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Revisiting autoimmune lymphoproliferative syndrome caused by Fas ligand mutations

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PII: S0091-6749(23)00001-5

DOI: https://doi.org/10.1016/j.jaci.2022.11.028

Reference: YMAI 15819

To appear in: *Journal of Allergy and Clinical Immunology*

Received Date: 11 August 2022

Revised Date: 1 November 2022

Accepted Date: 28 November 2022

Please cite this article as: Maccari ME, Schneider P, Smulski CR, Meinhardt A, Pinto F, Gonzalez-Granado LI, Schuetz C, Sica MP, Gross M, Fuchs I, Kury P, Heeg M, Vocat T, Willen L, Thomas C, Hühn R, Magerus A, Lorenz M, Schwarz K, Rieux-Laucat F, Ehl S, Rensing-Ehl A, Revisiting autoimmune lymphoproliferative syndrome caused by Fas ligand mutations, *Journal of Allergy and Clinical Immunology* (2023), doi: https://doi.org/10.1016/j.jaci.2022.11.028.

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1 **Revisiting autoimmune lymphoproliferative syndrome caused by Fas ligand**
2 **mutations**

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40 **Declaration of Interests**

41 The authors declare no competing interests.

42

43 **Word Count: 4675**

44 **Abstract word count: 249**

45 **Number of tables: 1**

46 **Number of figures: 4**

47 **Number of supplemental tables: 3**

48 **Number of supplemental figures: 1**

49

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57 **Abstract**

58 **Background:** Fas ligand (FasL) is expressed by activated T cells and induces death
59 in target cells upon binding to Fas. Loss-of-function *FAS* or *FASLG* mutations cause
60 autoimmune-lymphoproliferative syndrome (ALPS) characterized by expanded
61 double-negative T cells (DNT) and elevated serum biomarkers. While most ALPS
62 patients carry heterozygous *FAS* mutations, *FASLG* mutations are rare and usually
63 biallelic. Only two heterozygous variants were reported, associated with an atypical
64 clinical phenotype.

65 **Objective:** Revisit the significance of heterozygous *FASLG* mutations as a cause of
66 ALPS.

67 **Methods:** Clinical features and biomarkers were analysed in 24 individuals with
68 homozygous or heterozygous *FASLG* variants predicted to be deleterious. Cytotoxicity
69 assays were performed with patient T cells and biochemical assays with recombinant
70 FasL.

71 **Results:** Homozygous *FASLG* variants abrogated cytotoxicity and resulted in early-
72 onset severe ALPS with elevated DNT, raised Vitamin B12 and usually no soluble
73 FasL. In contrast, heterozygous variants impacted FasL function by reducing
74 expression, impairing trimerization or preventing Fas-binding. However, they were not
75 associated with elevated DNT and Vitamin B12 and did not affect FasL-mediated
76 cytotoxicity. The dominant negative effects of previously published variants could not
77 be confirmed. Even Y166C, causing loss of Fas-binding with a dominant-negative
78 effect in biochemical assays, did not impair cellular cytotoxicity nor caused Vitamin
79 B12 and DNT elevation.

80 **Conclusion:** Heterozygous loss-of-function mutations are better tolerated for *FASLG*
81 than for *FAS*, which may explain the low frequency of ALPS-FASLG.

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82 **Clinical Implication:** Based on current evidence, none of the reported heterozygous
83 *FASLG* mutations can be claimed to cause an inborn error of immunity.
84
85 **Capsule Summary:**
86 FasL function can tolerate haploinsufficiency and even dominant-negative inhibition.
87 This questions the clinical relevance of heterozygous *FASLG* variants and explains the
88 rarity of ALPS-FASLG.
89
90 **Keywords:** Autoimmune lymphoproliferative syndrome, Fas ligand, Fas, inborn error
91 of immunity, apoptosis
92
93 **Abbreviations:**
94 ALPS: autoimmune lymphoproliferative syndrome
95 DNT: double-negative T cells
96 FASLG, FasL: Fas ligand
97 sFasL: soluble Fas ligand
98 TNF: Tumor Necrosis Factor
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106 **Introduction**

107 Autoimmune-lymphoproliferative syndrome (ALPS) is a rare monogenic disorder
108 caused by defective Fas Ligand (FasL)/Fas signalling (1,2). This pathway induces
109 apoptosis in susceptible Fas expressing cells but can also mediate non-apoptotic
110 functions (3–5). One major function of the FasL/Fas pathway is the control of a small
111 population of highly proliferative T cells that expands in ALPS patients (6). These Fas-
112 controlled T cells accumulate primarily as TCR$\alpha\beta$+CD4-CD8- double-negative T cells
113 (DNT), a hallmark of the disease (7–9). Moreover, Fas signalling is important for the
114 elimination of autoreactive B cells (10,11). The main clinical manifestations are
115 therefore chronic benign lymphoproliferation and autoimmune cytopenia.

116

117 The most frequent genetic causes of ALPS are heterozygous germline or somatic
118 mutations in *FAS* (ALPS-FAS) (2,12–14). These mutations either impair expression,
119 resulting in haploinsufficiency or exert a dominant-negative effect on Fas signal
120 transduction. The low penetrance of heterozygous loss-of-expression *FAS* mutations
121 is increased by somatic second hits in *FAS* (14,15). Homozygous *FAS* mutations are
122 rare and lead to an early-onset, severe clinical phenotype (2,16–19). Typically, ALPS-
123 FAS patients have highly elevated serum Vitamin B12, IL-10 and soluble Fas ligand
124 (sFasL), which are used as diagnostic biomarkers (20–22).

125

126 Mutations in the gene encoding FasL (*FASLG*) occur in less than 1% of ALPS patients
127 (ALPS-FASLG) (23). FasL is a member of the TNF superfamily (TNFSF6). Its
128 extracellular, receptor-binding domain assembles as a homotrimer (24,25). While Fas
129 is expressed by many cell types, FasL is mainly expressed by activated T and NK cells
130 (26–28). Trimeric FasL can bind three receptor molecules (29), but apoptotic signalling

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131 requires at least two FasL trimers engaging receptors in close proximity, which is the
132 case for membrane-expressed but not for sFasL (30,31).
133
134 Only five homozygous and two heterozygous mutations in *FASLG* were reported to
135 cause ALPS in eleven individuals (32–38). Homozygous mutations were associated
136 with early-onset and severe clinical manifestations. These patients also presented with
137 high DNT frequencies and high Vitamin B12 levels. sFasL levels were very low or
138 undetectable. The three patients with heterozygous *FASLG* mutations (32,34) had
139 much later disease onset with atypical clinical manifestations and no clear biomarker
140 alterations. The rarity of heterozygous FasL deficiency despite increasing genetic
141 investigations calls for a re-evaluation of the pathogenic relevance of such
142 heterozygous variants, even if a dominant-negative mechanism has previously been
143 postulated.
144
145 We re-analyzed the pathogenicity of heterozygous *FASLG* mutations by a
146 comprehensive clinical, immunological and biochemical characterization. The study
147 included all previously reported patients, eight novel patients and five mutation carriers
148 with homozygous or heterozygous *FASLG* variants. Unexpectedly, all heterozygous
149 variants that impact FasL function *in vitro* by impairing expression, trimerization and/or
150 Fas-binding including one variant causing dominant-negative inhibition of Fas-binding
151 did not significantly affect FasL-mediated cytotoxicity of primary patient cells. Also, they
152 were observed in individuals with heterogenous clinical and immunological
153 manifestations and lacking biomarker elevations. Thus, FasL function appears to
154 tolerate molecular alterations better than Fas function. This may explain the low
155 frequency of ALPS-FASLG patients. Until more functional evidence is provided,

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156 heterozygous *FASLG* mutations remain variants of unknown significance which do not
157 cause ALPS.
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182 **Methods**

183 **Patients**

184 The study was approved by the local Ethics Committees in Freiburg and Paris (protocol
185 409/16 and 282/11; AL-PID study, German Clinical Trial Register: DRKS00000298 and
186 CPP Ile de France II) and the French Advisory Committee on Data Processing in
187 Medical Research (Paris, France). Written informed consent was obtained from all
188 individuals.

189

190 **sFasL ELISA and sFasL serum depletion**

191 sFasL was measured in patient sera with Quantikine® ELISA Human Fas
192 Ligand/TNFSF6 (R&D Systems). For the depletion assay, sera were depleted from
193 active FasL by incubation with Fas-Fc immobilized on beads, or EDAR-Fc as control.
194 FasL concentrations were then determined in the unbound fraction by ELISA. The
195 percentage of Fas-Fc-specific depletion relative to EDAR-Fc mock depletion was
196 calculated (100 – [Fas-Fc depleted FasL concentration/EDAR Fc depleted FasL
197 concentration *100]).

198

199 **Chromium release assay**

200 L1210 wild-type and L1210.FAS target cells were labelled with ^{51}Chromium (^{51}Cr). Day
201 7 T-cell blasts that were restimulated with PMA (Sigma Aldrich, 50 ng/ml) and
202 Ionomycin (Sigma Aldrich, 1 µg/ml) for 4h to induce optimal FasL expression were
203 used as effectors. To assess perforin-mediated cytotoxicity, day 7 blasts were
204 incubated with anti-CD3 antibody (Clone UCHT1, BD Pharmingen) for 1h before their
205 use in a redirected lysis assay on L1210 wild-type target cells. Cell killing capacity was
206 determined after incubation of effector and target cells at various ratios overnight,
207 followed by measurement of ^{51}Cr release (TopCount NXT; PerkinElmer).

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208 **Cell transfections and Western blot**

209 HEK-293T cells were transfected with polyethylenimine, Flag-tagged FASLG
210 expression plasmids and an EGFP expression plasmid as described (39). After 6 days
211 in serum-free OptiMEM medium, supernatants were concentrated in 30 kDa cut-off
212 protein concentrator devices to 400 µl (20x). Pellets were lysed in 200 µl of SDS-PAGE
213 sample buffer + 30 mM dithiothreitol, sonicated, and heated for 5 min at 95°C. 20 µl of
214 cell extracts or 0.5 to 10 µl of 20x supernatants, or 20 µl of fractions 7 to 20 from the
215 gel filtration column were loaded on 12% SDS-PAGE, transferred to nitrocellulose
216 membranes, and revealed with anti-Flag M2 monoclonal antibody (1 µg/ml), followed
217 by HRP-coupled goat anti-mouse antibody (1/5000) and enhanced
218 chemiluminescence.

219

220 **Size exclusion chromatography (SEC)**

221 200 µl of 20x supernatants were loaded on a HR10/30 Superdex 200 Increase gel
222 filtration column equilibrated in PBS and eluted at 0.55 ml/min with online UV
223 monitoring and with 1 ml fraction collection. The column was calibrated with a mix of
224 molecular weight standards. 5 µl of fractions 7 to 22 were analysed for binding to Fas-
225 Fc by ELISA, and 20 µl were used for Western blot anti-Flag .

226

227 **Receptor-binding ELISA**

228 Plates were coated with Fas-Fc (1 µg/ml) in PBS and blocked with block-buffer (4%
229 powdered skimmed milk in PBS 0.5% Tween 20) for 1 h at 37°C. In a preincubation
230 plate, 25 µl of 20x supernatant and 100 µl of incubation-buffer (block-buffer diluted 10
231 times in PBS) were added in well 1, and 5-fold serial dilutions were performed.
232 Samples were transferred in the ELISA plates. For SEC, 5 µl of fractions 7 to 22 plus
233 95 µl of incubation-buffer were added in the ELISA plates. Following steps were then

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234 performed: 1 h incubation (37°C). 3x wash-buffer (PBS, 0.05% Tween-20). 30 min
235 incubation with 100 µl biotinylated anti-Flag M2 (1/5000) (37°C). 3 washes. 30 min
236 incubation with 100 µl HRP-coupled streptavidin (1/5000) (37°C). 5 washes. Incubation
237 with 100 µl OPD solution. After colour development, addition of 50 µl 1N HCl.
238 Absorbance reading at 492 nm with an ELISA reader.
239

240 **Cytotoxic cellular assay**

241 In 96-flat-well-plates, 4-fold dilutions of Flag-FasL supernatants or purified Fc-FasL in
242 50 µl of RPMI 10%FCS were performed +/- anti-Flag M2 antibodies, at twice the
243 desired final concentration. 50 µl of Jurkat JC4 cells (25'000 to 50'000 cells/well) were
244 added. Plates were incubated for 16 h at 37°C in 5% CO_2 before addition of 20 µl of a
245 mix of phenazine methosulfate (PMS) at 45 µg/ml and 3-(4,5-dimethylthiazol-2-yl)-5-
246 (3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) at 2 mg/ml in PBS.
247 Absorbance at 492 nm was measured with an ELISA reader.
248

249 **Surface expression and receptor binding of FasL**

250 HEK-293T cells were transfected in 6 cm diameter plates with polyethylenimine, 0.1
251 µg of EGFP tracer plasmid, 2.3 µg of empty plasmid, and 0.2 µg of plasmids encoding
252 WT or mutants *FASLG* fused to the intracellular, transmembrane and stalk domain of
253 human BAFF. Fusion of TNF family ligands to the N terminal portion of human BAFF
254 generally ensures efficient surface expression (40). Three days later, cells were filtered
255 and spun in round-bottom 96-well-plate. Cells were stained for 20 min on ice with either
256 50 µl of Fas-Fc at 2 µg/ml in FBE-liq (PBS, 5% FCS, 2 mM EDTA, 1 IU/ml liquemin),
257 or with biotinylated rat IgG2a anti-hBAFF mAb Buffy1 that recognized the stalk region
258 of human BAFF (41)(Also commercially available from Enzo LifeSciences) at 2 µg/ml
259 in FBE-liq. Cells were washed twice with 200 µl FBE, then stained with 50 µl of anti-

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260 human-PE (1/500) or streptavidin-PE (1/500) in FBE-liq for 20 min on ice, washed with
261 200 µl of FBE, ressuspended in 100 µl of FBE and analysed by FACS using a Cytoflex
262 S apparatus (Beckman-Coulter) equipped with a CytoFRÖSTLI plate cooler (Cyto-
263 Service.ch, Châtel-St-Denis, Switzerland).
264
265 **Molecular dynamic simulation studies**
266 Trimeric wild type human FasL trimer was obtained from the protein data bank (PDB:
267 4MSV). Mutation L181P was introduced using PyMOL Molecular Graphics System,
268 Version 2.0 (Schrödinger, LLC). The mutation was generated in either one, two or three
269 subunits of the trimer. Molecular dynamic simulation was performed using wt FasL,
270 FasL 1x L181P, FasL 2x L181P and FasL 3x L181P. Simulations were carried out with
271 the GROMACS package version 2021.2 (42) using the CHARMM36 all-atom force field
272 (July 2021 version) (43). After the initial steps of minimization and equilibrations
273 (position restrained and free) the systems were simulated with a 2 fs time step at 310
274 K and 1 bar using the velocity rescaling thermostat and the semiisotropic Parrinello-
275 Rahman barostat (44). Every system was simulated for 300 nanoseconds. Each
276 simulation was analyzed at the level of each monomer or at the level of the trimer for
277 the root mean square deviation (RMSD), using PyMOL and for the hidden solvent
278 accessible surface using the output from 'sasa' tool of gromacs.
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286 **Results**

287 **Patients with heterozygous *FASLG* variants frequently present with**
288 **heterogenous clinical manifestations not typically seen in ALPS**

289 We collected all patients with autoimmunity and/or lymphoproliferation referred to our
290 centres who had *FASLG* variants predicted to be deleterious or of unknown
291 significance by at least one commonly used prediction program (Table E2). Eleven
292 patients with seven homozygous *FASLG* variants were identified, including three new
293 patients (P9-11) with two novel mutations (Table I, E1, Figure E1 and Online
294 Repository). As reported for the published cases, the three new patients had early-
295 onset severe lymphoproliferation within the first year of life. Eight patients including P9
296 had autoimmune cytopenia. Clinically, these patients represent a phenocopy of severe
297 ALPS-FAS. In addition, we identified eight individuals with heterozygous *FASLG*
298 variants, including five new patients (14-15; 17-19) with three novel mutations (Table
299 I, E1, Figure E1 and Online Repository). All patients had lymphoproliferation and three
300 had autoimmune cytopenia. Disease onset was variable between 18 months and 52
301 years. Seven of these 8 patients had symptoms not normally seen in ALPS patients
302 including granulomatous pneumonitis and autoimmune hepatitis (P12) psoriatic
303 arthritis (P13), sialadenitis (P14,P15), systemic lupus erythematosus (SLE)(P16),
304 recurrent respiratory infections and meningitis (P17) and tubular nephritis (P19). We
305 also analyzed five heterozygous parents of patients with homozygous mutations. All
306 parents were asymptomatic (Table I).

307

308 **Defective FasL-mediated cytotoxicity of primary T cells confirms the**
309 **pathogenicity of homozygous *FASLG* variants**

310 All analysed patients with homozygous *FASLG* mutations had significantly raised DNT
311 and Vitamin B12 levels. Serum sFasL was very low or undetectable in P10 and 11

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312 (V15Wfs*57), as previously described for P1 (A247E), P2 (F87fs*95), P7 and P8 (both
313 C202S). Values for the remaining four published cases were not reported. However,
314 P69Afs*71 is a frameshift mutation leading to complete loss-of-expression (36) and is
315 therefore expected to lead to absent sFasL in the serum. Interestingly, P9 (L181P) had
316 sFasL levels in the normal range (Figure 1A). P9 primary T cell blasts showed absent
317 FasL-dependent cytotoxicity (Figure 1B) similar to T cells from patients with the
318 homozygous loss of expression variants A247E (33) and V15Wfs*57 (Figure 1B),
319 indicating reduced protein function. Cytotoxic activity on Fas negative target cells was
320 absent (Figure 1C) and perforin-mediated cytotoxicity was normal (Figure 1D),
321 confirming assay specificity. Thus, defective FasL expression and/or function in
322 primary patient T cells confirmed the functional relevance of the two novel homozygous
323 mutations L181P and V15Wfs*57 for the clinical phenotype including DNT and Vitamin
324 B12 biomarker elevations. Of, note, accumulation of DNT and elevated Vitamin B12
325 due to increased expression of the Vitamin B12 carrier protein haptocorrin (45) are a
326 direct consequence of defective Fas dependent elimination of DNT and their
327 precursors (6).
328

329 **Patients with heterozygous *FASLG* variants show normal biomarkers and intact**
330 **FasL-induced cytotoxicity**

331 DNT and Vitamin B12 were normal in all patients with heterozygous *FASLG* variants
332 in whom these values were determined (Table I). DNT and Vitamin B12 were also not
333 elevated in the five heterozygous parents of patients with homozygous mutations.
334 Serum sFasL was normal in all patients except P17 (Y166C), who repeatedly showed
335 values above 3000 pg/ml (Figure 2A). Interestingly, sFasL levels were also increased
336 in the mother of P17 (heterozygous for Y166C) whereas the mother of P1 (A247E) and
337 the father of P10 (V15Wfs*57) showed sFasL levels below normal range. The

338 immunophenotype was variable in patients where this information was available. P17
339 had significantly reduced naïve CD4+ and CD8+ cells, 81% of CD8 T cells expressed
340 CD57 and 33% expressed HLA-DR. None of these abnormalities were observed in his
341 mother who carried the same mutation, nor in other carriers of heterozygous mutations.
342 In contrast to two previously reported cases with the same R156G mutation (34), FasL-
343 mediated cytotoxicity of primary T cell blasts was intact in P14 and P15 (R156G). It
344 was also unaffected in P17 and 18 (Y166C, Q130E) (Figure 2B, D, E). T cells blasts
345 of the mother/father of P1, 10 and 17 (A247E, V15Wfs*57 and Y166C) also mediated
346 normal cytotoxicity. Unexpectedly, T cell blasts of the healthy heterozygous parents of
347 P9 (L181P) showed reduced, but not absent FasL-mediated cytotoxic activity (Figure
348 2C-E).
349

350 **Biochemical analysis of recombinant FasL mutants**

351 For some patients, serum and primary cells were not available. Therefore, secreted
352 Flag-tagged FasL variants were expressed in HEK-293T cells. All mutants except
353 Q130E were over-represented in cell extracts, indicating folding or solubility defects in
354 this expression system. Solubility defects were mild for A247E, Y166C, R156G and
355 S155L, severe for C202S and G277S and apparently total for L181P and ∆158-185
356 which were undetectable in supernatants (Figure 3A). Wild type (WT) FasL and
357 mutants Q130E, S155L and R156G all bound recombinant Fas-Fc and, when cross-
358 linked with an anti-Flag antibody to mimic the active membrane-bound form (30), killed
359 Fas-expressing Jurkat cells (Figure 3B, C). All other mutants were either not secreted
360 and/or lacked receptor binding and cytotoxic activity.
361
362
363

364 **FasL-mediated apoptosis is resistant to haploinsufficiency**

365 Mutant A247E yielded discrepant results as it was released into the supernatant of
366 HEK-293T cells (Figure 3A), yet was undetectable in P1 serum (Figure 1A). We
367 characterized the native size of the secreted but inactive mutant A247E by size
368 exclusion chromatography (SEC) and found abnormally high molecular weight
369 multimers indicative of mutation-induced misfolding (Figure 3D). When soluble Flag-
370 tagged *FASLG* WT and A247E were co-transfected at different ratios, A247E had no
371 dominant-negative effect on the cytotoxic function of WT (Figure 3E), suggesting that
372 A247E does not co-associate with WT in the inactive high molecular form, should this
373 form be secreted or retained in cells. We hypothesized that the epitope recognized by
374 the anti-FasL antibody in the ELISA (Figure 1A) was disrupted by A247E. Indeed,
375 recombinant sFasL A247E could be detected by an anti-Flag antibody in the Western
376 Blot but could not be detected in the supernatant of A247E transfected HEK-293T
377 using this ELISA (Figure 3F). The lack of a dominant-negative effect of A247E in HEK-
378 293T cells is in line with the normal cytotoxicity of T cells of the asymptomatic
379 heterozygous mother of P1 (Figure 2C). Overall, the findings in the heterozygous
380 parents indicate that half of the normal amount of FasL is sufficient to preserve FasL
381 function as assessed by T cell cytotoxicity, biomarkers and clinical manifestations.

382

383 **A *FASLG* mutant abolishing Fas binding does not exert a dominant-negative**
384 **effect on membrane-bound FasL function**

385 In contrast to A247E, the inactive but soluble mutant Y166C had the same native size
386 as several active FasL variants, indicating no major folding/trimerization defect, but
387 rather specific impairment of receptor binding (Figure 3D). This makes Y166C a strong
388 candidate to exert a dominant-negative effect on co-expressed WT FasL. Indeed,
389 cytotoxicity of soluble Flag-tagged WT FasL cross-linked with anti-Flag was impaired

390 when it was co-expressed with an excess of Y166C mutant and it was around 50-fold
391 reduced at a 1:1 ratio (Figure 3E). However, since T cells from heterozygous P17 and
392 his asymptomatic mother (Y166C) had no cytotoxicity defect (Figure 2B,C), we infer
393 that cellular, membrane-bound FasL function remains sufficient despite the dominant-
394 negative effect in the soluble expression system (Figure 2A). We hypothesized that the
395 high sFasL levels found in P17 and his mother resulted at least in part from reduced
396 binding and consumption by Fas expressing cells. We tested this using immobilized
397 Fas-Fc to deplete FasL from patient serum. Serum sFasL from ALPS-FAS patients
398 was reduced by 60-80% after incubation with Fas-Fc compared to the negative control
399 EDAR-Fc. This reduction was only 40% in sera of P17 and his mother, confirming that
400 heterozygous Y166C leads to reduced Fas binding which may result in accumulation
401 *in vivo* (Figure 3G).

402

403 **A *FASLG* mutant predicted to impair trimer assembly is not associated with a**
404 **clinical or immunological phenotype**

405 Mutant L181P transfected in HEK-293T cells was insoluble (Figure 3A), yet sFasL was
406 not only present in P9 serum (Figure 1A), but was also able to bind Fas (Figure 3G),
407 indicating that sFasL expression of this particular mutant in HEK-293T cells does not
408 adequately mirror its physiology in patient cells. To analyze the *FASLG* variants in their
409 membrane-bound form, we generated fusion proteins between the N-terminal portion
410 of the TNF family ligand BAFF, including its transmembrane domain and a stalk portion
411 recognized by mAb Buffy1, and the C-terminal soluble domain of FasL that allows
412 assessing binding to Fas (Figure 4A). BAFF-N-FasL constructs were all expressed to
413 at least some extent at the cell surface, including L181P and, very weakly, ∆158-185,
414 as measured by Buffy1 staining (Figure 4B,C). Only WT, Q130E, S155L and R156G
415 were also obviously stained with Fas-Fc, confirming data obtained with Flag-FasL

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416 (Figure 3B and 4D). However, in this hemizygous expression system, the weakly
417 expressed L181P clearly showed residual binding to Fas-Fc that was not observed
418 with mutants A247E, C202S, R156G, G277S and Δ158-185 (Figure 4D). This suggests
419 that L181P is generated at low levels in P9, but because of deficient binding to Fas,
420 accumulates in serum up to “pseudo-normal” levels. Depletion on Fas-Fc (Figure 3G)
421 could be explained by the vast excess of immobilized Fas-Fc. When performing the
422 same experiment in a heterozygous system we detected a dominant-negative effect
423 on Fas-binding for mutant Y166C but not for other mutants observed in heterozygous
424 patients including Δ158-185 and R156G (Figure 4E,F).
425
426 We studied the impact of the L181P mutation on the stability of FasL in molecular
427 dynamic simulations comparing multimeric FasL WT with FasL carrying one, two or
428 three L181P mutated subunits. Important structural changes occurred in L181P trimer
429 relative to WT, even if only one or two monomers of the trimer were mutated (Figure
430 4G). As these differences were minimal when the analysis was performed for each
431 monomer (Figure 4H), L181P may destabilize the trimeric assembly with little or no
432 impact on the structure of the monomer. Accordingly, surfaces hidden at interaction
433 sites between monomers were reduced in the L181P variants (Figure 4I), indicative of
434 trimer opening. Thus, molecular dynamic simulations suggest that L181P, a conserved
435 residue in TNF family ligands, possibly relevant for their characteristic trimeric
436 assembly (Figure E1, (46)), mainly affects the stability of FasL trimers, which may
437 explain reduced Fas binding and cytotoxicity of T cell blasts in the asymptomatic
438 parents of P9.
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442 **Discussion**

443

444 ALPS caused by impaired FasL/Fas signalling includes autosomal-recessive and
445 autosomal-dominant inborn errors of immunity. Here, we performed the first
446 comprehensive clinical, immunological and biochemical investigation of a large group
447 of patients carrying germline mutations in the *FASLG* gene. We characterized eleven
448 patients with seven different homozygous and thirteen individuals with eight different
449 heterozygous *FASLG* variants. Two of the latter were biochemically demonstrated to
450 exhibit dominant-negative effects and three led to haploinsufficiency. However,
451 functional and clinical observations strongly argue against their causality for clinical
452 disease. This extends to the three previously published patients. Thus, FasL-mediated
453 cytotoxicity in primary cells can tolerate reduced expression and even dominant-
454 negative inhibition better than anticipated and none of the currently known
455 heterozygous *FASLG* mutations can be claimed to cause ALPS or another inborn error
456 of immunity.

457

458 Biochemically, two adjacent FasL trimers appear to be the minimal functional unit
459 required to activate apoptosis (31), and physiologically, membrane-bound FasL is
460 required to mediate T cell cytotoxicity (47). A heterozygous *FASLG* mutation leading
461 to loss-of-expression or expression of an inactive protein results in 50% reduction of
462 functional hexameric units of WT FasL (haploinsufficiency). If a heterozygous mutation
463 leads to expression of FasL that is inactive but can still heteromerize with WT, a
464 dominant-negative effect is expected. Clinically, fully penetrant ALPS-FASLG caused
465 by biallelic mutations presents with severe, early-onset lymphoproliferation and
466 autoimmune cytopenia. Our three newly reported patients provide no exception to this
467 rule and confirm that elevated DNT and Vitamin B12 are reliable biomarkers also for

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468 this disease. Published heterozygous *FASLG* mutations have been linked to disease
469 by their dominant-negative function on cytotoxicity. In principle, mechanisms other
470 than defective Fas mediated apoptosis such as FasL retrograde signaling (36,48–52)
471 could also be affected, but no evidence for their relevance *in vivo* has been provided
472 in patients so far. In particular, missense mutations in the intracellular domain of
473 *FASLG* with preserved expression but possible impact on retrograde signaling have
474 not been reported.
475
476 These considerations provide the framework to discuss the patients in this study and
477 from previous publications. In addition to the three frameshift mutations observed in
478 patients with homozygous mutations, the homozygous point mutations A247E, G277S
479 (as shown previously (33,38)) and L181P alter conserved residues in the TNF
480 superfamily, while C202S alters a disulphide bridge that bridges loops joining pairs of
481 anti-parallel beta-pleated sheets and stabilises the folding of FasL monomers (Figure
482 E1 C). Moreoever, C202 is highly conserved across veterbrate species down to fish
483 as are A247E, G277 and L181P (Figure E1 D). The severity of all these homozygous
484 mutations is consistent with a clear-cut cytotoxicity defect and a fully penetrant
485 phenotype including biomarker elevations.
486
487 The A247E mutation did not impair co-expressed WT FasL in several *in vitro* assays.
488 FasL-mediated cytotoxicity in relatives carrying this mutation or V15Wfs in
489 heterozygosity was normal and these individuals showed no clinical symptoms or
490 biomarker alterations. These findings and lack of reports on clinical symptoms in other
491 relatives of the reported homozygous patients support the concept that FasL
492 haploinsufficiency is not sufficient to cause disease. The Δ158-185 deletion removes
493 the conserved tryptophane 162 residue as well as beta sheets A' B' and B in the TNF

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494 homology domain that mediates binding to the receptor (46). Δ158-185 was previously
495 described in a patient with SLE and lymphoproliferation (32) but unfortunately
496 biomarkers were not reported. A causal link was postulated by demonstration of a
497 dominant-negative effect on cytotoxicity (32). In our expression system, the mutation
498 did not impair cell surface expression and receptor binding of a co-transfected WT
499 FasL protein. It is difficult to conceive how this deleterious allele should be able to exert
500 a dominant-negative effect, questioning the relevance of this heterozygous mutation
501 for the reported clinical phenotype.
502
503 Mutants R156G, S155L and Q130E showed normal production, secretion,
504 trimerization and cytotoxicity. Moreover, FasL-mediated cytotoxicity of primary patient
505 T cells was normal for two individuals with the heterozygous R156G and one with
506 heterozygous Q130E. However, R156G and S155L had more insoluble proteins than
507 WT and may therefore be produced in lower amounts than WT *in vivo*. Of note, our
508 two and the two previously reported patients with R156G presented with
509 lymphoproliferation in association with variable additional manifestations. None of
510 them had elevated biomarkers. A previous report demonstrated slightly reduced
511 cytotoxic activity of primary cells on Jurkat target cells and a dominant-negative effect
512 in HEK-293T cells overexpressing the mutant *FASLG* (34). In contrast, FasL-mediated
513 cytotoxicity of primary cells from our two patients was normal and no dominant-
514 negative effect on Fas-binding was observed with recombinant proteins. Atypical
515 clinical manifestations and lack of biomarker alterations further support that the
516 mutation is not disease-causing along the classical pathway. In addition, R156 was not
517 conserved among TNF families nor across other animal species (Figure E1 D, E).
518 While we cannot exclude that the R156G variant impinges on an activity of FasL

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519 different from apoptosis induction, such evidence would be required before linking it to
520 a disease phenotype.
521
522 The most informative mutations were Y166C and L181P. Y166 points directly to the
523 receptor (Figure E1 A) and soluble Y166C consistently showed loss of Fas-binding
524 despite normal assembly as trimers in its soluble form. When produced as sFasL,
525 Y166C exerted a dominant negative effect in the heterozygous state. At a 1:1 ratio of
526 WT and Y166C, cytotoxic activity of cross-linked recombinant proteins was reduced
527 about 50-fold. The dominant-negative effect in heterozygosity was confirmed in
528 experiments using BAFF-N-FasL constructs, which confirmed normal protein
529 expression on the cell surface but defective binding to Fas. Despite the strong
530 dominant-negative effect *in vitro*, primary patient T cells heterozygous for this mutation
531 were still fully cytotoxic. The patient carrying this variant had hypogammaglobulinemia,
532 severely reduced naïve T cells (Table E3) and infection susceptibility in addition to
533 lymphoproliferation and autoimmunity. Since Vitamin B12 and DNT were not elevated
534 and his mother carrying the same mutation was asymptomatic, these symptoms may
535 have another genetic cause. Whether the unusual elevation of serum sFasL in this
536 patient indeed results from decreased Fas binding and consumption and/or reflects a
537 hyperactivated T cell compartment with upregulated FASLG expression remains
538 unclear. This mutation provides an example that even a strong dominant-negative
539 effect of a heterozygous *FASLG* mutation can be tolerated and does not necessarily
540 result in ALPS. Exome sequencing also revealed a variant of unknown significance in
541 *NFKB2* in this patient, which is still under evaluation.
542
543 Finally, L181P was not secreted in the HEK-293T cell expression system, despite
544 normal serum sFasL levels even in the homozygous patient. Since non-cleavable

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545 L181P FasL was expressed on the cell surface, lack of secretion may be a
546 solubility/secretion problem of this variant in this transfection system. Molecular
547 dynamic simulation predicted an effect on the trimer stability. This is consistent with
548 the observed absent FasL-mediated cytotoxicity of T cells of the homozygous patient
549 and mildly reduced cytotoxicity of T cells of the heterozygous parents due to a
550 dominant-negative effect.

551

552 These considerations help explain why ALPS-FASLG is so rare compared to ALPS-
553 FAS. While similarly few patients with homozygous *FAS* mutations have been
554 described, several hundreds of patients with heterozygous *FAS* mutations and ALPS
555 have been reported. This is in part explained by *FAS* mutations affecting the
556 intracellular domain with a dominant-negative effect on signal transduction. However,
557 an equal number of patients has been reported with mutations affecting the
558 extracellular domain (14). Interestingly, in a relevant proportion of these cases, somatic
559 second events such as loss-of-heterozygosity significantly increase disease
560 penetrance (15). Such additional genetic events have so far not been reported for
561 *FASLG*. Notably, while intracellular retrograde FasL signaling has also been described
562 *in vitro*, its relevance for humans *in vivo* remains unclear. The impaired Fas receptor
563 mediated pro-apoptotic functions caused by complete FasL deficiency are sufficient to
564 explain the so far observed clinical phenotype, which fully mirrors that of complete Fas
565 deficiency. We have therefore focused on established cytotoxic functions of the FasL-
566 Fas interaction, that are unequivocally relevant for the clinical ALPS phenotype. Taken
567 together, the more profound effect on pro-apoptotic Fas-mediated signaling by *FAS*
568 variants as compared to *FASLG* variants likely contributes to the differences in their
569 clinical penetrance.

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571 From a clinical point of view, any *FASLG* mutation must be carefully functionally
572 investigated before it can be regarded as causative for an autoimmune-
573 lymphoproliferative disease. Absent FasL-mediated cytotoxicity of primary T cells is
574 currently the most reliable method to confirm functional insufficiency. Based on our
575 and previously published studies, currently no convincing evidence for linking
576 heterozygous *FASLG* mutations to any clinical phenotype has been presented. Thus,
577 the heterozygous *FASLG* mutations reported so far should not be regarded causative
578 for ALPS or any other inborn error of immunity. Importantly, the inappropriate
579 consideration of heterozygous *FASLG* variants as disease-causing discourages
580 physicians from looking for other diagnoses, some of which may offer personalized
581 treatment possibilities. Thus, in case of heterozygous *FASLG* variants of unknown
582 significance, an extended search for additional or alternative genetic alterations is
583 highly recommended.
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597 **Acknowledgements**

598 We are grateful to the patients and their families who made this study possible. We
599 thank the CCI Advanced Diagnostics Unit, the CCI Biobank and the Lighthouse Core
600 facility for excellent support. This work was funded by the German Federal Ministry of
601 Education and Research (BMBF 01 EO 0803 grant to the Center of Chronic
602 Immunodeficiency and BMBF 01GM1111B grant to the PID-Net Initiative) and the
603 Wilhelm Sander Stiftung (1 027 087 401). MEM was supported by an EXCEL-
604 Fellowship of the Faculty of Medicine, University of Freiburg, funded by the Else–
605 Kröner–Fresenius–Stiftung. PS is supported by the Swiss National Scientific
606 Foundation (grants 31003A_176256 and 310030_205196).

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623 **References**

624 1. Canale VC, Smith CH. Chronic lymphadenopathy simulating malignant
625 lymphoma. J Pediatr. 1967;70(6):891–9.
626 2. Rieux-Laucat F, Le Deist F, Hivroz C, Roberts IAG, Debatin KM, Fischer A, et
627 al. Mutations in fas associated with human lymphoproliferative syndrome and
628 autoimmunity. Science (80-). 1995;268(5215):1347–9.
629 3. Klebanoff CA, Scott CD, Leonardi AJ, Yamamoto TN, Cruz AC, Ouyang C, et al.
630 Memory T cell-driven differentiation of naive cells impairs adoptive
631 immunotherapy. J Clin Invest. 2016;126(1):318–34.
632 4. Le Gallo M, Poissonnier A, Blanco P, Legembre P. CD95/Fas, non-apoptotic
633 signaling pathways, and kinases. Front Immunol. 2017;8(SEP).
634 5. Peter ME, Budd RC, Desbarats J, Hedrick SM, Hueber AO, Newell MK, et al.
635 The CD95 Receptor: Apoptosis Revisited. Cell. 2007;129(3):447–50.
636 6. Maccari ME, Fuchs S, Kury P, Andrieux G, Völkl S, Bengsch B, et al. A distinct
637 CD38+CD45RA+ population of CD4+, CD8+, and double-negative T cells is
638 controlled by FAS. J Exp Med. 2020 Nov 10;218(2).
639 7. Sneller MC, Wang J, Dale JK, Strober W, Middelton LA, Choi Y, et al. Clinical,
640 immunologic, and genetic features of an autoimmune lymphoproliferative
641 syndrome associated with abnormal lymphocyte apoptosis. Blood.
642 1997;89(4):1341–8.
643 8. Sneller MC, Straus SE, Jaffe ES, Jaffe JS, Fleisher TA, Stetler- Stevenson M, et
644 al. A novel lymphoproliferative/autoimmune syndrome resembling murine lpr/gld
645 disease. J Clin Invest. 1992;90(2):334–41.
646 9. Oliveira JB, Bleesing JJ, Dianzani U, Fleisher TA, Jaffe ES, Lenardo MJ, et al.
647 Revised diagnostic criteria and classification for the autoimmune
648 lymphoproliferative syndrome (ALPS): Report from the 2009 NIH International
649 Workshop. Blood. 2010;116(14):35–40.
650 10. Hao Z, Duncan GS, Seagal J, Su Y, Hong C, Haight J, et al. Fas Receptor
651 Expression in Germinal-Center B Cells Is Essential for T and B Lymphocyte
652 Homeostasis. Immunity. 2008 Oct;29(4):615–27.
653 11. Janda A, Schwarz K, Van Der Burg M, Vach W, Ijspeert H, Lorenz MR, et al.
654 Disturbed B-lymphocyte selection in autoimmune lymphoproliferative syndrome.
655 Blood. 2016;127(18):2193–202.
656 12. Fisher GH, Rosenberg FJ, Straus SE, Dale JK, Middelton LA, Lin AY, et al.
657 Dominant interfering fas gene mutations impair apoptosis in a human
658 autoimmune lymphoproliferative syndrome. Cell. 1995;81(6):935–46.
659 13. Holzelova E, Vonarbourg C, Stolzenberg M-C, Arkwright PD, Selz F, Prieur A-
660 M, et al. Autoimmune Lymphoproliferative Syndrome with Somatic Fas
661 Mutations . N Engl J Med. 2004;351(14):1409–18.
662 14. Magerus A, Bercher-Brayer C, Rieux-Laucat F. The genetic landscape of the
663 FAS pathway deficiencies. Vol. 44, Biomedical Journal. 2021. p. 388–99.
664 15. Magerus-Chatinet A, Neven B, Stolzenberg MC, Daussy C, Arkwright PD,
665 Lanzarotti N, et al. Onset of autoimmune lymphoproliferative syndrome (ALPS)
666 in humans as a consequence of genetic defect accumulation. J Clin Invest.
667 2011;121(1):106–12.
668 16. Le Deist F, Emile JF, Rieux-Laucat F, Benkerrou M, Roberts I, Brousse N, et al.
669 Clinical, immunological, and pathological consequences of Fas-deficient
670 conditions. Lancet. 1996;348(9029):719–23.
671 17. Bettinardi A, Brugnoni D, Quiròs-Roldan E, Malagoli A, La Grutta S, Correra A,
672 et al. Missense mutations in the fas gene resulting in autoimmune
673 lymphoproliferative syndrome: A molecular and immunological analysis. Blood.

Journal Pre-proof

674 1997;89(3):902–9.
675 18. Kasahara Y, Wada T, Niida Y, Yachie A, Seki H, Ishida Y, et al. Novel Fas
676 (CD95/APO-1) mutations in infants with a lymphoproliferative disorder. Int
677 Immunol. 1998;10(2):195–202.
678 19. Van Der Burg M, De Groot R, Comans-Bitter WM, Den Hollander JC, Hooijkaas
679 H, Neijens HJ, et al. Autoimmune lymphoproliferative syndrome (ALPS) in a child
680 from consanguineous parents: A dominant or recessive disease? Pediatr Res.
681 2000;47(3):336–43.
682 20. Magerus-Chatinet A, Stolzenberg MC, Loffredo MS, Neven B, Schaffner C,
683 Ducrot N, et al. FAS-L, IL-10, and double-negative CD4 -CD8 - TCR α/β + T cells
684 are reliable markers of autoimmune lymphoproliferative syndrome (ALPS)
685 associated with FAS loss of function. Blood. 2009;113(13):3027–30.
686 21. Caminha I, Fleisher TA, Hornung RL, Dale JK, Niemela JE, Price S, et al. Using
687 biomarkers to predict the presence of FAS mutations in patients with features of
688 the autoimmune lymphoproliferative syndrome. J Allergy Clin Immunol. 2010
689 Apr;125(4):946-949.e6.
690 22. Rensing-Ehl A, Janda A, Lorenz MR, Gladstone BP, Fuchs I, Abinun M, et al.
691 Sequential decisions on FAS sequencing guided by biomarkers in patients with
692 lymphoproliferation and autoimmune cytopenia. Haematologica.
693 2013;98(12):1948–55.
694 23. Rao VK, Oliveira JB. How I treat autoimmune lymphoproliferative syndrome.
695 Blood. 2011;118(22):5741–51.
696 24. Suda T, Takahashi T, Golstein P, Nagata S. Molecular cloning and expression
697 of the fas ligand, a novel member of the tumor necrosis factor family. Cell. 1993
698 Dec;75(6):1169–78.
699 25. Takahashi T, Tanaka M, Inazawa J, Abe T, Suda T, Nagata S. Human fas ligand:
700 Gene structure, chromosomal location and species specificity. Int Immunol.
701 1994;6(10):1567–74.
702 26. Tanaka M, Suda T, Takahashi T, Nagata S. Expression of the functional soluble
703 form of human Fas ligand in activated lymphocytes. EMBO J. 1995;14(6):1129–
704 35.
705 27. Hahne M, Renno T, Schroeter M, Irmler M, French L, Bornand T, et al. Activated
706 B cells express functional Fas ligand. Eur J Immunol. 1996;26(3):721–4.
707 28. Lu L, Qian S, Hershberger PA, Rudert WA, Lynch DH TA. Fas ligand (CD95L)
708 and B7 expression on dendritic cells provide counter-regulatory signals for T cell
709 survival and proliferation. J Immunol. 1997;158(12):5676–84.
710 29. Schneider P, Bodmer J-L, Holler N, Mattmann C, Scuderi P, Terskikh A, et al.
711 Characterization of Fas (Apo-1, CD95)-Fas Ligand Interaction. J Biol Chem.
712 1997 Jul;272(30):18827–33.
713 30. Schneider P, Holler N, Bodmer JL, Hahne M, Frei K, Fontana A, et al. Conversion
714 of membrane-bound Fas(CD95) ligand to its soluble form is associated with
715 downregulation of its proapoptotic activity and loss of liver toxicity. J Exp Med.
716 1998;187(8):1205–13.
717 31. Holler N, Tardivel A, Kovacsovics-Bankowski M, Hertig S, Gaide O, Martinon F,
718 et al. Two Adjacent Trimeric Fas Ligands Are Required for Fas Signaling and
719 Formation of a Death-Inducing Signaling Complex. Mol Cell Biol.
720 2003;23(4):1428–40.
721 32. Wu J, Wilson J, He J, Xiang L, Schur PH, Mountz JD. Fas ligand mutation in a
722 patient with systemic lupus erythematosus and lymphoproliferative disease. J
723 Clin Invest. 1996;98(5):1107–13.
724 33. Del-Rey M, Ruiz-Contreras J, Bosque A, Calleja S, Gomez-Rial J, Roldan E, et

Journal Pre-proof

725 al. A homozygous Fas ligand gene mutation in a patient causes a new type of
726 autoimmune lymphoproliferative syndrome. Blood. 2006;108(4):1306–12.
727 34. Bi LL, Pan G, Atkinson TP, Zheng L, Dale JK, Makris C, et al. Dominant inhibition
728 of Fas ligand-mediated apoptosis due to a heterozygous mutation associated
729 with autoimmune lymphoproliferative syndrome (ALPS) Type Ib. BMC Med
730 Genet. 2007;8:1–14.
731 35. Magerus-Chatinet A, Stolzenberg MC, Lanzarotti N, Neven B, Daussy C, Picard
732 C, et al. Autoimmune lymphoproliferative syndrome caused by a homozygous
733 null FAS ligand (FASLG) mutation. J Allergy Clin Immunol. 2013;131(2):486–90.
734 36. Nabhani S, Hönscheid A, Oommen PT, Fleckenstein B, Schaper J, Kuhlen M, et
735 al. A novel homozygous Fas ligand mutation leads to early protein truncation,
736 abrogation of death receptor and reverse signaling and a severe form of the
737 autoimmune lymphoproliferative syndrome. Clin Immunol [Internet].
738 2014;155(2):231–7. Available from: http://dx.doi.org/10.1016/j.clim.2014.10.006
739 37. Ruiz-Garcia R, Mora S, Lozano-Sanchez G, Martinez-Lostao L, Paz-Artal E,
740 Ruiz-Contreras J, et al. Decreased activation-induced cell death by EBV-
741 transformed B-cells from a patient with autoimmune lymphoproliferative
742 syndrome caused by a novel FASLG mutation. Pediatr Res. 2015;78(6):603–8.
743 38. Sobh A, Crestani E, Cangemi B, Kane J, Chou J, Pai SY, et al. Autoimmune
744 lymphoproliferative syndrome caused by a homozygous FasL mutation that
745 disrupts FasL assembly. J Allergy Clin Immunol. 2016;137(1):324-327.e2.
746 39. Schneider P, Willen L, Smulski CR. Tools and techniques to study ligand-
747 receptor interactions and receptor activation by TNF superfamily members
748 [Internet]. 1st ed. Vol. 545, Methods in Enzymology. Elsevier Inc.; 2014. 103–
749 125 p. Available from: http://dx.doi.org/10.1016/B978-0-12-801430-1.00005-6
750 40. Bossen C, Tardivel A, Willen L, Fletcher CA, Perroud M, Beermann F, et al.
751 Mutation of the BAFF furin cleavage site impairs B-cell homeostasis and
752 antibody responses. Eur J Immunol. 2011;41(3):787–97.
753 41. Schneider P, Mackay F, Steiner V, Hofmann K, Bodmer JL, Holler N, et al. BAFF,
754 a novel ligand of the tumor necrosis factor family, stimulates B cell growth. J Exp
755 Med. 1999;189(11):1747–56.
756 42. Abraham MJ, Murtola T, Schulz R, Páll S, Smith JC, Hess B, et al. Gromacs:
757 High performance molecular simulations through multi-level parallelism from
758 laptops to supercomputers. SoftwareX. 2015;1–2:19–25.
759 43. Best RB, Zhu X, Shim J, Lopes PEM, Mittal J, Feig M, et al. Optimization of the
760 Additive CHARMM All-Atom Protein Force Field Targeting Improved Sampling
761 of the Backbone ϕ, ψ and Side-Chain χ 1 and χ 2 Dihedral Angles. J Chem
762 Theory Comput [Internet]. 2012 Sep 11;8(9):3257–73. Available from:
763 https://pubs.acs.org/doi/10.1021/ct300400x
764 44. Bussi G, Donadio D, Parrinello M. Canonical sampling through velocity rescaling.
765 J Chem Phys. 2007;126(1).
766 45. Bowen RAR, Dowdell KC, Dale JK, Drake SK, Fleisher TA, Hortin GL, et al.
767 Elevated vitamin B 12 levels in autoimmune lymphoproliferative syndrome
768 attributable to elevated haptocorrin in lymphocytes. Clin Biochem.
769 2012;45(6):490–2.
770 46. Bodmer JL, Schneider P, Tschopp J. The molecular architecture of the TNF
771 superfamily. Trends Biochem Sci. 2002;27(1):19–26.
772 47. A. O' Reilly L, Tai L, Kruse EA, Grabow S, Fairlie D, Haynes NM, et al.
773 Membrane-bound but not Secreted Fas Ligand Is Essential for Fas-Induced
774 Apoptosis and Prevention of Autoimmunity and Cancer. Nature.
775 2009;461(1):695–663.

Journal Pre-proof

776 48. Suzuki I, Fink PJ. Maximal proliferation of cytotoxic T lymphocytes requires
777 reverse signaling through Fas ligand. J Exp Med. 1998;187(1):123–8.
778 49. Desbarats J, Duke RC, Newell MK. Newly discovered role for Fas ligand in the
779 cell-cycle arrest of CD4+ T cells. Nat Med. 1998;4(12):1377–82.
780 50. Sun M, Lee S, Karray S, Levi-Strauss M, Ames KT, Fink PJ. Cutting Edge: Two
781 Distinct Motifs within the Fas Ligand Tail Regulate Fas Ligand-Mediated
782 Costimulation. J Immunol. 2007;179(9):5639–43.
783 51. Paulsen M, Mathew B, Qian J, Lettau M, Kabelitz D, Janssen O. FasL cross-
784 linking inhibits activation of human peripheral T cells. Int Immunol.
785 2009;21(5):587–98.
786 52. Lückerath K, Kirkin V, Melzer IM, Thalheimer FB, Siele D, Milani W, et al.
787 Immune modulation by Fas ligand reverse signaling: Lymphocyte proliferation is
788 attenuated by the intracellular Fas ligand domain. Blood. 2011;117(2):519–29.
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792
793
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826 **Figure legends**

827 **Figure 1: Homozygous *FASLG* variants. A,** sFasL of 95 patients with heterozygous
828 *FAS* mutations (ALPS-FAS) and of 7/11 patients with homozygous *FASLG* mutations.
829 Grey area = mean+/-SD (50 healthy donors (HD)). **B,** FasL-mediated cytotoxicity.
830 L1210.FAS = target cells (T); patient T-cell blasts = effectors (E). Grey area = mean+/-
831 2SD (21 HD). **C,** Cytotoxicity assay using L1210 wild type as target cells. **D,**
832 Cytotoxicity assay using L1210 wild type as target cells and patient T-cell blasts after
833 CD3 crosslinking as effectors.

834

835 **Figure 2: Heterozygous *FASLG* variants. A,** sFasL of heterozygous individuals. **B,**
836 **C,** FasL-mediated cytotoxicity. L1210.FAS = target cells (T); patient T-cell blasts =
837 effectors (E). **D,** Cytotoxicity assay using L1210 wild type as target cells. **E,** Cytotoxicity
838 assay using L1210 wild type as target cells and patient T-cell blasts after CD3
839 crosslinking as effectors.

840

841 **Figure 3: Expression, trimerization, binding and lytic activity of recombinant**
842 ***FASLG* mutants. A,** anti-Flag Western blot of cell supernatants (top) and cell extracts
843 (middle and bottom) of HEK-293T cells transfected with Flag-*FASLG* mutants. **B,**
844 Binding of Flag-tagged FasL to immobilized Fas-Fc by ELISA. **C,** Overnight cytotoxicity
845 of Flag-FasL mutants on Jurkat cells. **D,** Binding of Flag-FasL mutants to Fc-Fas by
846 ELISA after SEC (top panel). Anti-Flag Western blot of SEC fractions (bottom panel).
847 **E,** Cytotoxicity as in C with mutants co-transfected with different ratios of Flag-*FASLG*-
848 WT. **F,** sFasL in supernatants of transfected HEK-293T cells detected by the same
849 ELISA Kit used in Figure 1A. **G,** Fas-Fc-specific depletion of sFasL in sera of
850 individuals carrying homozygous (bold) or heterozygous (italics) *FASLG* mutations and
851 from ALPS-FAS patients.

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852 **Figure 4: Surface expression, trimerization and Fas binding of mutant *FASLG***
853 **alleles. A,** Schematic representation of BAFF-N-FasL chimeric membrane-bound
854 proteins, with the BAFF portion shown in red, and the FasL portion shown in black and
855 brown. The epitope recognized by mAb Buffy1 to monitor surface expression, and the
856 binding site to Fas-Fc used to measure receptor-binding are indicated. **B,** HEK-293T
857 cells co-transfected with the indicated *BAFF-N-FASLG* mutants and an EGFP tracer
858 were stained with Buffy1 or Fas-Fc (Y axes, GMFI of PE, 10e2 to 10e7). **C and D,**
859 Same as panel B, but showing PE intensity of Buffy1 (C) or FAS-Fc (D) staining on
860 EGFP-high cells (5 x 10e5 to 3x 10e6) in technical triplicates, gating as shown in B.
861 Browne, Forsythe and Welch One-way ANOVA, not assuming equal standard
862 deviations. **E and F,** Same as panel C,D but after co-transfecting HEK-293T cells with
863 both mutant and WT proteins. **G and H,** Root mean square deviation (RMSD) of L181P
864 at the level of the trimers (G) and of the monomers forming the trimer (H). **I,**
865 Quantification of the hidden solvent accessible surface shown as an average between
866 the three subunits.

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Table I. Genetics, clinical features and ALPS biomarkers of patients with homozygous (bold) and heterozygous (italic) *FASLG* variants investigated in this study.

P	Mutation	Ref	LPR	AIC	onset	DNT (% of CD3)	Vit B12 (pg/ml)	Non-ALPS typical features
1	**A247E**	(33)	+	+	6 wk	25	>2000	
2	**F87fs*95**	(35)	+	-	birth	87	1890	
3	**G277S**	(38)	+	+	7 mo	21	>3000	
4	**G277S**	(38)	+	+	birth	7	n.a.	
5	**P69Afs*71**	(36)	+	-	n.a.	17	>2000	
6	**P69Afs*71**	(36)	+	+	3 mo	29	>2000	
7	**C202S**	(37)	+	+	8 mo	8	>2000	
8	**C202S**	(37)	+	+	n.a.	n.a.	Increased	
9	**L181P**	new	+	+	6 mo	16	>2000	
10	**V15Wfs*57**	new	+	n.a.	3 mo	33	4000	
11	**V15Wfs*57**	new	+	n.a.	3 mo	49	4000	
12	*R156G*	(34)	+	+	18 mo	1.1	n.a.	Pneumonitis, autoimmune hepatitis
13	*R156G*	(34)	+	-	n.a.	0.5	n.a.	Psoriatic arthritis
14	*R156G*	new	+	-	5y	1.3	1021	Recurrent sialadenitis
15	*R156G*	new	+	-	2y	1.3	504	Recurrent sialadenitis
16	*D158-185*	(32)	+	-	52y	n.a.	n.a.	SLE
17	*Y166C*	new	+	+	13y	2.2	1194	Hypogamma, Meningitis
18	*Q130E*	new	+	+	10y	4	824	
19	*S155L*	new	+	-	n.a.	2	n.a.	Tubular nephropathy
Mo P1	*A247E*	new	-	-	-	1.8	238	
Mo P9	*L181P*	new	-	-	-	1	372	
Fa P9	*L181P*	new	-	-	-	1.8	288	
Fa P10	*V15Wfs*57*	new	-	-	-	1.6	414	
Mo P17	*Y166C*	new	-	-	-	1.6	427	

LPR = chronic benign lymphoproliferation. AIC = autoimmune cytopenia. DNT = double

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negative T cells. Vit B12 = Vitamin B12. n.a. = not available. Normal range for DNT: <2.5% of CD3+ T cells; normal range for Vitamin B12: 182-1090 pg/ml.

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Figure 1

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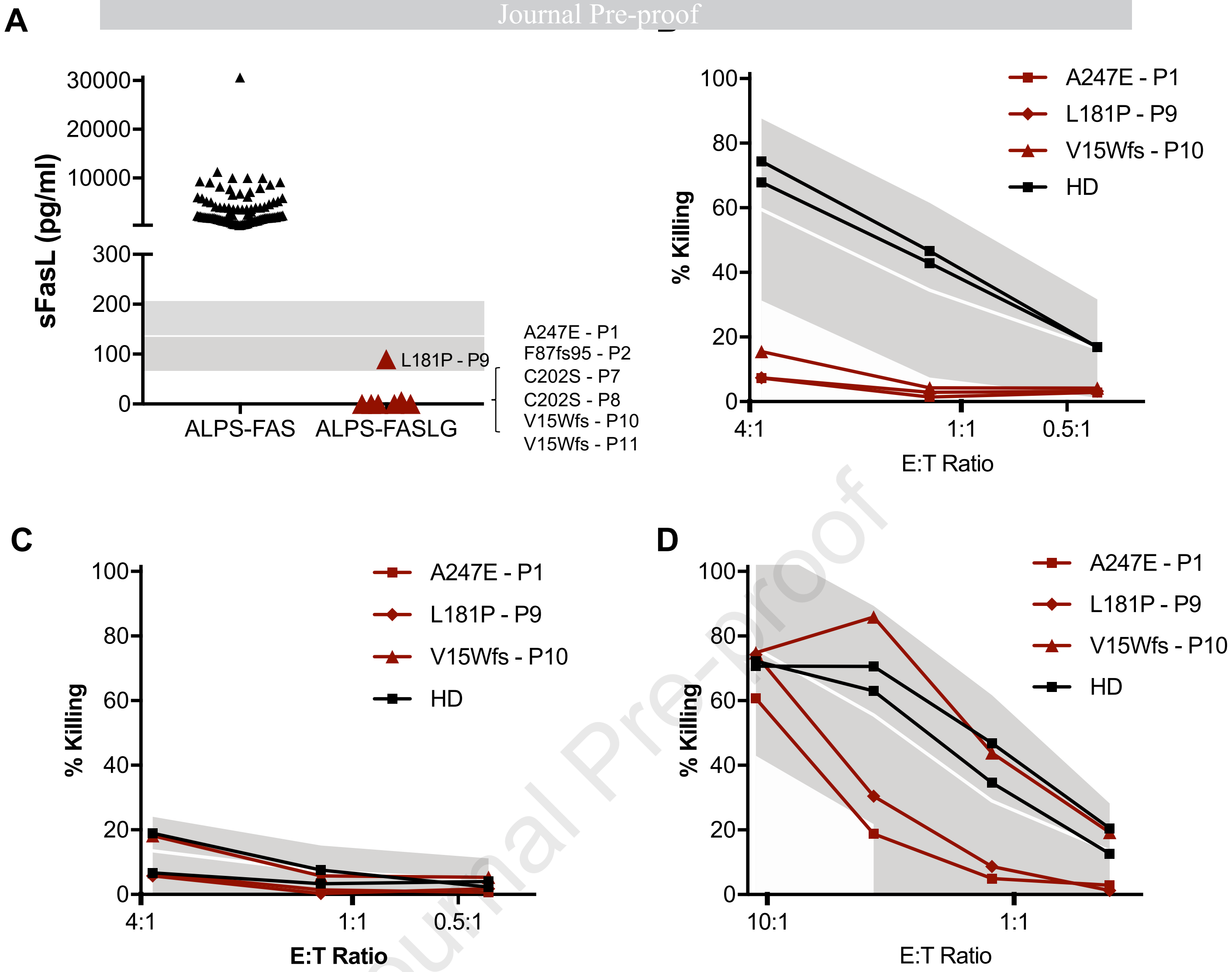

Figure 2

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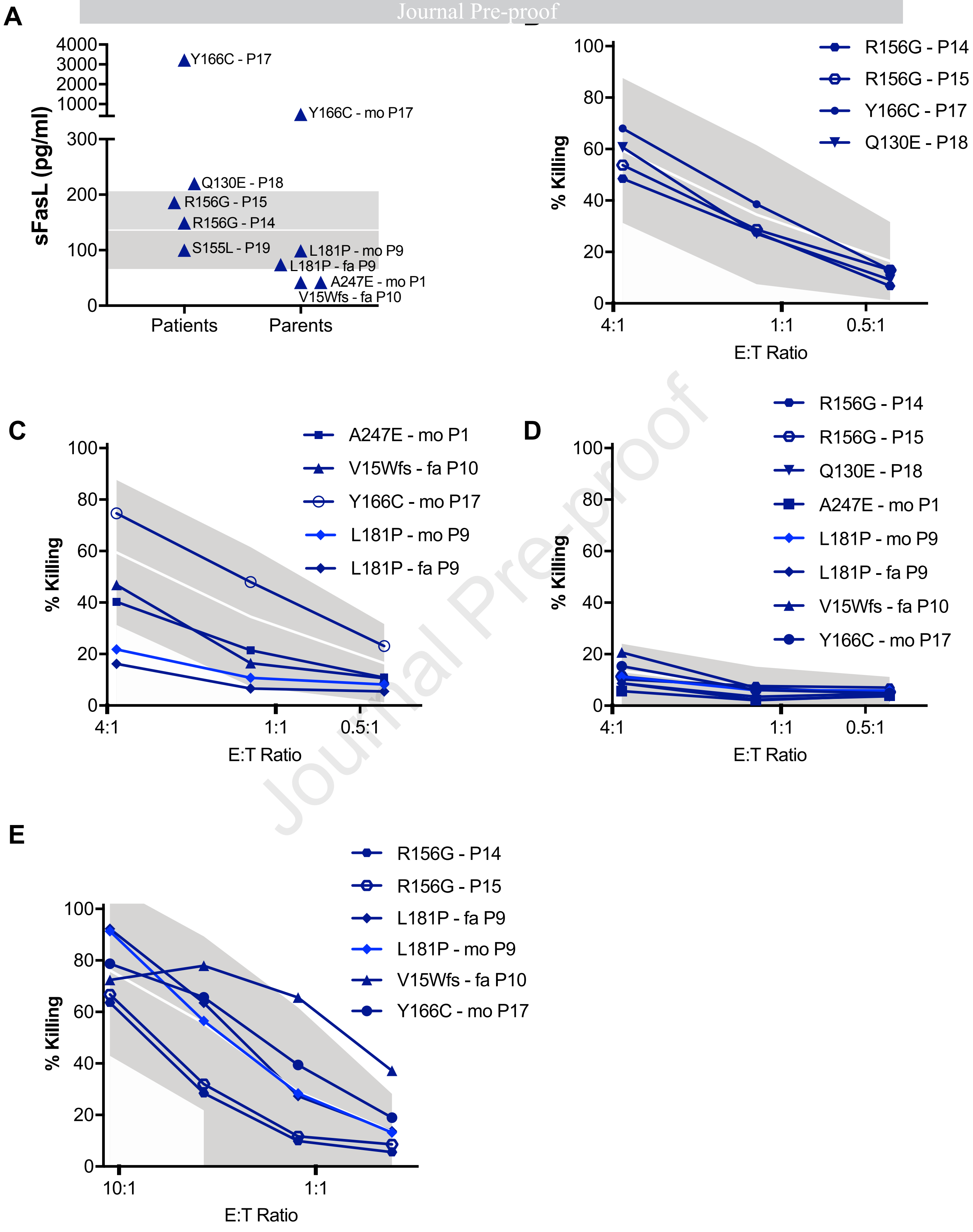

Figure 3

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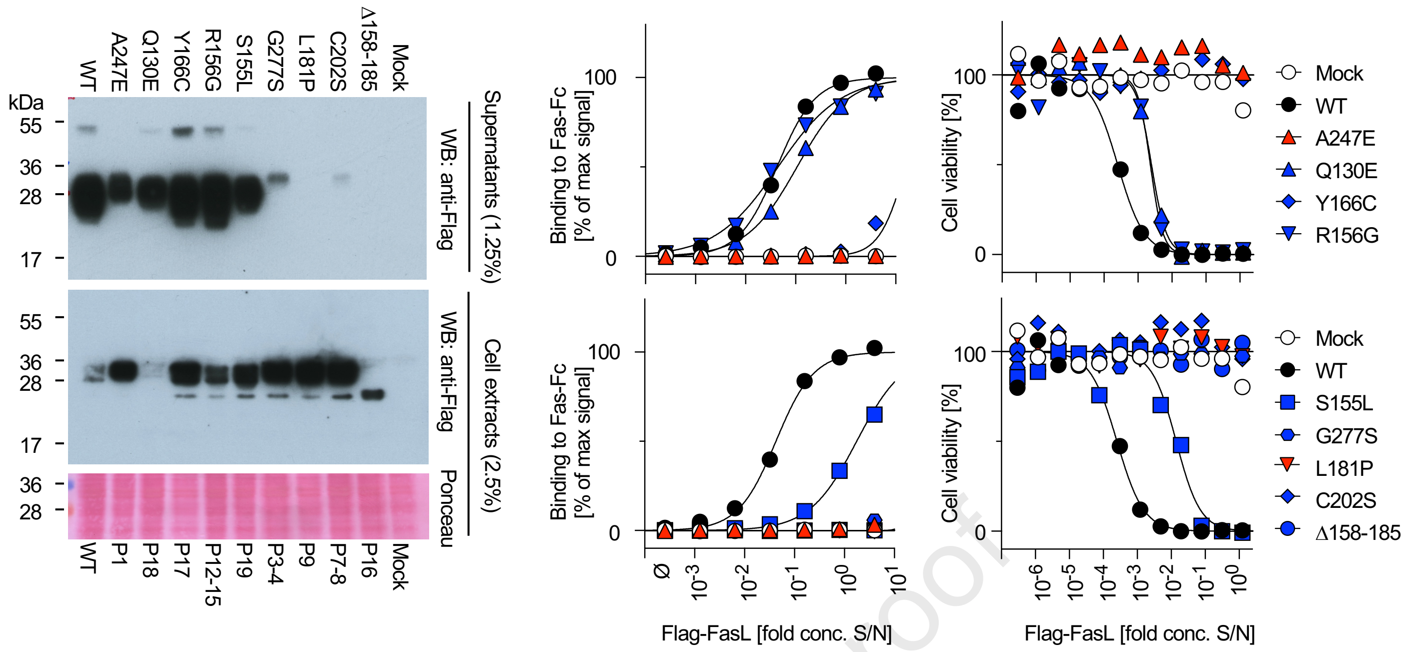

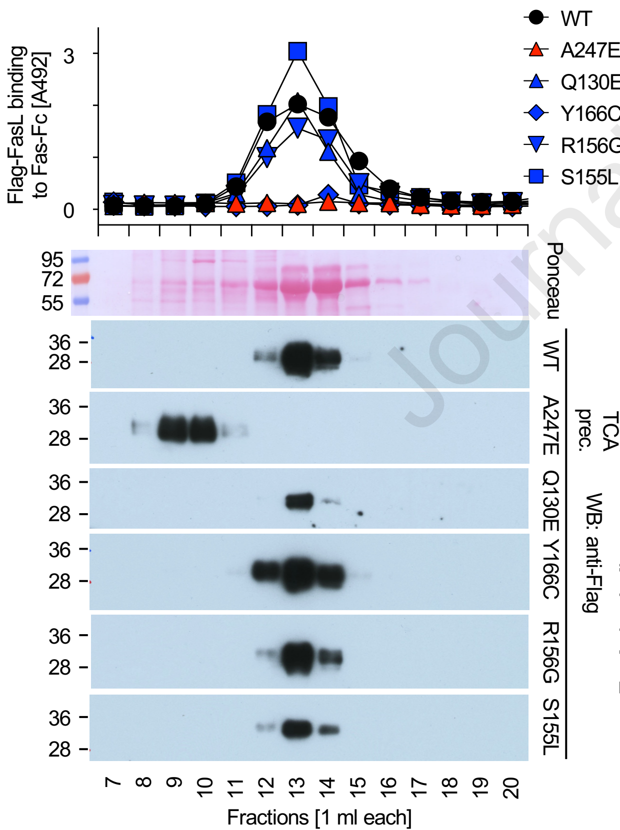

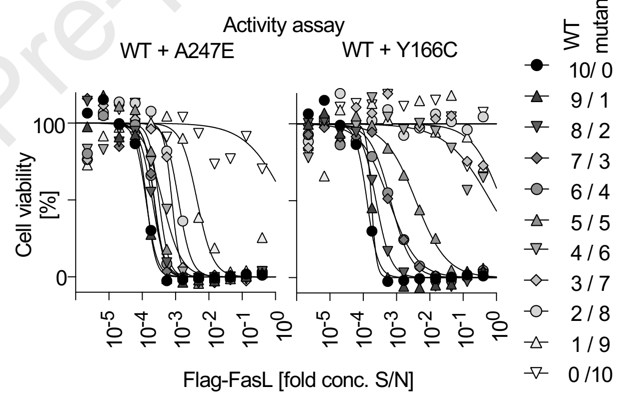

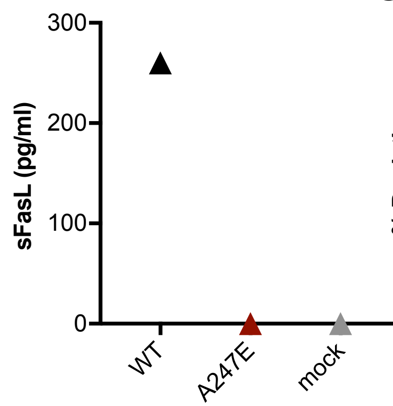

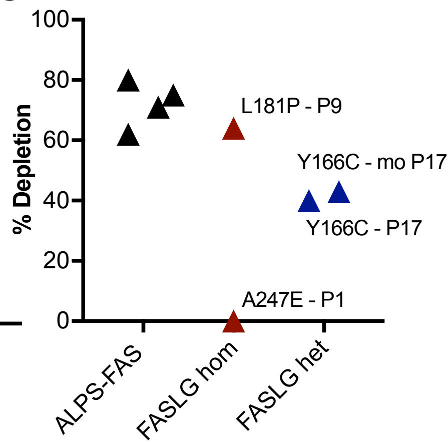

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Figure 4

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