

# B-cell activation by membrane-bound antigens is facilitated by the interaction of VLA-4 with VCAM-1

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**VCAM-1 is one of the main ligands of VLA-4, an integrin that is highly expressed on the surface of mature B cells. Here we find that coexpression of VCAM-1 on an antigen-bearing membrane facilitates B-cell activation. Firstly, this is achieved by mediating B-cell tethering, which in turn increases the likelihood of a B cell to be activated. Secondly, VLA-4 synergizes with the B-cell receptor (BCR), providing B cells with tight adhesion and enhanced signalling. This dual role of VCAM-1 in promoting B-cell activation is predominantly effective when the affinity of the BCR for the antigen is low. In addition, we show that the VCAM-1 ectodomain alone is sufficient to carry out this function. However, it requires the transmembrane domain to segregate properly into a docking structure characteristic of the B-cell immunological synapse (IS). These results show that the VLA-4/VCAM-1 interaction during membrane antigen recognition enhances B-cell activation and this function appears to be independent of its final peripheral localization at the IS.**

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## Introduction

The activation of B lymphocytes is triggered by the specific binding of antigen to B-cell receptor (BCR). While the degree of activation is determined by the affinity of this interaction (Lanzavecchia, 1985; Batista and Neuberger, 1998; Kouskoff *et al*, 1998), B-cell activation is also regulated by the way in which antigen is presented (Goodnow *et al*, 1988; Lang *et al*, 1996). B cells respond well to soluble antigens; however, antigens tethered on a cell surface are especially potent in driving B-cell activation (Batista *et al*, 2001). This difference in effectiveness between soluble and membrane-bound antigen is particularly important *in vivo* where antigens are often encountered in the form of immune complexes (ICs) tethered on a cell surface by complement or Fc receptors (Szakal *et al*, 1988; Haberman and Shlomchik, 2003; Kosco-Vilbois, 2003).

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We have recently shown that the interaction of leukocyte function-associated molecule-1 (LFA-1)/intercellular adhesion molecule-1 (ICAM-1) facilitates membrane antigen recognition by B cells (Carrasco *et al*, 2004). B-cell recognition of membrane-bound antigens leads to the formation of a mature immunological synapse (IS) where the BCR gathers antigen into a central cluster, surrounded by a peripheral ring of LFA-1 and ICAM-1 (Batista *et al*, 2001; Carrasco *et al*, 2004). This type of receptor distribution into defined supra-molecular activation clusters (SMACs) was originally observed in T cells (Monks *et al*, 1998; Grakoui *et al*, 1999; Krummel *et al*, 2000). While considerable effort has been made to dissect the role and compartmentalization of LFA-1/ICAM-1 in this structure, very little is known about the function and localization of other integrins, such as very late antigen-4 (VLA-4).

VLA-4 (CD49d/CD29;  $\alpha 4\beta 1$ ) belongs to the integrin family of cell adhesion molecules and is expressed on all leucocytes, including B cells (Springer, 1990). VLA-4 mediates leucocyte tethering, rolling and firm adhesion by interacting with vascular adhesion molecule-1 (VCAM-1) on the endothelium (Alon *et al*, 1995; Berlin *et al*, 1995). Such functional flexibility is achieved by adopting different states of activation (Chen *et al*, 1999). Particularly in B cells, VLA-4 has been shown to be involved in the compartmentalization of B cells into peripheral lymphoid tissue (Lu and Cyster, 2002; Lo *et al*, 2003). It has also been implicated in promoting the physical interaction of B cells and follicular dendritic cells (FDCs) (Koopman *et al*, 1991). In addition, increased adhesion through this integrin has been observed in response to chemokines and BCR crosslinking (McLeod *et al*, 2002; Spaargaren *et al*, 2003).

VLA-4 ligand, VCAM-1, is constitutively expressed on FDCs (Freedman *et al*, 1990; Koopman *et al*, 1991), in high endothelial venules of peripheral and mesenteric lymph nodes (Berlin *et al*, 1995) and on bone marrow stromal cells (Miyake *et al*, 1991). Its expression can also be induced in endothelium by various inflammatory cytokines (IFN $\gamma$ , TNF $\alpha$ ) (Osborn *et al*, 1989). The ability to facilitate leucocyte adhesion to endothelium implies that VCAM-1 is an important factor in the initiation of an inflammatory response. Indeed, conditional VCAM-1 mutant mice present an impaired humoral immune response against T-cell-dependent antigens (Leuker *et al*, 2001). Moreover, the upregulation of VCAM-1 expression on endothelium is associated with several chronic inflammatory diseases (van Dinther-Janssen *et al*, 1991; Belmont *et al*, 1994). However, the function of VLA-4/VCAM-1 in B-cell recognition of membrane antigen has not been investigated.

In this study, we show that the interaction of VLA-4 with VCAM-1 can synergize with the BCR to promote adhesion of B cells to antigen-bearing membranes and thereby facilitate antigen recognition. We also show that VCAM-1 can cooperate with ICAM-1 to supply firm B-cell adhesion upon BCR activation. Remarkably, under shear stress conditions, the

VLA-4/VCAM-1 interaction becomes a prerequisite for B-cell membrane-bound antigen recognition. This function is particularly striking when the affinity of the BCR for the antigen is low. Using target cells expressing VCAM-1 fused to green fluorescence protein (GFP), we show that this molecule participates in the formation of a docking structure that surrounds the BCR in the IS. However, VCAM-1 mutants lacking the transmembrane domain fail to be recruited to this docking structure or to be excluded from the central SMAC (cSMAC). These results point to a potential function for VLA-4/VCAM-1 in the recruitment and activation of B cells during an adaptive immune response.

## Results

The experimental system employed in this study is based on the use of wild-type (WT) B cells, and B cells obtained from two BCR transgenic models, 3-83 and MD4 (Goodnow *et al*, 1988; Russell *et al*, 1991), which allows us to compare our observations. The 3-83 transgenic BCR binds with high affinity to the H-2K<sup>k</sup> molecule, and also recognizes with substantially decreased affinity the peptides p31 and p11, which were derived from an M13 phage-display library (Table I; Kouskoff *et al*, 1998; Carrasco *et al*, 2004). B cells from MD4 transgenic mice carry an IgM<sup>+</sup>IgD<sup>+</sup> BCR, which binds hen egg lysozyme (HEL) with an affinity of  $5 \times 10^{10} \text{ M}^{-1}$  and mutated HELs with more than 10 000-fold decreased affinities (Table I; Batista and Neuberger, 1998; Carrasco *et al*, 2004).

### The interaction of VLA-4 with VCAM-1 promotes B-cell adhesion to antigen-bearing membranes

To study the role of VCAM-1 during membrane antigen recognition, we generated a glycosylphosphatidylinositol-anchored version of VCAM-1 (GPI-VCAM-1) and asked whether its interaction with VLA-4 enhanced the capacity of B cells to form antigen-dependent and -independent contacts with planar lipid bilayers. As a positive control, naive B cells were tested for adhesion to bilayers that contained an equivalent density of ICAM-1 ( $50 \text{ molec}/\mu\text{m}^2$ ). Quantification of the number of naive B cells attached to the membrane was carried out by interference reflection microscopy (IRM), which allows visualization of tight contact as dark areas (Figure 1A). At low densities of p31 (Table I), tight contacts were not detected unless VCAM-1 or ICAM-1 was present in

the lipid bilayers (Figure 1A). The presence of VCAM-1 was able to support the binding of a larger number of B cells than ICAM-1 (Figure 1B). However, the area of contact of the B cells on ICAM-1 was larger than on VCAM-1 (Figure 1C). High concentration of antigen supported B-cell adhesion, and the presence of VCAM-1 or ICAM-1 did not significantly affect the level of adhesion. These results showed that tight adhesion requires membranes containing p31 at a density higher than  $25 \text{ molec}/\mu\text{m}^2$ . In contrast, in the presence of VCAM-1 or ICAM-1, a reduced antigen density supported a similar level of adhesion (Figure 1B). Thus, similar to ICAM-1/LFA-1 (Carrasco *et al*, 2004), VCAM-1/VLA-4 facilitates adhesion under limited concentration of antigen and this function is regulated by BCR engagement of antigen.

### VLA-4 and VCAM-1 are involved during the first stages of the interaction

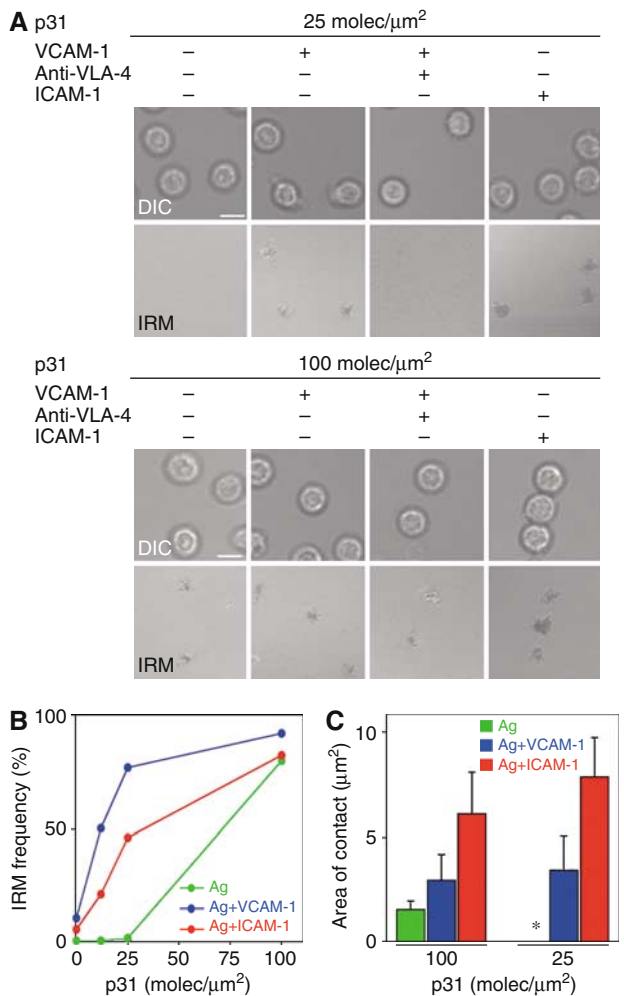
The area of contact between B cells and antigen-loaded membranes in the presence of ICAM-1 was around  $6\text{--}7 \mu\text{m}^2$ , whereas B cells bind to VCAM-1-containing membranes with areas of just  $3 \mu\text{m}^2$  (Figure 1C). To test whether these two integrin pairs have a different function, we compared the adhesion of naive splenic B cells to VCAM-1, ICAM-1 and antigen-containing membranes under shear stress. Glass slides containing planar lipid bilayers were connected to a syringe pump to establish a laminar flow, and B cells were injected. While the nature of flow through the lymphatic tissues is still under investigation, it is known to be lower than that observed in postcapillary venules ( $1\text{--}10 \text{ dyn}/\text{cm}^2$ ) (Atherton and Born, 1972). For this reason, we chose a low starting flow of  $0.4 \text{ dyn}/\text{cm}^2$ , which was gradually decreased to  $0.04 \text{ dyn}/\text{cm}^2$ . This allowed us to analyse the efficiency of B-cell binding to the membranes. We then slowly increased the shear stress to values up to  $2.5 \text{ dyn}/\text{cm}^2$ , which might not fully represent the physiological conditions in the lymphatic tissue, but allowed us to evaluate the strength of adhesion of the captured cells.

As shown in Figure 2 and Supplementary Movie S1, 20% of the naive splenic 3-83-injected B cells were able to either tether or firmly attach onto membranes loaded with VCAM-1 during the injection period. Phenotypic characterization of the bound cells revealed that marginal zone B cells were able to bind slightly better to VCAM-1 than follicular B cells under the conditions of our assay (Supplementary Figure 1A). The

**Table I** Antigen and integrin ligand affinities

Antigen	$K_A (\times 10^6 \text{ M}^{-1})$	Integrin ligand	$K_A (10^6 \text{ M}^{-1})$
3-83 binding		VCAM-1	
p31	65	High-affinity VLA-4	10
p11	7	Low-affinity VLA-4	~0.1
p0	—		
HyHEL10 binding		ICAM-1	
HEL <sup>WT</sup>	50 000	High-affinity LFA-1	~2
HEL <sup>RD</sup>	2080	Low-affinity LFA-1	~0.01
HEL <sup>KD</sup>	30		
HEL <sup>RKD</sup>	~3		

The  $K_A$  values for the different peptides/3-83 antibody and HEL mutants/HyHEL10 antibody were obtained using the Biacore, as previously described (Batista and Neuberger, 1998; Carrasco *et al*, 2004). The values derived represent the affinity of the dimeric antibody for the peptide in the case of 3-83. The values of HyHEL10 for HEL<sup>WT</sup> and the different HEL mutants account for the monomeric interaction. The  $K_A$  values for the integrins VLA-4 (Chigaev *et al*, 2003) and LFA-1 (Lollo *et al*, 1993) in high- and low-affinity states were previously reported.



**Figure 1** VLA-4/VCAM-1 interaction increases the adhesion of B cells to target membranes. (A) Differential interference contrast (DIC) and IRM images of 3-83 naive B cells settled onto planar lipid bilayers containing p31 antigen (100 molec/μm<sup>2</sup>, top panels; 25 molec/μm<sup>2</sup>, bottom panels) in the presence or absence of ICAM-1 (50 molec/μm<sup>2</sup>), VCAM-1 (50 molec/μm<sup>2</sup>) and/or neutralizing anti-mouse α4 mAbs. Scale bar, 5 μm. (B) Naive 3-83 B cells were evaluated for their capacity to form tight contacts on membranes loaded with p31 in the presence or absence of ICAM-1 or VCAM-1 at 50 molec/μm<sup>2</sup>. After 5–10 min, images were collected at each of the specified antigen densities and the number of cells showing tight contacts was determined. Data are representative of four different experiments. (C) Quantification of the area of B-cell contact with membranes containing p31 antigen at the indicated densities in the presence or absence of ICAM-1 or VCAM-1 at 50 molec/μm<sup>2</sup>. Data represent the mean of 40 cells in each case.

tethering of B cells under shear stress was dependent on the density of VCAM-1, with efficient attachment at densities greater than 50 molec/μm<sup>2</sup>. Under these conditions, only 2% of the flowing B cells were able to roll on VCAM-1-bearing membranes and generally detached afterwards (Supplementary Figure 1B). However, as the flow rate was increased, tethered B cells were rapidly washed away, indicating that the interaction with VCAM-1 in the membrane was not very tight. In contrast, naive B cells did not adhere to artificial bilayers containing either ICAM-1 or antigen alone under shear stress, even at high densities of 100 molec/μm<sup>2</sup>, although such density supports static binding of naive B cells (see above). These results indicate that VCAM-1 was more

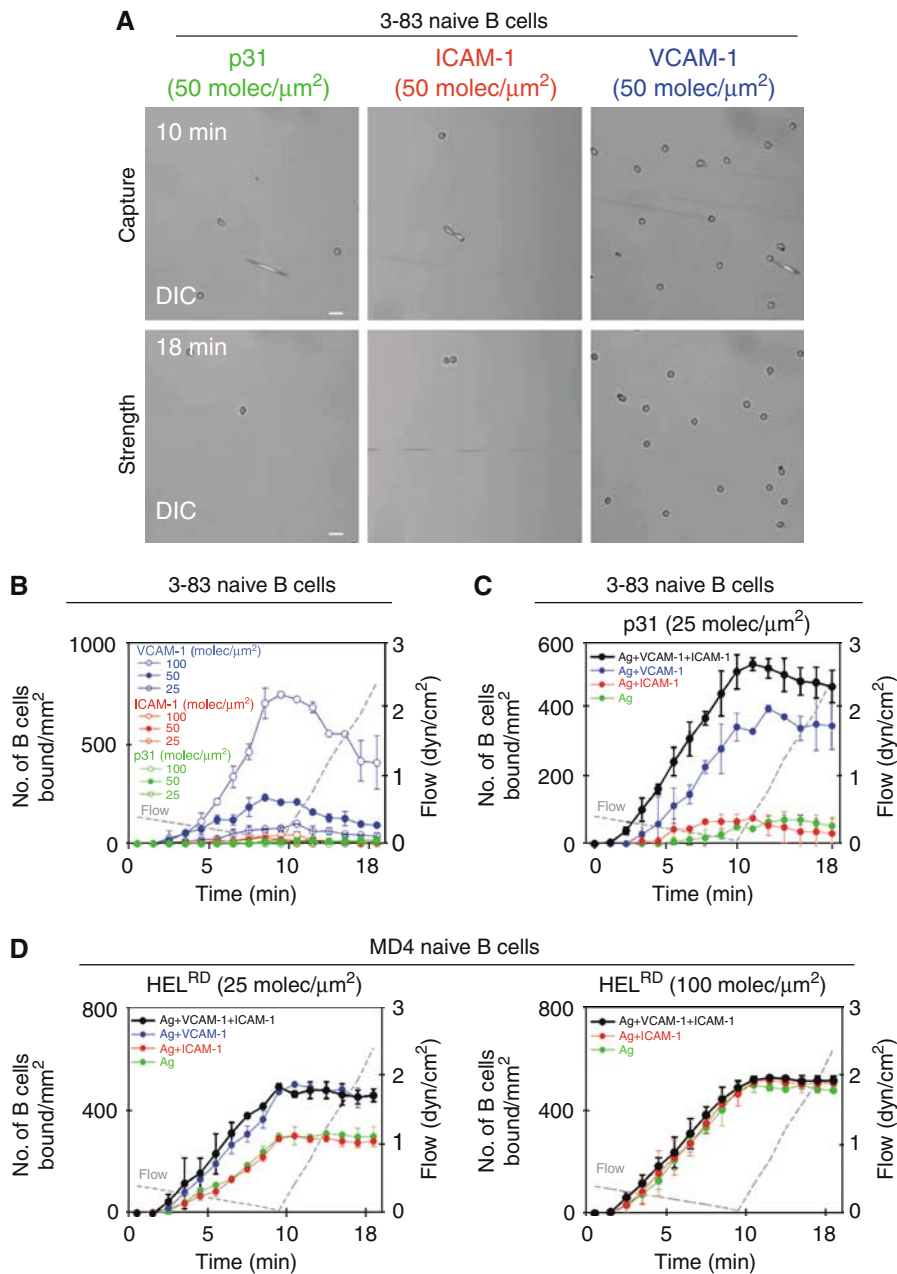
efficient at capturing naive B cells than ICAM-1 or antigen under shear stress, suggesting that it is involved during the first stages of the interaction.

We then analysed the adhesion of B cells to membranes containing different combinations of VCAM-1, ICAM-1 and antigen. As shown in Figure 2C, inclusion of VCAM-1 on bilayers containing limited amounts of antigen enhanced B-cell tethering and firm adhesion when compared with B cells interacting with membranes containing antigen alone. An additional increase in the number of cells attached to the bilayers was observed when naive B cells were perfused over membranes containing a combination of VCAM-1, ICAM-1 and antigen. However, no rolling leading to firm adhesion could be detected. This absence of rolling may be due to the low shear stress conditions in which the assays were performed. Leucocyte rolling was observed *in vivo* within a range of 1.5–4 dyn/cm<sup>2</sup> shear stress (Atherton and Born, 1972). B cells that had been activated by BCR crosslinking before perfusion on the membrane showed a greatly enhanced efficiency of adhesion to VCAM-1 and ICAM-1 (Supplementary Figure 1C). However, no enhanced attachment to the two integrin ligands was detected in the absence of BCR activation (Supplementary Figure 1D). From these data, we can conclude that VLA-4 can synergize with the BCR to promote B-cell capture and tight adhesion. Our experiments also show the inability of LFA-1 to initiate a functional contact and suggest that, by having different functions, the two integrins are able to cooperate but only in the presence of antigen.

To determine whether VCAM-1 assists B-cell attachment when the affinity of the BCR for antigen was much higher than for p31, we loaded lipid bilayers with a high-affinity lysozyme mutant HEL<sup>RD</sup> and measured adhesion of MD4 naive B cells under shear stress (Table I). As observed with p31 and 3-83 B cells, VCAM-1 synergized with HEL<sup>RD</sup> to induce more efficient binding of MD4 B cells. However, this was observed only at low antigen density (Figure 2D): equally high numbers of attached B cells were detected at high densities of antigen in the presence or absence of integrin ligands. This indicates that B cells reach an avidity above which VLA-4 binding does not contribute markedly to B-cell capture and attachment. Thus, as we previously showed for ICAM-1 (Carrasco *et al*, 2004), these results indicate that VCAM-1 is more efficient at increasing adhesion when the total avidity of the cell for the membrane is low.

#### VLA-4/VCAM-1 interaction facilitates B-cell activation

It is notable that VCAM-1 was particularly effective in mediating tethering of B cells to a target membrane and this could facilitate antigen recognition. To investigate this, we compared the frequency of B cells that raise their intracellular calcium levels when perfused, at low shear stress (0.1–0.05 dyn/cm<sup>2</sup>), over membranes containing different combinations of VCAM-1, ICAM-1 and antigen. As judged by the number of B cells that showed a calcium response, antigen-bearing membranes in the presence of VCAM-1 were far better at activating B cells than those containing ICAM-1 or antigen alone (Figure 3A and B, left panel). In contrast, no calcium flux was evident in B cells tethered to membranes in the absence of antigen. Interestingly, analysis of the magnitude of calcium response on single cells revealed a more robust signal when B cells recognized antigen in the presence



**Figure 2** VLA-4/VCAM-1 interaction facilitates the capture of naive B cells and membrane-bound antigen recognition under shear stress. (A, B) 3-83 naive B cells were perfused at decreasing shear stress for 10 min (capture step), followed by injection of buffer at increasing shear stress for 8 min (strength step). DIC images at two different time points of the same representative field of p31, ICAM-1 and VCAM-1 membranes are shown in (A). The capacity to capture B cells and the strength of binding of lipid bilayers containing VCAM-1, ICAM-1 or p31 antigen ( $K_A = 6.5 \times 10^7 M^{-1}$ ) at different densities (B), or of lipid bilayers containing p31 antigen in the presence or absence of VCAM-1 (50 molec/μm<sup>2</sup>) and/or ICAM-1 (50 molec/μm<sup>2</sup>) (C), were evaluated by counting B cells bound/mm<sup>2</sup> at the indicated time points. (D) Lipid bilayers containing HEL<sup>RD</sup> ( $K_A = 2 \times 10^9 M^{-1}$ ) at the indicated densities in the presence or absence of VCAM-1 (50 molec/μm<sup>2</sup>) and/or ICAM-1 (50 molec/μm<sup>2</sup>) were evaluated for their capacity to capture MD4 naive B cells and the strength of binding as previously described. The data in (A–D) are representative of at least three experiments. Scale bar, 20 μm.

of VCAM-1 than in its absence (Figure 3C, left panel). This calcium response was sufficient to fully activate the B cells, as demonstrated by the upregulation of CD86 or CD69 (Figure 3D).

These results indicate two mechanisms by which the interaction of VLA-4 with VCAM-1 might facilitate B-cell activation: firstly, by mediating B-cell tethering to the target membrane and thereby facilitating BCR/antigen engagement; and secondly, VLA-4 interaction with VCAM-1 appears to

synergize with the BCR to enhance the extent of the B-cell signalling, either by allowing more BCR engagement or by an independent signal through VLA-4. However, this role of VCAM-1 in increasing B-cell activation seems to operate only when the BCR affinity for the antigen was low. Indeed, when a high-affinity antigen (HEL<sup>RD</sup>; Table I) was loaded on the membranes, no significant difference in the percentage of B cells that sustained a calcium signal or in the strength of their response was observed either in the presence or

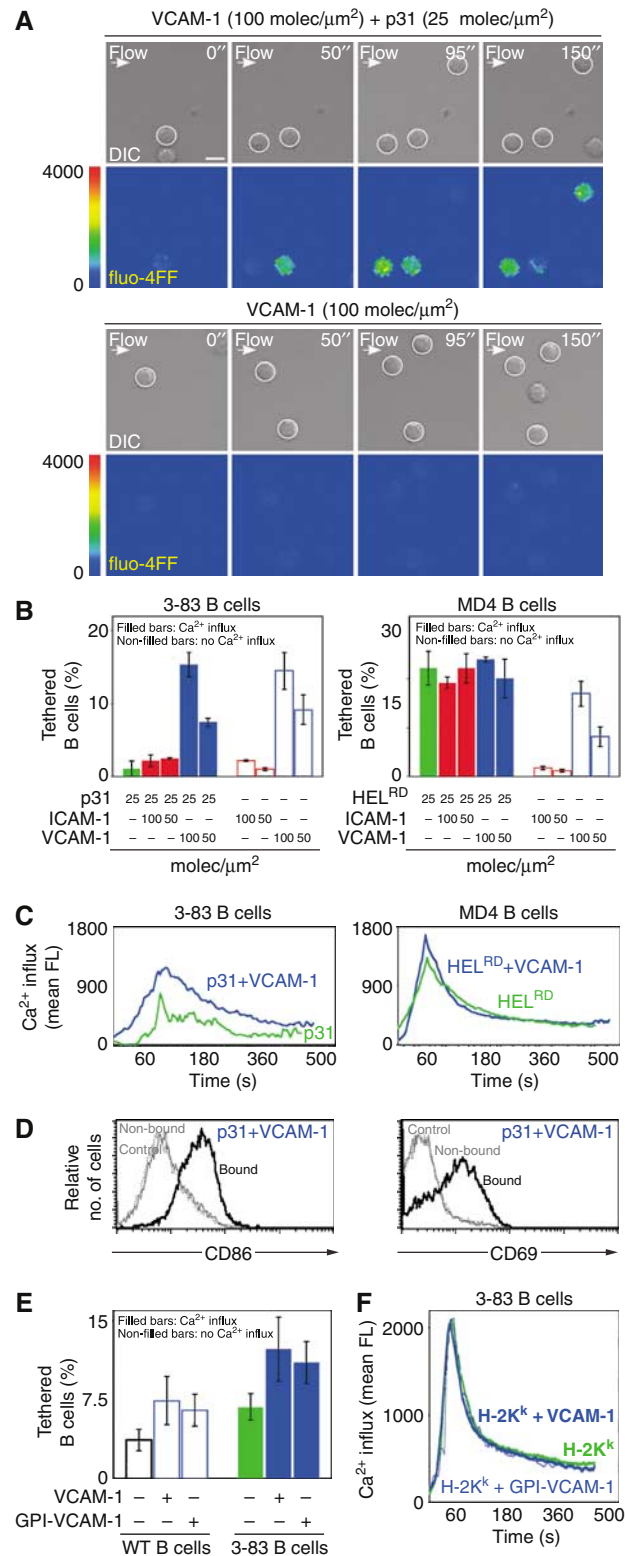
absence of integrin ligands (Figure 3B and C, right panels). These results indicate that VLA-4 enhances BCR signalling by providing further adhesion, not by providing an independent calcium response.

It was important to ascertain whether the ability of VCAM-1 to promote B-cell activation was also functional in the more complex and physiological context of a cell surface. To this

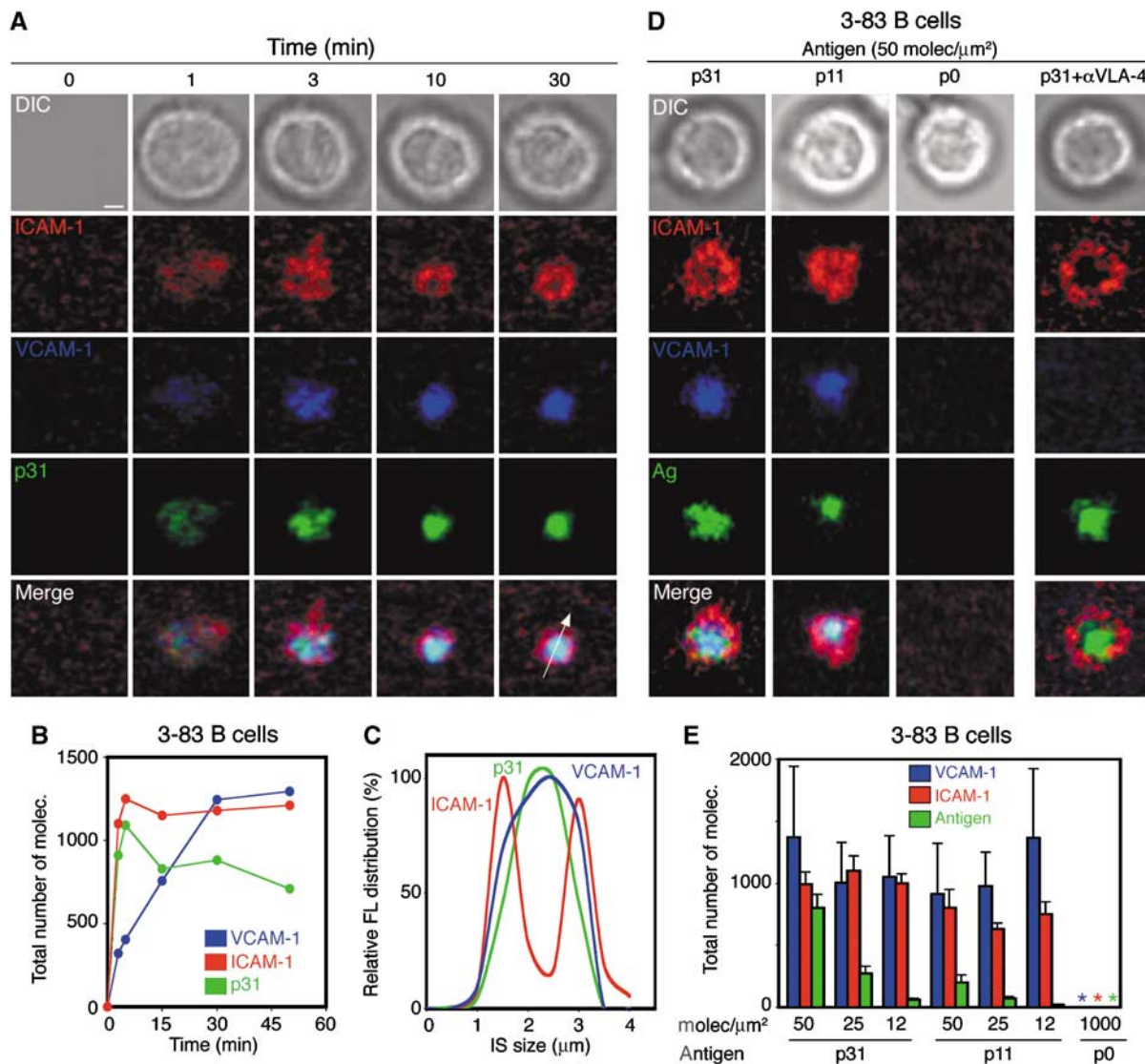
end, we initially compared the capacity of naive B cells to tether onto the surface of L cells, which express high levels of VCAM-1, or DCEK, a derived clone that lacks VCAM-1 expression. As shown in Figure 3E, naive WT B cells were significantly better at tethering on the surface of VCAM-1-expressing L cells than on DCEK cells. We then asked whether this increased tethering could promote B-cell activation. For these experiments, WT B cells were replaced with 3-83 transgenic B cells, as their BCR binds with high affinity to the H-2K<sup>k</sup> molecule, which is endogenously expressed on the surface of both cell lines. Higher numbers of transgenic B cells showed calcium flux when bound to L cells expressing VCAM-1 than on the DCEK cell line (Figure 3E); nonetheless, and probably owing to the high affinity of the 3-83 BCR for the H-2K<sup>k</sup> protein, no difference in the magnitude of the calcium response was observed (Figure 3F). A similar increase in B-cell tethering and degree of calcium signal was also observed when the GPI version of VCAM-1, used in the bilayer experiments, was expressed on the surface of DCEK (Figure 3E and F). In conclusion, and similar to the results obtained in the bilayer experiments, VCAM-1 can promote B-cell tethering and thereby facilitate antigen recognition.

### VLA-4 can drive the recruit of GPI-VCAM-1 to the cSMAC of the B-cell synapse

The IS is a general molecular structure for antigen recognition. Therefore, we wondered whether the contribution of VLA-4 to membrane antigen recognition was accompanied by localization at the synapse. To examine its recruitment and accumulation in the B-cell synapse, we allowed naive 3-83 B cells to interact with lipid bilayers that contained GPI-anchored Alexa 543-conjugated VCAM-1 (blue), GPI-



**Figure 3** VLA-4/VCAM-1-mediated tethering enhances B-cell activation under low shear stress. Naive B cells were perfused at low shear stress (0.1–0.05 dyn/cm<sup>2</sup>) over planar membranes containing VCAM-1 or ICAM-1 in the presence or absence of antigen. (A) Different time points of two representative experiments are shown. The upper panel in each horizontal pair shows DIC images in which tethered B cells are highlighted with a white circle. The bottom panels show their calcium response as fluo-4FF fluorescence in false colour scale. Scale bar, 5 μm. (B) The frequency of tethered B cells is represented as a percentage of the total number of perfused B cells under the different conditions analysed. The left panel shows 3-83 naive B cells on membranes loaded with the indicated densities of low-affinity p31 antigen, VCAM-1 or ICAM-1. The right panel shows the same but using MD4 B cells and HEL<sup>RD</sup> as high-affinity antigen. The proportion of activated B cells was determined based on the increased levels of fluo-4FF fluorescence (filled bars: activated B cells; non-filled bars: non-activated B cells). (C) The fluo-4FF fluorescence intensity was measured every 4 s for at least 50 cells in random fields. The average intensity was plotted as a function of time for 3-83 B cells (left panel) and MD4 B cells (right panel) upon tethering on antigen-bearing membranes containing p31 and HEL<sup>RD</sup> respectively, in the presence (blue line) or absence (green line) of VCAM-1. (D) Activation of the 3-83 naive B cells tethered (bound) versus the non-tethered B cells (non-bound) on membranes containing VCAM-1 and p31 as antigen. B-cell activation was evaluated after 24 h as the percentage of cells that upregulate CD86 and CD69, as described in Materials and methods. (E) As in (B), percentage of WT naive B cells or 3-83 naive B cells, which recognize H-2K<sup>k</sup> as antigen, that tether and/or raise their calcium levels over L cells (VCAM-1<sup>+</sup>/H-2K<sup>k</sup>), DCEK (VCAM-1<sup>-</sup>/H-2K<sup>k</sup>) or DCEK transfectants (GPI-VCAM-1<sup>+</sup>/H-2K<sup>k</sup>). (F) As in (C), calcium response of 3-83 B cells under the conditions specified in (E).



**Figure 4** VLA-4 can drive the recruitment of GPI-VCAM-1 to the cSMAC of the IS. Naive 3-83 B cells were settled onto planar lipid bilayers containing GPI-linked ICAM-1 (red) at  $50 \text{ molec}/\mu\text{m}^2$ , GPI-linked VCAM-1 (blue) at  $25 \text{ molec}/\mu\text{m}^2$  and p31 antigen (green) at  $50 \text{ molec}/\mu\text{m}^2$ . (A) Time-lapse fluorescent images show the accumulation of p31 (green), ICAM-1 (red) and VCAM-1 (blue) in the pattern of a mature synapse at the specified time points. The top panels show DIC images at the same time points. (B) Quantification of the total number of molecules of VCAM-1, ICAM-1 and p31 antigen during synapse formation of a representative 3-83 B cell. (C) Distribution pattern of the antigen ICAM-1 and VCAM-1 fluorescent signal across a section of the IS, shown as a white arrow in (A), at 30 min. (D) Naive 3-83 B cells were settled onto membranes containing ICAM-1 (red), VCAM-1 (blue) ( $50$  and  $25 \text{ molec}/\mu\text{m}^2$ , respectively) and different antigens (green) at  $50 \text{ molec}/\mu\text{m}^2$ : p31 ( $K_A = 6.5 \times 10^7 \text{ M}^{-1}$ ), p11 ( $K_A \sim 7 \times 10^6 \text{ M}^{-1}$ ) and p0 (null). DIC, fluorescence and merged images from a representative B cell are shown. (E) Quantification of the number of molecules of VCAM-1, ICAM-1 and antigen recruited in the B-cell synapse shown in (D). Data represent the mean of 50 cells in each case. Scale bar,  $1 \mu\text{m}$ .

anchored Alexa 488-conjugated ICAM-1 (red) and Alexa 633-conjugated p31 (green) under static conditions. Synapse formation was then visualized by confocal fluorescence microscopy. Upon B-cell contact, ICAM-1 and antigen (p31) could be found segregated within the interface, forming a classical mature IS. Surprisingly, in all the synapses analysed, VCAM-1 mainly localized together with the engaged antigen in the central cluster (Figure 4A). Whereas ICAM-1 and antigen were engaged with similar fast kinetics in the absence (Carrasco *et al*, 2004) or presence of VCAM-1, it took several minutes for VCAM-1 to reach equilibrium (Figure 4B). The final density ( $\sim 300 \text{ molec}/\mu\text{m}^2$ ) and total number of VCAM-1 molecules engaged ( $\sim 1200$  molecules; Figure 4B) within the central cluster of the IS were similar in all cells analysed. As

shown in Figure 4D and Supplementary Figure 2A, altering the affinity of the BCR for the antigen present in the bilayer did not alter the distribution of VCAM-1. Quantitative analysis revealed that, independent of the amount of antigen present in the bilayer, similar quantities of VCAM-1 were recruited in all cases (Figure 4E and Supplementary Figure 2B). However, the engagement of the BCR was essential, as no recruitment of VCAM-1 was observed with p0, a peptide for which the affinity of this BCR is below the threshold of B-cell activation. Furthermore, preincubation of B cells with blocking anti-VLA-4 monoclonal antibodies showed that the recruitment of VCAM-1 to the IS was indeed completely dependent on VLA-4 (Figure 4D and Supplementary Figure 2A). Thus, these results suggested that VCAM-1 can be

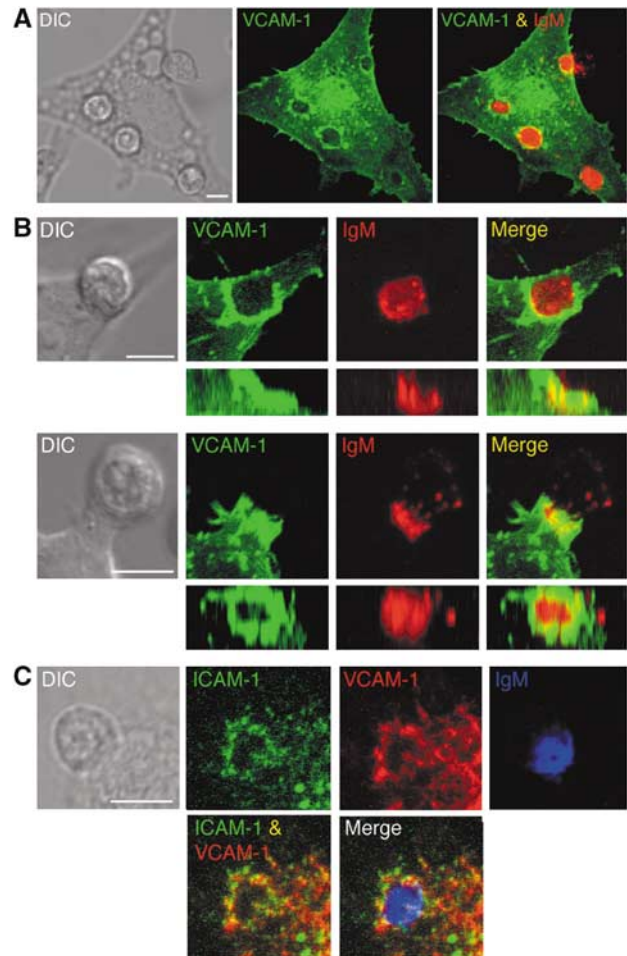
recruited to the cSMAC of the IS by VLA-4 via a mechanism that was dependent on BCR engagement. They also indicate that VLA-4 follows a different pattern of aggregation than LFA-1.

### **VCAM-1 is part of the B-cell-antigen-presenting cell docking structure**

While these observations shed light on the favoured localization of VLA-4 in the cSMAC, they do not provide any insight as to whether the association of VCAM-1 with other proteins of the target cell may control its distribution in the IS. To this end, we studied the interaction of naive 3-83 B cells with L cells expressing a GFP-linked full-length VCAM-1 as antigen-presenting cells. In parallel, the BCR localization was assessed by staining B cells with non-blocking fluorescently labelled Fab fragments against IgM, before antigen exposure. Three-dimensional reconstruction of confocal images showed that, in at least 90% of the conjugates analysed, VCAM-1-GFP participated in the formation of a docking structure surrounding the BCR/antigen and was completely excluded from the cSMAC (Figure 5A and B and Supplementary Movie S2). Furthermore, these observations were confirmed by immunostaining of the endogenous VCAM-1 expressed by the L cells, which showed a similar pattern of distribution (data not shown). Interestingly, with target cells expressing ICAM-1-GFP, we observed a good colocalization of the two integrin ligands in the cup structure (Figure 5C). These results showed the participation of VCAM-1 in the docking structure that we previously described for the B-cell synapse (Carrasco *et al.*, 2004). Furthermore, they also indicate that its localization in the IS was not simply the result of the interaction with VLA-4, but it was likely to involve association of VCAM-1 with other proteins in the target cell.

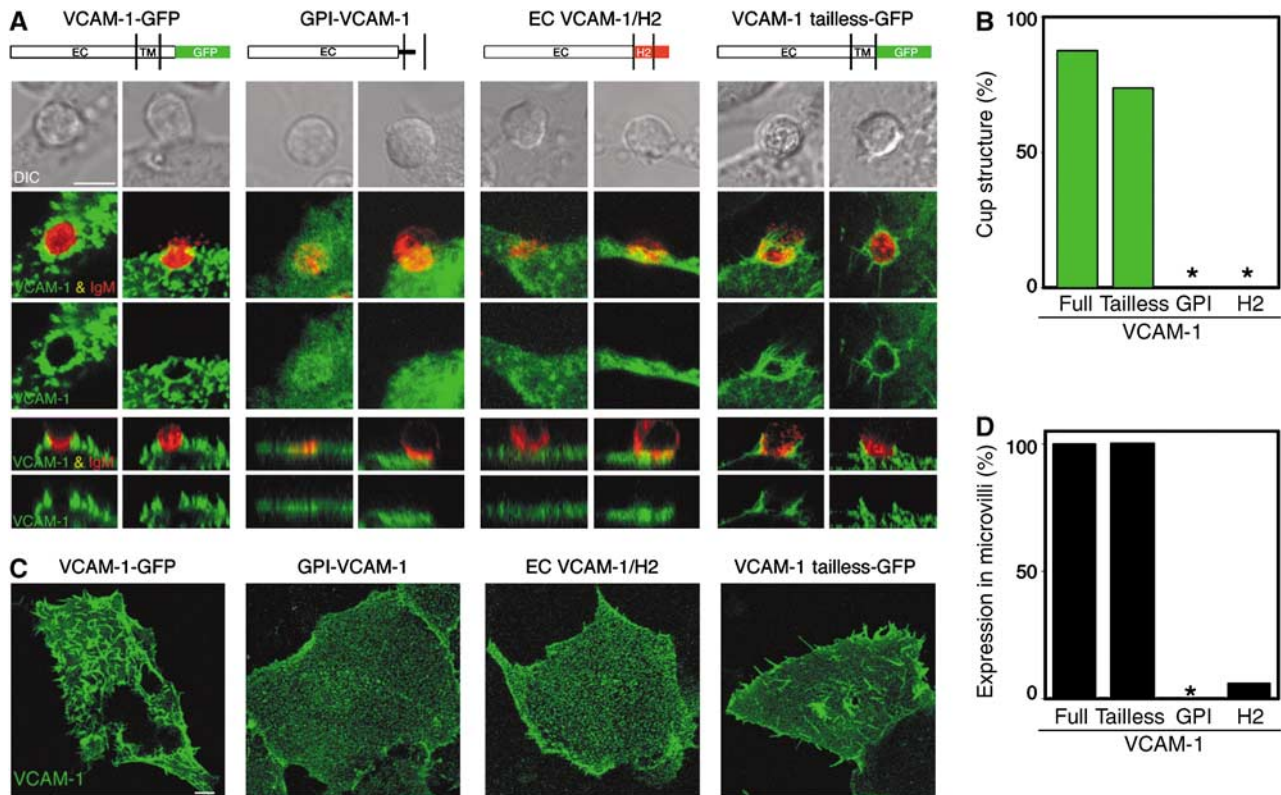
### **Localization of VCAM-1 in the docking structure and its exclusion from the cSMAC are dependent on its transmembrane domain**

The fact that full-length VCAM-1 was recruited and segregated into the pSMAC of the IS when expressed on target cells, but the GPI-VCAM-1 used in the lipid bilayer was recruited but not properly segregated, suggested that its proper segregation relies upon the transmembrane domain and/or cytoplasmic tail. If this interpretation was correct, one would expect that a truncated form of VCAM-1 expressed on the surface of target cells should fail to be distributed to the pSMAC. As shown in Figure 6A, this was indeed the case. Mislocalization represented as lack of exclusion from the cSMAC was observed when synapse formation was analysed with target cells that expressed either a truncated form of VCAM-1, in which its extracellular part has been linked to the H-2K<sup>b</sup> transmembrane domain and a short intracytoplasmic tail (EC VCAM-1/H2), or GPI-VCAM-1 (Figure 6A and B). In contrast, an intracytoplasmic tailless version of this molecule (VCAM-1 tailless-GFP) was excluded, which indicates that the transmembrane domain of the protein was responsible for the proper localization in the IS. Support for this observation also came from studying the distribution of the different VCAM-1 mutants on the target cells. It was noticeable that full-length VCAM-1-GFP was usually expressed on the surface of microvilli (Figure 6C and D). In contrast, GPI-VCAM-1 or EC VCAM-1/H2 was distributed evenly across the mem-



**Figure 5** Full VCAM-1 localizes in the periphery of the B-cell synapse. (A) Interaction in real time of naive 3-83 B cells stained with Cy5-conjugated non-blocking anti-IgM Fabs (red) with L cells expressing VCAM-1-GFP (green) was analysed by confocal microscopy. Separate and merged three-dimensional projections of the confocal fluorescence image stacks taken after 20 min incubation are shown. DIC image at the same time point is shown in the left panel. (B) Side and top views of the three-dimensional reconstruction of the docking structure formed by VCAM-1-GFP in the B-cell synapse. Two different examples of DIC, separate and merged fluorescent images of VCAM-1-GFP and IgM are shown. (C) 3-83 B cells interacting with ICAM-1-GFP target cells were fixed, permeabilized and stained for endogenous VCAM-1 and IgM with anti-VCAM-1 (red) and anti-IgM antibodies (blue). DIC, separate and merged three-dimensional projection of the confocal fluorescence image stacks of VCAM-1, ICAM-1 and IgM are shown. Scale bar, 5 μm.

brane, indicating that these forms are differentially associated with the cytoskeleton of the target cells. Finally, treating target cells with inhibitors of actin polymerization, like cytochalasin D, disrupted VCAM-1-GFP's microvilli distribution (data not shown). These results suggest that VCAM-1 is linked to the actin cytoskeleton via the interaction of its transmembrane domain with another protein. While, as shown in Figure 3, the link between VCAM-1 and the cytoskeleton of the target cell did not seem to be highly significant in mediating B-cell tethering and facilitating activation, it appeared to be a requirement for the exclusion of VCAM-1 from the cSMAC and the sorting of this molecule in the cup-like structure that characterizes the B-cell IS.



**Figure 6** The transmembrane domain of VCAM-1 determines its distribution in the IS. **(A)** Interaction of 3-83 naive B cells with DCEK cells transfected with the different VCAM-1 constructs. 3-83 B cells interacting with DCEK cells transfected with GPI-VCAM-1 or EC VCAM-1/H2 were fixed, permeabilized and stained with antibodies against VCAM-1 and IgM, as indicated in Materials and Methods. 3-83 B cells, previously stained with Cy5-conjugated non-blocking anti-IgM Fabs, were in contact with VCAM-1-GFP and VCAM-1 tailless-GFP DCEK transfectants for 25 min at 37°C. Then, confocal live-imaging was acquired. Two different examples are shown for each VCAM-1 construct. The middle panels show top views of fluorescent images representing a projection of several horizontal confocal sections. The bottom panels show a sagittal section of the top view in each case. DIC images are shown in the top panel. Schematic illustrations of the different VCAM-1 constructs are shown on top of the corresponding panels. Scale bar, 5 µm. **(B)** The four VCAM-1 constructs were evaluated for their capacity to be excluded and form a docking structure in the B-cell synapse. Data obtained from at least 30 B-cell synapses were analysed in each case. **(C)** COS transfectants of the different VCAM-1 constructs were fixed and stained with anti-VCAM-1 mAb (green). Each panel represents a projection of the confocal fluorescence image stack. Scale bar, 5 µm. **(D)** The four VCAM-1 constructs were evaluated for their capacity to be expressed on microvilli at the surface of DCEK cells. Data obtained from at least 30 DCEK transfected cells were analysed in each case.

## Discussion

This study describes the role of VLA-4/VCAM-1 interaction during B-cell recognition of membrane-bound antigens. We find that, under static conditions in synergy with the BCR, VLA-4 enhances B-cell adhesion to an antigen-bearing membrane. We also show that, under low shear stress, VCAM-1 becomes a prerequisite for antigen recognition, suggesting that this adhesion pair is involved during the first stages of the interaction between the B cell and the target membrane. Furthermore, we establish that, when the affinity of the BCR for the antigen is low, the interaction of VLA-4 with VCAM-1 facilitates B-cell activation. This is achieved by two independent mechanisms: by mediating B-cell tethering to the target membrane and thereby facilitating antigen/BCR engagement, and by enhancing the level of signalling.

It is likely that this increase in B-cell signalling is mainly owing to the capacity of VCAM-1 to promote tight adhesion. As a consequence of this, more antigen engagement of the BCR might occur. This presumption is based on our experimental observation, which indicates that the enhanced adhesion by VLA-4/VCAM-1 allows better antigen gathering under static conditions (Figure 1 and data not shown), as previously

described for the LFA-1/ICAM-1 interaction (Carrasco *et al*, 2004). Furthermore, no difference in the levels of calcium response can be detected when the affinity of the BCR for the antigen was high. However, at this point, it is not possible to completely rule out whether VLA-4 is able to deliver an independent signal that could account for the increased calcium response or may even follow a calcium-independent pathway. In this line, it has been shown that the interaction of VLA-4 with VCAM-1 can protect germinal centre or peripheral blood B cells from apoptosis (Koopman *et al*, 1994; Hayashida *et al*, 2000). In the latter case, this seems to be mediated by an upregulation of Bcl-X<sub>L</sub>.

We also provide evidence that the ectodomain of VCAM-1 alone is sufficient to perform these functions in the absence of any linkage with the cytoskeleton of the target cell. Using the planar bilayers system, we find that a GPI version of VCAM-1 is sufficient to mediate B-cell tethering and enhance signalling. Similarly, expression of the GPI-VCAM-1 on the surface of target cells can also promote B-cell activation as well as a full VCAM-1 molecule. These results are intriguing as the topological distribution of selectins and adhesion molecules in the microvilli of leucocytes has been shown to be critical in enhancing their tethering capacity (von Andrian



*et al*, 1995). However, it is important to point out that this increase in tethering was observed only under shear rates that prevail in microvessels, which are much higher than those utilized in our study. Indeed, differences in adhesion, depending on the topological distribution, were not detected under static conditions. Furthermore, and in line with our results, it has been shown that firm adhesion of leucocytes mediated by VLA-4 and LFA-1 is not affected by the mislocalization of their respective ligands, VCAM-1 and ICAM-1, due to the disruption of the endothelium cytoskeleton (Carman *et al*, 2003).

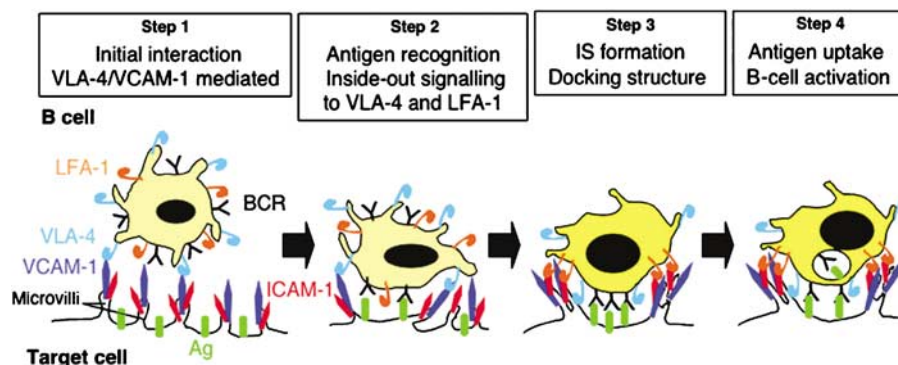
The role of VCAM-1 in promoting antigen recognition seems to be independent of its final localization at the IS. Thus, this function in enhancing B-cell activation is likely to precede the sorting of the molecule in the IS. Upon antigen recognition, VCAM-1 segregates to form a docking structure that is characteristic of the B-cell IS. The transmembrane domain of the protein determines this localization. This conclusion is based on our lipid bilayer experiments where a GPI-VCAM-1 form is recruited to the cSMAC by VLA-4. Furthermore, in cell-to-cell interaction experiments, EC VCAM-1/H2 and GPI-VCAM-1, but not VCAM-1 tailless-GFP, fail to be excluded from the cSMAC. Interestingly, the group of Sanchez-Madrid (Barreiro *et al*, 2005) has recently shown that VCAM-1 is involved in the interaction with tetraspanin proteins that probably link VCAM-1 and ICAM-1 on the surface of a target cell. In a previous study, these authors showed that the same two integrin ligands participate in the formation of a 'cup-like' structure that anchors leucocytes to the endothelium (Barreiro *et al*, 2002). In this structure, ICAM-1 and VCAM-1 appear to be linked to the actin cytoskeleton by the association with ERM proteins. In addition, the group of Springer has shown that ICAM-1 and VCAM-1 are associated with actin-rich microvilli in the 'cup-like' structure and has suggested that this provides directional guidance to leucocytes for extravasation (Carman *et al*, 2003; Carman and Springer, 2004). Both groups speculate that such a structure facilitates leucocyte migration. This study and previous evidence (Carrasco *et al*, 2004) show that a similar structure is formed in the context of the B-cell synapse. This docking structure is likely to play a role in providing firm adhesion to the central cluster of BCR/antigen and we suggest that, in this context, it may facilitate antigen extraction for presentation to T cells.

The peripheral localization of LFA-1/ICAM-1 is one of the characteristic features of the IS (Monks *et al*, 1998; Wulfig

*et al*, 1998; Grakoui *et al*, 1999). The retention of LFA-1 in the pSMAC seems to be mediated by its association with the cytoskeleton through talin (Monks *et al*, 1998). Our results suggest that VLA-4 follows an inverse pattern of distribution. Consistent with this notion, it has recently been shown that, in the presence of blocking antibodies, VLA-4 also localizes in the cSMAC of the CD4 T cell (Mittelbrunn *et al*, 2004); however, this was observed in the absence of detectable VCAM-1. These lines of evidence strongly argue that VLA-4 is likely to associate differently from LFA-1 with the cell cytoskeleton. Such a differential association may well relate with an overlapping but sequential function of VLA-4 and LFA-1.

Several studies using T cells have highlighted the capacity of VLA-4 to mediate tethering and strong adhesion within the multistep model of leucocyte trafficking (Butcher, 1991; Springer, 1994; Alon *et al*, 1995; Berlin *et al*, 1995; Grabovsky *et al*, 2000). In this model, the first step involves transient and reversible adhesion that, when followed by a second event, results in strong and sustained attachment owing to the activation of integrins. Chemokines have been proposed as the mediators of the second step; however, a possible role for other receptors was anticipated. Indeed, recently, Fc $\gamma$ RIII has been shown to mediate leucocyte adhesion in response to ICs *in vivo* and *in vitro* (Coxon *et al*, 2001; Stokol *et al*, 2004). It is conceivable that the BCR may play a similar role in the recruitment and activation of B cells. In this line, VCAM-1 is highly expressed on the surface of FDCs where it has been shown to enhance the interaction with B cells (Freedman *et al*, 1990; Koopman *et al*, 1991). Similarly, a role for VLA-4 in promoting antigen recognition may well be important in determining B-cell recruitment and activation in pathological situations such as in rheumatoid arthritis, systemic lupus erythematosus and glomerulonephritis. These diseases are characterized by the accumulation of ICs in cartilage surface (Matsumoto *et al*, 2002), synovial arterioles (Schaller *et al*, 2001) and postcapillary venules (Smedegard *et al*, 1985), together with high levels of expression of adhesion molecules, such as VCAM-1 (van Dinther-Janssen *et al*, 1991; Belmont *et al*, 1994).

Thus, incorporating our results into a model of B-cell membrane antigen recognition (Figure 7), VCAM-1 expressed on the surface of target cells (FDC, vascular endothelium) may capture the B cell through its interaction with VLA-4 under conditions of restrictive/limiting cellular interactions (germinal centre, shear stress). The absence of antigen



**Figure 7** Model of B-cell membrane antigen recognition.

recognition will result in B-cell detachment from the target cell within seconds. However, the encounter of specific antigen by the BCR will result in inside-out signalling to VLA-4 and LFA-1 in order to strengthen the interaction with VCAM-1 and promote the interaction with ICAM-1, respectively. This firm attachment to the target cell would allow the B cell to form a mature IS and become activated. This model may well apply to autoimmune disorders, in which the chronic expression of VCAM-1 could facilitate B-cell triggering by low-affinity self-antigens.

## Materials and methods

### Mice and splenic B-cell purification

Three different mouse strains were used: C57BL/6 WT mice and the BCR transgenic lines MD4 (HEL-specific BCR; Goodnow *et al*, 1988) and 3-83 (H-2K<sup>k</sup>-specific BCR; Russell *et al*, 1991). Splenic naive B cells were purified by negative selection, as previously described (Carrasco *et al*, 2004). An enrichment of 95–98% B cells was obtained by this procedure.

### 3-83 peptide ligands and recombinant lysozymes

The three different monobiotinylated peptide ligands (p31, p11 and p0) and the lysozyme mutants (HEL<sup>RD</sup>, with amino-acid positions 21 and 101 mutated to alanine; HEL<sup>KD</sup>, with amino-acid positions 97 and 101 mutated to alanine; and HEL<sup>RKD</sup>, with amino-acid positions 21, 97 and 101 mutated to alanine; Batista and Neuberger, 1998) used in this study were obtained as described previously (Carrasco *et al*, 2004).

### Generation of VCAM-1 constructs

The GPI-linked VCAM-1 construct was generated as follows. The extracellular domain of murine VCAM-1 was amplified by PCR with cDNA obtained from murine 3T3 NIH fibroblast as a template, using the sense primer 5'-GGGGTACCCAGAGACTTGAAATGCCTG-3' and the antisense primer 5'-CCGAATCCACTACCTGAATAGTCCTGTGTTGTTCTTTTCC-3'. The fragment obtained was cloned into *KpnI*/*EcoRI* restriction sites on pcDNA3 expression vector containing the GPI domain coding sequence (Carrasco *et al*, 2004). The GPI-linked VCAM-1 construct was transfected into J558L cells and positive clones were selected in the presence of geneticin.

The complete murine VCAM-1 and VCAM-1 tailless coding sequences were amplified using the same sense primer 5'-TCCCCCGGGCCTTCCACCTCCACTCACTTTGGATTCTGTGC-3' and antisense primer 5'-TCCCCCGGGAAGCTTTTCTTGCAAATAAAC-3' and then

cloned into *KpnI*/*XmaI* in the pEGFP-N1 expression vector. The EC VCAM-1/H2 construct was generated by replacement of the coding sequence of the GPI domain in the GPI-linked VCAM-1 construct with the coding sequence of a Ser/Gly linker, plus the H-2Kb transmembrane region, plus a cytoplasmic tail obtained from a lysozyme construct described by Batista *et al* (2001).

### Planar lipid bilayers and laminar shear stress assays

The planar lipid bilayers containing the different proteins were prepared as previously described (Grakoui *et al*, 1999; Carrasco *et al*, 2004). The chambers were blocked with PBS 2% FCS, followed by antigen loading in PBS 0.5% FCS. The different antigens for 3-83 and MD4 B cells were loaded in the bilayers using Alexa 633-streptavidin (Molecular Probes), as previously described (Carrasco *et al*, 2004). For lamina shear stress assays, the flow chamber was connected to a pump in order to control the shear stress applied and B cells were perfused at  $1 \times 10^6$ /ml. For the Ca<sup>2+</sup> measurements, B cells were previously stained with 1  $\mu$ m fluo-4FF (Molecular Probes). The assays were performed in PBS 0.5% FCS, 0.5 mM Mg<sup>2+</sup>, 0.5 mM Ca<sup>2+</sup> and 1 g/l D-glucose at 37°C. A series of three DIC images, with a second delay between each, were taken every minute during all shear stress experiments. Images were acquired on a Zeiss Axiovert LSM 510-META inverted microscope and analysed by Volocity (Improvision, UK) and ImageReady (Adobe) softwares. The number of B cells bound per mm<sup>2</sup> was calculated by counting the B cells that remain in the same position on the three images of each series. The fraction of B cells bound, Ca<sup>2+</sup> fluxing or rolling was estimated by counting these events within the total number of B cells flowing. For the activation assay, bound and not bound fractions of B cells were incubated overnight inside the flow chamber and in a dish, respectively. Then, they were collected and analysed by FACS for the expression of CD86 and CD69. The shear stress applied in dyn/cm<sup>2</sup> was calculated by the Hagen-Poiseuille equation.

### Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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