Organization of the Integrin LFA-1 in Nanoclusters Regulates Its Activity\textsuperscript{D}

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The \(\beta_2\)-integrin LFA-1 facilitates extravasation of monocytes (MOs) into the underlying tissues, where MOs can differentiate into dendritic cells (DCs). Although DCs express LFA-1, unlike MOs, they cannot bind to ICAM-1. We hypothesized that an altered integrin organization on the DC plasma membrane might cause this effect and investigated the relationship between membrane organization and function of LFA-1 on MOs and DCs. High-resolution mapping of LFA-1 surface distribution revealed that on MOs LFA-1 function is associated with a distribution in well-defined nanoclusters (100–150-nm diameter). Interestingly, a fraction of these nanoclusters contains primed LFA-1 molecules expressing the specific activation-dependent L16-epitope. Live imaging of MO–T-cell conjugates showed that only these primed nanoclusters are dynamically recruited to the cellular interface forming micrometer-sized assemblies engaged in ligand binding and linked to talin. We conclude that besides affinity regulation, LFA-1 function is controlled by at least three different avidity patterns: random distributed inactive molecules, well-defined ligand-independent proactive nanoclusters, and ligand-triggered micrometer-sized macroclusters.

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Springer, 1990). Recently, endothelial cells were shown to form upright microvilli-like projections that are enriched in ICAM-1 molecules (Carman et al., 2003). Transmigrating MOs present regions of increased density of LFA-1 molecules that form linear clusters and colocalize with the ICAM-1–enriched projections (Carman et al., 2003). As precursors of antigen-presenting immature dendritic cells (DCs; Banchereau and Steinman, 1998), MOs can differentiate in DCs upon reverse transmigration over endothelial monolayers (Randolph et al., 1998) or when cultured in vitro in the presence of GM-CSF and IL-4 (moDCs; Romani et al., 1996). Although moDCs express LFA-1, unlike MOs, adhesion to ICAM-2 and -3 is completely mediated by the C-type lectin DC-SIGN (CD209; Geijtenbeek et al., 2000a, 2000b). These observations suggest that the plasma membrane organization of adhesion receptors on MOs and DCs must change dynamically, along with the specific alterations occurring during the development from precursors toward DCs. Our previous studies showed that distribution of DC-SIGN at the cell surface dynamically changed during DC development along with its binding properties (Cambi et al., 2004). This prompted us to investigate and compare the relationship between cell membrane organization and adhesiveness of LFA-1 on MOs and DCs. Here, we demonstrate that LFA-1–mediated binding to ICAM-1 is completely lost during development of moDCs. This coincides with gradual exclusion of LFA-1 from lipid microdomains. We used high-resolution transmission EM (TEM) on whole-mount samples of MOs and DCs to map the cell surface distribution of LFA-1 at submicrometer level. Our findings demonstrate the existence of three levels of avidity for LFA-1: randomly distributed inactive molecules, well-defined ligand-independent nanoclusters, and ligand-triggered micrometer-sized macroclusters.

**MATERIALS AND METHODS**

**Monoclonal Antibodies and Chemicals**

The mouse mAbs against α, chain: NKI-L15, NKI-L16, and TS2/4 (kindly provided by E. Martz). Activating KIM185, kindly provided by M. Robinson, Celltech (Slough, United Kingdom), and the blocking NKI-L19 recognize the β2 chain. REK-1 is a mAb anti-ICAM-1. CD46 and CD55 were detected by labeling with polyclonal (PL)–coated Fort IX (1 h at 37°C), washed to remove unbound cells, and immediately fixed with 1% PFA for 15 min. Subsequent Ab and gold labeling was performed as previously described (Cambi et al., 2004). When labeling was performed using NKI-L16, no pre-fixation with 1% PFA was performed to prevent loss of NKI-L16 binding. Therefore, after adhesion on the substrate, cells were washed in ice-cold buffer and incubated on ice with cold NKI-L16. Isotype-specific controls were always included. It should be noted that the effect of temperature changes on NKI-L16 labeling is negligible (Supplementary Figure 2).

**Analysis of Gold Particle Distribution Pattern**

For flow cytometry analysis, cells were incubated (30 min, 4°C) in PBS, 0.5% BSA, and 0.01% sodium azide, with different mAbs (5 μg/ml), followed by incubation with FITC–labeled goat anti-mouse IgG or IgM (Caltag, Becton Dickinson, San Francisco, CA) for 30 min at 4°C. The relative fluorescence intensity was measured on a FACSCalibur. Isotype-specific controls were included. Analysis was done with a Bio-Rad MRC1024 Confocal Laser Scanning Microscope (Richmond, CA). Signals were collected sequentially to avoid bleed-through. To prevent loss of NKI-L16 binding in the double- or triple-labeling experiments, cells were not fixed before adding the primary Abs. They were allowed to adhere on FN-coated coverslips and stained with anti-LFA-1 (NKI-L16 or NKI-L15, or TS2/4) and anti-ICAM-1 (10 μg/ml) at 4°C, after prolonged incubation in CLSM buffer to minimize a specific binding. After removing unbound Abs by extensive washing in ice-cold PBS, cells were quickly fixed in 1% PFA, and after a blocking step in CLSM buffer, secondary staining for LFA-1 and ICAM-1 was performed using isotype-specific Alexa-conjugated goat anti-mouse antibodies. Manders coefficient M1 was calculated as already reported (Costes et al., 2004) and reflects the amount of LFA-1 colocalizing with GM1 separately for each cell.
1–binding capacity from MOs to DCs, we measured LFA-1–mediated binding to ICAM-1–Fc-coated beads during DC development (Geijtenbeek et al., 1999). MOs and DCs profoundly differ in their capacity to bind ICAM-1–coated fluorescent beads (Figure 1A). More than 60% of MOs spontaneously bound ICAM-1, and this interaction was predominantly β2-integrin mediated, as shown by the effective block in the presence of anti-β2 blocking mAb NKI-L19. The use of anti-ζL blocking mAb (NKI-L15) indicated that on MOs, binding to ICAM-1 was predominantly mediated by LFA-1 (40%), whereas the other two β2-integrins MAC-1 and p150.95 mediated the residual binding.

On differentiation of MOs into DCs, spontaneous binding to ICAM-1 gradually decreased. On DCs, residual binding to ICAM-1 was barely detectable and slightly inducible by the anti-β2–activating mAb KIM185. Similarly, other stimuli known to activate LFA-1, such as Mn2+, cytochalasin D, thapsigargin, or PMA, were unable to enhance LFA-1–binding capacity (our unpublished data). Furthermore, no LFA-1–mediated binding was observed to soluble ICAM-1 molecules nor to ICAM-1 coated onto a plate (our unpublished data), further indicating that LFA-1 on DCs is unable to bind to its ligand independently of the assay used. Any involvement of Fc receptors in the binding to ICAM-1 Fc was excluded by preincubating the cells with a Fc receptor–blocking agent that showed no effect in the bead assay (our unpublished data).

To exclude that the decreased binding to ICAM-1 on DCs was due to decreased expression levels, we detected LFA-1 expression by flow cytometry. Figure 1B shows that LFA-1 expression levels, detected with the mAb NKI-L15, remained unaltered during DC development.

The transition of LFA-1 from an inactive into an active state is known to depend on extracellular Ca2+ ions, and Ca2+ occupancy is reported by the α chain–specific mAb NKI-L16, which detects the L16 epitope (Keizer et al., 1988; van Kooyk et al., 1991, 1994). Recent studies more specifically showed that NKI-L16 recognizes a Ca2+-bound extended conformation of the α subunit, typical of primed LFA-1 (Xie et al., 2004). Therefore, we analyzed the L16 epitope expression on MOs and DCs. As shown in Figure 1B, the L16 epitope was highly expressed on MOs but was barely detectable on DCs.

It should be noted that the high expression level of the other β2 integrins on both MOs and DCs prevented us from analyzing other known activation reporter epitopes that involve the β2 subunit to monitor LFA-1 activation state.

Together, these observations indicate that major changes in LFA-1 adhesiveness occur during DC development.

**Involvement of Lipid Rafts on LFA-1 Activity on MOs and DCs**

The recruitment of active LFA-1 into specific cholesterol- and glycosphingolipid-enriched microdomains, known as lipid rafts, was proposed as additional mechanism to regulate integrin activity (Krauss and Altevogt, 1999; Leitinger and Hogg, 2002; Marwali et al., 2003). Therefore, we investigated whether lipid rafts played a role in LFA-1 adhesiveness on MOs and DCs.

On MOs, the effect of MCD, a lipid raft–disrupting agent that extracts membrane cholesterol, was tested on the ICAM-1 bead adhesion assay (Figure 2A). MCD treatment inhibits binding for ~40%, indicating that lipid raft disruption partially affected LFA-1–binding capacity. Furthermore, we examined the codistribution of LFA-1 and the lipid raft marker GM1, a glycosphingolipid, on MOs and DCs, by Ab patching and confocal microscopy (Figure 2B). When cocapping was induced on MOs, LFA-1 completely colocalized with GM1 to the same extent as for the lipid raft–associated GPI-anchored protein CD55. By contrast, on DCs, LFA-1 is completely excluded from the lipid raft fraction.

Gradual exclusion of LFA-1 from these lipid microdomains parallels the loss of L16 epitope and binding capacity. Already after 3 d of monocyte differentiation, on the majority of the cells, LFA-1 no longer colocalized with GM1 (our unpublished data).

On MOs, we quantified the colocalization of either NKI-L15 (total LFA-1) or NKI-L16 (primed LFA-1) with GM1 by calculating the Manders coefficient (Costes et al., 2004). No significant differences in colocalization with lipid rafts were observed between primed and total LFA-1 (Figure 2C), suggesting that on MO LFA-1 resides in lipid rafts even if the molecule is not in the Ca2+-bound extended primed conformation. Finally, the colocalization coefficient for LFA-1 and GM1 on DCs was found to be 0.14 ± 0.24 (n = 25), where 1 indicates full colocalization, thus indicating a significant exclusion of LFA-1 from lipid raft domains on DCs.

**LFA-1 Is Organized in Nanoclusters on MOs**

The organization of the cell membrane has been described as a mosaic of numerous lipid and protein microdomains ranging from 20 to 500 nm (Mayor and Rao, 2004). The fact that
Figure 2. LFA-1 resides in lipid rafts on MOs, not on DCs. (A) LFA-1–mediated adhesion to ICAM-1–coated beads was measured on MOs after cholesterol depletion by preincubation with 20 mM MCD for 30 min at 37°C. Data shown are means ± SD of one representative experiment (out of three) performed in triplicate. (B) Confocal microscopy analysis of copatching of LFA-1 (NKI-L15 labeled) and GM1 on MOs and DCs. Receptor copatching and staining were performed as described in Material and Methods. CD55 and CD46 are positive and negative lipid rafts marker, respectively. Results are representatives of multiple cells in three independent experiments. Bar, 5 μm. (C) To quantify the degree of colocalization between either NKI-L15 (total LFA-1) or NKI-L16 (primed LFA-1) with GM1 on MOs, the Manders coefficient (M1) was calculated. M1 can vary between 0 and 1 (1 = colocalization). Receptor copatching and staining were performed as described in Material and Methods, and cells were analyzed by confocal microscopy (n = 22).
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**Figure 3.** LFA-1 is clustered on MOs and random on DCs. MOs and DCs were specifically labeled with 10-nm gold and treated for TEM (see Materials and Methods). Results represent multiple cells in several independent experiments. The top pictures represent whole cells imaged by TEM. Middle and bottom pictures are higher magnifications where 10-nm gold particles are visible. Bar, 200 nm unless otherwise indicated.

LFA-1 differentially associates with lipid rafts on MOs and DCs (Figure 2) prompted us to investigate the cell membrane organization of LFA-1 at high resolution.

We exploited TEM to study whole-mount samples of MOs and DCs, after specific labeling of LFA-1 with gold particles. Recently, we and others demonstrated the potential of this method to map and quantitate the distribution patterns of cell membrane receptors (Vereb et al., 2000; Panyi et al., 2003; Cambi et al., 2004).

MOs and DCs were allowed to adhere and stretch before labeling with mAb against LFA-1 and gold particles. It should be noted that the stretched cells are so thin (down to 200 nm at cell periphery) that sectioning is not required, thus making the whole dorsal membrane available for gold labeling and subsequent TEM analysis. To exclude any effect of the substrate on LFA-1 distribution, TEM analysis was also performed on cells that were gold-labeled in suspension and then mounted onto PLL. No differences were seen between cells stretched on the support or cells adhering to PLL (our unpublished data). We found that LFA-1 distribution changed dramatically during DC development (Figure 3).

Although on MOs LFA-1 is organized in well-defined nanoclusters, on DCs the gold particles are evenly distributed over the cell surface.

To quantitatively describe LFA-1 distribution pattern, nearest neighbor (nn) distance values among the gold particles were calculated applying spatial-point-pattern analysis (Cambi et al., 2004). On MOs, almost 80% of the gold particles resides within 50-nm distance from its nearest neighbor (Figure 4A). In contrast, on DCs, nn distance values are almost equally distributed in all distance categories, indicating no preferential organization of gold particles.

The relative partitioning of gold particles in clusters of various sizes (i.e., number of particles/cluster) was also quantified (Figure 4B). Although on MOs only 20% of gold particles were detected as single features, on DCs up to 70% of gold particles were found as isolated single features on the cell membrane.

LFA-1 nanoclusters appear either as round or slightly elongated features, with an indicative average diameter of 150 nm, and are randomly localized on the cell. Similarly to NKI-L15, NKI-L16 staining also showed clustered distribution of gold-labeled LFA-1 on MO cell membrane (Figure 5A). However, when LFA-1 was labeled by NKI-L16, the number of gold particles per \( \mu m^2 \) was approximately fourfold lower when compared with NKI-L15 labeled cells (Figure 5B). Labeling with TS2/4 mAb, which recognizes another (inert) LFA-1 epitope, gave results similar to NKI-L15 (our unpublished data).

To exclude that differences in the amount of gold particles per \( \mu m^2 \) were due to differences in Ab labeling efficiency, we also calculated the density of LFA-1 clusters on the cell surface (Figure 5C). NKI-L16 labeled only one third of LFA-1 clusters detectable on MO cell membrane. Again, TS2/4 labeling gave similar results when compared with NKI-L15 (our unpublished data). In addition, all mAbs showed similar distributions of nn distance values among gold particles (Figure 5D) and comparable cluster sizes (Figure 5E). This indicates that LFA-1 nanoclusters labeled by NKI-L16 are similar to the NKI-L15 negative nanoclusters in terms of density and size, but represent a primed fraction of LFA-1 that strongly binds Ca\(^{2+}\) and is in an extended (primed) conformation. MOs express LFA-1 and ICAM-1, facilitating the formation of aggregates of cells. Because it was suggested that ICAM-1 ligation might trigger LFA-1 microclusters formation (Kim et al., 2004), cells were seeded at low density to avoid stimulation by homotypic aggregation and were extensively washed allowing LFA-1 distribution to equilibrate before labeling for TEM was performed. Furthermore, inhibition of LFA-1/ICAMs interactions in cis does not alter LFA-1 nanocluster organization (Supplementary Figure 3).

Therefore, on MO LFA-1 nanoclusters are formed in a ligand-independent manner. Although most LFA-1 nanoclusters are in a resting state, ~25% are in a primed state (L16 epitope expressed). Notably, L16 epitope expression is confined to a subset of nanoclusters rather than equally distributed over all clusters, suggesting the existence of clusters representing distinct states of integrin activation. MO differentiation into DC leads to complete dispersion of the nanoclusters and subsequent random distribution of single inactive LFA-1 molecules.

**Primed LFA-1 Nanoclusters Readily Bind to ICAM-1**

To further investigate the difference between primed and resting LFA-1 nanoclusters, we performed double labeling of LFA-1 using NKI-L16 and TS2/4 (nonoverlapping epitopes). As shown in Figure 6A, all LFA-1 nanoclusters are detectable by TS2/4, but only a subpopulation of these also shows expression of the L16 epitope (indicated in yellow). Interestingly, when MOs were seeded at high density,
primed L16 epitope expressing LFA-1 was specifically enriched at the contact zone between adjacent cells, indicating that binding of ligand leads to preferential recruitment of primed LFA-1 clusters (Figure 6B).

To test if the primed LFA-1 fraction at the contact site between adjacent cells was specifically involved in the binding to ICAM-1 on the opposing cell, we allowed the formation of conjugates between individual MOs and performed triple labeling to detect total LFA-1, primed LFA-1, and ICAM-1 simultaneously. The primed LFA-1 fraction completely colocalized with ICAM-1 at the contact sites, forming macroclusters up to a size of several micrometers (Figure 6C).

Although several proteins have been identified that bind to the cytoplasmic tails of integrins (Liu et al., 2000), accumulating evidence suggests that talin plays a central role in activating LFA-1 (Kupfer and Singer, 1989; Monks et al., 1998; Sampath et al., 1998). In verifying which cytoplasmic regulators of LFA-1 were present at the contact sites, we observed that LFA-1 macroclusters engaged in binding with ICAM-1 completely colocalize with talin, which is clearly enriched at the contact sites (Figure 6D). Although we did not find a similar enrichment of other known regulators of LFA-1 such as cytohesin-1 (Geiger et al., 2000) and RapL (Katagiri et al., 2003; Figure 6E and F), we cannot exclude that these proteins may have either a transient or a weaker role in regulating LFA-1 adhesion on MOs.

Next, we investigated whether the interaction of LFA-1 with talin is essential in the formation of primed nanoclusters even in absence of ligand binding. Ligand-independent Ab-induced capping of primed LFA-1 did not result in clear colocalization of the cytoplasmic proteins talin, cytohesin, and RapL, suggesting that the expression of the L16 epitope is not the result of pre-existing ligand-independent association of the cytoplasmic tails of LFA-1 with any of its known regulators (Supplementary Figure 1).

From these results we conclude that on the cell membrane of MO LFA-1 molecules are organized in nanoclusters formed without prior engagement of ligand and independent from known intracellular regulators such as talin.

**Figure 4.** Quantitative analysis of the distribution of gold particles labeling LFA-1. The digital images were processed by a custom-written software based on Labview. Gold labels were counted, and coordinates were assigned to each feature. Interparticle distances were calculated using a nearest neighbor (nn) distance algorithm. (A) nn distance values were calculated for each image, and the data of several independent experiments were pooled. Subsequently, the nn distances were divided into three classes: 0–50, 50–100, and >100 nm, and the percentage of nn distance values falling into each class was plotted. (B) The partitioning of gold labels in clusters of various size (i.e., number of particles/cluster) was also quantified. Clusters were defined when gold particles were <50 nm apart from a neighboring particle. The percentage of gold particles involved in the formation of a certain cluster size was calculated. The insets are two representative processed digital images, where each type of cluster is shown in a different color. One representative experiment out of three is shown.
primed LFA-1 molecules can be observed at the contact area between MOs and T-cells from 270 to 810 s after contact initiation. Importantly, the presence of neither TS2/4 nor NKI-L16 significantly increases binding of MOs (nor of DCs) to ICAM-1–coated beads (our unpublished data), suggesting that the cell–cell interaction cannot be attributed to the presence of these Abs. When the interaction between MOs and T-cells occurred in presence of the blocking mAb NKI-L15, hardly any conjugate was observed, and no recruitment of primed LFA-1 could be detected (Figure 7B).

Finally, we quantified the recruitment of primed LFA-1 fraction (L16 positive) with respect to the total LFA-1 (labeled by TS2/4 or NKI-L15) by calculating the recruitment index (RI), which we defined as the fluorescence intensity at the contact area divided by the fluorescence intensity of the whole cell (Figure 8). Figure 8A shows that primed LFA-1 is significantly enriched at the cell–cell contact site with respect to the total LFA-1 fluorescence signal. In presence of the blocking Ab NKI-L15, no clear interaction between MOs and T-cells could be observed, and no specific recruitment of LFA-1 was observed at the cell–cell contact (Figure 8B).

Together, these results indicate that ligand-independent primed LFA-1 nanoclusters are preformed LFA-1 platforms rapidly recruited to the cell–cell contact where they fuse to originate micrometer-sized macroclusters. The LFA-1 macroclusters are engaged in ligand binding and specifically interact with the cytoskeleton via talin.

**DISCUSSION**

The leukocyte specific β2 integrin LFA-1 regulates cell adhesion, migration, and immunological synapse formation. Here
we demonstrate for the first time, exploiting high-resolution TEM and spatial-point pattern analysis, that LFA-1–mediated cell adhesion is directly correlated with submicrometer-sized avidity changes of LFA-1 when MOs differentiate into DCs. On DC LFA-1 Is Inactive

MOs can readily differentiate into antigen-presenting DC upon reverse transmigration of endothelial monolayers (Randolph et al., 1998) or when cultured in vitro in presence of GM-CSF and IL-4 (Romani et al., 1996). Therefore, by using MOs and moDCs instead of LFA-1 transfectants we followed changes in the behavior of endogenous adhesion receptors in a semiphysiological system. Moreover, moDC are currently manipulated ex vivo for antitumor and antiviral therapy (Figdor et al., 2004). Detailed characterization of the migratory and adhesive properties of moDCs is therefore very important and can have direct consequences for the currently executed clinical trials.

Although MOs and DCs express equivalent levels of LFA-1, there is a marked difference in LFA-1 activation state on the two cell types (Figure 1A). High-resolution mapping of LFA-1 cell surface distribution patterns by TEM and quantitative spatial point pattern analysis now revealed that on DCs the resting LFA-1 molecules are randomly distributed on the cell membrane as single features (Figure 3). Similar observations were made on K562 cells transfected with LFA-1 (K-LFA-1), which do not bind ICAM-1 and exhibit a random cell surface distribution of LFA-1 (our unpublished data). These data are in agreement with recent observation that the absence of FRET-detectable microclustering corresponds with lack of binding activity on K-LFA-1 cells (Kim et al., 2004).

Crystallography, NMR, and negative stain EM showed different conformational states of integrin fragments, revealing structural rearrangements of both β and α subunits upon activation (Beglova et al., 2002; Takagi et al., 2002; Xiao et al., 2004). In the low-affinity conformation, the headpiece of the integrin faces down toward the cell membrane; in the high-affinity conformation, the headpiece extends upward. This molecular switch occurs at the “genu” of the integrin subunits (Takagi and Springer, 2002). In particular, the α subunit leg has been shown to extend at the Ca²⁺-dependent epitope near the thigh–genu interface (Xie et al., 2004). This epitope appears only in the extended conformation and in the case of αL specifically recognized by the conformation-sensitive mAb NKI-L16 (Keizer et al., 1988; Xie et al., 2004). We have shown that LFA-1–binding capacity is directly correlated to the expression of this epitope (Figure 1B). Because in the αL inactive conformation the L16 epitope is shielded from Ab recognition, the complete lack of L16 epitope on DCs suggests that all LFA-1 molecules are in the “off,” i.e., bent conformation. A recent publication of van Gisbergen et al. (2005) suggests that on DCs inactive LFA-1 may be triggered upon interaction of DCs with T-cells (van Gisbergen et al., 2005). Further studies are needed to unravel the exact signaling pathways that may trigger resting LFA-1 molecules on DCs.

LFA-1 Nanoclusters Are Either Resting or Primed

On MOs, LFA-1 is organized in primed nanoclusters (L16 positive) and resting nanoclusters (L16 negative). Because no apparent differences were found in gold particles proximity or cluster size when comparing resting and primed nanoclusters (Figure 5), we hypothesize that clustering and...
priming represent two distinct events and LFA-1 molecules must all adopt either the bent inactive or the extended primed conformation within a nanocluster. The underlying mechanism remains unknown, but it is tempting to speculate that a cooperative effect between the packed LFA-1 molecules may exist. Recently, Nishiuchi et al. (2005) de-

Figure 7. The primed LFA-1 nanoclusters are rapidly recruited at the MO–T-cell contact. (A) MOs were allowed to adhere at low density onto a glass coverslip for 30 min at 37°C. After washing with PBS, MOs were labeled with anti-al mAbs NKI-L16 and TS2/4, as described in Materials and Methods. After removal of unbound Abs, Jurkat T-cells were added. The formation of spontaneous conjugates between MOs and T-cells was followed at 37°C with a Zeiss LSM 510 microscope. (B) In presence of the blocking mAb NKI-L15, the number of conjugates was lower, and no enrichment of NKI-L16–positive LFA-1 molecules at the cell–cell contact was observed.

Figure 8. Quantification of the recruitment of the primed LFA-1 fraction at the MO–T-cell contact site. As shown in the cartoon, the recruitment index (RI) of each Ab was defined as the fluorescence intensity at the contact site divided by the fluorescence intensity of the whole cell. Data in A and B are referred to the images shown in Figure 7, A and B, respectively, and are representative of multiple cells in three independent experiments.
Figure 9. Major changes in LFA-1 avidity and adhesiveness occur during DC development. This cartoon summarizes the major phenotypical changes observed on DCs compared with their precursor MO. Although on immature DCs, LFA-1 is unable to bind to its ligand, is randomly distributed at the cell surface and completely excluded from the lipid rafts, on MO LFA-1 is organized in well-defined submicrometer-sized nanoclusters. LFA-1 nanoclusters reside in lipid rafts but differ in terms of activation state: a fraction is primed and expresses the L16 epitope, the rest is in a bent resting conformation. By interaction with ICAM-1-bearing cells, the primed nanoclusters are recruited at the contact site, generating micrometer-sized macroclusters. There, LFA-1 binds to the counterreceptor ICAM-1 and establishes additional interactions with the cytoplasmic protein talin. In cell types other than MO, other regulators, such as RapL, might be then recruited from the cytoplasm and further strengthen binding and modulate signaling.

Described the role of the tetraspanin CD151 in modulating the integrin α3β1 adhesiveness by stabilizing its active conformation through physical interaction. Other tetraspanins were shown to be involved in the regulation of LFA-1 adhesiveness (Shibagaki et al., 1999; Van Compernolle et al., 2001). We are currently investigating the role of these tetraspanins in the formation of LFA-1 nanoclusters.

The arrangement of LFA-1 in nanoclusters may also guarantee an efficient binding and rebinding of LFA-1 to the ICAM-1 homodimers expressed on the endothelium. Biochemical studies showed that a divalent LFA-1 is the minimum configuration for forming efficient bonds with ICAM-1 (Sarantos et al., 2005). LFA-1 nanoclusters may further increase the likelihood of LFA-1 binding to ICAM-1. Shamri et al. (2005) recently showed that on rolling lymphocytes LFA-1 activation occurs locally and abruptly and that priming of LFA-1 molecules (i.e., L16 epitope induction) is a prerequisite for ICAM-1-induced activation of the α subunit I-domain.

LFA-1 Nanoclusters Are Formed Independently of Ligand Binding

It has long been debated whether clustering of integrins triggers or rather results from ligand binding. Here, we present "snapshots" of the MO cell membrane, in the absence of ligand binding, where LFA-1 shows a well-defined clustered distribution pattern. Because integrin activity on circulating leukocytes is upregulated within seconds (Shamri et al., 2005), organization of LFA-1 in small predefined nanoclusters would favor instant interaction with the endothelial surface. Carman and colleagues showed that on MO linear clusters of LFA-1 colocalize with ICAM-1-containing microvilli on the endothelium before MO extravasation (Carman and Springer, 2004). Our observations extend their findings and suggest that on MO cell surface, preformed LFA-1 nanoclusters may facilitate or accelerate the interaction of MOs with these endothelial ICAM-1-enriched projections.

Although it was suggested that localized clustering of LFA-1 at the cell surface might simply result from previous encounters of LFA-1 with ICAM ligands exposed by adjacent cells (Kim et al., 2004), our current findings suggest a more complex mechanism. Kim et al. (2004) showed that only binding of multimeric ligand (i.e., ICAM-1Fc/Anti-IgA complexes) to LFA-1-induced FRET-detectable microclustering on K-LFA-1 cells. We observed that when MOs are allowed to adhere at low density to prevent cell contact with ICAM-1 molecules from adjacent cells, nanoclusters are still present. Moreover, inhibition of LFA-1/ICAMs interactions in cis does not alter LFA-1 nanocluster organization (Supplementary Figure 3).

Furthermore, DCs, which do not have LFA-1 nanoclusters, cannot be induced to form LFA-1 aggregates, even when cultured at high cell density (our unpublished data). However, we do not exclude that upon ligand binding a further increase in receptor packing might occur within the LFA-1 nanoclusters that would induce FRET-detectable changes in intradomain receptor proximity. Further high-resolution analysis of the integrin molecules within these assemblies is needed to provide detailed insight in the molecular and spatial organization of LFA-1 nanoclusters.

LFA-1 Macroclusters Derive from the Recruitment of Pre-existing Nanoclusters

We investigated the interaction between MOs double-labeled for LFA-1 (NKI-L16 and TS2/4) and unlabeled ICAM-1–bearing T-cells by live imaging and quantified LFA-1 accumulation at the cell–cell contact (Figures 7 and 8). The RI of primed LFA-1 molecules at the MO–T-cell contact site is significantly higher than the RI of the total LFA-1 (Figure 8). This indicates that the preformed L16-positive nanoclusters act as "primed" scaffolds of LFA-1 molecules that readily bind the ligand. These scaffolds are recruited at the contact site where larger micrometer-sized macroclusters are subsequently formed (Figure 9).

Further adhesion strengthening might result from interactions of LFA-1 cytoplasmatic tails with known β2- or αL-interacting cytoplasmatic regulatory proteins. We could clearly detect a specific enrichment of talin at the cell–cell interface where LFA-1 binds to ICAM-1 (Figure 6D). Strikingly, neither interactions with cytohesin-1 nor RapL were observed (Figure 6, E and F), suggesting that the mechanism(s) regulating LFA-1 activity on MOs may differ from...
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