Supporting Information

Design of split superantigen fusion proteins for cancer immunotherapy

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Running title: *Split superantigen for cancer immunotherapy*

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Supporting Material Included:

Supporting Figures 1-9

Supporting Tables 1-2

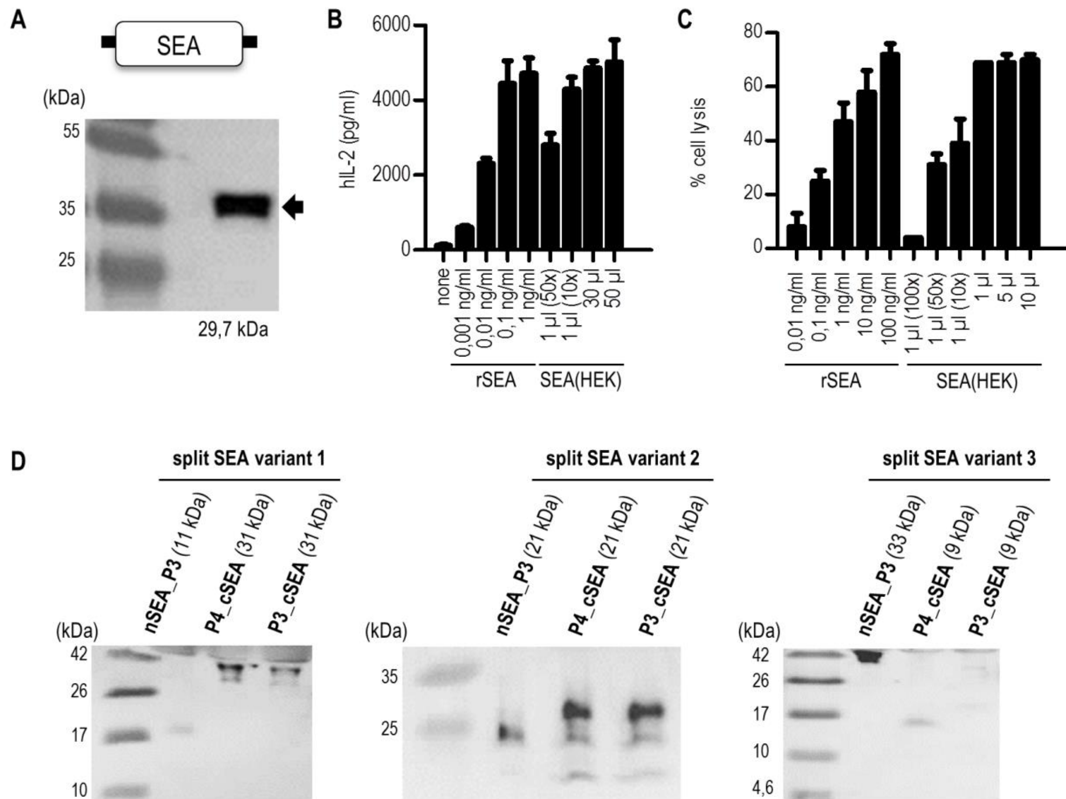

Figure S1. (A) A schematic diagram of wild type SEA in pFLAG-CMV3 vector containing signal peptide for secretion and FLAG tag for detection of expression and western blot analysis of wild type SEA present in HEK293T supernatant 3-5 days after transfection. (B) Stimulation of PBMCs with recombinant (rSEA) and with supernatant of HEK293T containing recombinant wild type SEA. After 24 hour incubation at 37 °C, supernatant was collected and the production of human IL-2, as an indicator of T-cell activation, was measured by commercially available ELISA assay. (C) SEA (expressed in HEK293T supernatant) mediated cytotoxicity in comparison to recombinant SEA (rSEA) cytotoxicity against BCWM cells. In brief, the target cell line BCWM was incubated with different concentrations of recombinant SEA and different volumes of HEK293T supernatant, also diluted (100X, 50X, 10X), containing recombinant wild type SEA. SEA-reactive T cell line was used as effector cell line in ratio 1:10. After overnight incubation the percentage of cell lysis of the BCWM cell line was determined by IVIS Lumina. (D) Western blot analysis of split SEA coiled-coil fusion proteins present in HEK293T supernatant 3-5 days after transfection.

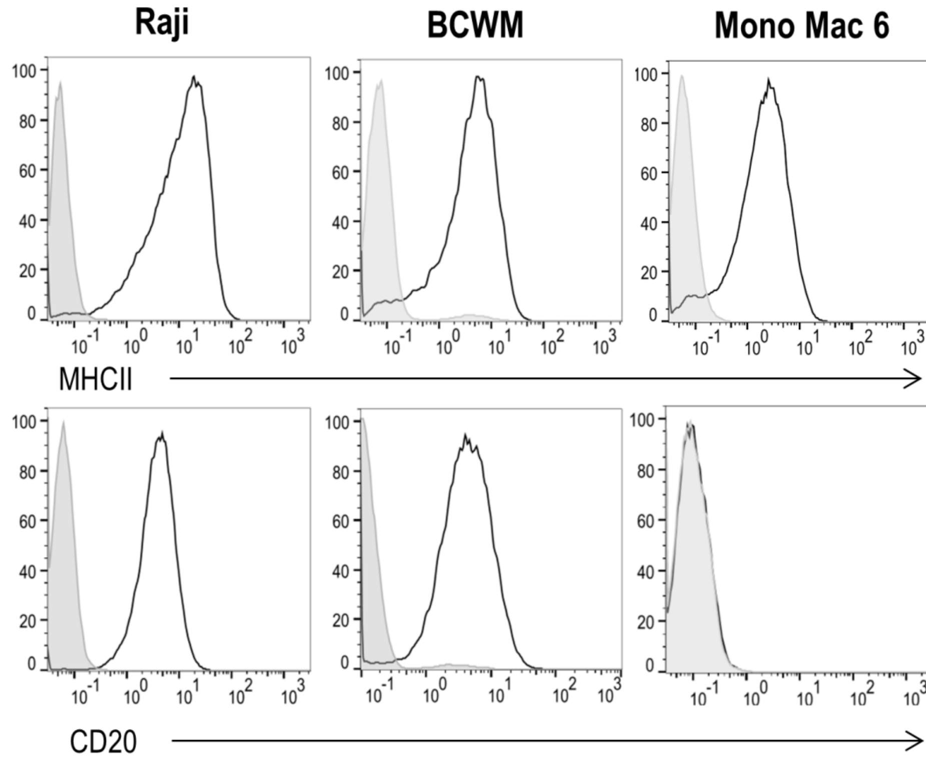

Figure S2. Surface expression of MHC class II and CD20 on cell lines used in this study. Cells were stained with anti-HLA-DR, DP, DQ/APC or anti-CD20/VioBlue, respectively. The shaded curves on histogram show unstained cells.

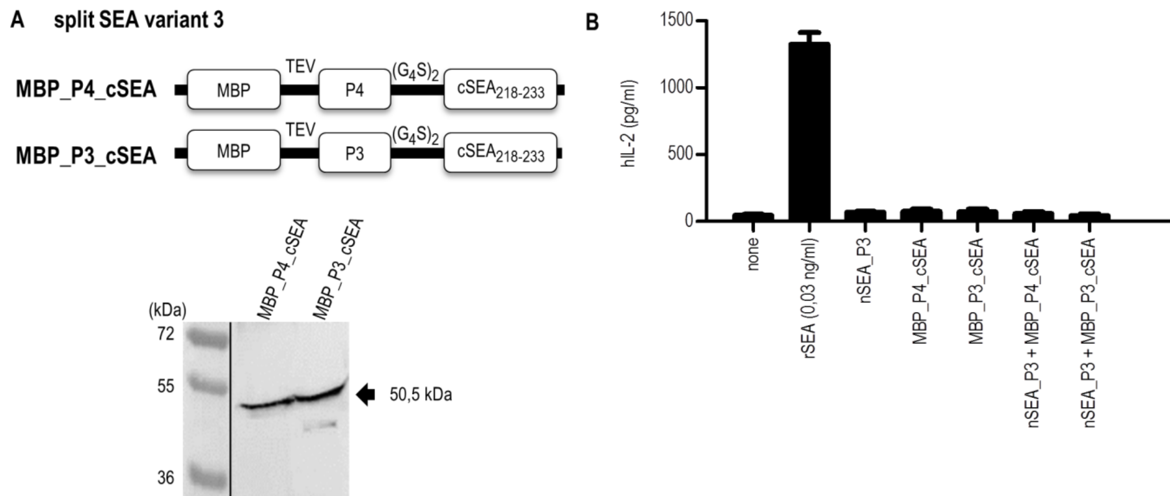

Figure S3. Improving the production of split SEA variant 3 fusion proteins. (A) A schematic diagram of DNA constructs and western blot analysis of P4_cSEA and P3_cSEA fusion proteins of split variant 3 fused with MBP present in HEK293T supernatant 3-5 days after transfection. DNA constructs were cloned into pFLAG-CMV3 vector containing signal peptide for secretion and FLAG tag for detection of expression. (B) PBMCs were stimulated with 0,03 ng/ml of recombinant SEA (rSEA) or with 50 µl of supernatant collected from HEK293T cells containing MBP_P4_cSEA and/or MBP_P3_cSEA fusion proteins of split variant 3. After 24 hour incubation at 37 °C, supernatant was collected and the production of human IL-2, as an indicator of T-cell activation, was measured by commercially available ELISA assay.

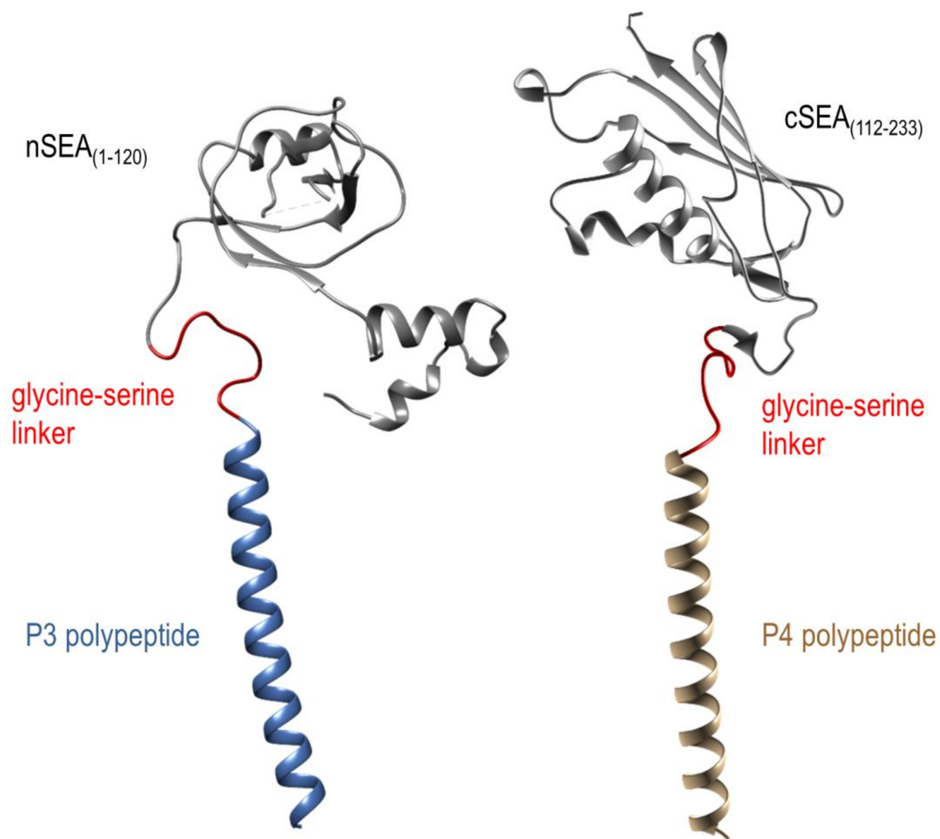

Figure S4. Molecular model represents a split SEA variant 2 (SEA; pdb1ESF.ent) fused to P3 and P4 coiled-coil dimers.

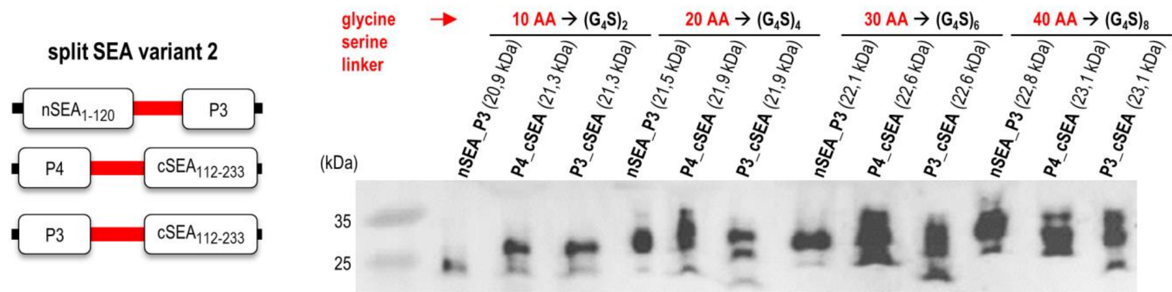

Figure S5. Production of split SEA variant 2 coiled-coil fusion proteins. A schematic diagram of DNA constructs (left) and western blot analysis (right) of split SEA variant 2 fused with either P3 or P4 polypeptide through glycine-serine linker (10, 20, 30 or 40 amino acids) present in HEK293T supernatant 3-5 days after transfection. All constructs were cloned into pFLAG-CMV3 vector containing signal peptide for secretion and FLAG tag for detection of expression. For clarity, fusion proteins with 10 amino acid linkers (the same as in Figure S1) were also included.

A **nSEA$_2$_P3**

MSALLILALVGAAVADYKDDDDKLSEKSEEINEKDLRKKSELQGTALGNLKQIYYYNEKAKTENKESHDQFLQHTILFKGFFTDHS
WYNDLLVDFDSKDIVDKYKGKKVDLYGAYYGYQCAGGTPNKTACMYGGVTLHDNNRLTGGGGSGGGGSSPEDEIQQLEEEIA
QLEQKNAALKEKNQALKYG*

P4_cSEA$_2$

MSALLILALVGAAVADYKDDDDKLSPEDKIAQLKQKIQALKQENQQLEEENAALEYGGGGGSGGGGSTLHDNNRLTEEKKVPINL
WLDGKQNTVPLETVKTNKKNVTVQELDLQARRYLQEKYNLYNSDVFDGKVQRGLIVFHTSTEPSVNYDLFGAQGQYSNTLLRIY
RDNKTINSENMHIDIYLYTS*

P3_cSEA$_2$

MSALLILALVGAAVADYKDDDDKLSPEDEIQQLEEEIAQLEQKNAALKEKNQALKYGGGGGSGGGGSTLHDNNRLTEEKKVPINL
WLDGKQNTVPLETVKTNKKNVTVQELDLQARRYLQEKYNLYNSDVFDGKVQRGLIVFHTSTEPSVNYDLFGAQGQYSNTLLRIY
RDNKTINSENMHIDIYLYTS*

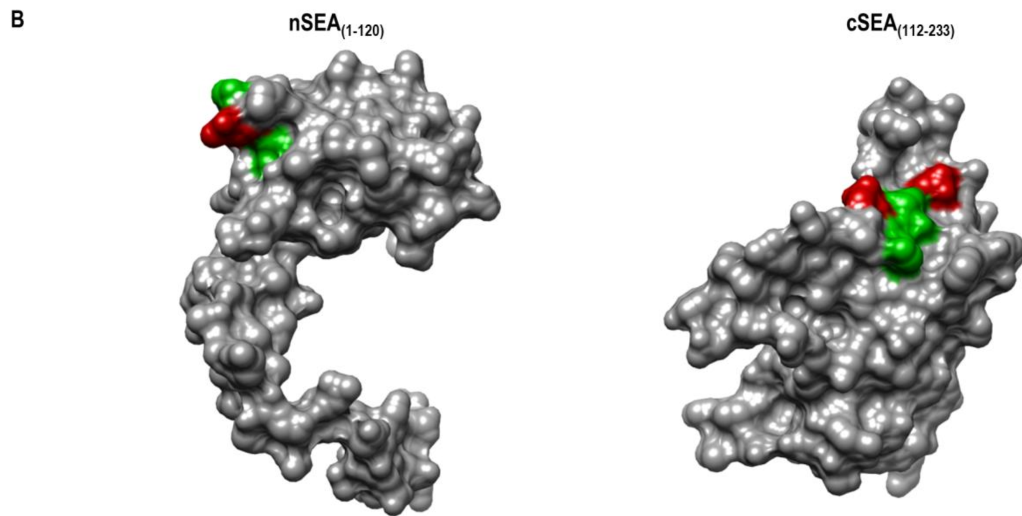

Figure S6. Potential N-glycosylation sites in SEA$_2$-coiled coil fusion proteins. (A) Amino acid sequence of split SEA$_2$-coiled-coil fusion proteins, where Asn-Xaa-Ser/Thr motifs in the sequences are highlighted in green. Asparagine residues predicted to be N-glycosylated are highlighted in red. (B) 3D model of split SEA$_2$ fragments based on the crystal structure. Color code is the same as in the sequence panel.

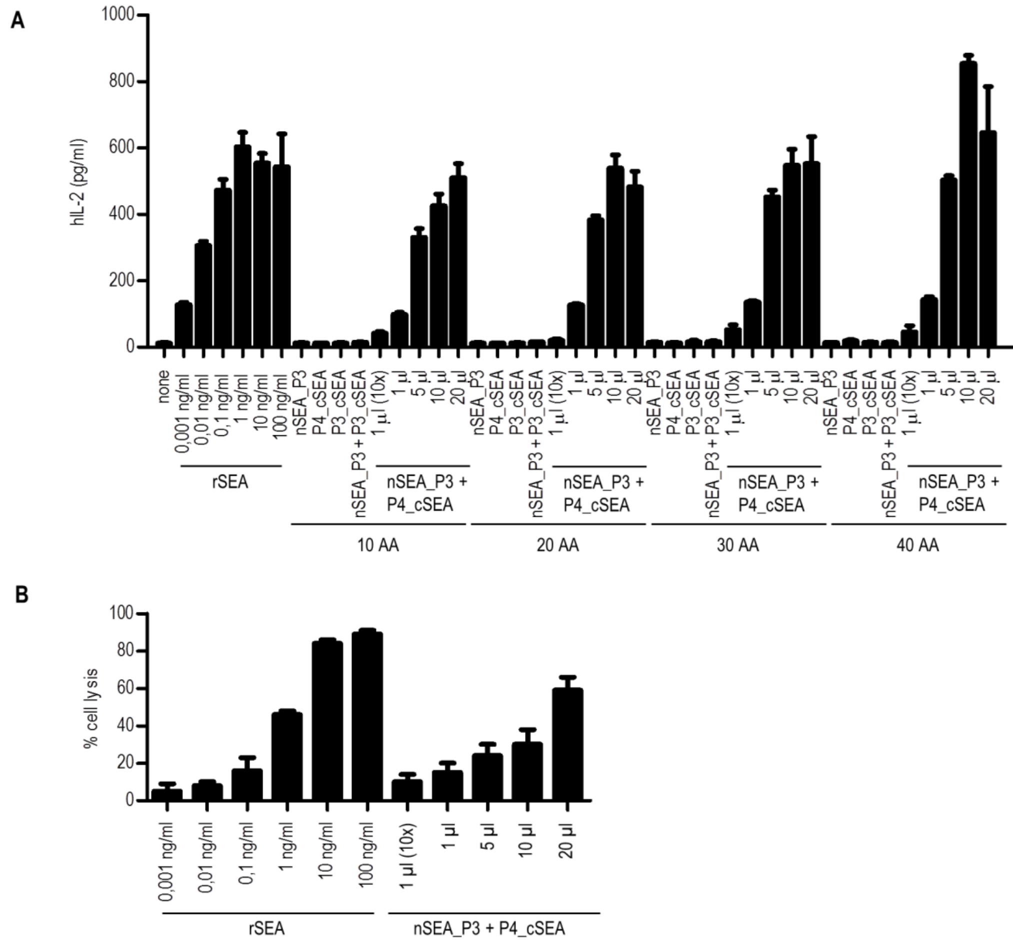

Figure S7. Stimulation and cytotoxicity assay with different amount of HEK293T supernatant containing SEA_2-coiled coil fusion proteins. (A) Stimulation assay. Briefly, PBMCs were stimulated with different concentrations of recombinant SEA and with different amounts (as indicated) of HEK293T supernatant containing nSEA_P3 and P4_cSEA. As control cells were stimulated with 20 µl of HEK293T supernatant containing each fusion protein nSEA_P3, P3_cSEA or P4_cSEA. After 24 hour incubation at 37 °C, supernatant was collected and the production of human IL-2, as an indicator of T-cell activation, was measured by commercially available ELISA assay. (B) Cytotoxicity assay. In brief, the target cell line BCWM was incubated with different concentrations of recombinant SEA or with different amounts (as indicated) of supernatant collected from HEK293T cells containing split SEA_2-coiled-coil fusion proteins (with 10 amino acid long glycine-serine linker). SEA-reactive T cell line was used as effector cell line in ratio 1:10. After overnight incubation the percentage of cell lysis of the BCWM cell line was determined by IVIS Lumina.

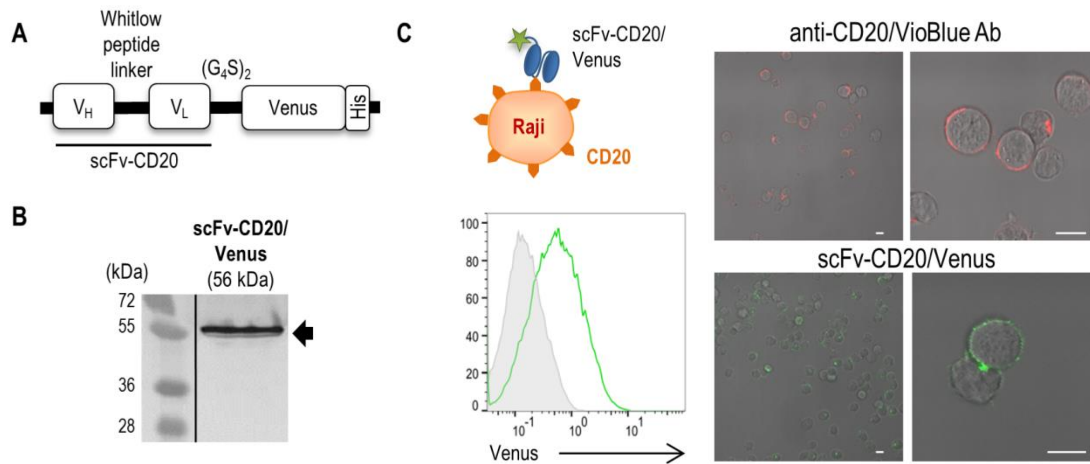

Figure S8. B-cell targeting of scFv-CD20. (A) Construct showing fusion protein scFv-CD20/Venus. V_H, variable region of heavy chain; V_L, variable region of light chain; G_4S_2, glycine-serine linker; His, histidine tag. DNA construct was cloned into pFLAG-CMV3 vector containing signal peptide for secretion. (B) Western blot analysis of scFv-CD20/Venus fusion protein present in HEK293T supernatant 3-5 days after transfection. (C) Histogram (left) and confocal images (right) demonstrates surface binding of the scFv-CD20/Venus present in the HEK293T supernatant on target Raji cells. The shaded curves on histogram show unstained target Raji cells. Bar for all images represents 10 µm. In confocal imaging anti-CD20/VioBlue was used as a positive control.

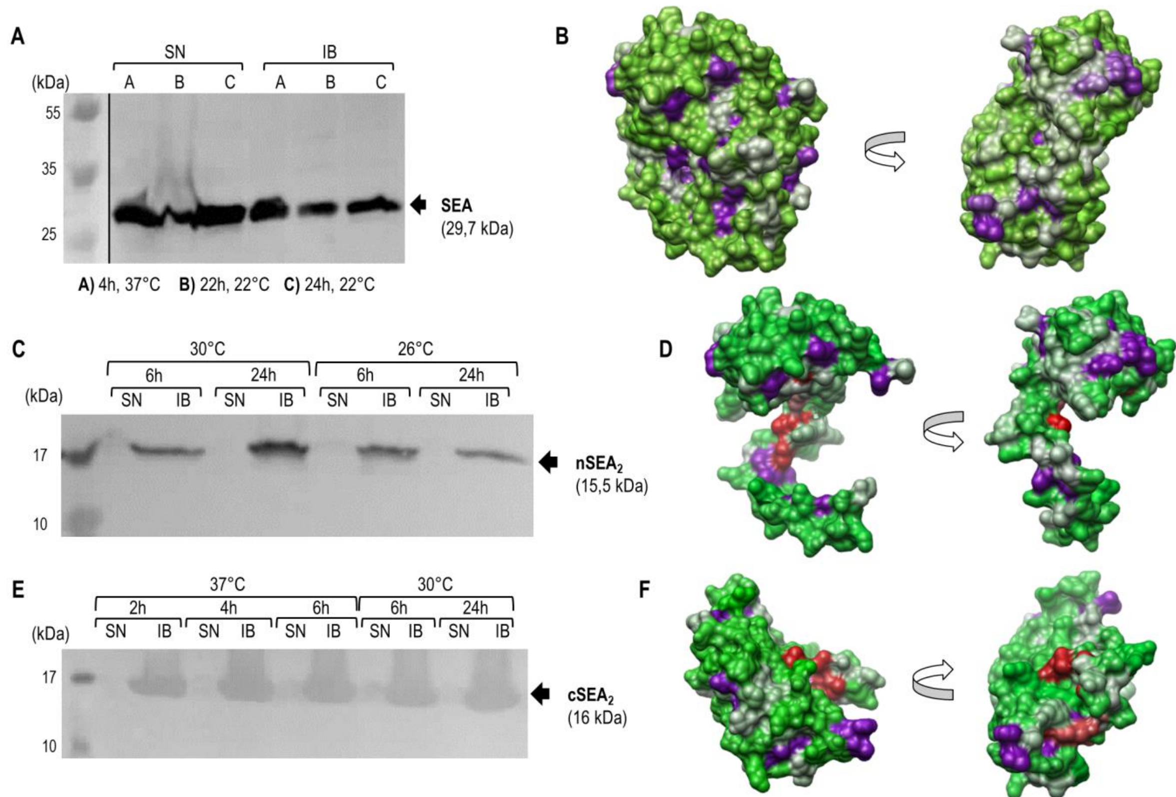

Figure S9. Production and exposed hydrophobic surface of SEA and split SEA_2 fragments. (A, C, E) Western blot analysis of wild type SEA (A) and split SEA_2 fragments, $nSEA_2$ (C) and $cSEA_2$ (E) produced in *E.coli* after induction with 0.2 mM IPTG. Representative soluble (SN) and insoluble (IB) fractions after production at different temperatures and times are shown. (B, D, F) Solvent exposed hydrophobic surface of wild type SEA (B) and split SEA_2 fragments, $nSEA_2$ (D) and $cSEA_2$ (F). Hydrophilic residue surface is colored green, while hydrophobic regions are colored purple and newly surface exposed regions in split SEA_2 fragments in comparison to wild type SEA are colored red.

Name	Short name	Amino acid sequence
Staphylococcal enterotoxin A	SEA	SEKSEEINEKDLRKKSELQGTALGNLKQIYYYNEKAKTENKESHDQFLQHTI LFKGFFTDHSWYNDLLVDFDSKDIVDKYKGKKVDLYGAYYGYQCAGGTPN KTACMYGGVTLHDNNRLTEEKKVPINLWLDGKQNTVPLETVKTNKKNVTV QELDLQARRYLQEKYNLYNSDVFDGKVQRGLIVFHTSTEPSVNYDLFGAQ GQYSNTLLRIYRDNKTINSENMHIDIYLYTS
N-fragment (split SEA variant 1)	$nSEA_{1-36}$	SEKSEEINEKDLRKKSELQGTALGNLKQIYYYNEKA
C-fragment (split SEA variant 1)	$cSEA_{28-233}$	QIYYYNEKAKTENKESHDQFLQHTILFKGFFTDHSWYNDLLVDFDSKDIVDK YKGKKVDLYGAYYGYQCAGGTPNKTACMYGGVTLHDNNRLTEEKKVPINL WLDGKQNTVPLETVKTNKKNVTVQELDLQARRYLQEKYNLYNSDVFDGKV QRGLIVFHTSTEPSVNYDLFGAQGGQYSNTLLRIYRDNKTINSENMHIDIYLYT S
N-fragment (split SEA variant 2)	$nSEA_{1-120}$	SEKSEEINEKDLRKKSELQGTALGNLKQIYYYNEKAKTENKESHDQFLQHTI LFKGFFTDHSWYNDLLVDFDSKDIVDKYKGKKVDLYGAYYGYQCAGGTPN KTACMYGGVTLHDNNRLT
C-fragment (split SEA variant 2)	$cSEA_{112-223}$	TLHDNNRLTEEKKVPINLWLDGKQNTVPLETVKTNKKNVTVQELDLQARRY LQEKYNLYNSDVFDGKVQRGLIVFHTSTEPSVNYDLFGAQGGQYSNTLLRIY RDNKTINSENMHIDIYLYTS
N-fragment (split SEA variant 3)	$nSEA_{1-226}$	SEKSEEINEKDLRKKSELQGTALGNLKQIYYYNEKAKTENKESHDQFLQHTI LFKGFFTDHSWYNDLLVDFDSKDIVDKYKGKKVDLYGAYYGYQCAGGTPN KTACMYGGVTLHDNNRLTEEKKVPINLWLDGKQNTVPLETVKTNKKNVTV QELDLQARRYLQEKYNLYNSDVFDGKVQRGLIVFHTSTEPSVNYDLFGAQ GQYSNTLLRIYRDNKTINSENMHI
C-fragment (split SEA variant 3)	$cSEA_{218-233}$	TINSENMHIDIYLYTS
glycine-serine linker	$(G_4S)_x$	GGGGSGGGGS
polypeptide P3	P3	SPEDEIQQLEEEIAQLEQKNAALKEKNQALKYG
polypeptide P4	P4	SPEDKIAQLKQKIQALKQENQQLEEENAALEYG
anti-CD20 single chain variable antibody fragment	scFv-CD20	QVQLQQSGAEVKKPGSSVKVSCKASGYTFTSYNMHWVKQAPGQGLEWI GAIYPGNGDTSYNQKFKGKATLTADESTNTAYMELSSLRSEDTAFYYCARS TYYGGDWYFDVWGQGTTVTVSSGSTSGSGKPGSGEGSTKGDIQLTQSPS SLSASVGDRVTMTCRASSSVSYIHWFQQKPGKAPKPWIYATSNLASGVPV RFSGSGSGTDYTFTISSLQPEDIATYYCQQWTSNPPTFGGGTKLEIK
maltose binding protein	MBP	KIEEGKLVIWINGDKGYNGLAEVGKKFEKDTGIKVTVEHPDKLEEKFPQVAA TGDGPDIIFWAHDRFGGYAQSGLLAEITPDKAFQDKLYPFTWDAVRYNGKL IAYPIAVEALSLIYNKDLLPNPPKTWEEIPALDKELKAKGKSALMFNLQEPYF TWPLIAADGGYAFKYENGKYDIKDVGVDNAGAKAGLTFLVDLIKNKHMNAD TDYSIAEAAFNKGETAMTINGPWAWSNIDTSKVNYGVTVLPTFKGQPSKPF VGVLSAGINAASPNKELAKEFLENYLLTDEGLEAVNKDKPLGAVALKSYEEE LAKDPRIAATMENAQKGEIMPNIPQMSAFWYAVRTAVINAASGRQTVDEAL KDAQTRITK
cleavage site for TEV (Tobacco Etch Virus) protease	TEV	ENLYFQG
fluorescent protein Venus	Venus	VSKGEELFTGVVPILVELDGDVNGHKFSVSGEGEGDATYGKLTLKLICTTG KLPVPWPTLVTTLGYGLQCFARYPDHMKQHDFFKSAMPEGYVQERTIFFK DDGNYKTRAEVKFEGDTLVNRIELKGIDFKEDGNILGHKLEYNYNSHNVYIT ADKQKNGIKANFKIRHNIEDGGVQLADHYQQNTPIGDGPVLLPDNHYLSYQ SKLSKDPNEKRDHMVLLEFVTAAGITLGMDELYK

Table S1. Amino acid sequences of constructs used in this study.

Protein	Surface area of solvent accessible hydrophobic patches (Å^2)	Surface area of solvent accessible hydrophobic patches compared to total surface (%)
SEA	924	10,4
$nSEA_2$	1123	19,5
$cSEA_2$	865	15,5

Table S2. Exposed hydrophobic patches for each protein.