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Research Article

Formation of the B cell synapse: retention or recruitment?

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Abstract. Interaction of B cells with membrane antigen results in the formation of the B cell synapse: the B cell receptor (BCR) and antigen concentrate in the contact zone while CD45/B220 and the phosphatase SHP-1 are excluded. This study shows that, unlike in T cells, synapse formation does not require active transport processes (while subsequent antigen extraction and IgM downregulation do). The synapse architecture depends on the available protein ligands in the contact zone. Thus Syk, IgM and Fc receptor accumulation require the presence of ITAM-bearing BCRs, membrane antigen and membrane (IgG-containing) immune complexes, respectively. Remarkably, non-bound proteins are frequently not only homogeneously distributed but excluded from the contact zone. These results suggest that proteins mainly reach the contact zone by undirected diffusion, and in order not to be expelled by molecular crowding they require capture by and fixation to a binding protein.

Key words. B cell; synapse formation; affinity discrimination; Fc receptor; Syk.

Cell-cell interaction is important for immunological surveillance. Detailed studies of the contact zones between lymphocytes (B cells, T cells and NK cells) and their target cells have revealed large protein segregation structures [1], which have been shown to be important for lymphocyte activation [2]. Moreover, protein transfer between the cells has frequently been observed [3–8]. In analogy to neural synapses, these structures have been termed the 'immunological synapse' (IS) [9]. The B cell synapse is characterized by IgM and antigen concentration in the contact zone, from which the inhibitory proteins CD45/B220, CD22 and SHP-1 are excluded [4]. About the mechanism by which the B cell synapse forms little is known.

T helper cell synapse formation has been studied in greater detail and has been shown to require active cytoskeletally driven movement of proteins [9–15]. The importance of active transport processes is corroborated by a study that found the range of T cell receptor (TCR) velocities to significantly exceed the speed expected from undirected diffusion [16]. These observations stand only in apparent contradiction to mathematical models which showed that the receptor pattern observed during T cell synapse formation can be generated spontaneously [17, 18]: the 'active' change of biophysical properties (e.g. transport velocity of the TCR, membrane stiffness) that is observed in experiments can be expected to be necessary to generate conditions similar to those used in the models. Signaling precedes synapse formation [12, 19], and is likely to trigger the cytoskeletal polarization and receptor redistribution [20]. Here it is important to note that despite the failure to form a mature synapse, small protein segregation structures can still be observed in contact zones when cytoskeletal processes are inhibited [12, 13]. Segregation of proteins in these transient small (cytochalasin D-resistant) clusters is likely to be driven by the different lengths of the extracellular protein domains [20]. Clusters of CD3ζ may then initiate T cell signaling [12].

Receptor densities, ligand affinities as well as the extracellular length of receptor-ligand complexes differ greatly between the T and B cell synapse and mechanistic details

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of synapse formation may thus be very different. In contrast to what is observed in T cells, this study shows that (while antigen acquisition is compromised), the B cell synapse can still form when signal initiation at the B cell receptor (BCR) is prohibited and active transport processes are impaired. Moreover, the segregation pattern of both membrane and cytoplasmic proteins are shown to depend on the availability of binding sites within the contact zone while a dependency on the length of the extracellular domain as suggested by the topological model [21] could not be noticed. Together, these data suggest a diffusion and capture mechanism for B cell synapse formation: receptors mainly reach the contact zone by diffusion and require a binding partner in order retained and not excluded by molecular crowding. Implications for affinity discrimination by B cells are discussed.

Materials and methods

Reagents

Goat anti-mouse IgM and rat anti-CD45/B220 were bought from Southern Biotechnology, PE-anti-FcγRII/III was obtained from Pharmingen, and rabbit anti CD72, -SHP-1 and -Syk antibodies were all purchased from Santa-Cruz Biotechnology. Anti-hen egg lysozyme (HEL) antibodies were prepared from the F10 hybridoma which was a kind gift of Smith-Gill. Biotinylated anti-rabbit antibody was obtained from Amersham Pharmacia Biotech, FITC-streptavidin from Dako, and FITC-anti-rat, latrunculin A and genestein from Sigma. Phalloiding-rhodamine was purchased from Molecular Probes. Primary antibodies were used at 10 µg/ml. Secondary antibodies were diluted to up to 2 µg/ml to avoid unspecific reactivity.

Cells and mice

MD4 [22] as well as HEL, IgM/β (bet), IgM/$\beta^{Y\rightarrow L}$ (bmut) mice [23] and J558L[mHEL] target cells expressing membrane HEL [4] have been described before. Target cells expressing membrane HEL fused to intracellular GFP were obtained by transfecting SBM3.1 cells with plasmid FDB172 (kind gift of F. D. Batista), where the HEL sequence had been inserted into the pEGFP(N1) vector from Invitrogen. J558L[mIC] target cells were generated by incubating J558L[mHEL] with 10 µg/ml mouse IgG1 anti-HEL antibody for 30 min on ice prior to use.

Inhibitor study

For inhibitor studies, MD4 or HEL lymphocytes were incubated with 1 µM latrunculin A, 0.25 mM genestein or 10 mM sodium azide for 2 h prior to as well as during the experiments. Both 1 µM latrunculin A and 10 mM sodium azide did not increase the signal for propidium iodide (PI) staining as judged by FACS; 0.25 mM genestein has been found before to be the optimal concentration for inhibiting B cell signaling [24] but leads to a small increase in the PI signal.

Confocal microscopy

10^7/ml lymphocytes and 5×10^6/ml target cells were mixed and incubated at 37 °C on poly-L-lysine coated slides for the times indicated before being fixed with either 4% paraformaldehyde at room temperature (RT) or with ice-cold methanol on ice. Cells (except for CD72 staining) were further permeabilized with 0.3% Triton X-100 and blocked overnight in 1% BSA. Cells were stained with the primary antibodies for 1–2 h at RT, washed, incubated with a secondary antibody for 45 min and with the third reagent for 30 min where applicable. Confocal images were acquired using a Nikon E-800 microscope attached to a BioRad Radiance Plus scanning system equipped with 488-nm and 543-nm lasers, as well as differential interference contrast for transmitted light. Images were processed using BioRad Lasersharp 2000 software. Statistics were based on the analysis of at least five confocal fields, each harboring in general between 20 and 60 B cells. The error bars indicate the standard deviation between means obtained for each field. Results are representative of at least three independent experiments.

FACS analysis

All reactions (100 µl of 10^6/ml lymphocytes with 100 µl of 5×10^5/ml J558L[mHEL] target cells or as indicated) were incubated for the indicated times in 96-well plates at 37 °C. The reactions were stopped by placing the cells on ice. Cells were subsequently stained for 25 min on ice and analyzed by FACSCalibur as triplicates immediately after staining was complete. Error bars indicate the standard deviation between the triplicates. Results are representative of at least three independent experiments.

Results

Active transport processes are involved in membrane antigen acquisition but appear dispensable for B cell synapse formation

Active transport processes were inhibited by treating B cells with either sodium azide, which depletes cells of ATP, or with latrunculin A, which inhibits actin polymerization [25–27]; new tyrosine phosphorylation after BCR-linked activation was inhibited with genestein [28]. Treatment of B cells with 10 mM sodium azide, 1 µM latrunculin A or 0.25 mM genestein prior to and during the co-incubation with antigen-displaying cells neither prohibited IgM and antigen concentration in the contact zone (fig. 1A–B) nor the exclusion of CD45/B220 (fig. 1C) and SHP-1 (fig. 1D). A difference could, however, be noted in the rapidity and extent of CD45 and SHP-1 exclusion as well as in the kinetics of synapse dissolution. Thus pro-

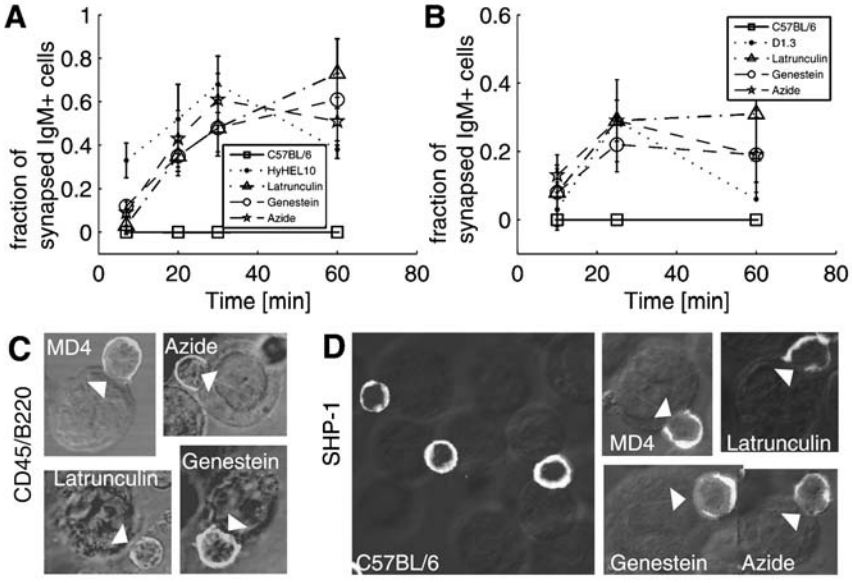

Figure 1. Latrunculin, azide, and genestein do not prohibit B cell synapse formation. Time dependent fraction of MD4 (*A*) or HEL (*B*) splenic B cells that form synapses with J558L[mHEL] target cells. CD45/B220 (*C*) and SHP-1 (*D*) are excluded from contact zones formed by MD4 B cells with J558L[mHEL] target cells (contact zones were defined by IgM accumulation and are marked by arrowheads). C57BL/6 B cells are unspecific for the antigen. (Inhibitor treatment is indicated in each panel.)

tein exclusion appeared slightly less efficient in the presence of the inhibitors (especially with genestein) and a quantitative analysis of B cell synapse formation revealed that synapse dissolution was retarded in the presence of inhibitors (fig. 1B, C). The quantitative analysis was conducted by determining the fraction of IgM-positive lymphocytes that had concentrated antigen in a contact zone with the antigen-displaying target cells. The retarded synapse dissolution was observed with both splenic B cells from transgenic MD4 mice expressing IgM specific for the HEL HyHEL10 epitope (fig. 1A) and with splenic B cells from HEL mice expressing IgM specific for the HEL D1.3 epitope (fig. 1B). The different extent to which MD4 and HEL B cells formed synapses correlated well with the affinity difference for the antigen HEL (HyHEL10 $K_a = 5 \times 10^{10}$ M^{-1} [29–31] and D1.3 $K_a = 3 \times 10^8$ M^{-1} [32–35]).

Despite successful synapse formation, azide, latrunculin A and genestein reduced the efficiency of membrane antigen-induced IgM downregulation as judged by FACS analysis (fig. 2A, B) and affected membrane antigen acquisition (Fig. 2C, D). IgM downregulation was assessed by incubating B cells with J558L[mHEL] target cells for the indicated times and staining the cells with FITC-anti-CD45/B220 and PE-anti-IgM on ice for 25 min. IgM surface expression was altered in the presence of inhibitors (fig. 2A) such that the inhibitory effect can be judged better when relating the IgM loss to the initial BCR density (fig. 2B). Negative times refer to times cells were kept on ice for staining. Antigen acquisition was determined by analyzing the transfer of GFP-fused antigen from target cells to CD45/B220-stained lymphocytes by FACS. The proportion of B cells that adsorbed antigen increased rapidly at the beginning of the reaction and more slowly toward the end (fig. 1D). The total amount of antigen associated with B cells increased within the first 60 min, before the level declined (presumably due to antigen degradation) (fig. 2C). Azide and genestein delayed antigen acquisition and impaired a subsequent decline in the antigen levels (and thus presumably antigen uptake and/or degradation). Such an energy dependence of internalization has previously been reported for NK cells [5]. On the other hand, latrunculin A treatment caused a relative increase in the antigen levels on B cells. This is unlikely to reflect facilitated antigen acquisition but rather hints at a block in antigen uptake (and thus subsequent degradation).

Effective antigen acquisition thus requires energy and general cell signaling but not the remodeling of the cytoskeleton, while antigen uptake and degradation require both. A similar dependency on active transport processes was not noted for B cell synapse formation.

ITAM sequences are not required for synapse formation but for Syk recruitment to the contact zone

Although synapse formation proceeds at inhibitor concentrations that compromise IgM downregulation and antigen acquisition, the use of inhibitors always leaves the possibility that effective concentrations are lethal, and that the antigen extraction process is only more sensitive. This especially applies to genestein, effective concentrations (0.25 mM) of which had already a rather strong general

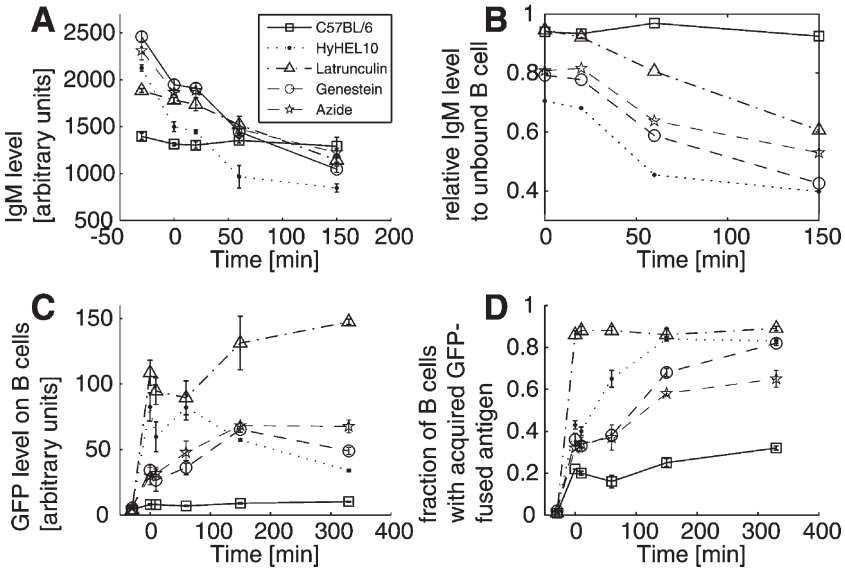

Figure 2. Latrunculin azide, and genestein impair IgM downregulation and antigen acquisition. IgM surface levels (*A*, *B*) and the level of GFP-fused antigen acquisition (*C*, *D*) were determined for C57BL/6 and MD4 B cells that had been incubated with J558L[mHEL] target cells for the indicated times under the specified conditions. IgM levels are either given in absolute values (*A*) or relative to the initial staining (*B*). Note that the inhibitors affected the initial staining intensity for IgM. Time dependent HEL-GFP acquisition by B cells (*C*). Fraction of B cells with acquired GFP-fused antigen over time (*D*). All results are representative of at least three independent experiments.

detrimental effect compared with latrunculin A or azide. Doubling the concentration of genestein led to considerable cell death and fully inhibited synapse formation (data not shown). The impact of cell signaling on B cell synapse formation was therefore also analyzed using splenic B cells bearing a signaling-incompetent HEL-specific BCR (D1.3 epitope). The BCR expressed in these transgenic mice is a chimera composed of mouse μ and rat κIg chains directly linked through a hydrophobic transmembrane segment to the cytoplasmic domains of either Igβ, or a mutated Igβ whose ITAM tyrosines are substituted by leucines (IgM/$\beta^{Y\rightarrow L}$) [23]. This substitution leads to the destruction of the recruitment site for the kinase Syk. The transmembrane segment (which derives from the mouse H-2K^b gene) confers sheath-independent surface transport [36] and the receptors do not show detectable association with endogenous Igα or Igβ chains [37].

The IgM/$\beta^{Y\rightarrow L}$ B cells had previously been reported to be signaling defective and not to upregulate CD86 upon IgM cross-linking [23]. Figure 3 confirms that membrane-bound antigen (J558L[mHEL]) also cannot activate IgM/$\beta^{Y\rightarrow L}$. Note that activation is also severely compromised (though not impossible) in IgM/β B cells, which is in agreement with experiments that report an important role for Igα in B cell activation [38].

Despite the failure of IgM/$\beta^{Y\rightarrow L}$ to support B cell activation, synapse formation could still be detected. IgM and antigen concentration in, as well as CD45/B220 and SHP-1 exclusion from the contact zone of IgM/$\beta^{Y\rightarrow L}$ B cells (fig. 4A–C) were at least as strong as with IgM or IgM/β B cells; SHP-1 exclusion from the IgM/β synapse was

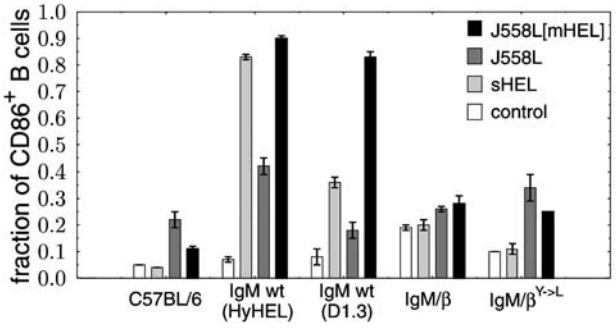

Figure 3. CD86 upregulation of B cells in response to soluble or membrane-bound antigen. Cultured splenocytes from transgenic mice expressing either unspecific BCR, IgM (HyHEL10), IgM (D1.3), IgM/β (D1.3), or IgM/$\beta^{Y\rightarrow L}$ (D1.3) were incubated for 24 h with medium alone, in the presence of 1 μM HEL, 5×10^5/ml J558L or 5×10^5/ml J558L[mHEL] target cells. Splenocytes were stained with PE-anti-CD45/B220 and FITC-anti-CD86. The profiles were gated by size and for CD45/B220+ lymphocytes. All results are representative of at least three independent experiments.

generally only observed once IgM had disappeared from the contact zone, suggesting that SHP-1 exclusion is slower, as loss of IgM from the contact zone generally marks a later step during B cell synapse maturation. The main defects of the IgM/$\beta^{Y\rightarrow L}$ synapse are a strongly retarded dissolution (fig. 4D) and a much lower efficiency in acquiring membrane antigen (fig. 4E). Moreover, Syk only accumulated in the IgM and IgM/β synapse but not in the IgM/$\beta^{Y\rightarrow L}$ synapse (fig. 4F). Remarkably, Syk was, however, not only found to be homogeneously distributed in IgM/$\beta^{Y\rightarrow L}$ B cells but was frequently excluded from the contact zone 30 min after mixing the cells (fig. 4F).

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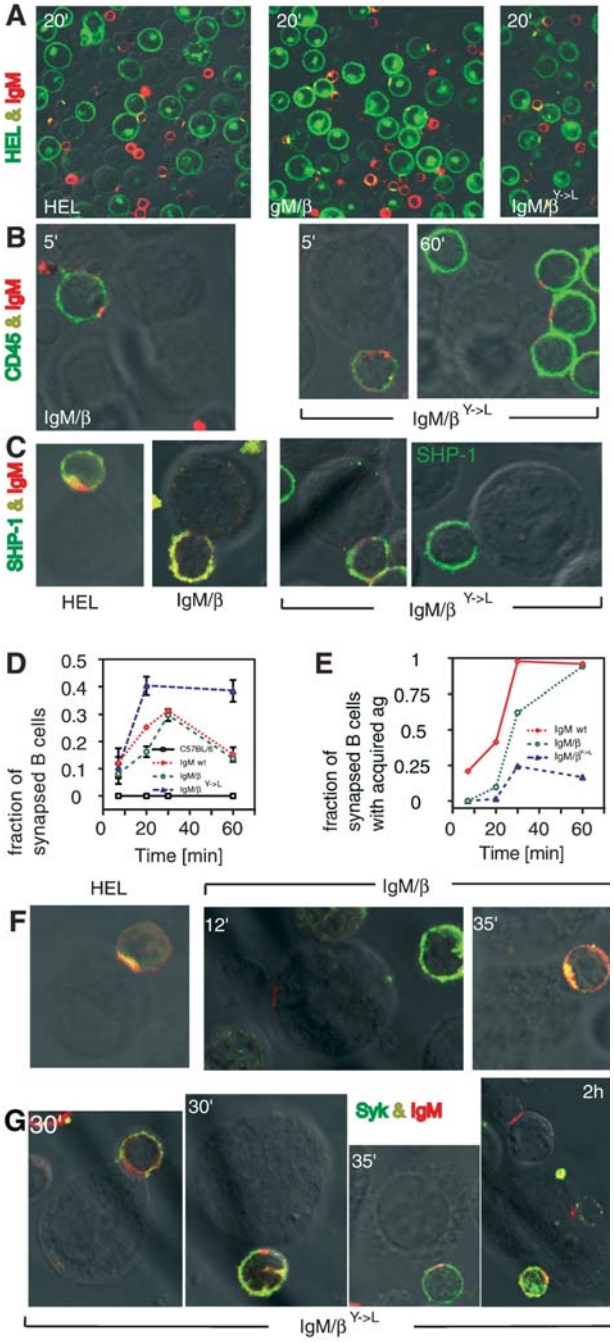

Figure 4. A signaling-competent BCR is not required for B cell synapse formation. B cells (smaller cells) expressing either IgM/β (D1.3), or IgM/$\beta^{Y\to L}$ (D1.3) concentrate (*A*) IgM (red) and antigen (HEL, green) in the contact zone with J558L[mHEL] (larger cells), from which CD45/B220 (green) (*B*) and SHP-1 (green) (*C*) are excluded. (*D*) Fraction of B cells expressing either an unspecific BCR (black line), or HEL-specific IgM (D1.3) (red line), IgM/β (D1.3) (green line), IgM/$\beta^{Y\to L}$ (D1.3) (blue line) which form synapses upon co-incubation with J558L[mHEL] target cells for the indicated times. (*E*) Fraction of synapsed B cells expressing either HEL-specific IgM (D1.3) (red line), IgM/β (D1.3) (green line), or IgM/$\beta^{Y\to L}$ (D1.3) (blue line), which have acquired antigen after the indicated times. These results represent the average from at least 30 counted synapses. (*F*) HEL-specific IgM (D1.3) and IgM/β (D1.3) B cells accumulate Syk (green) in the contact zone with J558L[mHEL]. (*G*) Syk is excluded from IgM/$\beta^{Y\to L}$ (D1.3) synapses.

IgM/$\beta^{Y\to L}$ lacks the ITAM sequences required for Syk binding and this observation thus suggests that retention of a protein in the contact zone requires the availability of a binding side, and unbound proteins may become excluded by molecular crowding. An observation that is illustrated by one image of the IgM/β synapse but which applies to all three B cell types is that Syk was frequently invisible in synapsed B cells (while being well visible in adjacent non-synapsed B cells). This may be due to a conformational change in Syk which may alter the epitope to which the Syk-specific antibody binds. Syk degradation is a less likely explanation.

Differential segregation of proteins in the BCR and Fc synapse

If synapse formation is indeed mainly enabled by protein diffusion and subsequent capture in the contact zone, synapses (though with a different architecture) should also form in response to non-cognate IgG-containing immune complexes, which will engage the Fc receptor on B cells. Indeed presenting B cells with non-cognate IgG-containing immune complexes resulted in the accumulation of immune complexes (fig. 5A) and FcγRIIb1 (fig. 5D) but not of IgM (fig. 5D) in the contact zone. This synapse type will be referred to as an Fc synapse. In the BCR

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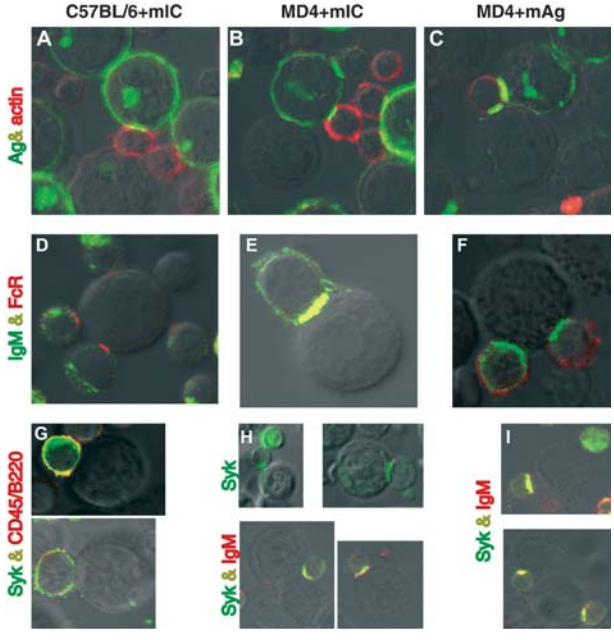

Figure 5. The Fc synapse. Unspecific B cells or HEL-specific (HyHEL10) B cells (MD4) (smaller cells) were incubated with either J558L[mAg] or J558L[mIC]. Antigen (green) is concentrated in the contact zones formed between unspecific B cells and J558L[mIC] target cells, and HEL-specific B cells with either J558L[mAg] or J558L[mIC] target cells. (*A–C*) IgM (green) (*D–F*) and Syk (green) (*G–I*) are concentrated in the contact zone formed by HEL-specific (*E–I*) but not by HEL-unspecific B cells (*D*, *G*), while FcR (red) is only concentrated in contact zones formed with J558L[mIC] (*D*, *E*) but not with J558L[mAg] (*F*).

synapse, which forms upon encounter with (uncomplexed) membrane antigen, IgM is included in and FcγRIIb is excluded from the contact zone (fig. 5F). Both receptors are included in the contact zone formed with cognate IgG-containing membrane immune complexes (fig. 5E). As before, non-bound receptors were frequently not only homogeneously distributed but rather excluded from the contact zone. Similar observations were made with the kinase Syk, which only concentrated in the IgM-containing contact zones, but not in those from which IgM was excluded (fig. 5G–I).

Discussion

The results presented here show that B cell synapses can still form even if BCR-initiated signaling processes are prohibited and active transport processes are inhibited to a degree that IgM downregulation, antigen transfer and synapse dissolution are impeded. The analysis of signaling-defective BCR mutants excludes the possibility that these observations only reflect secondary effects of employed inhibitors. Thus, the proteins that accumulate in the contact zone between B cells and antigen-displaying target cells apparently get there mainly by undirected diffusion. Retention in the contact zone requires the availability of binding partners and removal of such a binding site frequently results in the exclusion (and not only the homogeneous distribution) of the protein. Protein exclusion from the contact zone may therefore be driven by molecular crowding, and thus be also mainly undirected. Such a diffusion and capture mechanism is not unprecedented [39] and offers an explanation for how B cells discriminate between a wide range of binding affinities as is discussed below.

Other passive mechanisms such as segregation of protein complexes according to their extracellular length (topological model [21]) or by association with certain lipid domains, termed rafts [40], are less likely to play a role. While topographical differences can, in principle, assist receptor segregation [17, 18, 21], the extracellular domain of IgM-antigen/immune complexes is rather long, which will reduce such a driving force for protein expulsion. The minor impact of receptor length on receptor localization in the B cell synapse is illustrated particularly well by the distribution of IgM and FcγRIIB1 in the B cell synapse formed with either membrane antigen or unspecific IgG-containing membrane immune complexes. While the exclusion of IgM from the Fc synapse may be in agreement with a topographic model which suggests that longer receptors are excluded, exclusion of FcR from the BCR synapse would certainly not be. These results agree with the observation that a large segregation pattern in the T cell synapse requires active transport processes [9–15].

While the role of lipid rafts in driving lymphocyte synapse formation and signaling remains controversial [41–43], the fact that changes in the extracellular binding partner (antigen versus immune complexes) and a mutation in the cytoplasmic motif far away from the membrane (both of which are unlikely to affect the lipid environment directly) did alter the localization of proteins suggests that the impact of lipid domains on protein localization is at least not very strong. Indirect effects, in that recruitment of a factor is necessary for subsequent raft recruitment, would limit the effects of rafts to an amplifying process. Of interest in this context is to note that viable concentrations (2 mM) of the raft-disrupting, cholesterol-extracting drug β-methylcyclodextrin neither affected B cell synapse formation nor antigen acquisition while antigen degradation was impaired [D. Iber, unpublished observations]. Given the high sensitivity of B cells to this drug, one cannot however exclude that degradative processes are more sensitive than B cell synapse formation or antigen acquisition and that a removal of raft lipids sufficient to impair B cell synapse formation is lethal.

That B cells but not T cells can form synapses in the absence of active cytoskeletally mediated transport processes is likely to be accounted for by the differences in affinity and density of interactions. Only at high antigen density and high interaction affinity will the density of receptors be large enough to exclude other unbound proteins from the contact zone. In agreement with this, synapse formation appears to be weaker if the interaction affinity is reduced (fig. 1B, C) as well as if antigen is displayed at a lower density [44, D. Iber, unpublished observations]. Of interest will be to investigate the importance of active transport processes in a low-affinity, low-density system, as this would correspond to the physiological situation at the onset of an immune response.

Antigen internalization, on the other hand, clearly depends on active processes, even though the initial adsorption process appears not to require remodeling of the cytoskeleton (an observation similar to one made with T cells [45]). The extent of antigen internalization as well as the efficiency of targeting to processing compartments is thus likely to reflect the strength of B cell signaling that is elicited by the antigen.

How the affinity of antigen binding is translated into a differential cellular signal is an enigma. This paper provides evidence that B cell synapse formation is mainly driven by passive processes and suggests that proteins that lack a binding partner in the contact zone become expelled by molecular crowding. All proteins that have so far been reported to localize to the contact zone exhibit an activating function (such as Syk, PLC-γ) while excluded proteins are known to be inhibitory (such as SHP-1, CD22, CD45). A spatial separation may trigger B cell signaling by having a similar effect as a direct phosphatase inhibition, which has been shown to be sufficient for B cell activation even in

the absence of antigen or anti-IgM antibodies [46, 47]. B cells may discriminate between different affinities of binding on the basis of the different extent to which protein segregation is triggered. Lower-affinity interactions will engage fewer receptors in the contact zone and thus lead to less inhibitor expulsion. According to such a model, the affinity threshold would correspond to the affinity that enables sufficient protein segregation to trigger B cell signaling, and the affinity ceiling would correspond to the affinity for which maximal protein segregation is obtained, and thus the B cell response does not improve any further with increasing BCR affinity.

This model of B cell activation provides an explanation for the different discrimination ranges observed for soluble antigen and for antigen presented either on a small particle or a large surface [48]. Displaying antigen on a surface (independent of its size) will facilitate receptor segregation and thus lower the affinity threshold compared to soluble antigen. The affinity ceiling on the other hand can be expected to be attained at a lower affinity if antigen is presented on a small particle as compared to a large surface, since the smaller contact zone will be filled with IgM already at a lower affinity, thus resulting in maximal inhibitor exclusion already at a lower affinity. Factors that interact with inhibitory proteins in the contact zone (e.g. α2,6-linked sialoglycoconjugates with CD22, and IgG antibodies with FcR) will increase the affinity threshold by opposing exclusion of the inhibitor by molecular crowding.

While the existence of the Fc synapse provides support for the notion that co-receptor engagement can affect the detailed B cell synapse architecture and thereby presumably tune B cell signaling and responsiveness, it also raises new questions. Under physiological conditions the majority of B cell contacts will be with immune complexes for which the B cell is not specific. In that case, Fc synapse formation will lead to the homoaggregation of Fc receptor which has been reported to result in B cell apoptosis [49–51]. Mechanisms will therefore need to exist to avoid random loss of B cells from the peripheral pool in response to Fc receptor engagement by unspecific immune complexes. Fc receptor downregulation has been reported for germinal center B cells [52, 53] and it will be important to see how far this extends to other peripheral B cells and what the signals are for a downregulation.

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