Membrane mobility and clustering of Integrin Associated Protein (IAP, CD47)—Major differences between mouse and man and implications for signaling

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Abstract

Integrin Associated Protein (IAP, CD47) is a ubiquitous integral membrane protein implicated in processes (in mice) that range from inhibiting clearance by phagocytes [Oldenborg et al., \textit{Science} 2000; Gardai et al., \textit{Cell} 2005] to neutrophil motility [Lindberg et al., \textit{Science} 1996]. SIRP\textsubscript{α} is CD47’s main receptor on phagocytes plus a number of other cell types, and SIRP\textsubscript{α}-CD47 interactions in clusters are believed to mediate signaling. However, considerable species differences in CD47 sequence as well as differences in CD47 extractability from mouse cells versus man motivate a characterization of mobility, clusterability, and kinetics under force of CD47-SIRP\textsubscript{α}. Despite similar levels of CD47 on red cells from mouse and man, we find an effective avidity of SIRP\textsubscript{α}-CD47 for mouse appears higher than for human. Both mouse and human CD47 show clustering by multivalent SIRP\textsubscript{α} complexes, but only mouse cells aggregate with CD47 concentrating at cell–cell contacts. This proves consistent with fluorescence imaged micro-deformation, which indicates near-complete mobility of CD47 on mouse cells compared to only about 30–40% mobility on normal human cells. To qualify the method, we also show that disrupting cellular F-actin dramatically increases the mobility of integral membrane proteins. Furthermore, atomic force microscopy probing of cell membranes with human SIRP\textsubscript{α} confirms the species-specific interactions and provides evidence of clustering and adhesion on short time scales, but it also shows surprisingly strong forces in detachment for a signaling complex. The results thus highlight major species differences in CD47-SIRP\textsubscript{α} interactions and CD47 integration, suggesting that signaling by CD47 in man may be qualitatively different from mouse.

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Introduction

Protein–protein interactions, in their biological context, depend not only on kinetics of association and affinity but also on protein concentration and mobility of the proteins, particularly if the proteins are membrane proteins as studied here. For example, mobility constraints on the immunological synapse between T cells and antigen presenting cells strongly influences cell–cell signaling [1]. A “phagocytic synapse” [2] that is now being proposed between phagocytes and their various target cells should be governed by similar factors. However, one might expect recognition aspects to be faster in the phagocytic synapse than in the immunological synapse since the latter takes 10–30 min to develop, whereas phagocytes can completely engulf a target cell such as a yeast cell in 30 s under physiological conditions [3]. A putative “marker of self” interaction between CD47 on the target cell and SIRP\textsubscript{α} on the phagocyte (Fig. 1) [4,5] should therefore signal quickly against engulfment.

CD47–knockout mice are immuno-compromised but viable [6], and while there seem to be no clear cases yet of human RBC with total absence of CD47 [7,8], a key role for human–CD47 inhibiting phagocytosis of human cells has
yet to be adequately demonstrated. Target cells where CD47 seems to have a role include mouse RBC and also cells that apoptose [4]. Phagocytes studied thus far have also been derived from mouse. CD47-deficient RBC are cleared rapidly in wild-type mice by splenic red pulp macrophages [5], and so the time scales and physical setting in vivo that are relevant to a putative phagocytic synapse are those of an RBC being driven through the narrow sinusoidal capillaries of the spleen. Our aim in vitro here is to map out some of the key physical as well as biochemical differences between mouse and human CD47 and CD47’s interactions with SIRPα. Ultimately, we show that there are remarkably similar densities of CD47 on mouse and human RBC, and that the CD47-SIRPα interaction can engage and co-cluster within seconds, but we also find major species differences in specificity and mobility that are likely to have functional implications for signaling.

Materials and methods

Chemicals

The following reagents were from Sigma-Aldrich (St. Louis, MO): poly-L-lysine hydrobromide MW 2900, phosphate-buffered saline (PBS) tablets (0.01 M phosphate buffer/0.0027 M potassium chloride/0.137 M sodium chloride, pH 7.4), PKH67 dye, and bovine serum albumin (BSA). All solutions were made in filtered double-distilled water. p-chloromercuriphenylsulphonic acid (PCMS) was a gift of Dr. W. Gratzer, London, UK.

Production of recombinant human and mouse SIRPα

COS-1 cells (ATCC, Manassas, VA) were transfected with pcDNA3-based vector (Invitrogen, Carlsbad, CA) encoding a human SIRPα extracellular domain fused to GST [9] using

Table 1
Supplier and concentration information for RBC labeling reagents

<table>
<thead>
<tr>
<th>Labeled component</th>
<th>Reagent</th>
<th>Supplier</th>
<th>Stock</th>
<th>Final concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phospholipid</td>
<td>FL-DHPE</td>
<td>Invitrogen Corp., Carlsbad, CA</td>
<td>0.25 mM in EtOH</td>
<td>12.5 μM</td>
</tr>
<tr>
<td>Band 3</td>
<td>Eosin-5-Maleimide</td>
<td>Invitrogen Corp.</td>
<td>2 mg/ml in PBS</td>
<td>0.02 mg/ml</td>
</tr>
<tr>
<td>Mouse CD47</td>
<td>FITC-labeled mIAP301</td>
<td>BD Biosciences, San Jose, CA</td>
<td>0.6 mg/ml</td>
<td>0.12 mg/ml</td>
</tr>
<tr>
<td>Mouse CD47a</td>
<td>mSIRPα&lt;sup&gt;a&lt;/sup&gt;</td>
<td>this laboratory</td>
<td>~0.4 mg/ml in PBS</td>
<td>~70 μg/ml (1 μM)</td>
</tr>
<tr>
<td>Human CD47</td>
<td>FITC-labeled B6H12</td>
<td>BD Biosciences, San Jose, CA</td>
<td>0.1 mg/ml</td>
<td>~70 μg/ml (1 μM)</td>
</tr>
<tr>
<td>Human CD47&lt;sup&gt;a&lt;/sup&gt;</td>
<td>hSIRPα&lt;sup&gt;a&lt;/sup&gt;</td>
<td>This laboratory</td>
<td>~0.7 mg/ml in PBS/10% glycerol</td>
<td>(1 μM)</td>
</tr>
<tr>
<td>GST</td>
<td>Rabbit anti-GST Alexafluor 488 or 647&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Invitrogen Corp., Carlsbad, CA</td>
<td>2 mg/ml</td>
<td>0.2 mg/ml</td>
</tr>
<tr>
<td>Human Glycophorin C</td>
<td>FITC-labeled BRIC-10</td>
<td>IBGRL, Bristol, UK</td>
<td>1 mg/ml</td>
<td>~0.1 mg/ml</td>
</tr>
</tbody>
</table>

<sup>a</sup> CD47 labeling using SIRP requires the addition of anti-GST.

<sup>b</sup> AlexaFluor 647 conjugation to anti-GST was done using a protein labeling kit from Invitrogen.
Lipofectamine 2000 (Invitrogen) as per manufacturer’s instructions. Secreted SIRPα1-GST (referred as hSIRPαex) was affinity-purified using glutathione sepharose 4B (Amersham Biosciences, Piscataway, NJ) as per manufacturer instructions and dialyzed against PBS (Invitrogen). The protein was stored at −20°C with or without addition of 10% v/v glycerol (Fisher Scientific, Hampton, NJ). The extracellular domain of mouse SIRPα [10] was also prepared as a GST fusion and will be referred as mSIRPαex.

Fluorescent labeling of RBC membrane lipid and proteins

10–20 μl C57 mouse blood collected in heparinized tubes (Covance, Denver, PA) or fresh human blood obtained from pin-pricks on healthy volunteers was washed 3–4× in 0.5 ml PBS (lipid and Band 3 labeling) or PBS/1% BSA (PBSA). 0.5–1.0 μl of packed RBC was used typically in 50 μl labeling reactions and incubated at ambient temperature for 30 min–1 h. RBC were spun, and the pellet was washed 2–3× in 0.5 ml PBSA (except SIRP labeled samples which were resuspended in 0.5–1.0 ml cold PBSA right before analysis). Table 1 provides details of labeling reagents and concentrations used.

Detection and clustering SIRPα

To simulate SIRPα clustering on phagocytes, a multivalent complex of SIRPαex and anti-GST was formed with excess anti-GST with incubation at 37°C. Gel filtration showed that the complex has a molecular weight \( M = 1 \) MDa, and dynamic light scattering (DLS) indicated a complex with a mean hydrodynamic radius \( R_h \approx 12 \) nm (by mass). Manufacturer-supplied measures of \( R_h \) for two large proteins (immunoglobulin-G: 160 kDa, 7.1 nm) and (thyroglobulin: 650 kDa, 10.1 nm), yield a power-law fit of \( R_h = 2.0 \times 10^{0.25} \), and this correlation implies a molecular weight for the (SIRPαex+anti-GST) complex of \( M \approx 1.3 \) MDa, consistent with gel filtration. Assuming two SIRPα to two anti-GST, this molecular weight would imply approximately six SIRPα per complex.

Fluorescence-imaged microdeformation

Capillary tubes of 1.0 mm inner diameter (World Precision Instruments, Sarasota, FL) were pulled into micropipettes using a Flaming-Brown Micropipette Puller (Sutter Instrument, Novato, CA) and cut to various diameters using a deFonbrune-type microforge (Vibratome, St. Louis, MO). Micropipettes were attached to a dual-stage water manometer with reservoirs of adjustable height. RBC were swollen using 70% PBSA 30% dH2O, and the micropipettes were passivated in the same buffer to reduce interaction between RBC and the glass surface. Suction was then applied using a syringe for a duration of 1–30 min, during which fluorescence images were acquired.

Microscopy

FIMD images were acquired on a Nikon TE300 inverted microscope with a 60× or 100× oil objective and recorded using a liquid nitrogen cooled CCD camera (Roper Scientific, Trenton, NJ, USA) or a Cascade CCD camera (Photometrics, Tuscon, AZ). All other images were acquired on a Olympus IX71 inverted microscope with a 60× oil objective and a recorded using a Cascade CCD camera (Photometrics, Tuscon, AZ). Image acquisition was performed with Image Pro software (Media Cybernetics, Silver Spring, MD). All image analysis was done using Image J 1.31v (http://rsb.info.nih.gov/ij/).

Flow cytometry

For flow cytometry, forward scatter, side scatter and fluorescence (FL1, FL2, FL3, FL4 channels in logarithmic mode)
Preparation of spread cells

One-hundred microliters of 10 mg/ml poly-L-lysine solution was allowed to adsorb for 10 min to a clean glass slide, and excess solution was drained away. The poly-L-lysine-coated slide was allowed to dry under vacuum for at least 2 h. Fresh human blood was obtained from finger pricks of healthy donors. Rat blood was obtained from Covance (Princeton, NJ). Twenty microliters of fresh blood was washed three times in PBS containing 1% BSA at room temperature to get packed RBC. Packed human RBC was fluorescently labeled with PKH67, washed, and resuspended in PBS. For studying CD47-SIRPα interactions, equal quantities of human and rat RBC mixed, and 100 μl of mixed RBC, were allowed to adhere to each poly-L-lysine-coated glass slide for 10 min.

Unattached cells were removed by gentle rinsing of the slide several times with PBS solution, and an additional volume of PBS was added for the experiments. For the other experiments, only human RBC was used.

AFM and preparation of functionalized tips

Force curves in indentation and retraction of spread erythrocytes were obtained with an Asylum Research AFM. This was mounted on the Nikon Eclipse TE 300 inverted microscope (Nikon, Tokyo, Japan). For studying adhesion between CD47 and SIRPα, blunt tips with nominal spring constants of 60 pN/nm (microlevers, Park Scientific, Sunnyvale, CA) were first silanized by immersion in a solution of 1.25% Allyltrichlorosilane in toluene. The silanized tips were then immersed in recombinant human SIRPα ex solution of desired concentration for 10 min. The functionalized tips were then washed thoroughly in 1% BSA (Sigma-Aldrich, St. Louis) solution in PBS to remove loosely attached protein. The retraction profiles seen in the adhesion experiments typically showed sawtooth patterns of force versus extension, with asymmetric peaks in force that were counted as peaks when the drop-off in force reached at least 50% toward the baseline. Values for the cantilever spring constants were obtained by a manufacturer supplied thermal noise method and were used in all calculations.

Table 2
Man and Mouse CD47 densities and cross-species SIRPα interactions

<table>
<thead>
<tr>
<th></th>
<th>Human RBC</th>
<th>Mouse RBC</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD47 levels per RBC</td>
<td>25,000a</td>
<td>16,500</td>
<td>1.54</td>
</tr>
<tr>
<td>Mean RBC Area (μm²)</td>
<td>140b</td>
<td>90c</td>
<td>1.55</td>
</tr>
<tr>
<td>Human-SIRPα ex bindingd</td>
<td>25</td>
<td>&lt;0.5</td>
<td>&gt;50-fold specific</td>
</tr>
<tr>
<td>Mouse-SIRPα ex bindingd</td>
<td>3</td>
<td>64</td>
<td>22-fold specific</td>
</tr>
</tbody>
</table>

a Mouro-Chanteloup et al., 2003 [11].
b Hategan et al., 2003 [12].
c Murdock et al., 2000 [13].
d Median signal from flow cytometry after subtraction of baseline signal (~5–10).

Results and discussion

Similar expression levels on mouse and man but distinct SIRPα interactions

CD47 concentrations, affinities for SIRPα, and mobilities in the membranes of human RBC versus mouse RBC are the primary variables that we seek to compare. On human RBC, it is already known that the number of copies of CD47 is approximately 25,000 per cell [11]. To determine CD47 levels in mouse RBC, we titrated binding of fluorescent monoclonal antibodies against CD47 (Table 1) for mouse and human RBC and compared the binding profiles from flow cytometry measurements of median intensities. The association with
mouse and human RBC both fit a simple binding isotherm (Fig. 2A) with apparent affinities of μM or less and no evidence of cooperativity. The saturation levels of binding—which indicate CD47 levels per RBC—were smaller on mouse RBC versus human RBC, but the ratio is essentially identical to the ratio of average RBC areas because human RBC are considerably larger (Table 2). This common surface density of CD47 translates to an effective CD47 concentration of 10–100 μM within a phagocytic synapse of 100 nm² and ~10-nm gap height (using the dimensions of immunoglobulin domains in CD47 and SIRPα per Fig. 1).

To assess SIRPα association with human and mouse red cells, both human and mouse recombinant SIRPα extracellular were expressed as GST fusion constructs (SIRPαex) and used at ~1 μM concentrations. Additionally, to mimic clustering of SIRPα on phagocytes which will tend to increase the avidity of interactions [14], the SIRPαex was made a multivalent ligand using polyclonal anti-GST. The antibody was also fluorescent so that SIRPαex binding to RBC could be detected by flow cytometry. Measurements were generally done within 1 min of mixing, since a time series after mixing with human RBC revealed spontaneous dissociation with a time constant of 15 min. Additionally, in the presence of a high affinity anti-CD47 antibody (B6H12) that would limit SIRPαex rebinding, the dissociation time was reduced to 7 min. These diffusion-limited time constants of minutes for multivalent SIRPα ligands are long compared to measurements of blood flow through the spleen where red cells have been seen to make point attachments to resident cells that last for up to 10 s but certainly not minutes [15]. One should keep in mind, however, that red cells flowing through the spleen experience significant hydrodynamic forces, and the dissociation time is generally expected to decrease exponentially with force [16], motivating our atomic force microscopy studies below.

Though physiological context is clearly key to understanding protein kinetics, cross-species interactions are comparatively simpler to assess and clearly show here that SIRPα binding is species-specific for both human and mouse protein.

Fig. 4. Fluorescence-imaged microdeformation (FIMD) of human and mouse red cells show mouse CD47 has high mobility. (A) Disrupting the actin cytoskeleton in human RBC with PCMS results in patchy and dim labeling of F-actin with rhodamine phalloidin. This disruption reduces the cytoskeletal connectivity of proteins such as Glycophorin C and thus decreases the gradient from entrance-to-cap. (B) On mouse RBC, Band 3 appears largely connected to the cytoskeleton, whereas CD47 appears completely mobile, accumulating at the cap (c) of the aspirated portion of the cell. Lipid appears uniformly distributed with equal entrance (e) and cap intensities.
Human SIRPα<sup>ex</sup> binds human RBC with an apparent affinity >50-fold more specific than binding to mouse cells (Table 2). Mouse SIRPα<sup>ex</sup> binds mouse RBC with an apparent affinity about 22-fold more specific than binding to human cells. The results also suggest that mouse SIRPα generally binds more strongly than human SIRPα. The monovalent affinities could be different or, more likely based on results below, the multivalent association is strengthened by higher mobility of CD47 on mouse RBC.

Imaging of the human RBC labeled with human SIRPα<sup>ex</sup> as above shows a dozen or more distinct fluorescent clusters per cell (Fig. 3A). The fluorescence bleaches rapidly and proves challenging to image (see Materials and methods). In contrast, mouse RBC labeled with mouse SIRPα<sup>ex</sup> show a distinct tendency to aggregate RBC that is also evident in a strong forward scatter in flow cytometry measurements. Furthermore, the regions of cell–cell contact appear highly fluorescent (Fig. 3B), and while fluorescent heterogeneities are also apparent on mouse RBC, the brightest portions of the membrane rim by several-fold are the large sectors of cell–cell contact. The images are typical of those seen in cadherin-mediated adhesion of epithelial cells [17,18] and suggest higher mobility for clustered CD47 in the mouse RBC membrane compared to human.

**CD47 is freely mobile on mouse cells but not on human cells**

Because rapid photobleaching limits a clear assessment of cluster mobility, fluorescence imaged micro-deformation (FIMD) was applied to the mouse RBC as already applied to human RBC [8,19]. The method involves fluorescent labeling of a specific cell component and then taking a single image of the cell, while it is deformed within a glass micropipette [20]. Recent studies with anti-CD47 and normal human RBC have demonstrated that on average 40% of CD47 is mobile. Such estimations are based on the slope of the intensity gradient from entrance to cap and are illustrated here by chemically collapsing this strong density gradient that usually forms with Glycophorin-C (Fig. 4A). This integral membrane protein is normally attached via protein 4.1 to the spectrin-actin cytoskeleton that resists the strain of aspiration into the micropipette [20]. However, addition of PCMS, which destabilizes F-actin in the cytoskeleton [21] and leads to only spotty F-actin labeling with rhodamine-phalloidin, relaxes the gradient of Glycophorin-C by more than 10-fold.

Mouse RBC also exhibit the range of FIMD results presented above with human RBC. Labeling of mouse Band 3 with fluorophores previously used on human cells [20] shows a similar entrance-to-cap gradient in FIMD as seen with human RBC.
human Band 3. The gradient is shallower than that found for the cytoskeleton and is consistent with 20–40% mobile Band 3 [22]. Labeling of mouse RBC with a lipid probe shows no gradient, consistent with a fluid bilayer. Although these are among the first FIMD results reported for mouse RBC, the most important findings in context here are the results with CD47 – labeled with anti-CD47 (mIAP301) – that show a gradient inverted from that of Band 3 and consistent with past findings for completely mobile proteins such as GPI-linked CD59 [20]. The accumulation at the cap is understood to reflect steric exclusion from the rest of the projection by Band 3 and other cytoskeleton-attached proteins. The results thus show that CD47 is >80% mobile on mouse RBC, contrasting with CD47 on human RBC.

For comparison to mouse RBC, Fig. 5 summarizes past and present measurements of CD47 expression levels and %mobile CD47 on human red cells, both normal and human variants. Rh variants among normal cells and Protein 4.2 deficiencies have

![Image](image_url)

**Fig. 6.** Force probe studies of red cells show multiple, strong CD47-SIRPα attachments. (A) hSIRPα<sup>ex</sup>-functionalized AFM tip used for probing CD47 on red cells. Lower image shows AFM contact mode height image of fixed red cells. (B) A typical force curve obtained when an unfixed human red cell is probed with a human SIRPα<sup>ex</sup>-functionalized tip coated at 0.5 mg/ml protein. (C) Unbinding forces for human SIRPα<sup>ex</sup> and human or rat red cells at either dilute (0.05 mg/ml) or concentrated (0.5 mg/ml) coating concentrations.

![Image](image_url)

**Fig. 7.** Rate-dependent interactions between CD47 and SIRPα. (A) Cross-over from two peaks to single peak with increasing rate of indentation and shorter contact times. (B) First and second peak forces obtained with contact times.
been found to have a significant influence on both CD47 expression and mobile protein on human RBC. However, the mobile fraction on human cells is always far less than that found here for mouse. The clear picture that emerges is that protein 4.2 attaches most of CD47 (to Band 3 and spectrin-actin in human RBC and that Rh also mediates some attachment [8,19], but neither attachment occurs in mouse. This picture is consistent with 4.2−/− mice showing no difference from wild-type in CD47 extractability, even though human variants showed significant perturbations [11].

SIRPα binding detected by AFM studies leads to clusters that grow in seconds

A principal physiological setting for RBC–phagocyte interactions is within sinusoids of the spleen. There the RBC make transient contact with a diverse array of phagocyte receptors as the RBC are driven by the flow of blood. The shear stresses and pressures on the red cells equate to net forces on these cells and to detachment forces on any adhesive attachments that the cells might form in the spleen. To begin assessing the molecular dynamics of the CD47-SIRPα interaction under force, we have begun to probe cells with sharpened AFM nano-tips pre-coated with human SIRPαex (Fig. 6A). Human and rat red cells were first immobilized on polylysine-coated coverslips to form a tensed spherical cap [12], and then the cells were probed with the SIRPαex-coated AFM tip. Generally, a first phase of indentation is followed by retraction in which – if binding has occurred within the time of contact – one or more forced unbinding events are evident (Fig. 6B). We used rat RBC in these studies because sequence comparisons with mouse CD47 would suggest a similar lack of human SIRPαex interaction as seen above with mouse RBC (see Table 2). Indeed, rat cells show no interaction with human SIRPαex, but the human cells do show unbinding forces of either ∼70 pN when using AFM tips coated in dilute SIRPαex solutions or else average forces of 600–1500 pN for multi-peak detachment profiles when tips bear more concentrated SIRPαex (Fig. 6C). The dilute limit result is certainly reasonable for single molecule adhesion in comparison to other cell adhesion molecules and where multi-valent clustering is intentionally avoided [16]. Nonetheless, forces imposed as here on multi-molecular clusters of SIRPα-CD47 are likely to be more physiologically relevant, so that, as modeled recently [23], each unbinding peak reflects collective detachment from a finite number of CD47-SIRPα adhesions.

The fact that a timescale of 7–15 min for spontaneous cluster dissociation (see above) is accelerated to a timescale of less than 1 s for forced detachment highlights the strong influence of force on protein kinetics. Indeed, intravital microscopy studies cited earlier [15] show individual RBCs in Ringer-perfused mouse spleens exhibiting “stop and go” motion and spending from 0.015 s to 9.71 s in any RBC-size segment of capillary. At some locations, RBCs were seen to adhere to splenic reticular cells by point attachments, and the RBC were clearly detained by surface interactions rather than by entrapment in narrow channels. This in vivo timescale range of 0.01 to 10 s certainly seems to accommodate the moderately strong CD47-SIRPα adhesions, whether these clusters signal as proposed (Fig. 1) or not.

The frequency of detecting one peak or else two or more peaks in retraction of the AFM tip from human RBC depends on the probing rate. The slower the rate (forward and back), the more that multiple clusters are likely to form and be disrupted (Fig. 7A). The cross-over rate from one cluster to more than one is close to 1 μm/s and equates to a contact time of about 1 s. Hence, 1 s or slower seems relevant enough in vivo for the results here to suggest the possibility of multiple clusters of CD47-SIRPα. The unbinding forces of each cluster also grow with time (Fig. 7B), and simple exponential fits yield time constants of ~1 s for the first cluster and ~2.5 s for the second cluster. Interestingly, with most single molecule systems, longer times for stress application lead to lower forces of detachment [16], but we see the opposite effect here most likely because the cluster assembly step during tip contact dominates the unbinding force profiles.

Conclusions

Although we have not yet assessed by AFM mouse SIRPαex interactions with mouse RBC, we can certainly expect to see the same type of species specificity of Fig. 6C. We might also see with mouse SIRPαex and mouse RBC higher forces and broader peak forces as more CD47 are able to diffuse and bind to the AFM tip (per Fig. 7). What seems clear, however, is that CD47 interacts and integrates very differently in human and mouse cell membranes, and so the putative phagocytic synapse that has arisen mainly from studies in mouse (most recently, mouse-derived J774 phagocytes in Gardai et al. [4]) may not translate so simply to human. The protein–protein interactions seem ubiquitous, but they also seem likely to have important functional differences across species.

Acknowledgments

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References


