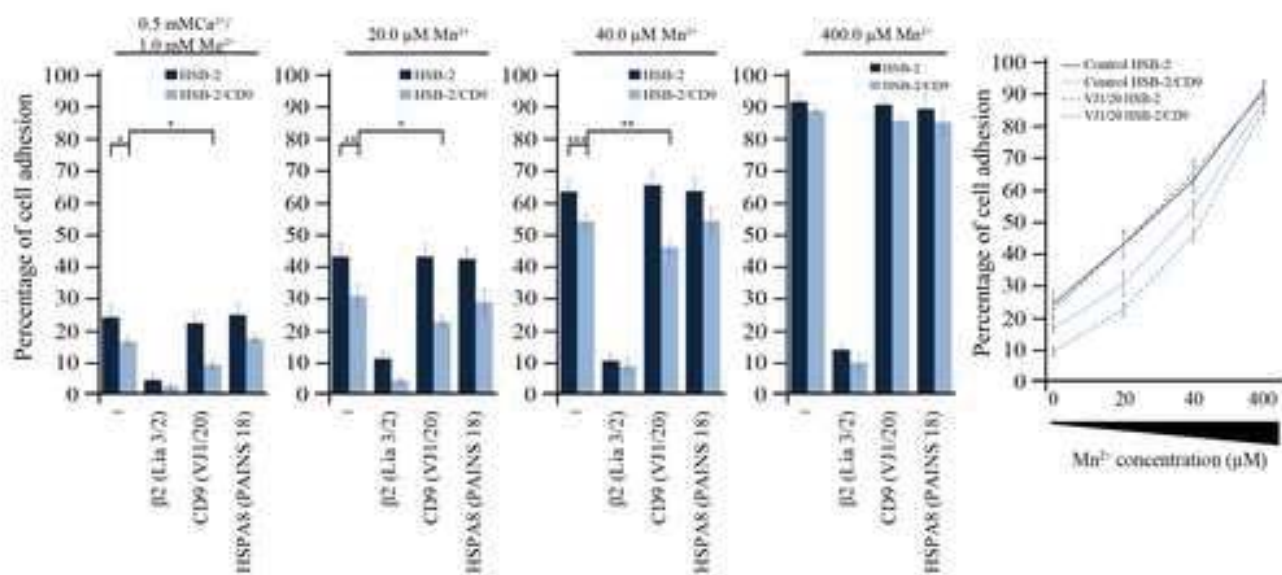


FIGURE 6

Figure 7
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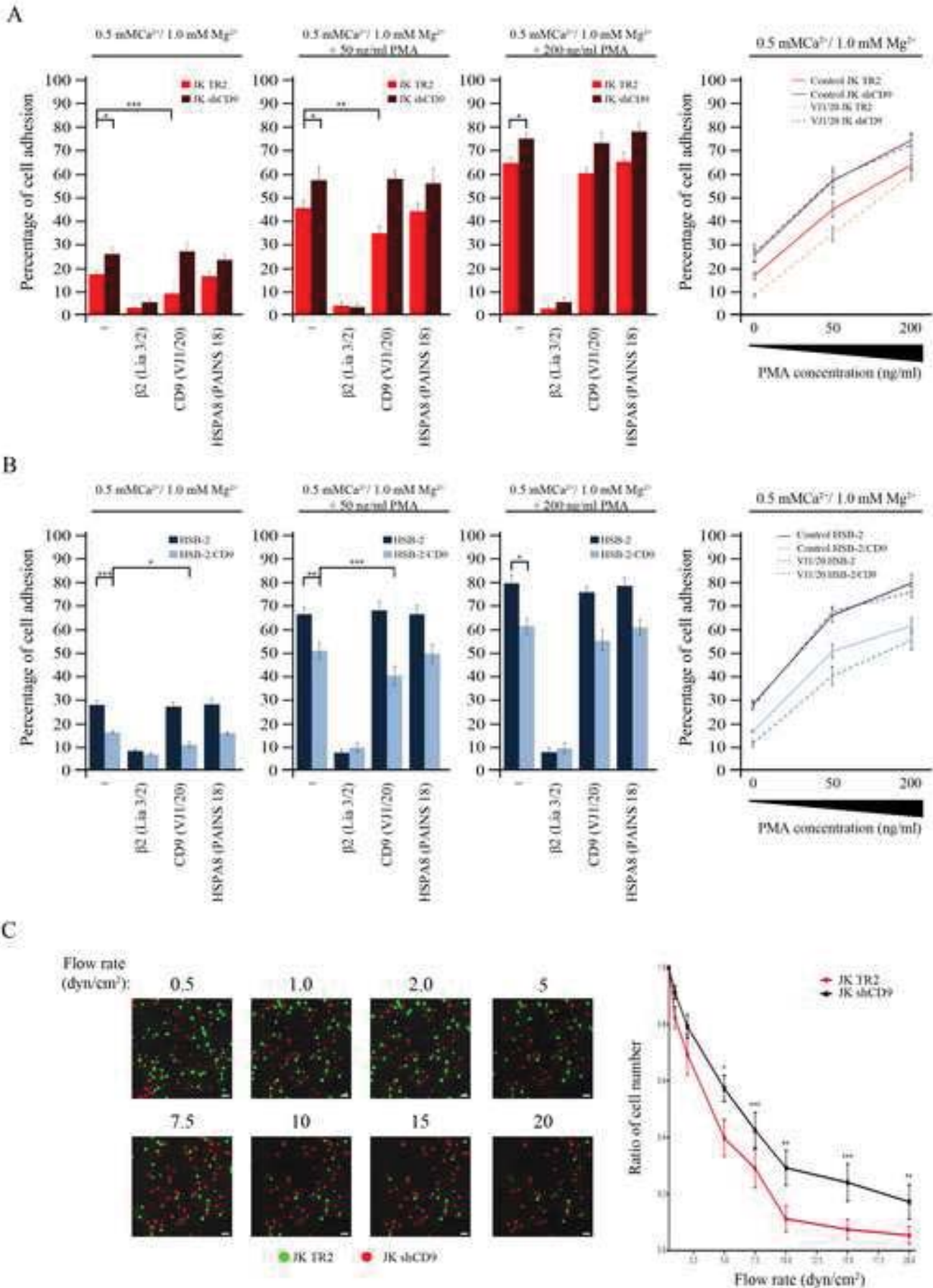


FIGURE 7

Figure 8
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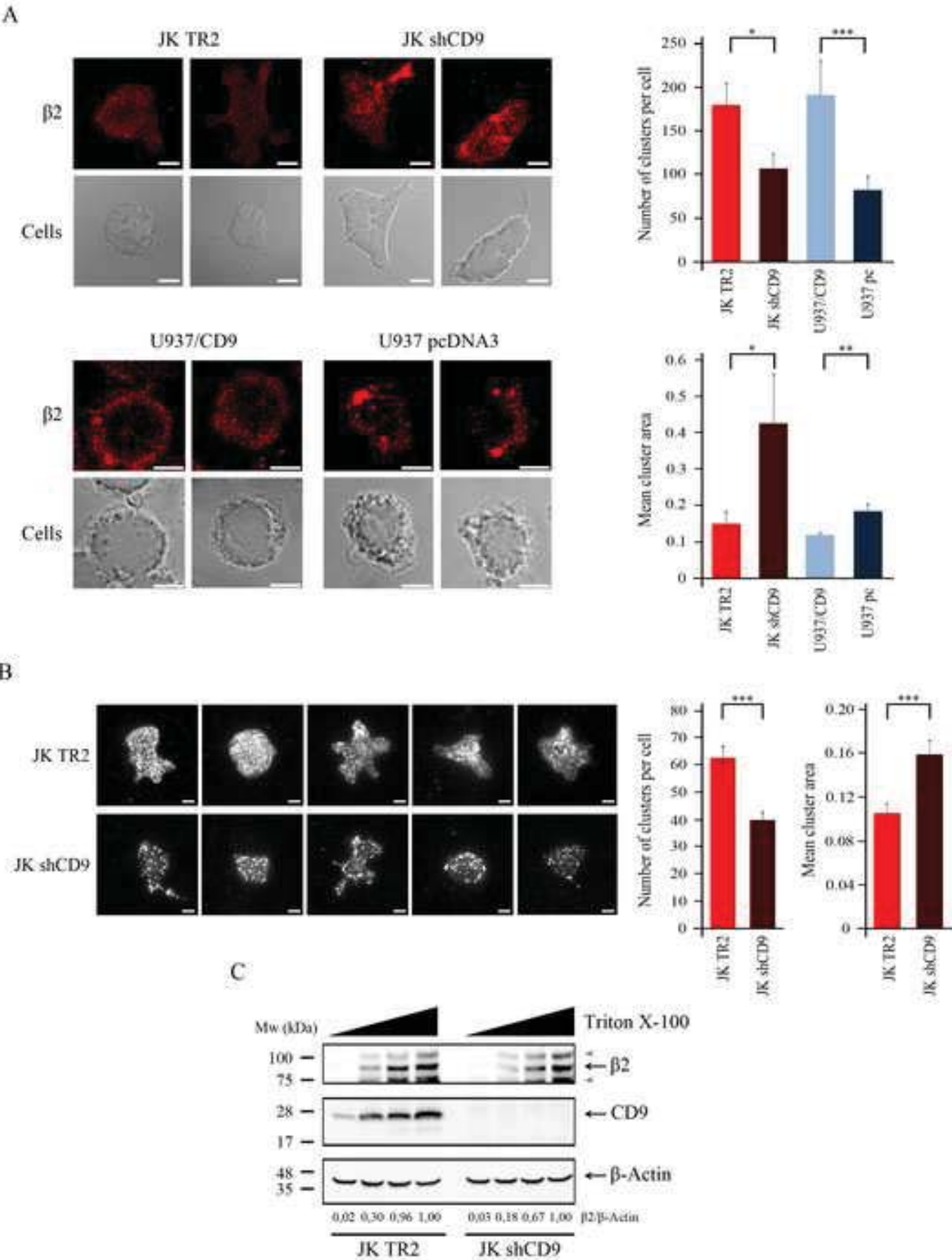


FIGURE 8

Figure 9
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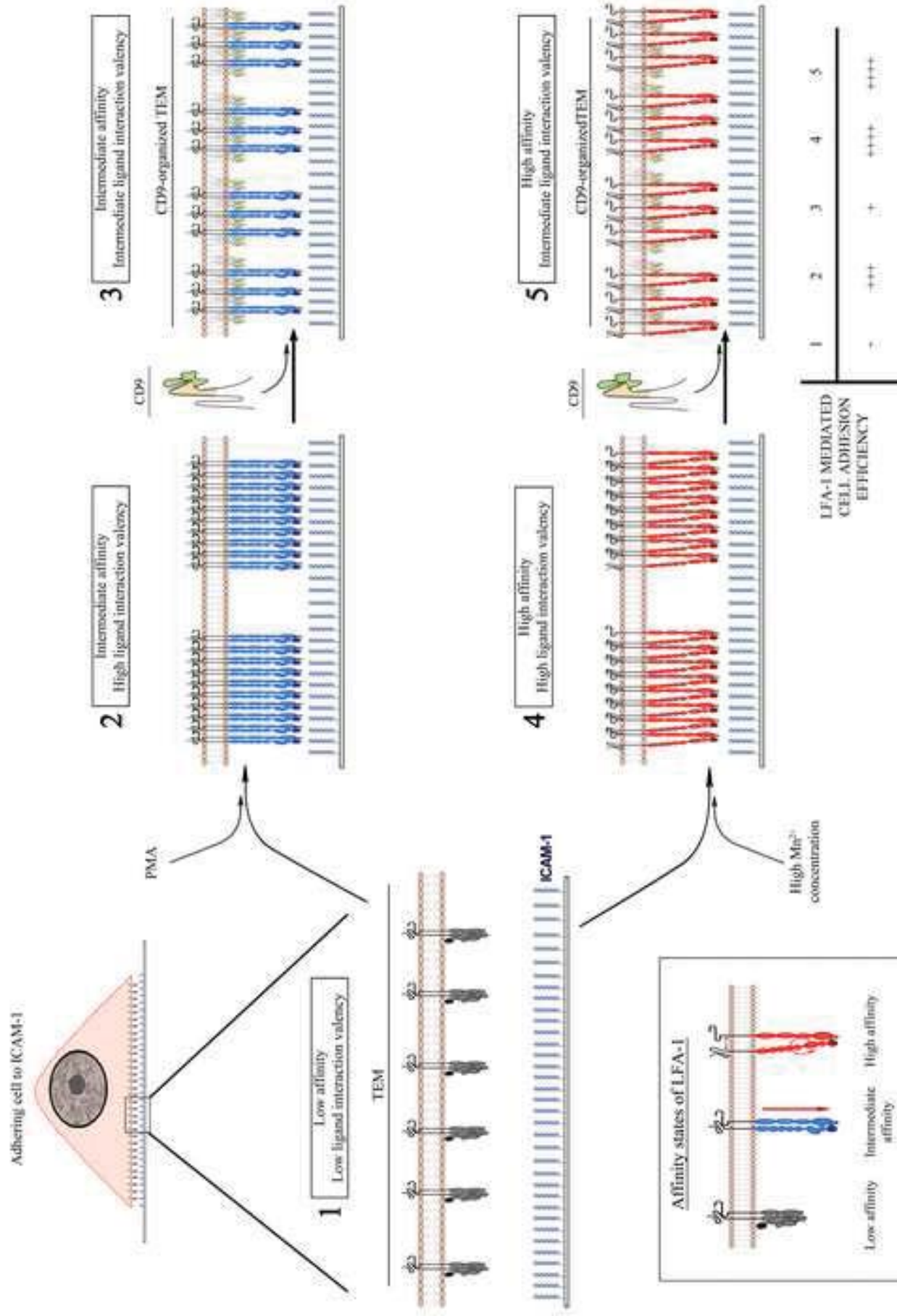
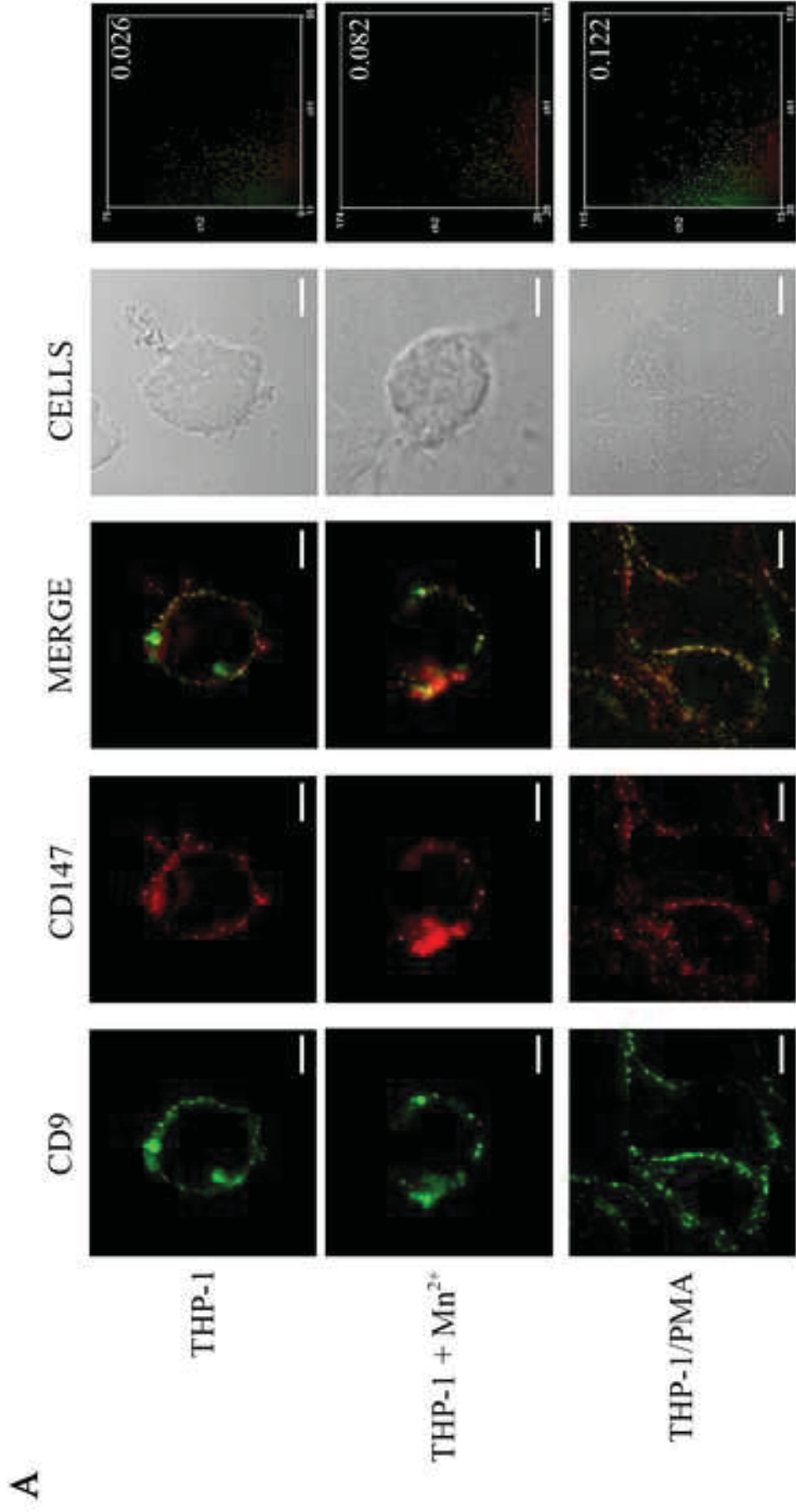
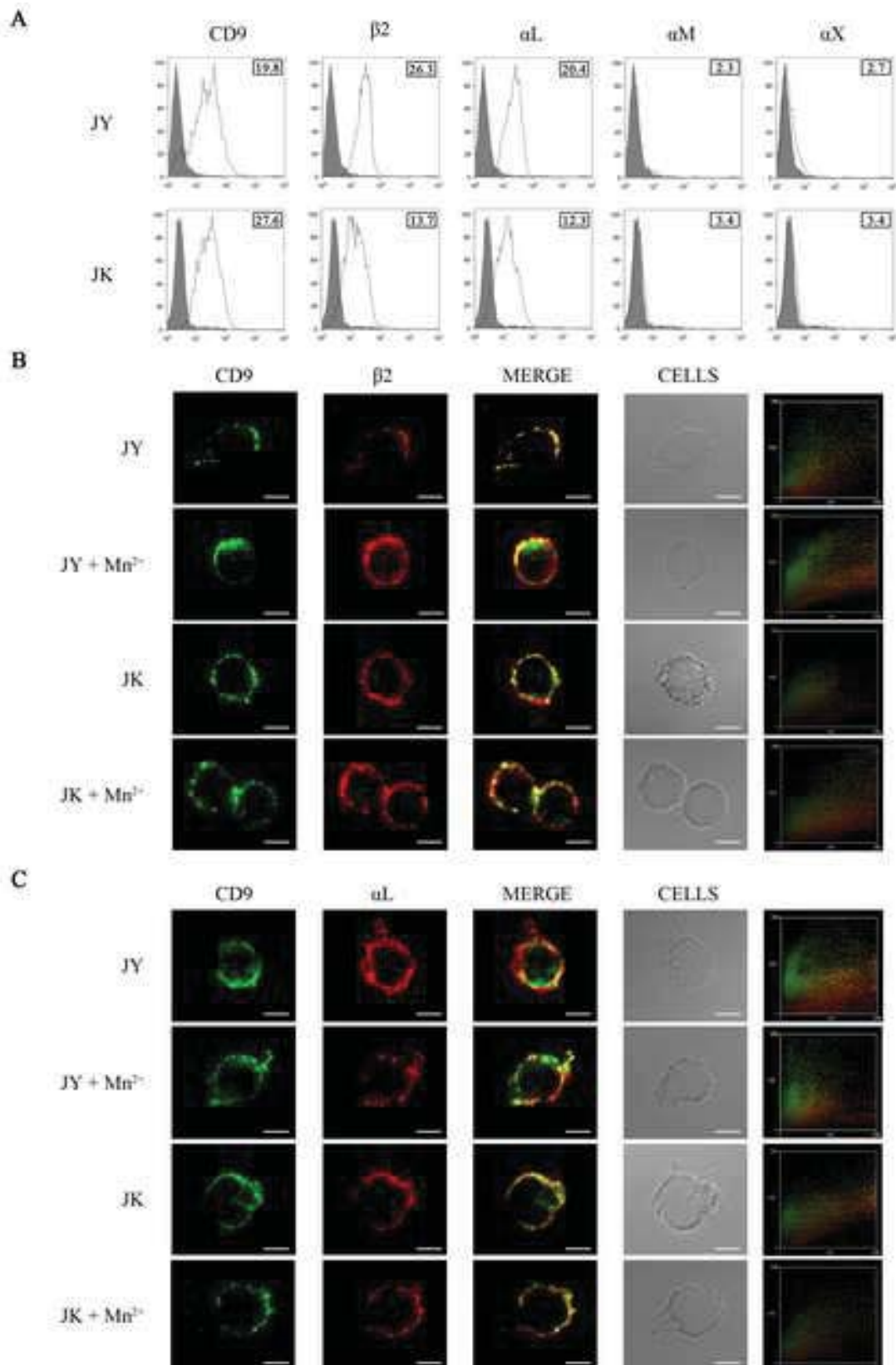


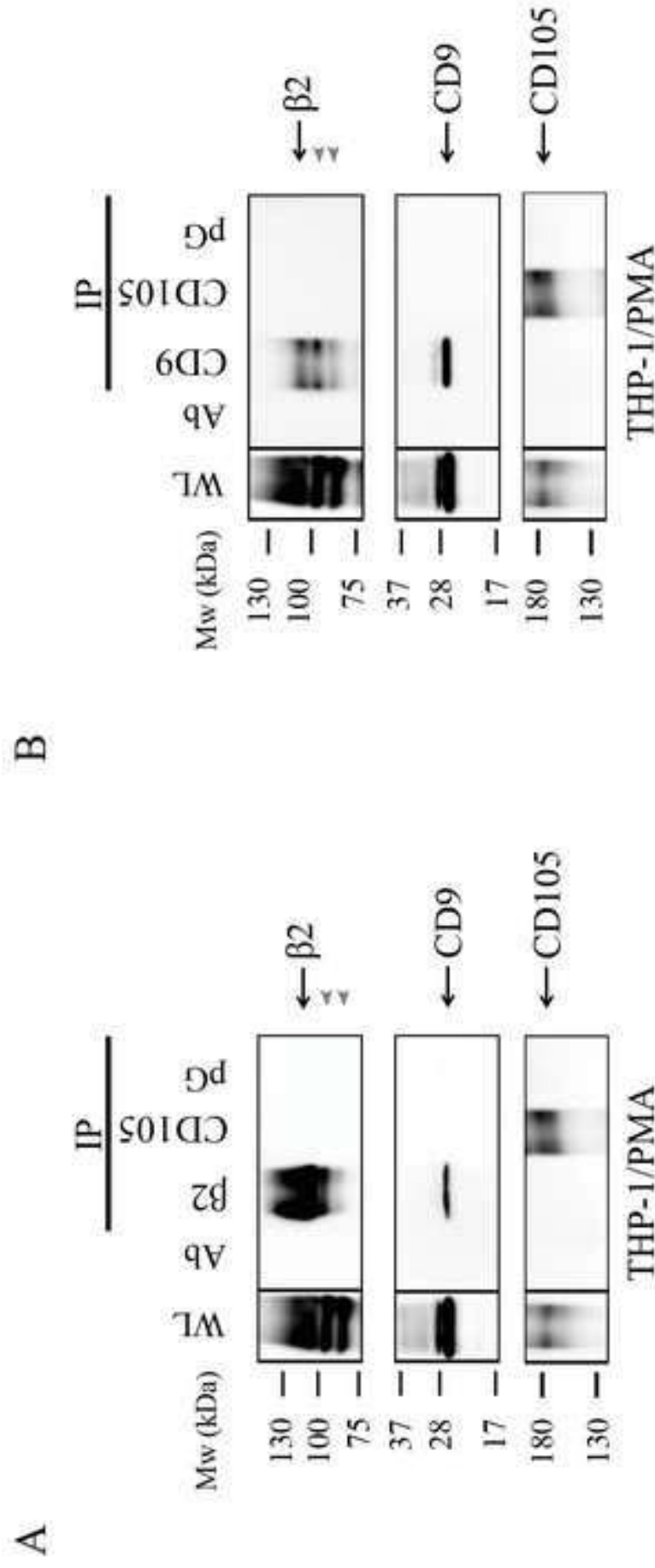
FIGURE 9



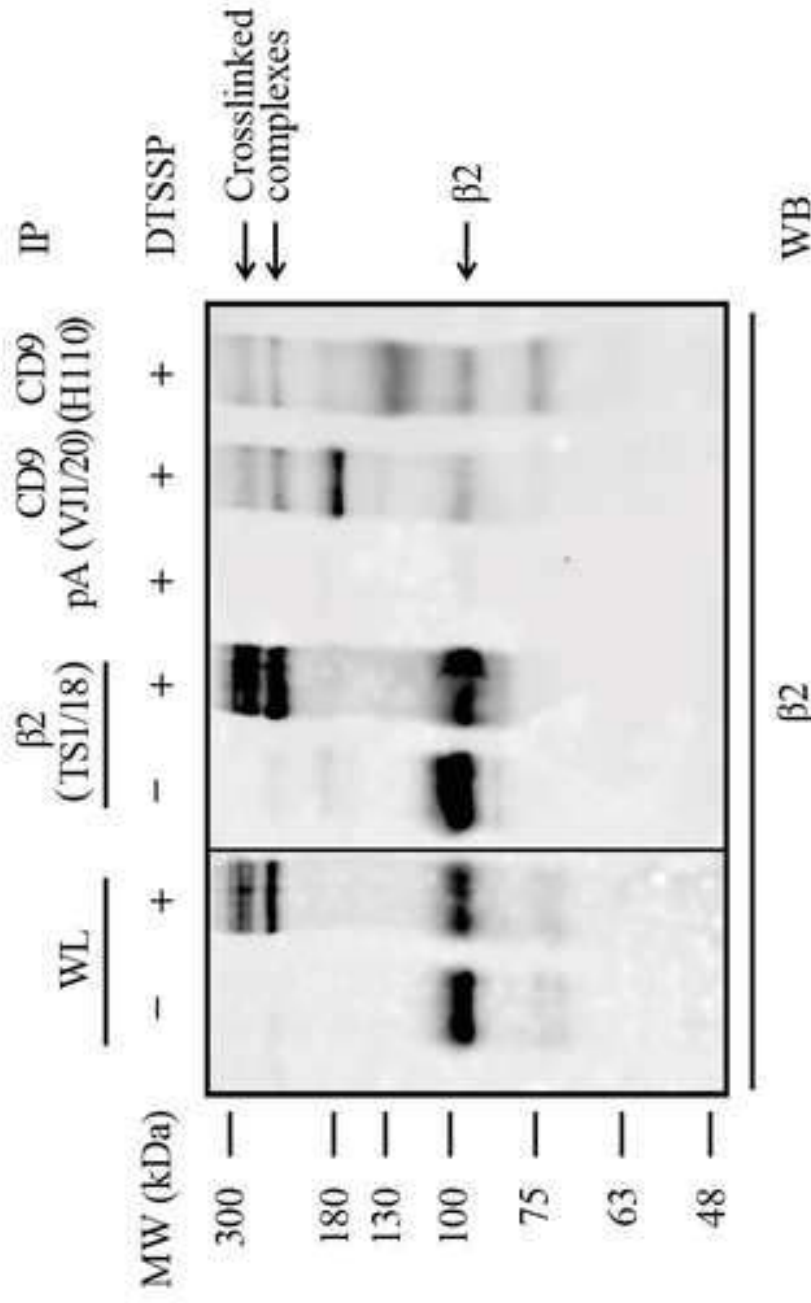
SUPPLEMENTARY FIGURE 1. Confocal microscopy of THP-1, THP-1 in the presence of 0.4 mM Mn²⁺ and THP-1/PMA differentiated cells showing marginal co-localization of CD9 (green) and CD147 (red), as a control for the specific co-localization between CD9 and $\beta 2$ integrin shown in Figure 1. Representative images of confocal sections for each channel (green or red) and merged channels are shown together with co-localization histograms (right panels) showing Pearson co-localization values. Scale bars = 5 μ m.



SUPPLEMENTARY FIGURE 2. CD9 co-localizes with LFA-1 on the surface of different leukocytic cell lines. **A)** The expression of CD9 (mAb VJ1/20), $\beta 2$ (mAb TS1/18), αL (mAb TP1/40), αM (mAb Bear-1) and αX (mAb HC1/1) molecules was analyzed by flow cytometry on JY B-lymphocytic cells (upper panels) or JK T-lymphocytic cells (lower panels). Negative controls are represented by gray filled histograms; empty histograms show the expression of the indicated molecules. The numbers in boxes represent M.F.I. values. **B)** and **C)** Confocal microscopy of JY and JK cell lines showing co-localization at the cell surface of CD9 in green and $\beta 2$ (panel B) or αL (panel C) in red. Representative images of confocal sections for each channel (green or red) and merged channels are shown together with co-localization histograms (right panels). Scale bars = 5 μm .



SUPPLEMENTARY FIGURE 3. Co-immunoprecipitation analysis of the association between LFA-1 and CD9. THP-1/PMA cells were incubated at 4°C with the immunoprecipitating mAbs TS1/18 (anti- β 2) in panel A, and VJ1/20 (anti-CD9) in panel B, prior to their lysis in 1% Triton-X100-containing lysis buffer. Protein immunocomplexes were precipitated with Protein-G-sepharose, then resolved by 8% (for detection of β 2) or 12% (for detection of CD9) SDS-PAGE under non-reducing conditions, and immunoblotted with anti- β 2 (MEM-46) (upper panels) or anti-CD9 (VJ1/20) (middle panels) biotin-conjugated mAbs or with anti-CD105 control protein (lower panels). Blots are representative of three different experiments. In some cell lysates, several β 2 bands (indicated by grey arrowheads) are immunodetected. WL: whole cell lysate, pG: Protein-G-sepharose without precipitating antibody.



SUPPLEMENTARY FIGURE 4. Analysis of LFA-1 and CD9-containing cross-linked complexes. JY cells were lysed in 1% Brij-97 lysis buffer and aliquots were either treated with DTSSP cross-linker or left untreated. Triton X-100 was then added to a final concentration of 1% (v/v) to cross-linked (+) and to non-cross-linked lysates (-). Samples were immunoprecipitated with mAb TS1/18 (anti- $\beta 2$), mAb VJ1/20 (anti-CD9) or with a rabbit polyclonal antibody H110 (anti-CD9), resolved on 7% SDS-PAGE under non-reducing conditions and subsequently immunodetected with biotinylated anti- $\beta 2$ integrin (MEM-48) mAb followed by streptavidin-HRP.

***Conflict of Interest**

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