Macrophages: micromanagers of antagonistic signaling nanoclusters

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How cells integrate antagonistic receptor signaling events is enigmatic. Using superresolution optical microscopy, Lopes et al. (2017. *J. Cell Biol.* https://doi.org/10.1083/jcb.201608094) demonstrate the nanometer-scale molecular reorganization of antagonistic signaling receptors in macrophages, after engagement by the receptors of activating and inhibitory ligands. They propose that large-scale rearrangements of this type underpin decision-making by these cells.

When leucocytes encounter ligand-bearing targets, tens to hundreds of receptors of different types are triggered, leading to downstream signaling. How receptor triggering occurs is uncertain (van der Merwe and Dushek, 2011), and even less is known about how the distinct signals the receptors produce are integrated, allowing "go/no-go" response choices to be made. What is clear is that many of the receptors have antagonistic effects, i.e., some receptors are directly activating, whereas others are tasked with suppressing the activators. In new work appearing in this issue, Lopes et al. make the case that signal integration, in macrophages at least, relies on the nanometer-scale (or nanoscopic) reorganization of local groups or nanoclusters (NCs) of receptors (Lopes et al., 2017).

Cleverly, Lopes et al. (2017) reduce the problem of signal integration to its simplest form. They study the interplay between an activating receptor (Fcγ receptor I [FcγRI]) and an inhibitory regulator, signal reduction protein α (SIRPα); together, these molecules play a major role in controlling macrophage activation and phagocytosis (Barclay and van den Berg, 2014; Getahun and Cambier, 2015). FcγRI binds to pathogen-immobilized antibodies, leading to receptor phosphorylation by, for example, the Src-family kinases (Fig. 1 A), and the recruitment of activating downstream signaling effectors. In contrast, the SIRPα receptor binds not to antibodies but to a "don't eat me" signaling ligand expressed by most human cells called CD47 (Barclay and van den Berg, 2014). The SIRPα/CD47 axis is such an important modulator of macrophage function that it is now a promising target for cancer immunotherapy (Ngo et al., 2016). CD47 engagement also results in SIRPα phosphorylation, but in this case SIRPα recruits a phosphatase, SHP-1, that reduces FcγRI-dependent signaling, perhaps by acting directly on the receptor (Barclay and van den Berg, 2014). Using this system, Lopes et al. (2017) set out to observe first how the receptors are organized in the nonactivated state, and then how this changes when one or both receptors are bound to ligands.

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Dissecting the complex interplay of these receptors required observational methods that were up to the task. The direct imaging of signaling responses in immune cells is challenging, however. Typical problems are: adequate labeling of the proteins of interest; controlling the initiation of responses so that imaging can be performed in good time; achieving the time and spatial resolution necessary for unraveling molecular reorganization; and obtaining good statistics. Lopes et al. (2017) sought to control the triggering status of their human macrophages by plating them onto either poly-L-lysine– (nonactivating) or human IgG (activating)–coated microscope cover glass surfaces and fixing the cells after a predetermined interval (10–30 min). Although the signaling effects of glass substrates (Chang et al., 2016) might otherwise have prompted some degree of skepticism, Lopes et al. (2017) were able to reprise their main findings using macrophages settled onto nonactivating and ligand-presenting supported lipid bilayers. The use of specific fluorescent primary antibodies in combination with multicolor superresolution optical microscopy (direct stochastic optical reconstruction microscopy [dSTORM]) and careful image analysis allowed molecular reorganization to be observed down to 50-nm spatial resolution with high statistical confidence. The authors also took care to avoid the overcounting of blinking fluorophores, a very important control when using dSTORM (Baumgart et al., 2016). Although some of the organizational changes observed were rather subtle, the use of rigorous controls and state-of-the-art image analyses coupled with data simulation allowed Lopes et al. (2017) to conclude that the changes were real.

What Lopes et al. (2017) found first is that FcγRI and SIRPα form NCs under both nonactivating and activating conditions that were ∼40–70 nm in diameter, engaged 75–80% of all the receptors, and were distributed at a density of 3–6 NCs/μm^2. Only minor changes were observed for activating versus nonactivating conditions, however. NC sizes slightly decreased (SIRPα) or remained constant (FcγRI), with the fraction of NC-associated molecules increasing by a small amount and the density slightly decreasing. Although the change in NC density was attributed to increased internalization of both receptors (as measured by flow cytometry), no functional sequelae were attributed to this or the other changes. The main finding, therefore, was that nanoclustering of FcγRI (and SIRPα) is constitutive, in the manner claimed for many other receptors, but contrary to previous work on Fc receptors (Jaumouillé et al., 2014).

The Rockefeller University Press $30.00
J. Cell Biol. Vol. 216 No. 4 871–873
https://doi.org/10.1083/jcb.201702028

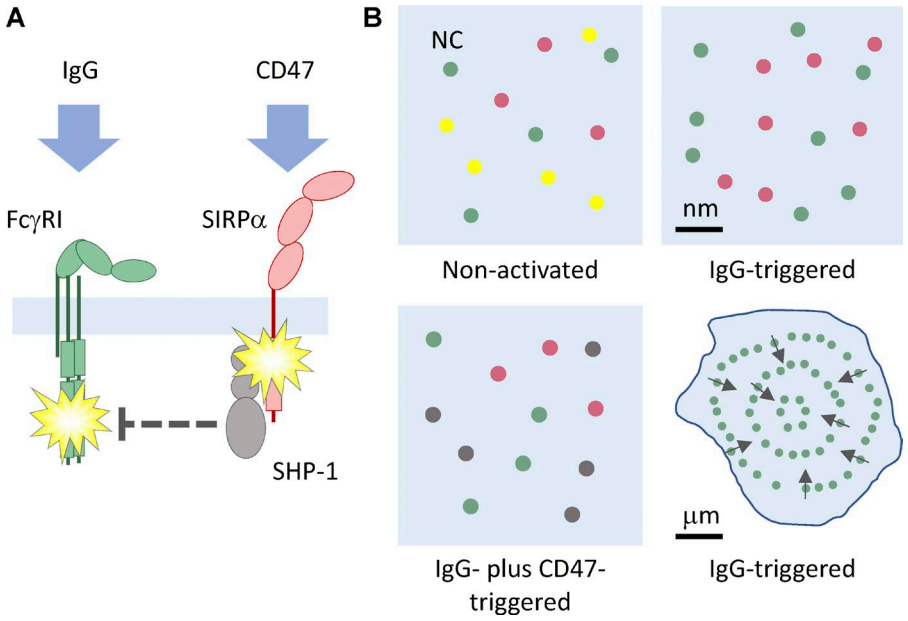

Figure 1. **NC-based signal integration at the macrophage cell surface.** (A) FcγRI binds human IgG leading to receptor phosphorylation (yellow flash) and then downstream signaling. Ligation of SIRPα by CD47 leads to recruitment of SHP-1, a cytosolic phosphatase that blocks signaling, perhaps by dephosphorylating FcγRI directly. (B) Distribution of FcγRI (green circles) and SIRPα (red) NCs in nonactivated and triggered (or activated) cells at the nanometer scale. In the nonactivated cell, the FcγRI and SIRPα NCs exhibit a tendency to be constitutively associated (producing yellow NCs; top left), limiting signaling. After IgG-induced receptor triggering the NCs segregate (top right), enhancing signaling. After IgG plus CD47 ligation (bottom left), the receptors exhibit less segregation and instead recruit SHP-1 (gray circles), blocking signaling. At the micrometer scale (bottom right), after triggering with glass-immobilized IgG, concentric rings of FcγRI NCs form as the "frustrated" macrophage attempts to phagocytose the glass surface.

When Lopes et al. (2017) brought two-color superresolved imaging of the relative positions of the FcγRI and SIRPα NCs into play, matters started to get more interesting. The two-color analysis showed that a substantial fraction of FcγRI and SIRPα NCs are constitutively associated under nonactivating conditions (i.e., <50-nm nearest-neighbor distances; Fig. 1 B, top left, yellow circles) and that the NCs moved apart in the presence of FcγRI-binding IgG (i.e., ∼100-nm nearest-neighbor distances; Fig. 1 B, top right). Because the cells were fixed it was unclear whether the NC couples were stable or formed transiently. Regardless, the authors concluded that FcγRI/SIRPα proximity is regulated upon activation, in such a manner that the SIRPα-mediated inhibition of FcR signaling becomes less likely, reinforcing the activation step. Building on these findings, Lopes et al. (2017) went on to investigate the changes in organization after the ligation of SIRPα by recombinant CD47 ligand. Whereas FcγRI and SIRPα nanoclustering was preserved on IgG- and CD47-coated surfaces, the local segregation of the NCs was abrogated and, instead, phosphorylated SHP-1 phosphatase was now recruited to a subset of the receptors (Fig. 1 B, bottom left, gray circles). Collectively, these findings showed that the absence and the activation of macrophage signaling was correlated with the micromanagement of receptor organization; i.e., colocalization of FcγRI/SIRPα in the former case and segregation in the latter.

Complementing the superresolution-based analysis, Lopes et al. (2017) went on to characterize the macroscopic organization of FcγRI NCs under nonactivating/inhibitory and activating conditions. Whereas cell spreading was asymmetric with FcγRI NCs exhibiting random distributions in the absence of ligands, the macrophages spread with uniform, radial symmetry on antibody-presenting surfaces and, somewhat surprisingly, the FcγRI NCs assembled into concentric rings (Fig. 1 B, bottom right). The authors linked concentric ring formation to frustrated attempts by the macrophage to phagocytose the IgG-coated microscope cover glass. Concentric ring formation (as well as NC segregation) persisted over long (30 min) activation periods and coincided with cytokine secretion. Live-cell imaging confirmed that the formation of concentric FcγRI NC rings was not a cell-fixation artifact. Treatments with actin cytoskeleton–disrupting drugs or with inhibitors of specific elements of macrophage signaling pathways revealed that both the formation of the concentric FcγRI NC rings and the local changes in organization of FcγRI and SIRPα NCs were at least in part regulated by the actin cytoskeleton, particularly by formins, as well as being highly dependent on Src-family kinase signaling. Finally, by making comparisons with a low-affinity receptor (FcγRII) and by using two IgG isotypes that differentially trigger FcγRI and FcγRII receptors, Lopes et al. (2017) showed that NC reorganization required direct receptor ligation and was not simply a consequence of general macrophage activation and, interestingly, that FcγRII NCs do not coassociate with SIRPα NCs. Overall, these experiments suggested that signal integration involving high-affinity, presumably strongly signaling receptors depends on the colocalization of signaling NCs and that, at the time of signaling, macrophages move NCs of antagonistic receptors out of reach of each other.

Like all important studies, the work of Lopes et al. (2017) raises as many questions as it answers. Among the new mysteries are: why do just a subset of the FcγRI and SIRPα NCs coassociate in the nonactivated state, and why is it that the organization of the signaling proteins into NCs is a more efficient way to control signaling than simple ad-mixtures of freely diffusing molecules, as was otherwise expected according to imaging experiments published elsewhere (Jaumouillé et al., 2014)? A related question is: why is the high-affinity FcγRI receptor regulated in this way but not its low-affinity counterpart? One possibility is that the threshold for signaling by high-affinity receptors is set relatively high and signaling is suppressed by even low levels of NC colocalization and that the movement of relatively large blocks of molecules into and out of position allows very robust control of signaling around this threshold. In contrast, lower-affinity receptors may be kept under tonic control by the small fraction of freely diffusing inhibitory receptors. A much subtler question, for which there does not yet seem to be a ready answer, is: how is signaling initiated if it is blocked until FcγRI and SIRPα NCs segregate (i.e., what is it that triggers the displacement of the inhibitory SIRPα NCs)? And, finally: why is there a need for this displacement during macrophage responses, given that pathogens do not express CD47 and are therefore unlikely to trigger SIRPα

phosphorylation and SHP-1 recruitment anyway? Clearly, more exciting work is set to follow.

Acknowledgments

We thank the UK Medical Research Council and the Wellcome Trust for generous funding.

The authors declare no competing financial interests.

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