TIRF and photoactivation microscopy reveal the recruitment of the sorting nexin 9 to CD28 microcluster in activated T cells

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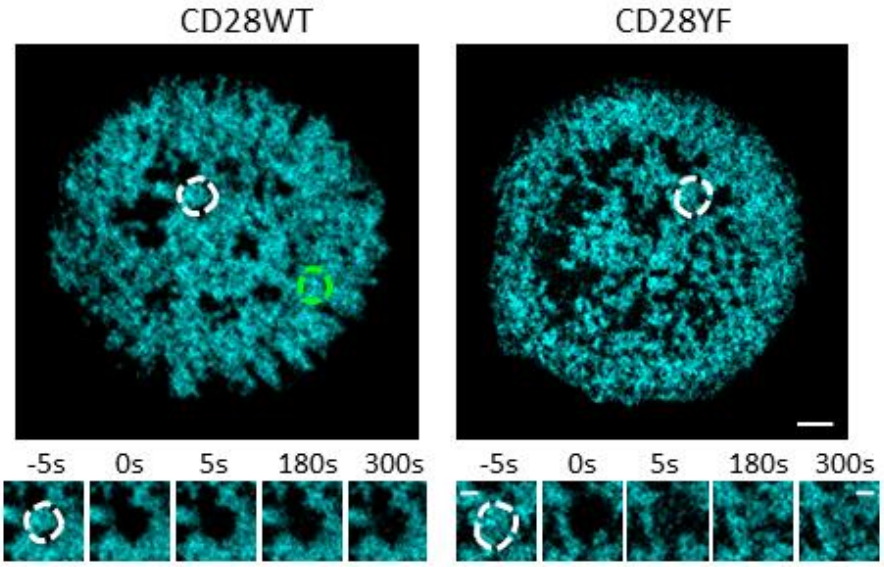
T cell activation is mediated by the T cell receptor (TCR) and various co-receptors including CD28. Binding to their ligand at the surface of antigen presenting cells triggers the phosphorylation of intracellular domains as well as the internalization of both TCR and CD28. CD28 co-stimulation is required for full T cell activation but little is known of the mechanisms mediating its internalisation upon engagement. However it has been proposed that CD28 internalization is markedly enhanced by overexpression of the endocytic protein sorting nexin 9 (SNX9).

Using life cell TIRF microscopy we show that CD28, which assembled in very stable microclusters at the plasma membrane upon T cell activation. We observed that SNX9 was recruited in these microclusters where it did not appear to promote CD28 internalising. To investigate how CD28 signalling realte to the recruitment of SNX9 we made CD28 (WT) 'non-activatable' by making Tyrosine->Phenylalanine (YF) point mutations of residues that are phosphorylated upon receptor activation. Photoactivated CD28WT/YF tagged with PA-mCherry accumulated in both CD28WT and YF with SNX9 microclusters, indicating that phosphorylation of CD28 is not required for SNX9 recruitment.

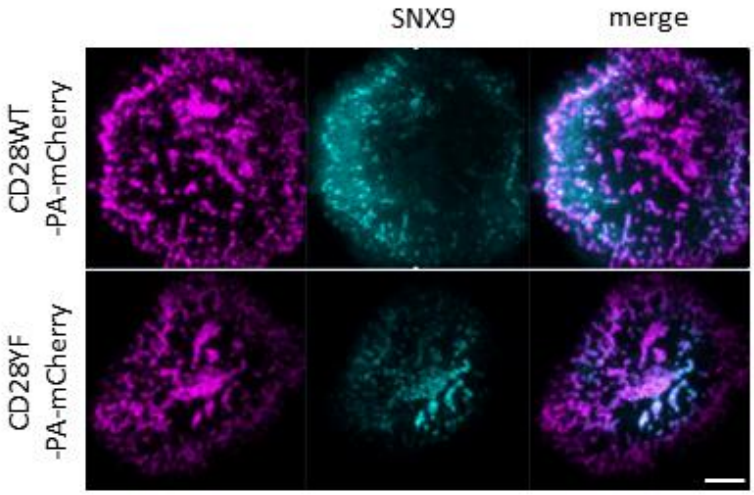
SNX9 possesses a PX domain (connection to endosomes through phosphoinositide-binding) or the SH3 domain (link to actin polymerisation through binding of proline-rich domain). In order to understand which interactions controls SNX9 recruitment to CD28 clusters, we knocked out SNX9 in the Jurkat T cell line by CRIPR/Cas9 gene edition and re-expressed SNX9 mutants lacking either the PX or the PH domain. We showed that neither SNX9 mutants localised in CD28 microclusters, which suggests that SNX9 needs a connection to membranes and to the actin cytoskeleton to be recruited to CD28 microclusters. In turn, CD28 microclusters in activated cells were not affected by the absence of SNX9 or by the overexpression of the binding mutants, suggesting that SNX9 does not contribute to shape CD28 microclusters. To further evaluate if there is a difference in the mobility of CD28WT and YF microcluster we use a two photon FRAP approach using confocal microscopy where we bleach a single CD28 microcluster and obesrved that CD28YF recovered faster than the phosphorylatable CD28, indicating that CD28 activation contribute to the stability of its microclusters.

Altogether we show that SNX9 is rapidly recruited to the IS upon T cell activation where it gets recruited into already existing CD28 microclusters. SNX9 is not regulating CD28 endocytosis or cluster formation. We are further investigating why SNX9 is rapidly recruited into CD28 clusters, and the potential role SNX9 may play in signalling and non-signalling CD28 mobility within membrane microclusters.

Two-photon FRAP of CD28 microcluster



Photoactivation of CD28WT/YF in TIRF



SNX9 recruitment to CD28 microclusters