MOLECULAR BASIS OF BIOCOMPATIBILITY: CELLULAR ENGINEERING ANALYSES

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ABSTRACT

Foreign objects in the body are invariably targeted by phagocytes for removal or isolation. While central to our health, this process is often a limitation to biocompatibility of tissue implants or effective drug delivery. What mechanisms underlie distinctions of foreign from self? CD47 has been hypothesized to act as a "marker of self" through molecular interactions with SIRP-α on macrophages [1]. We first study CD47's integration in the erythrocyte membrane with respect to both cytoskeletal attachment and association with other key membrane components. By fluorescence methods that include imaged microdeformation, CD47 is found be mostly connected to the cytoskeleton and it is seen to colocalize with immunomodulating Rh proteins. The cytoskeletal attachment ensures that an immobile fraction of CD47 remains homogeneously distributed on the cell membrane at all times, while a small unconnected fraction diffuses to reinforce or amplify any "self" signal. In parallel studies aimed at clarifying the "self" signal, CD47's lone Ig domain is expressed on the surface of yeast. Yeast lack Rh and are generally rapidly phagocytosed. Upon contact with neutrophils in autologous plasma, wild type yeast are indeed readily phagocytosed, whereas CD47-yeast show dramatically reduced adhesion, activation, and phagocytosis by neutrophils. The fundamental insights into CD47's integration into a biomembrane as well as CD47's function as a phagocyte inhibitor are being actively pursued to inhibit phagocytic responses to biomedical implants and drug delivery systems.

INTRODUCTION

CD47 (also known as IAP) is a ubiquitously expressed five-span transmembrane protein with a single extracellular immunoglobulin (IgV) domain and an intracellular tail. CD47 in non-erythroid cells appears to be involved in a variety of functions ranging from adhesion to signal transduction.

Association of CD47 on erythrocytes with proteins of the Rh membrane complex is suggested by the observation that Rh_{null} erythrocytes, which lack Rh and Rh-associated glycoprotein (RhAG), express significantly less CD47[2]. A physical or functional association between CD47 and the Rh complex has not otherwise been directly demonstrated.

CD47 on mature erythrocytes appears to mediate cell-cell interactions with SIRP-_ of splenic macrophages. This association is thought to inhibit a phosphorylation cascade that blocks phagocytosis and prevents erythrocyte clearance from the circulation[1]. Furthermore, CD47 proved ineffectual when the leucocytes were incubated with anti-SIRPα antibodies, indicating that the signaling partner for CD47 was SIRP-α[1]. Specifically, conformation of the IgV domain of CD47 seems to be dependent on effective SIRP-_ signaling. Additional erythrocyte components may contribute to this cell signaling and motivates a better understanding of CD47's associations within the erythrocyte membrane.

METHODS AND MATERIALS

1) Visual colocalization of CD47 with Rh protein complex by induced clusters of crosslinking antibodies: Erythrocytes were labeled with a crosslinking monoclonal antibody to CD47, Phycoerythrin-BRIC126 (PhE-BRIC126). Additional surface proteins were tagged with monoclonal antibody and followed by a FITC secondary antibody. Images were taken at the equatorial position of the cell, with one image per fluorophore. The images were mathematically overlaid such that only pixels in common were counted, and the coincident intensity index was determined as the common pixels divided by the total pixels less the common (Fig. 1).

2) FIMD determination of CD47 cytoskeletal connectivity: Central features of fluorescence imaged micro-deformation (FIMD) methods can be found in Discher et al.[3]. Membrane components of intact cells were labeled with fluorescent antibody. The cells were then aspirated into a micropipette of 1-2 _m diameter. Analysis of the resulting image focused on the fluorescent gradient of the aspirated projection of the cell, which can be used to determine cytoskeletal connectivity.

3) Surface expression of CD47 IgV domain on S. cerevisiae: CD47 was expressed as an N-terminus fusion construct to the yeast agglutinin Aga2P with a $(Gly_4Ser)_{3-5}$ linker separating the two proteins [4]. Aga2P is linked extracellularly by a pair of disulphide bonds to Aga1P which is covalently cross-linked to the yeast cell wall. A free amino terminus of IgV-CD47 provides a native orientation.

4) Neutrophil mediated phagocytosis of S. cerevisiae using laser tweezers: S. cerevisiae induced to express IgV-CD47, green fluorescent protein (GFP), or nothing (wild type) were placed with plasma-diluted whole blood in a closed chamber under an optical microscope with an attached laser tweezer. Neutrophils were identified and yeast cells were brought into contact (Fig. 2a). Cell-cell adhesion strengths were benchmarked against the trapping force of the laser. Neutrophil adhesion, activation, and phagocytosis times, if any, were recorded.

RESULTS

CD47 colocalization with Rh and RhAG:

Visual colocalization was based on a scale set by double labeling of CD47 with non-competing antibodies and labeling of CD47 and the lipid bilyer (Fig. 1). PhE-BRIC126 labeled CD47 with phalloidin labeled cytoskeletal F-actin results in no significant coincidence. Band 3 and Glycophorin C do not show disproportionate fluorescence colocalization to CD47. However, RhAG appears coincident to the CD47 clusters suggesting that RhAG and CD47 colocalize and associate on the cell surface.

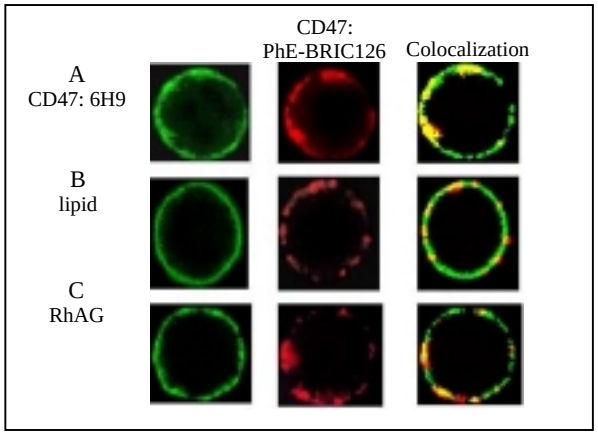

Fig. 1: Visual colocalization of CD47 with RhAG. (A) Positive colocalization: double labeling of CD47 with a PhE-BRIC126 and non-competing monoclonal antibody, 6H9. (B) Negative colocalization: uniform labeling of the lipid bilayer FL-PE. (C) Yellow spots in the colocalization image indicate significant overlap of antibody labeled RhAG (green) with PhE-BRIC126 (red).

CD47 Connection to the Erythrocye Spectrin-Actin Cytoskeleton:

CD47 and RhAG show similar FIMD fluorescent gradients along the aspirated projection, suggesting similar cytoskeletal connectivity. CD47 on Rh_{null} cells, shown to have no RhAG or Rh and reduced expression of CD47 by immunolabeling, exhibit a very similar FIMD gradient to CD47 on normal erythrocytes.

Characterization of CD47's Ig Domain on *S. cerevisiae*:

The presence and concentration of CD47 IgV domain were observed with fluorescent antibody labeling with either Fab-BRIC126 or 6H9. Flow cytometry was used for quantification and surface expression was confirmed with visual fluorescence microscopy of edge brightness.

Effect of CD47 Surface Expression in Neutrophil Adhesion, Activation, and Phagocytosis of *S. cerevisiae*:

80-85% of yeast cells expressing the extracellular domain of CD47 avoided phagocytosis by neutrophils (Fig 1b), even after enforced contact of minutes. The few yeast from CD47+ cultures that were phagocytosed were engulfed much more slowly than wild type (Fig 2c). Wild type and GFP-expressing control cells adhered strongly to neutrophils within 1-2 seconds and were engulfed in less than 1 min. In contrast, CD47+ yeast that avoided engulfment showed delayed weak adhesion and release.

CONCLUSIONS:

With the ability to inhibit phagocytosis, CD47's role as a "marker of self" can extend the stealth capabilities of modern drug delivery systems, or shield any implanted devices under constant immunological attack. The neutrophil is the most abundant circulating cell of the immune system, and can used as a cell model for gauging foreign body responses. CD47 seems to be sufficient to inhibit phagocytosis of yeast by neutrophils in a majority of cases.

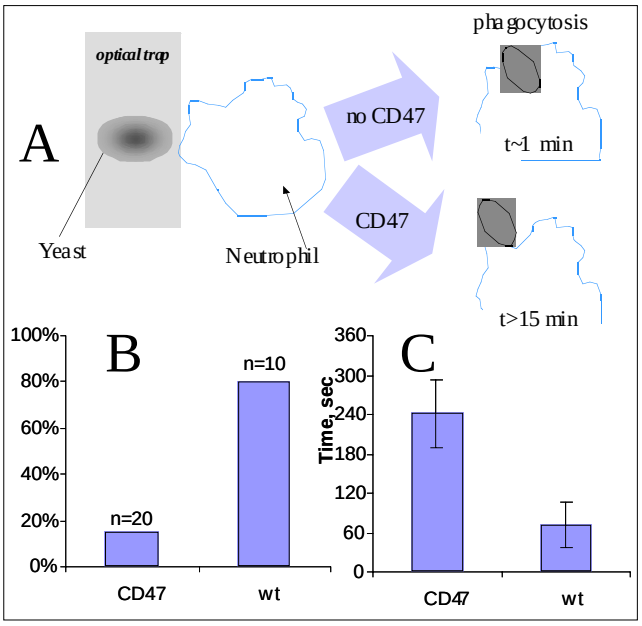

Fig. 2: (A) Schematic of yeast-neutrophil interactions. Yeast are brought into contact with neutrophils and are observed. Phagocytosis, if any, is usually observed immediately. (B) CD47 expressing yeast are phagocytosed 80% less than wild type. (C) Phagocytosis times, for those yeast that are eaten. Note wild type yeast are eaten more rapidly.

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