Supplementary Materials

Receptor homodimerisation significantly prolongs the lifetime of ligand-induced crosslinking of CLEC-2 but not GPVI

Joanne C. Clark[1,2*], Eleyna M. Martin[1,2], Alexandre Slater[1,2], Davide Calebiro[2,3], Zsombor Koszegi[2,3,*] Steve P. Watson[1,2]

[1]Department of Cardiovascular Sciences, School of Medical Sciences, College of Medicine and Health, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

[2]Centre of Membrane Proteins and Receptors (COMPARE), The Universities of Birmingham and Nottingham, The Midlands, UK.

[3]Department of Metabolism and Systems Science, School of Medical Sciences, College of Medicine and Health, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

***Corresponding authors:** Dr. Joanne C Clark[1], Email: j.clark.5@bham.ac.uk; Dr Zsombor Koszegi[2], Email: z.koszegi@bham.ac.uk

[1]Department of Cardiovascular Sciences, Level 1 IBR, School of Medical Sciences, College of Medicine and Health, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK.

[2]Department of Metabolism and Systems Science, School of Medical Sciences, College of Medicine and Health, University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

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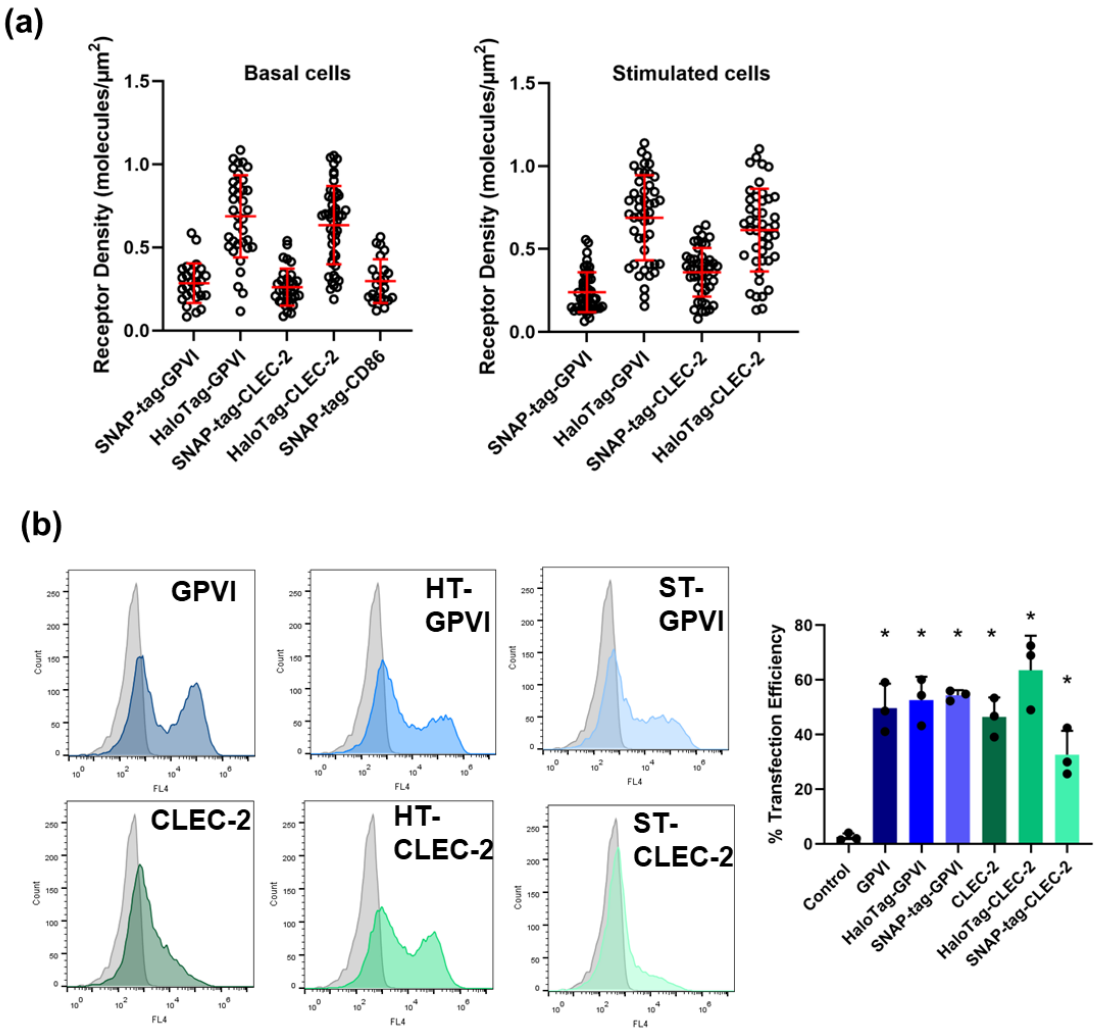
Antibodies

Anti-GPVI HY101, and anti-mouse IgG Alexa Fluor 647 were purchased from Invitrogen, ThermoFisher Scientific (Paisley, UK). The generation of the CLEC-2 monoclonal antibody AYP1 has been reported.[1] AYP1 was produced in house using the generated hybridoma cell lines and purified using protein G affinity chromatography.

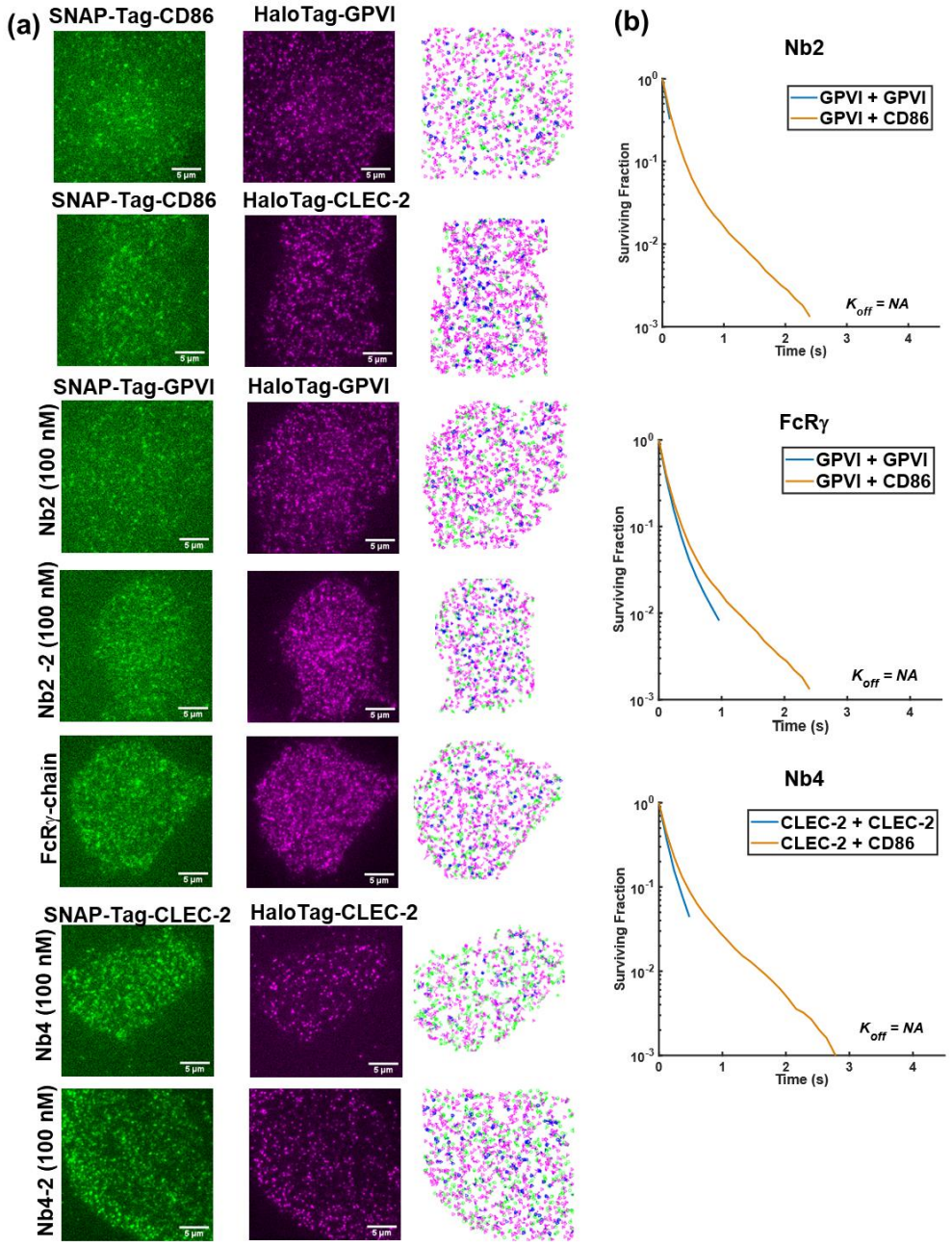
Flow cytometry to measure GPVI and CLEC-2 expression

To measure expression of the GPVI and CLEC-2 constructs (described above) used in NFAT assays on the surface of DT40 cells, a sample of each transfection was stained with HY101 anti-GPVI antibody (1.25 µg/ml) or AYP1 anti-CLEC-2 antibody (66 nM) followed by anti-mouse Alexa Fluor-647 secondary antibody staining (1:400). The samples were acquired and analysed in an Accuri C6 flow cytometer (BD Biosciences, USA). Cell populations were gated on cell size using forward scatter (FSC) vs side scatter (SSC) to distinguish them from electronic noise. The light scatter and fluorescent channel (FL4) were set to logarithmic gain and 10,000 events per sample were analysed. Data expressed as MFI (a.u) and histograms were made using FlowJo v10.0.7 (Eugene, OR). Statistical analysis was by a one-way ANOVA with a Bonferroni post-hoc test.

Supplementary Figures



Supplementary Figure 1. Comparison of expression levels of GPVI and CLEC-2 constructs used in experiments in the study. (a) Receptor density (molecules/um^2) of SNAP- or Halo-tag GPVI or CLEC-2 and SNAP-tag-CD86 constructs expressed in CHO cells under basal and stimulatory conditions used in single particle tracking studies (n=6 independent experiments). The average molecule density was calculated by counting and averaging the localised particles in frames 20 to 200 in the given area of the cell. (b) Expression of wild-type GPVI or CLEC-2 and SNAP- or Halo-tagged GPVI and CLEC-2 in DT40 chicken B-cells used in the NFAT assay, measured by flow cytometry using anti-GPVI HY101 (1.25 µg/ml) or anti-CLEC-2 AYP1 antibody (66 nM) with anti-mouse Alexa Fluor-647 secondary staining. Grey histograms show non-specific secondary staining alone. Flow cytometry data presented as % transfection efficiency of the GPVI and CLEC-2 constructs showing the percentage of positive cells. Significance was measured with a one-way ANOVA with a Bonferroni *post-hoc* test where $P \leq 0.05$. Data presented as mean±SD (n=3 independent experiments).



Supplementary Figure 2. Single-particle tracking of GPVI and CLEC-2. (a-b) Total internal reflection fluorescence (TIRF) microscopy was used to image live CHO cells for 400 frames (~12 seconds) expressing the receptor combinations: Halotag-GPVI + SNAP-tag-GPVI, Halotag-CLEC-2 + SNAP-tag-CLEC-2, Halotag-GPVI + SNAP-tag-CD86 and Halotag-CLEC-2 + SNAP-tag-CD86. CD86 was used as a non-interacting control protein. Generated videos were tracked to generate trajectories and analysed as described in the methods section. (a) Two-colour single particle tracking was used to analyse GPVI-GPVI and CLEC-2-CLEC-2 interactions under basal conditions and following ligand addition. Representative images of CHO cells imaged using TIRF microscopy under basal conditions and following stimulation with the labelled treatments, where green = SNAP-tag-receptors labelled with Alexa Fluor-647 and magenta = Halotag-receptors labelled with Janelia Fluor-549 and the corresponding

dual channel tracking analysis showing the trajectories of SNAP- and Halo-tag-receptors. Blue shows colocalisation of the two channels (scale bar: 5 mm for all images). (b) Interaction survival curves for GPVI-GPVI or CLEC-2-CLEC-2 (blue) and GPVI/CLEC-2-CD86 (orange) interactions following monovalent nanobody (Nb2 or Nb4, 100 nM) stimulation and FcRγ-chain expression (GPVI). No true interactions could be detected following deconvolution with SNAP-tag-CD86 (estimate random colocalisations) and no kinetic parameters (K_{on} and K_{off}) could be determined (NA).

References

1. Gitz, E. *et al.* CLEC-2 expression is maintained on activated platelets and on platelet microparticles. *Blood* **124**, 2262-2270, doi:10.1182/blood-2014-05-572818 (2014).