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Supplementary Information for

CD52 glycan binds the pro-inflammatory B box of HMGB1 to engage the Siglec-10 receptor and suppress T-cell function

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Supplementary text

Figs. S1, S2 and S3

References for SI reference citations

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SI Materials and Methods

Reagents. Recombinant human HMGB1 (disulfide form), HMGB1 mutant (3S-HMGB1, in which cysteines are replaced by serines) and HMGB1 Box A and Box B were purchased from HMGBiotech (Milan, Italy) and recombinant human Siglec-10-Fc from R&D (Pittsburgh, Pa). Recombinant human CD52-Fc and Fc control protein were produced in HEK 293 cells as previously described (1). Recombinant human HSP 90 and HSP 70 were purchased from Enzo Life Sciences (East Farmingdale, N.Y.). Antibodies used in this study were to: CD52 (FL-61), CD3ζ (D-4) (Santa Cruz Biotechnology, Calif.), phosphotyrosine (4G10) (Millipore, Billerica, Mass.), HMGB1 (mouse monoclonal 3E8 blocking antibody [2]), Siglec-10 (APC-labeled) (5GC) and mouse IgG (APC-labeled) isotype control (Biolegend, San Diego, Calif.), Siglec-10 (AF2130) (R&D systems, Minneapolis, Minn.), and phosphorylated SHP1 (Tyr564) (D11G5) (Cell Signaling, Danvers, Mass.), CD4-Pacific blue (RPA-T4) (BD Pharmingen, San Jose, Calif.), IFN-γ (1-D1K; biotinylated clone B6-1 (Mabtech, Melbourne, Australia), poly-clonal goat antibody to rabbit immunoglobulin G (7074) and horse antibody to mouse immunoglobulin G (7076) each conjugated to horseradish peroxidase (HRP) (Cell Signaling, Danvers, Mass). Anti-human CD4 and CD3 antibody microbeads (130-045-101) and LS columns were from Miltenyi Biotec (Macquarie Park, NSW, Australia), human T-activator CD3/CD28 Dynabeads were from Gibco (Waltham, Mass.) and neuraminidase (*Clostridium perfringens* type V) from Sigma-Aldrich (St. Louis, Mo.); *Sambucus nigra* agglutinin lectin (SNA), *Maackia amurensis* lectin I and lectin II (MAAI and MAAII) were from Vector Laboratories (Burlingame, Calif.), ultrapure LPS from *E.coli*, and Pam3CSK4 were from Invivogen (San Diego, Calif.), [^{3}H] thymidine from

ICN Biomedicals (Aurora, OH) and tetanus toxoid from CSL (Melbourne, Victoria, Australia). Fetal calf serum (FCS) was from Sigma-Aldrich (St. Louis, Mo.). Iscove's modified Dulbecco's medium (IMDM) and serum-free AIMV medium were from ThermoFisher Scientific (Waltham, Mass.) and Roswell Park Memorial Institute (RPMI) 1640 medium was from Gibco (Waltham, Mass.), protease and phosphatase inhibitors were from Roche Diagnostics (Basel, Switzerland). The Bio-Plex Pro Human Cytokine panel from Bio-Rad Laboratories (Hercules, Calif.)

Blood donors, cell purification and culture. De-identified venous blood was obtained from healthy young adult donors, with informed consent, through the Volunteer Blood Donor Registry of The Walter and Eliza Hall Institute of Medical Research (WEHI), following approval by WEHI and Melbourne Health Human Ethics Committees. Peripheral blood mononuclear cells (PBMCs) were isolated on Ficoll/Hypaque (Amersham Pharmacia, Uppsala, Sweden), washed in phosphate-buffered saline (PBS) and re-suspended in IMDM medium containing 5% pooled, heat-inactivated human serum (PHS; Australian Red Cross, Melbourne, Australia), 100 mM non-essential amino acids, 2 mM glutamine and 50 μM 2-mercaptoethanol (IP5 medium).

Bioactivity of HMGB1. PBMCs cells ($2x10^5$/well) were cultured in IP5 medium and THP-1 cells ($2x10^5$/well) in RPMI 1640 medium supplemented with 10% (v/v) fetal calf serum (Sigma-Aldrich, St. Louis, Mo), in 96-well plates. Cells were stimulated with 50 ng/ml ultrapure *E. coli* LPS or 2 μg/ml Pam3CSK4 (Invivogen, San Diego, Calif) in the presence and absence of two different recombinant HMGB1 isoforms, HMGB1-disulfide or HMGB1-3S (HMGBiotech, Milan, Italy). HMGB1-disulfide pre-treated with DTT (1mM) for 2 hours was also included as a second negative control for HMGB1-disulfide.

Media were harvested after 14 hours for assay of TNF-α and IL-6 by Bio-Plex Pro Plex human Cytokine, Assay (Bio-Rad Laboratories Ltd, Hercules, Calif) with the Bio-Plex 200 System.

PBMC proliferation assay. PBMCs were seeded into a 96-well round bottom plate ($2x10^5$ cells/well) and incubated in triplicate with CD52-Fc (10 μg/ml) ± HMGB1 (20 μg/ml) and anti-CD3 antibody (OKT3; 5 μg/ml) in IP5 medium or serum-free (AIM V) medium for 48 h. [^{3}H] thymidine (0.5 mCi) was added to the wells during the last 16 h. Cells were then harvested onto glass fiber filters, washed, dried and counted in a scintillation counter to determine ^{3}H-thymidine incorporation into DNA.

ELISpot assay. PBMCs (2 x 10^5 cells/well) were cultured in 200 μl of IP5 medium in triplicate wells of a 96-well ELISpot plate (PVDF MultiScreen) from Merck Millipore (Bayswater, Australia) containing anti-IFN-γ monoclonal antibody pre-bound (1μg/ml) at 4°C. PBMCs were incubated with tetanus toxoid (10 Lfu/ml) added to the wells together with CD52-Fc (10 μg/ml) and either anti-HMGB1 mAb (20 μg/ml) or HMGB1 Box B (10 μg/ml) or HMGB1 Box A (10 μg/ml). After 24 h, cells were removed by washing and IFN-γ spots were developed by incubation with biotinylated anti-IFN-γ antibody (1μg/ml) followed by streptavidin-alkaline phosphatase and BCIP/NBT colour reagent (Resolving Images, Melbourne, Australia).

Binding studies. 96-well flat-bottom plates (Corning, N.Y.) were coated (50 μl/well) overnight at 4°C with CD52-Fc (10 μg/ml), Siglec-10-Fc (10 μg/ml), HMGB1 (20 μg/ml), HMGB1 Box B (20 μg/ml) or HMGB1 Box A (20 μg/ml). The plates were washed three times with PBS and blocked with binding buffer (20 mM HEPES, 2% BSA, 150 mM NaCl, 3 mM $MnCl_2$, 1 mM $CaCl_2$, 1 mM $MgCl_2$, pH 7.6) (10) for 1 h at room

temperature (RT). After washing, proteins diluted in binding buffer were added (50 µl/well, 10 μg/ml) in triplicate and incubation continued for 1 h. Unbound proteins were removed by washing 3 times with PBS at RT. Specific primary antibodies to CD52, HMGB1 or Siglec-10 diluted to 1 μg/ml in 50 µl binding buffer were added for one hour at RT. After washing, HRP-conjugated secondary antibodies, diluted 1/1000 in 50µl binding buffer, were added for 1 h, at RT, followed by washing with PBS. Chromagen substrate solution, 3,3′, 5,5′-tetramethylbenzidine (TMB) was then added and colour development stopped by addition of 0.5M H_2SO_4. Absorbance was measured at 450 nm in a Multiskan Ascent 354 microplate photometer (Thermo Labsystems, San Francisco, Calif.).

Solution binding was performed by incubating CD52-Fc or Fc (each 20 μg/ml) and Siglec-10-Fc (10 µg/ml) in binding buffer, with HMGB1, HSP 90 or HSP 70 (each 20 μg/ml) overnight at 4°C. CD52-Fc (or Fc) was then precipitated with Streptactin beads, the pellet washed with binding buffer and subjected to SDS-PAGE followed by immunoblotting with anti-Siglec-10 antibody.

Binding of CD52-Fc to HMGB1 was also measured by surface plasmon resonance in a BIAcore 3000 instrument (BIAcore Inc. Zilina Region, Slovakia). Binding of CD52-Fc to Siglec-10 was measured in a GE Healthcare BIAcore™ T100 instrument. Research grade CM5 dextran sensor chips from GE Healthcare (Little Chalfont, United Kingdom) were activated with the coupling reagents 0.2 M 1-ethyl-3-3-diethylaminopropyl carbodiimide hydrochloride (EDC) and 0.05 M N-hydroxysuccinimide (NHS) mixed in equal volumes at double final concentrations just before use. After binding of HMGB1 or Siglec-10, 1M ethylenediamine pH 9.2 (HMGB1) or pH 8.0 (Siglec-10-Fc) was used to block remaining

activated carboxyl groups, pH scouting was performed and maximum immobilisation in 10mM sodium acetate was observed at pH 5.0 for HMGB1 and pH 5.5 for Siglec-10-Fc. HMGB1 (100 μg/ml) was immobilized onto an activated chip in flow cell 2, to yield 4,875 Response Units. As a control for binding to HMGB1, Box A protein (100 μg/ml) was similarly coupled to flow cell 1. Recombinant human Siglec-10-Fc (25 μg/ml) was immobilized onto an activated chip in flow cell 2 to yield 1,990 Response Units. As a control for binding to Siglec10-Fc, Fc protein (25 μg/ml) was coupled to flow cell 1, to yield 7,000 Response Units. Dilutions and binding reactions were in running buffer (10 mM HEPES, 150 mM NaCl, 3.4 mM EDTA, 0.005% Tween 20, pH 7.4) filtered through a 0.22 μm filter prior to being degassed by sonication. Proteins were serially diluted in running buffer and passed over the chip at a flow rate of 20 µl/min at 25 °C. Response curves were calculated by the BIAevaluation 4.0.1 software (BIAcore Inc.).

Lectin ELISA. A 96-well flat-bottom plate was coated with 20 μg/ml of different lectins (MAAI, MAAII and SNA) overnight at 4 °C and subsequently blocked with 1 % BSA for 1h. After washing with PBS, CD52-Fc (20 μg/ml) was added and incubated at RT for 1 h and washed twice with PBS. After washing with PBS, a 1:1000 dilution of HRP-conjugated antibody to CD52 (Campath H1; 1µg/ml) was added and incubated at RT for 1 h. TMB was added and colour development stopped by addition of 0.5M H_2SO_4. Absorbance measured at 450 nm in a Multiskan Ascent 354 microplate photometer (Thermo Labsystems, San Francisco, Calif.).

De-sialylation and re-sialylation of recombinant CD52-Fc protein. De-sialylation and re-sialylation of CD52-Fc were performed by a modification of the method of Paulson and Rogers (3). Briefly, CD52-Fc (1 mg) was incubated with *Clostridium perfringens*

type V neuraminidase (50 mU/ml) for 3 h at 37 ºC to remove sialic acids. CD52-Fc was then passaged through a Protein G-Sepharose column, which was washed twice with PBS before eluting bound protein with glycine-HCl 0.1 M, pH 2.8 into 1M Tris-HCl pH 8.0, followed by dialysis against PBS. Binding to MAAI lectin was performed to confirm removal of sialic acids. CD52-Fc was then incubated with either of two sialyltransferases, PdST6GalI which restores sialic acid in α-2,6 linkage with underlying galactose or CstII which restores sialic in α-2,3 linkage with galactose, in the presence of the substrate CMP-N-acetylneuraminic acid sodium salt (0.46 mM-0.90 mM; Carbosynth, Compton Berkshire, United Kingdom) for 3 h at 37 °C. The two CD52-Fc proteins with different linkages (α-2,3 or α-2,6) were passed through Protein G-Sepharose columns, washed twice with PBS and eluted with 0.1 M glycine-HCl, pH 2.8, into 1M Tris-HCl pH 8.0, followed by dialysis against PBS. Samples were freeze-dried, re-suspended in PBS at 200 μg/ml and stored at -20 °C.

Immunoprecipitation and immunoblotting. To determine if CD52-Fc induced phosphorylation of Siglec-10, PBMCs (5 x 10^6/ml) were cultured in IP5 medium in a 6-well flat bottom plate with anti-CD3/CD28 antibody Dynabeads at 37°C ± CD52-Fc (40 μg/ml) for 5, 10 or 30 min at 37°C. Ice cold PBS was added and cells washed at 4°C and extracted in lysis buffer (1% Triton X-100, 25mM Hepes pH 7.2, 0.5mM $MnCl_2$, 5mM $MgCl_2$, 1.0 mM sodium vanadate, 7.5mM ATP) on ice for 30 minutes. After centrifugation, the supernatant was incubated overnight at 4°C with pan-phosphotyrosine antibody 4G10 (5 μg/ml) and precipitated with Protein G-Sepharose beads for 2 h, at 4 °C. After centrifugation and extensive washing with NETN buffer (0.5% NP-40, 1 mM EDTA, 20 mM Tris–HCl at pH 8.0) supplemented with protease inhibitors, pellets were

re-suspended in 4X SDS sample buffer, heated for 10 minutes at 95 °C and centrifuged for 10 minutes at 10,000 rpm, then the supernatant (precipitate) was transferred to another tube and then analysed in the automated, quantitative Western blot analysis (Wes) instrument from Protein Simple (San Jose, Calif.), according to the user guide. Samples were mixed with 5X Master Mix (DTT, fluorescent-labelled marker, SDS) and incubated at 70°C for 5 min, then together with a biotin- labelled protein ladder loaded into individual wells of the sample plate. Antibodies were diluted with antibody diluent buffer. Briefly, the capillaries were filled with separation matrix for 200 s, stacking matrix for 15 s and finally with sample for 9 s. Separation was performed at 375 V for 25 minutes. Capillaries were then exposed to UV light, activating the proprietary linking chemistry and locking the separated proteins to the capillary walls. Subsequently, the matrix was removed and washed 3 times with washing buffer. The capillaries were then blocked with antibody diluent to prevent non-specific binding, incubated with primary antibody, then secondary antibody and HRP-linked antibody for 30 min each. After washing, the detection substrate was introduced into the capillary to generate chemiluminescence via the catalysis of HRP. The signal was captured at 15 s, integrated by Compass software (Proteinsimple, San Jose, Calif.) and plotted.

To identify proteins that associate with CD52-Fc after its binding to cells, purified $CD4^+$ T cells ($1x10^6$) were cultured with anti-CD3/CD28 antibody Dynabeads at 37°C in the presence of CD52-Fc (40 μg/ml) for 5, 15, 30 or 60 min. Ice cold PBS was added and cells were washed at 4°C, pelleted and extracted with 1% digitonin lysis buffer (1% digitonin, 10 mM iodoacetamide, in Tris-buffered saline [TBS]) supplemented with protease and phosphatase inhibitors). Recombinant CD52-Fc contains a Strep-tag

sequence and was therefore extracted by mixing lysates (500 µl) for 1 h at 4 °C with Strep-Tactin-Sepharose beads (50 µl). After centrifugation and extensive washing with NETN buffer (0.5% NP-40, 1 mM EDTA, 20 mM Tris–HCl at pH 8.0) supplemented with protease inhibitors, beads were re-suspended in 4X SDS sample buffer, and heated for 5 minutes at 95 °C, briefly centrifuged to remove the beads and fractionated in a 4-20% NuPAGE gel (ThermoFisher Scientific, Waltham, Mass.). Fractionated proteins were transferred to an Immobilon PVDF membrane (Millipore, Billerica, Mass.) and after blocking with 5% non-fat milk in TBS buffer the membrane was incubated with antibodies (1 μg/ml) to CD52, HMGB1, Siglec-10, p-SHP1 (Y564) or TCR CD3zeta, overnight at 4°C, washed, incubated with secondary HRP-conjugated antibodies and visualized by enhanced chemiluminescence (GE Healthcare, Parramatta, Australia).

Statistical analyses Data are expressed as mean ± standard deviation (SD).

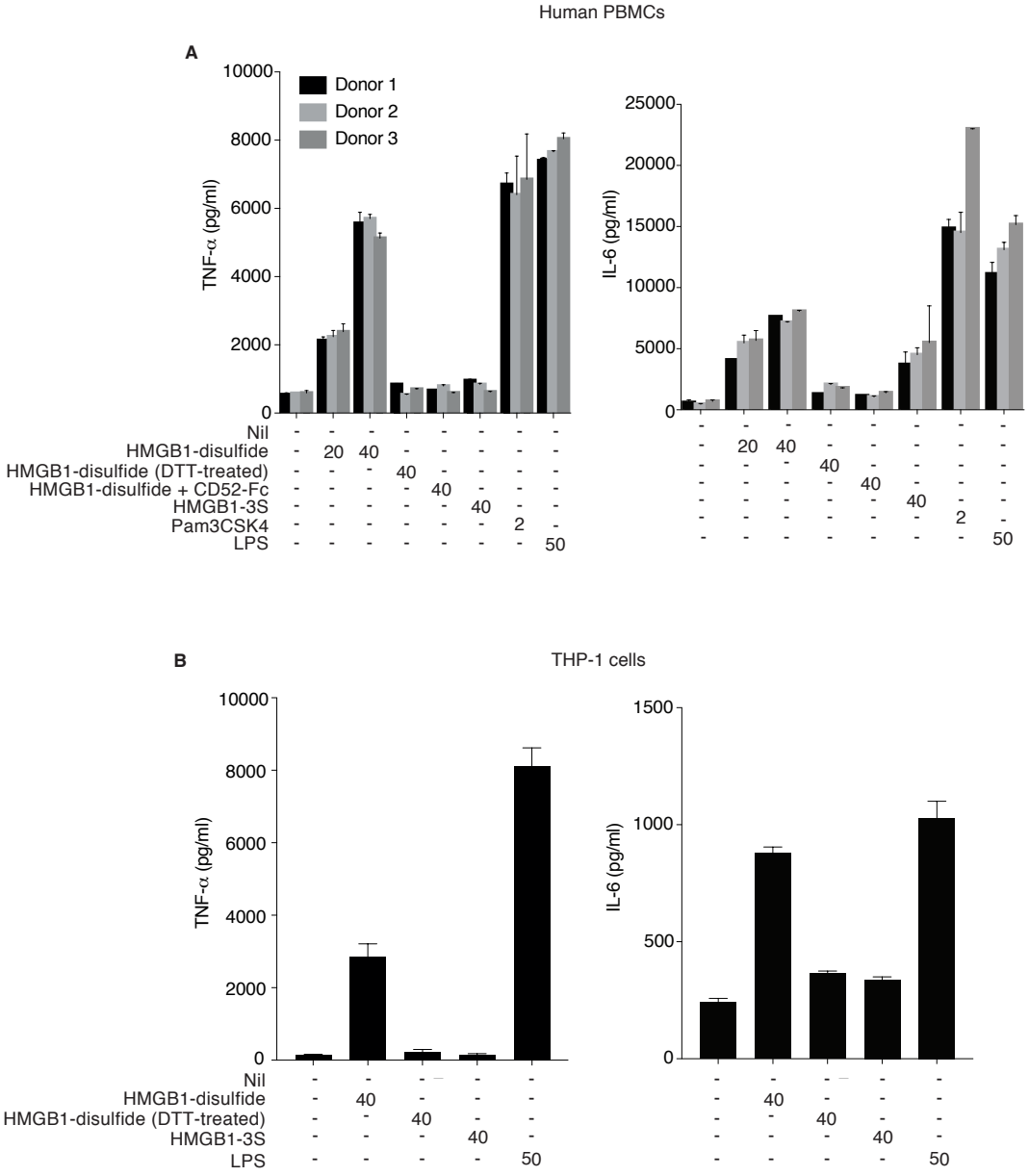

Fig. S1. HMGB1-disulfide stimulates secretion of cytokines. Human PBMCs from three donors **A**) or human monocyte line THP-1 cells **B**) were cultured with HMGB1-

disulfide (20 or 40 μg/ml) ± CD52-Fc (10 μg/ml), HMGB1-disulfide pre-treated with DTT (1mM) (40 μg/ml) or HMGB1-3S (40 μg/ml), or PamCSK3 (2 μg/ml) or LPS (50 ng/ml), for 14 hours at 37 °C. TNF-α or IL-6 in the medium, shown as mean±SD, was measured with the Bio-Plex 200 System.

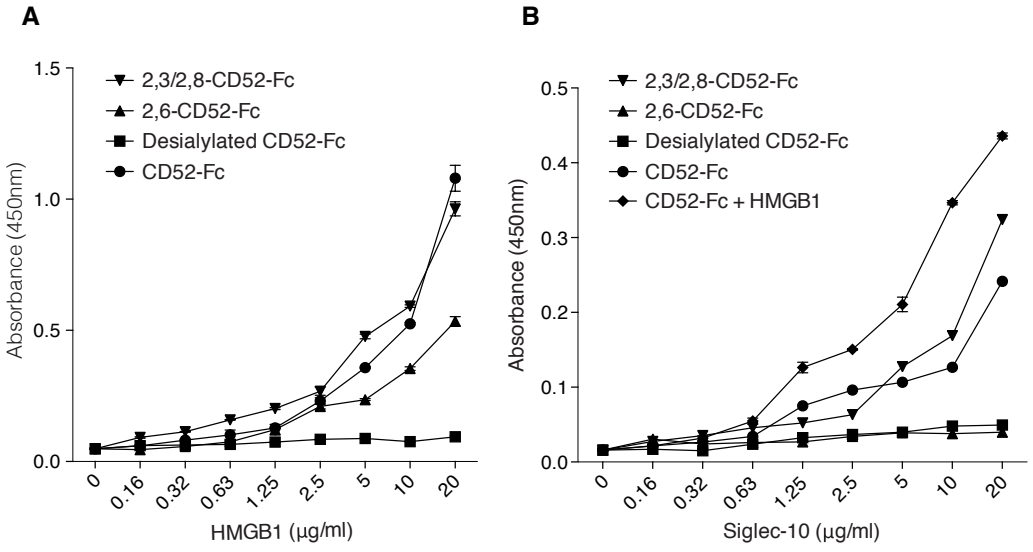

Fig. S2. CD52-Fc glycan with α2–3 sialic acid binds to HMGB1 and Siglec-10

Effects of de-sialylation and re-sialylation with specific α-2,3 or α-2,6 sialic acid-galactose linkages on binding of CD52-Fc to HMGB1 or Siglec-10. CD52-Fc was de-sialylated with neuraminidase and re-sialyated by either α-2,3 sialyltransferase (α-2,3-CD52-Fc) or α-2,6 sialyltransferase (α-2,6-CD52-Fc). Binding of modified CD52-Fc forms (10 μg/ml) to increasing concentrations of immobilized **A**) HMGB1 or **B**) Siglec-10-Fc as shown was performed in microtiter plates as described in Materials and Methods and legend to Figure 2.

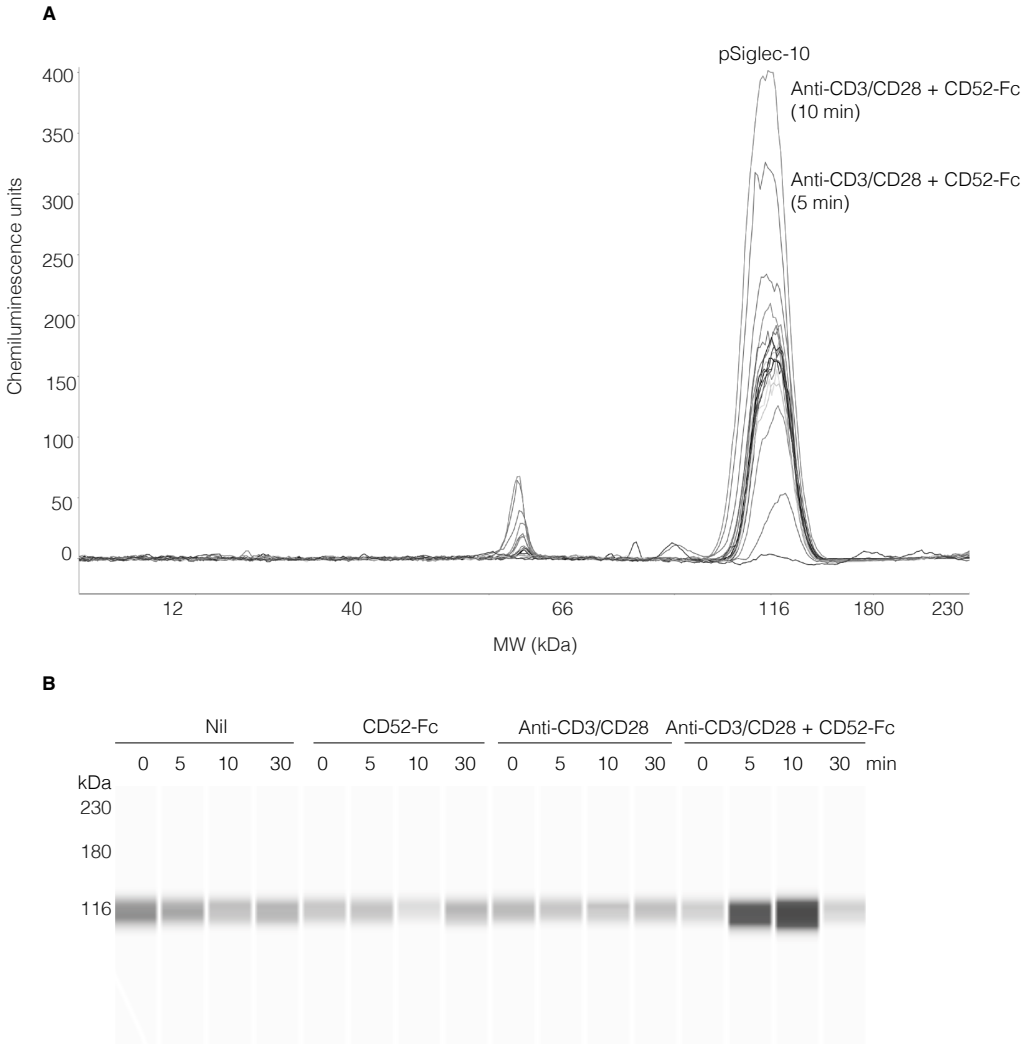

Fig. S3. CD52-Fc induces tyrosine phosphorylation of Siglec-10 in activated T cells

PBMCs were incubated with anti-CD3/CD28 antibody beads ± CD52-Fc (10 μg/ml) for 5, 10 or 30 minutes at 37 °C, then washed and re-suspended in lysis buffer on ice for 30 minutes. The cleared lysate was incubated with anti-phosphotyrosine antibody (4G10) overnight at 4 °C, precipitated with Protein G-Sepharose beads and the precipitate immunoblotted with antibody to Siglec-10 in the Wes capillary apparatus. Results are shown as **A**) chromatogram of Siglec-10 detected after immunoprecipitation with anti-phosphotyrosine antibody, and **B**) virtual blot generated from the chromatogram.

References for SI reference citations

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