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1 **Easy Pan-Detection of Human IgA Immunoglobulins**

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27

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29 **Abstract**

30 IgA antibodies are key immune effectors against invading pathogens but also possess
31 essential immunoregulatory functions. Detecting and quantifying human IgA⁺ B-cell subsets
32 and secreted IgA molecules is needed for investigating the protective, modulatory and
33 pathophysiologic roles of IgAs. Here, we produced a recombinant tagged trimeric form of the
34 streptococcal IgA-binding peptide (SAP) by transient transfection-based eukaryotic
35 expression system. The trimeric SAP (tSAP) probe had a higher production yield and
36 apparent binding affinity to human IgA1 and IgA2 immunoglobulins when compared to the
37 dimeric SAP molecule classically used to purify IgAs. tSAP bound both monomeric and
38 dimeric IgAs, and allowed immunoblot detection and ELISA quantification of serum IgA
39 antibodies in humans and non-human primates. Fluorescently labeled tSAP also permitted
40 an accurate quantification of circulating human blood IgA-expressing memory B cells by flow-
41 cytometric analyses. Thus, the easy-to-produce high affinity recombinant tSAP probe we
42 developed is a versatile and valuable tool to quantify secreted and membrane-bound human
43 but also primate IgA immunoglobulins.

44 **1. Introduction**

45 Immunoglobulin A (IgA) is found in animal vertebrates of the Reptilia (birds and some reptiles
46 *i.e.*, crocodiles) and Mammalia classes (Magadan-Mompo et al., 2013; Kaetzel and Russell,
47 2015). In humans, IgA divides into two subclasses, IgA1 and IgA2, which distinguish in terms
48 of sequence/structure, glycosylation state, body fluid distribution, synthesis rate/half-life,
49 biophysical binding properties (*e.g.* bivalent interactions), and biological activities (Woof and
50 Mestecky, 2015). Human B-cell populations expressing membrane-bound surface IgAs
51 predominate in mucosal tissues, and IgA⁺ memory B-cell subsets are also abundant in blood
52 (Berkowska et al., 2015; Prigent et al., 2016). Secreted IgAs prevail as dimeric
53 immunoglobulin molecules in mucosa-associated secretions but circulate mainly as antibody
54 monomers in blood (Kaetzel, 2007). IgA antibodies play a key protective function as immune
55 effectors against invading pathogens but also as immunosuppressors of pro-inflammatory
56 responses, and immunomodulators of the gut microbiota (Mkaddem et al., 2014; Russell et
57 al., 2015; Pabst and Slack, 2020). Conversely, IgA-antigen complexes can promote
58 inflammatory processes, and IgA antibodies are pathogenic mediators in certain auto-
59 inflammatory and autoimmune disorders (Hansen et al., 2019). Because of their paramount
60 role in body homeostasis, protective and pathogenic humoral responses, as well as their
61 growing interest as therapeutics (Leusen, 2015), new tools are needed to study IgA biology
62 and functions. To this end, we recently developed a methodological strategy to efficiently
63 clone, produce and purify recombinant monoclonal IgA antibodies from single human B cells
64 (Lorin and Mouquet, 2015). By generating and charactering hundreds of IgA monoclonals,
65 this approach greatly facilitated investigating IgA humoral responses and functions (Prigent
66 et al., 2016; Lorin et al., 2017; Planchais et al., 2019).

67 The M22 surface protein of *Streptococcus pyogenes* and its peptide derivatives bind
68 the Fc region of human IgA molecules, and were used to create specific IgA-binding
69 reagents for antibody detection and purification (Johnsson et al., 1999; Sandin et al., 2002).
70 A synthetic M22-derived 50 amino acid dimeric peptide referred as the streptococcal IgA-
71 binding peptide (SAP) has a high-affinity for human IgAs (Sandin et al., 2002), and is

72 therefore currently used to fabricate commercially available affinity chromatography resin
73 products. Taking advantage of the SAP property, here we developed of a recombinant
74 tagged trimeric SAP protein as an easy-to-make versatile probe for detecting human IgAs in
75 various binding assays.

76

77 **2. Methods**

78 *2.1. Cloning and production of recombinant streptococcal IgA-binding proteins (SAPs)*

79 Codon-optimized nucleotide fragments encoding the IgK immunoglobulin signal peptide, the
80 SAP followed either by a single cysteine residue (dSAP) or the trimerization motif of T4
81 bacteriophage fibrin (tSAP), and 6xHis- and Avi-tags in C-terminal were synthesized
82 (Invitrogen GeneArt Gene Synthesis, Thermo Fisher Scientific) and digested using Anza 5
83 and 11 restriction enzymes (Thermo Fisher Scientific). Digested DNA fragments were gel
84 purified using NucleoSpin® Extract II kit (Macherey-Nagel) and cloned into
85 pcDNA™3.1/Zeo⁽⁺⁾ expression vector (Thermo Fisher Scientific). Transformed-DH10β
86 bacteria (New England Biolabs) were screened by PCR and plasmid DNA purifications were
87 performed from positive clones grown in 100 µg/ml ampicillin-containing Luria-Bertani broth
88 cultures using NucleoBond® Xtra Maxi kit (Macherey-Nalgel). Freestyle™293-F suspension
89 cells (Life Technologies) were cultured in EX-CELL® serum free medium (Sigma-Aldrich)
90 supplemented with phenol red sodium salt (5 µg/ml, Sigma), L-glutamine (2×) and penicillin–
91 streptomycin (0.2×; 10,000 U/ml, Life Technologies). 293-F cells were grown in suspension
92 at 37 °C in a humidified 5% CO₂ incubator on a Celltron shaker platform (Infors HT) rotating
93 at 130 rpm (for 250 ml media in 1 l culture flasks) or 180 rpm (for 20 ml media in 50 ml
94 bioreactors). Twenty-four hours before transfection, cell density was adjusted at 1.5×10^6
95 cells/ml, and culture grown overnight in the same conditions as mentioned above to reach
96 $\sim 2.5 \times 10^6$ cells/ml the day of transfection. Cells were harvested by centrifugation at 1250
97 rpm for 5 min, and resuspended in fresh FreeStyle™ 293 Expression Medium (Life
98 technologies) without antibiotics at a density of 2.5×10^6 cells/ml. Plasmid DNA coding for
99 SAPs were then added to 293-F cells at a final concentration of 2 µg/ml of culture medium.

100 The cultures were swirled 5 min on shaker in the culture incubator. Linear polyethylenimine
101 (PEI) (25 kDa, Polysciences) solution (at 1 mg/ml in distilled water and 0.2 μ m-filtered) was
102 diluted 1:1 with FreeStyle™ medium and added drop by drop to the 293-F cell cultures (final
103 concentration in medium of 9 μ g/ml). The cultures were grown under agitation in the 37 °C
104 incubator with 5% CO₂. Twenty-four hours' post-transfection, cells were diluted 1:1 with EX-
105 CELL® serum free medium, without antibiotics and supplemented with L-glutamine and
106 phenol red sodium salt to obtain a final transfection volume of 20 ml or 250 ml. Transfected
107 cells were cultivated for 5 days after transfection. Supernatants were then harvested,
108 centrifuged at 4200 rpm for 30 min and 0.2 μ m-filtered to remove cellular debris.

109

110 *2.2. Purification and coupling of recombinant SAPs*

111 Recombinant tagged-dSAP and -tSAP were purified by affinity chromatography by high-
112 performance chromatography using the Ni Sepharose® Excel Resin according to
113 manufacturer's instructions (Thermo Fisher Scientific). Beads were washed once with 50 ml
114 of PBS, and incubated on a rolling wheel overnight at 4 °C with SAP probes-containing
115 supernatants (500 μ l for 250 ml of culture supernatant). Beads were harvested by
116 centrifugation at 2000 rpm for 10 min without break, placed into a Poly-Prep®
117 chromatography column (Bio-Rad) pre-equilibrated with equilibrium/wash buffer (50 mM
118 Na₂HPO₄, 300 mM NaCl, 10mM Imidazole; pH 7.4), and washed 3 times with
119 equilibrium/wash buffer. SAPs were eluted from the resin with 5 x 500 μ l of elution solution
120 (50 mM Na₂HPO₄, 300 mM NaCl, 150mM Imidazole - pH 7.4). For each fraction, protein
121 concentration was measured using the NanoDrop 2000 instrument (Fisher Scientific). SAP-
122 containing fractions were pooled, and dialyzed overnight against PBS using Slide-A-Lyzer®
123 dialysis cassettes (10 kDa MW cut-off, Thermo Fisher Scientific). Protein purity was
124 evaluated by in-gel protein silver-staining using Pierce® Silver Stain kit (Thermo Fisher
125 Scientific) following protein separation of samples heated 95°C for 5 min in reducing and
126 non-reducing conditions by SDS-PAGE using NuPAGE™ 12% Bis-Tris gels (Life
127 Technologies, Thermo Fisher Scientific). Purified SAPs were biotinylated using BirA biotin-

128 protein ligase bulk reaction kit (Avidity, LLC) or directly coupled to Dylight™ 650 Dye using
129 Dylight™ 650 NHS ester labeling kit (Thermo Fisher Scientific) according to manufacturer's
130 instructions.

131

132 *2.3. SAP-based IgA ELISA assays*

133 For recombinant SAP-binding ELISA analyses, high-binding 96-well ELISA plates (Costar)
134 were coated overnight with 250 ng/well of purified recombinant 10-1074 IgA1, IgA2 and
135 dimeric IgA1 monoclonal antibodies (Lorin and Mouquet, 2015). For serum IgA detection,
136 sera from healthy humans (Etablissement Français du Sang - EFS), mouse, rat, guinea-pig,
137 rabbit, chicken, goat, cow (Sigma), and non-human primates (a kind gift from Drs Nicolas
138 Huot and Michaela Muller-Trutwin, Institut Pasteur) were buffer-exchanged against PBS
139 using Amicon® Ultra Centrifugal Filters (100 kDa MW cut-off, Merck Millipore) to partially
140 deplete albumin, the most abundant serum protein in mammals (Moore, 1945), whose
141 presence could interfere with protein separation by SDS-PAGE as well as with protein
142 immobilization on ELISA plates, and as a result with the subsequent IgA detection. High-
143 binding 96-well ELISA plates (Costar) were coated overnight with 2.5 µg/well of serum
144 proteins. After washing with 0.05% Tween-PBS (PBST), plates were blocked for 2 h with 2%
145 BSA, 1 µM EDTA, 0.05% Tween-PBS (blocking buffer), and then incubated for 2 h with SAP-
146 containing supernatant, purified SAPs, and biotinylated dSAP and tSAP (560 nM), and seven
147 consecutive 1:4 dilutions in PBS. After PBST washings, plates were revealed by addition of
148 horseradish peroxidase (HRP)-conjugated mouse anti-6xHisTag antibodies or HRP-
149 conjugated streptavidin (1 µg/ml in blocking solution, BD Biosciences), and HRP
150 chromogenic substrate (ABTS solution, Euromedex). All experiments were performed in
151 triplicate at room temperature, and using HydroSpeed™ microplate washer and Sunrise™
152 microplate absorbance reader (Tecan Männedorf, Switzerland).

153

154 *2.4. Infrared fluorescent immunoblotting*

155 Purified SAP proteins (125 to 500 ng /well) heated at 95°C for 5 min in reducing and non-
156 reducing were separated by SDS-PAGE using NuPAGE™ 12% Bis-Tris gels (Life
157 Technologies, Thermo Fisher Scientific), and then electrotransferred onto nitrocellulose
158 membranes. Membranes were then saturated overnight at 4°C in PBS-0.05% Tween 20
159 (PBST)-5% dry milk. After washing 3 times with PBST, membranes were incubated with
160 mouse anti-6xHis antibody (1µg/ml; BD biosciences) in PBST-5% dry milk for 2 h.
161 Membranes were then washed with PBST and incubated for 1 h with 1:10,000-diluted IRDye
162 800CW-conjugated goat anti-mouse IgG (LI-COR Biosciences) in PBST-5% dry milk.

163 For SAP-based IgA detection, total serum proteins (20 µg/well) heat-treated for 5 min
164 at 95°C were separated by SDS-PAGE using NuPAGE™ 4-12% Bis-Tris gels (Invitrogen,
165 Thermo Fisher Scientific) in non-reducing conditions, and electrotransferred onto
166 nitrocellulose membranes. Saturated membranes were PBST-washed 3 times, and
167 incubated for 2 h with biotinylated-tSAP (1 µg/ml) in PBST-5% BSA. After washings,
168 membranes were incubated for 1 h with 1:10,000-diluted Alexa fluor™ 790-conjugated
169 streptavidin (Thermo Fisher Scientific) in PBST-5% BSA. Finally, membranes were PBST-
170 washed and scanned with the Odyssey Infrared Imaging system (LI-COR Biosciences).

171

172 *2.5. IgA flow cytometric staining by SAP probes*

173 Peripheral blood mononuclear cells from healthy donors (Etablissement Français du Sang -
174 EFS) were purified by Ficoll density gradient centrifugation according to the manufacturer's
175 instructions (GE Healthcare), and then washed twice with 1% BSA-PBS (FACS buffer). Two
176 millions cells in 200 µl of FACS buffer per tube were incubated for 30 min at 4°C with
177 LIVE/DEAD fixable dead cell stain kit (Molecular Probes, Thermo Fisher Scientific) to
178 exclude dead cells. After washing with FACS buffer, cells in 200 µl of FACS buffer were
179 incubated for 30 min at 4°C with Dylight™ 650-conjugated SAP probes (ranging from 14 to
180 520 nM), and with a cocktail of mouse anti-human antibodies: CD19-A700 (HIB19, BD
181 Biosciences, San Jose, CA), IgG BV786 (G18-145, BD Biosciences) and IgA FITC (IS11-
182 8E10, Miltenyi Biotec, Bergisch Gladbach, Germany). Finally, cells were washed with FACS

183 buffer and resuspended in 400 µl 1% paraformaldehyde (PFA)-PBS solution. Following a
184 lymphocyte and single cell gating, live cells were gated on CD19⁺ B cells. FACS analyses
185 were performed using a FACS Aria II cytometer (BD Biosciences) and FlowJo software
186 (v10.3, FlowJo LLC, Ashland, OR).

187

188 **3. Results**

189 *3.1. Design and production of recombinant streptococcal IgA-binding proteins*

190 Dimeric SAP display higher binding affinity to human IgAs than its monomeric counterpart
191 (Sandin et al., 2002). Thus, we designed two multimeric SAP gene constructs for mammalian
192 cell-based expression of recombinant proteins, each containing a N-terminal leader peptide
193 for secretion, and C-terminal 6xHis- and Avi-tags for purification and biotinylation,
194 respectively (**Fig. 1A**). Dimeric SAP (dSAP) corresponding to the original di-sulfide bonded
195 peptide M dimer (Sandin et al., 2002), and a trimeric form of the SAP (tSAP) for which we
196 replaced the C-terminal cysteine residue in dSAP by the trimerization motif of T4
197 bacteriophage fibrin (**Fig. 1A**) were produced. To this end, FreeStyle™ 293-F cells were
198 transiently transfected with purified dSAP and tSAP vector DNA, and cultivated for five days.
199 The concentration of produced recombinant SAPs in harvested supernatants measured by
200 sandwich ELISA showed a ~ 20-fold higher production yield for tSAP compared to dSAP
201 (44.4 µg/ml vs 2.3 µg/ml in average, respectively) (**Fig. 1B**). Accordingly, purification of
202 histidine-tagged SAPs by high-performance immobilized metal ion affinity chromatography
203 (IMAC) directly from supernatants allowed recovering higher amounts of protein products for
204 tSAP compared to dSAP (*per* 20 ml culture, 686 µg vs 117 µg in average, respectively). The
205 purity of SAPs in phosphate buffered saline (PBS) solution was confirmed by visualizing
206 specific protein bands on silver-stained SDS-PAGE gels, and by infrared immunoblotting
207 using anti-HisTag antibody detection (**Fig. 1C**). As expected, purified dSAPs were well
208 detected as dimers both on the stained gel and immunoblot under non-reducing conditions
209 (**Fig. 1C**). To compare the reactivity of dSAP and tSAP against human IgA molecules, we
210 performed ELISA experiments using as antigens purified IgA monoclonal antibodies (Lorin

211 and Mouquet, 2015). ELISA binding analyses showed although both SAPs recognize human
212 monomeric IgA1 and IgA2 as well as dimeric IgA1 (dIgA1) antibodies, tSAP displayed a
213 higher relative affinity to human IgAs (30-, 13- and 9-fold greater than dSAP for IgA1, IgA2
214 and dIgA1, respectively) (**Fig. 1D**).

215

216 *3.2. Sensitive detection of human and non-human primate IgAs by SAP trimers*

217 The M22 protein of *S. pyogenes* has been shown to interact with two loops in the C α 2-C α 3
218 interdomain of the human IgA Fc region (Pleass et al., 2001). Interestingly, the ²⁵⁷LLG²⁵⁹ loop
219 motif in C α 2 is well conserved across several mammal species known to possess circulating
220 IgAs (Kaetzel and Russell, 2015), whereas the ⁴⁴⁰PLAF⁴⁴³ loop motif in C α 3 is only found in
221 primate and bovine IgAs (**Fig. 2A**). We thus evaluated the capacity of purified recombinant
222 SAPs to bind serum IgAs from these various mammals using AviTag-biotinylated dSAP and
223 tSAP as ELISA probes. Binding analyses showed that SAPs react with immobilized IgA-
224 containing serum proteins from human and macaque but not from goat, cow, chicken, rabbit,
225 guinea pig, rat and mouse (**Fig. 2A**). In agreement, SAP probes were also able to bind
226 serum IgAs by ELISA from other non-human primate species: African green monkey,
227 Mandrill, Cercopithecus cephus, Golden-bellied and Black crested mangabeys, Allen's
228 swamp and Hamlyn's monkeys (**Fig. 2B**). Consistent with the data obtained for human IgAs,
229 tSAP exhibited a higher relative binding affinity than dSAP against non-human primate IgAs
230 (**Figs. 2A and 2B**). To confirm the recognition of IgAs in total serum proteins by SAPs, we
231 next performed an infrared immunoblotting using biotinylated tSAP as a detection probe. As
232 expected, tSAPs revealed immunoreactive protein bands with molecular weights
233 corresponding to IgAs in sera from the human subject and non-human primates but not from
234 the other tested mammals (**Fig. 2C**).

235

236 *3.3. Efficient flow cytometric capture of IgA-expressing B cells by SAP probes*

237 Human B-cell subsets expressing IgA B-cell receptors are abundant in the blood and
238 mucosa-associated lymphoid tissues. To investigate whether SAP probes also recognize

239 membrane-bound IgAs on B cells, human peripheral blood mononuclear cells from healthy
240 individuals (n=3) were stained with a concentration range of Dylight™650-conjugated
241 (DyL⁶⁵⁰)-SAP probes, as well as FITC- and Alexa 786-conjugated anti-human IgA and IgG
242 antibodies, respectively (**Fig. 3**). Flow cytometric analysis showed that fluorescent SAP
243 probes bind to blood IgA⁺ B cells in a dose dependent manner (**Fig. 3B** and **3C**). All
244 SAP⁺CD19⁺ cells were IgA⁺ and IgG⁻ indicating that SAP probes bind specifically IgA-
245 expressing B cells, without competition with fluorescently labeled anti-human IgA antibodies
246 ordinarily used in flow cytometry (**Fig. 3B**). Optimal binding of human IgA⁺ B cells was
247 achieved by staining with DyL⁶⁵⁰-tSAP at a final concentration of 520 nM (mean 97.5%, CV
248 11%; **Fig. 3C**).

249

250 **4. Concluding remarks**

251 tSAP is a novel, easy to produce, high-affinity IgA-binding reagent, which possesses superior
252 activity compared to the originally-described SAP molecule. Recombinant SAP trimers can
253 be utilized for a sensitive detection and quantification of human but also non-human primate
254 IgAs in various immunoassays. tSAP is a small non-glycosylated protein secreted in the
255 culture cell supernatant, which may thus also be easily produced using prokaryote-based
256 expression systems. Moreover, considering its very high production yield and binding affinity,
257 coupling tSAPs to polysaccharide beads would permit a low-cost and efficient purification of
258 IgAs by affinity chromatography. Finally, due to its binding capacity to non-human primate
259 IgA antibodies, the tSAP probe constitutes a very useful tool to investigate IgA-mediated
260 immunity in infected or vaccinated monkeys (Amos et al., 2015; Nelson et al., 2016;
261 Ackerman et al., 2018; Eudailey et al., 2018).

262

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273

274 **Author Contributions**

275 C.P. performed and analyzed experiments, and wrote the manuscript. H.M. conceived and
276 supervised the study, designed experiments, and wrote the manuscript.

277

278 **Declaration of Interests**

279 The authors declare no competing interests.

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368

369 **Figure legends**

370 **Figure 1. Production of recombinant streptococcal IgA-binding proteins.** (A) Schematic
371 diagram shows the expression-cloning construct for producing trimeric streptococcal IgA-
372 binding proteins. (B) Dot plots comparing the production yield of the dimeric and trimeric
373 streptococcal IgA-binding protein (dSAP and tSAP, respectively), determined by ELISA in
374 cell culture supernatant and after affinity purification. Values of each dot correspond to the
375 means of intra-assay duplicate measurements. (C) Silver-stained SDS-PAGE gels (top) and
376 infrared immuoblots (bottom) show purified dSAPs and tSAPs (ranging from 125 to 500 ng)
377 in non-reducing (not red.) and reducing conditions. Representative stained gel and infrared
378 immunoblot images (from two independent experiments) are shown. Asterisks indicate SAP
379 dimers under non-reducing conditions. (D) ELISA graphs comparing the dSAP and tSAP
380 binding to purified monomeric and dimeric IgA1, and monomeric IgA2 monoclonal antibodies
381 (left). Means \pm SD from intra-assay triplicate measurements are shown. Dot plots comparing
382 the relative binding affinity of recombinant SAPs against monomeric and dimeric IgAs (right).
383 Fold differences of K_D values between dSAP and tSAP against IgA antibodies are indicated
384 on the graph.

385

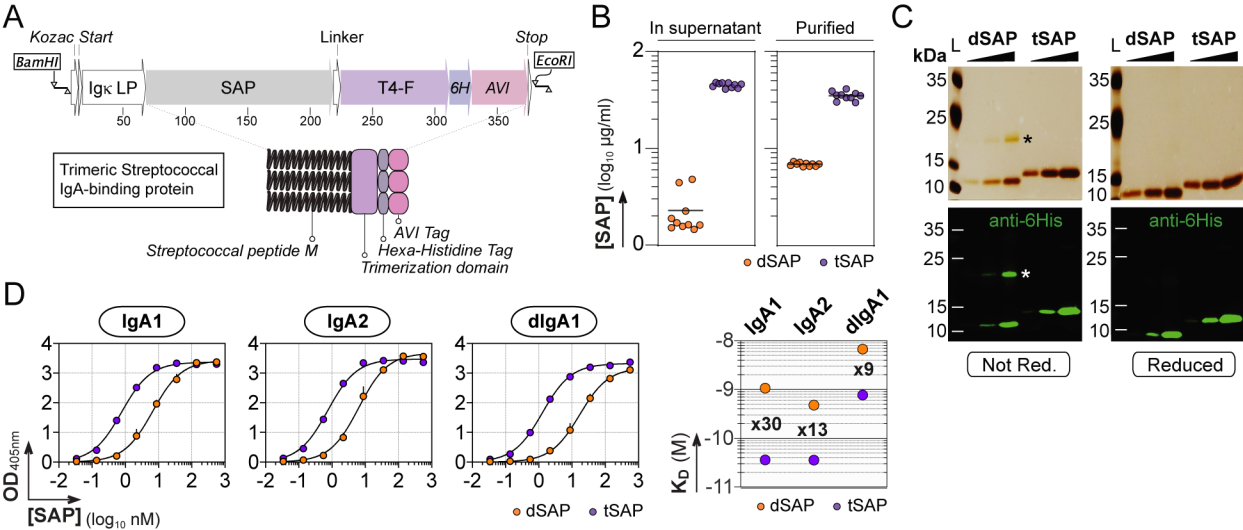
386 **Figure 2. SAP binding capacity to mammalian serum IgA antibodies.** (A) Circular
387 cladogram built from the amino acid alignment of immunoglobulin α 1 chains from various
388 mammal species (top). Gui. Pig, Guinea pig. Ribbon diagrams showing the crystal structure
389 of the human IgA1 Fc (1OW0) (Herr et al., 2003) (bottom). Amino acid stretches predicted to
390 interact with *S. pyogenes* M22 protein are depicted in orange and red in the C α 2 and C α 3
391 domain, respectively. Amino acid alignments the ²⁵⁷LLG²⁵⁹ and ⁴⁴⁰PLAF⁴⁴³ loop motifs from
392 the selected mammal species are shown. (B) ELISA graph comparing the reactivity of dSAP
393 and tSAP against serum IgA antibodies from selected mammals as shown in (A). Means \pm
394 SD from intra-assay triplicate measurements in one representative experiment (from two
395 independent experiments) are shown. (C) ELISA graph showing the binding of dSAP and
396 tSAP to serum IgAs from selected non human-primate species. Human serum IgAs were

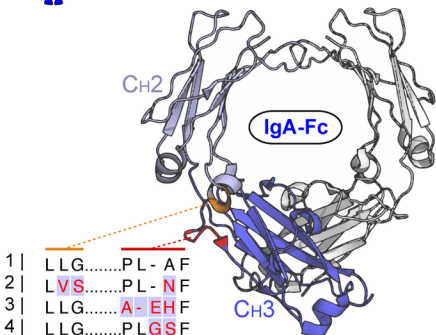
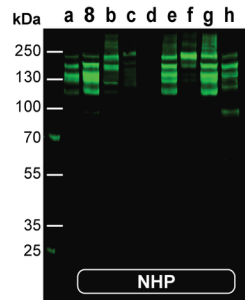
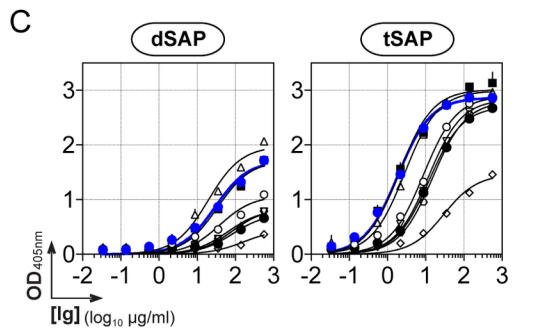
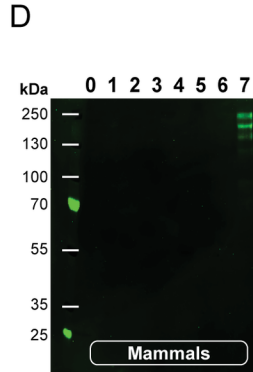
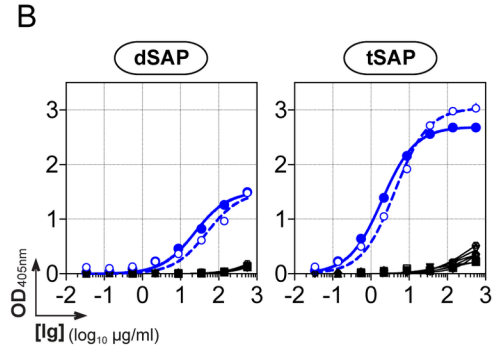
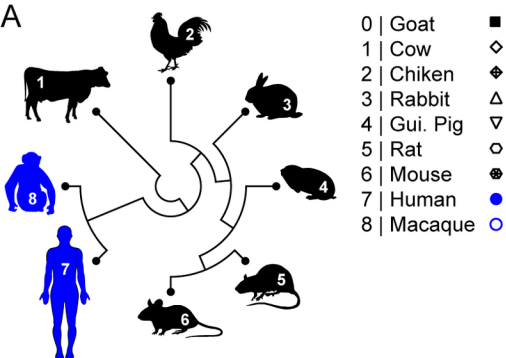
397 used as positive control (blue lines). Means \pm SD from intra-assay triplicate measurements
398 are shown. **(D)** Infrared immunoblotting showing the reactivity of biotinylated tSAPs against
399 serum IgA antibodies from selected mammal (left) and primate (right) species.

400

401 **Figure 3. SAP-based flow cytometric detection of human IgA-expressing B cells. (A)**

402 Representative cytograms showing the flow cytometric gating strategy to determine
403 frequencies of circulating blood IgA⁺ B cells from healthy individuals (n=3) by fluorescent
404 SAP probes' staining. **(B)** Titration of fluorescently labeled dSAP and tSAP (concentrations
405 varying from 14 to 520 nM) to quantify human IgA⁺ B cells. Data from one representative
406 healthy subject are shown. Commercially available anti-IgA antibody was used as positive
407 control. **(C)** Graphs comparing the frequency of circulating blood IgA⁺ B cells revealed using
408 commercial anti-IgA antibodies simultaneously bound by the SAP probes at different
409 concentrations. Mean values obtained from duplicate measurements in 3 healthy individuals
410 are shown. Error bars indicate SEM.





1 | LLG.....PL- AF
 2 | LVS.....PL- NF
 3 | LLG.....A- EHF
 4 | LLG.....PLGSF
 5 | LLG.....PM- SF
 6 | LLG.....PM- NF
 7 | LLG.....PL- AF
 8 | LLG.....PL- AF

257 259 440 443

a | AGM ●
 b | Cercopithecus cepus ■
 c | Mandrill ○
 d | Black crested mangabey ◇
 e | Allen's swamp monkey ▽
 f | Hamlyn's monkey ○
 g | Golden-bellied mangabey △

