

1 TITLE

2  
3 Autoantibody discovery across monogenic, acquired, and COVID19-associated autoimmunity  
4 with scalable PhIP-Seq

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150 ABSTRACT

151

152 Phage Immunoprecipitation-Sequencing (PhIP-Seq) allows for unbiased, proteome-wide  
153 autoantibody discovery across a variety of disease settings, with identification of disease-specific  
154 autoantigens providing new insight into previously poorly understood forms of immune  
155 dysregulation. Despite several successful implementations of PhIP-Seq for autoantigen  
156 discovery, including our previous work (Vazquez et al. 2020), current protocols are inherently  
157 difficult to scale to accommodate large cohorts of cases and importantly, healthy controls. Here,  
158 we develop and validate a high throughput extension of PhIP-seq in various etiologies of  
159 autoimmune and inflammatory diseases, including APS1, IPEX, RAG1/2 deficiency, Kawasaki  
160 Disease (KD), Multisystem Inflammatory Syndrome in Children (MIS-C), and finally, mild and  
161 severe forms of COVID19. We demonstrate that these scaled datasets enable machine-learning  
162 approaches that result in robust prediction of disease status, as well as the ability to detect both  
163 known and novel autoantigens, such as PDYN in APS1 patients, and intestinally expressed  
164 proteins BEST4 and BTNL8 in IPEX patients. Remarkably, BEST4 antibodies were also found in  
165 2 patients with RAG1/2 deficiency, one of whom had very early onset IBD. Scaled PhIP-Seq  
166 examination of both MIS-C and KD demonstrated rare, overlapping antigens, including CGNL1,  
167 as well as several strongly enriched putative pneumonia-associated antigens in severe COVID19,  
168 including the endosomal protein EEA1. Together, scaled PhIP-Seq provides a valuable tool for  
169 broadly assessing both rare and common autoantigen overlap between autoimmune diseases of  
170 varying origins and etiologies.

171

172 INTRODUCTION

173

174 Autoantibodies provide critical insight into autoimmunity by informing specific protein  
175 targets of an aberrant immune response and serving as predictors of disease. Previously,  
176 disease-associated autoantigens have been discovered through candidate-based approaches or  
177 by screening tissue specific expression libraries. By contrast, the development of proteome-wide  
178 screening approaches has enabled the systematic and unbiased discovery of autoantigens.

179 Two complementary approaches for proteome-wide autoantibody discovery include  
180 printed protein arrays and Phage-Immunoprecipitation Sequencing (PhIP-seq) (Jeong et al.,  
181 2012; Larman et al., 2011; Sharon and Snyder, 2014; Zhu et al., 2001). While powerful, printed  
182 protein arrays can be cost- and volume-prohibitive and are not flexible to adapting or generating  
183 new antigen libraries. On the other hand, PhIP-seq, originally developed by Larman *et al.*, uses  
184 the economy of scale of arrayed oligonucleotide synthesis to enable very large libraries at  
185 comparatively low cost (Larman et al., 2011). However, phage-based techniques have remained  
186 hindered by labor-intensive protocols that prevent broader accessibility and scaling.

187 Recently, we and others have adapted and applied PhIP-Seq to detect novel, disease  
188 associated autoantibodies targeting autoantigens across a variety of autoimmune contexts  
189 (Larman et al., 2011; Mandel-Brehm et al., 2019; O'Donovan et al., 2020; Vazquez et al., 2020).  
190 However, both technical and biological limitations exist. From a technical standpoint, PhIP-Seq  
191 libraries express programmed sets of linear peptides, and thus this technique is inherently less  
192 sensitive to detect reactivity to conformational antigens, such as Type I interferons (IFNs) and  
193 GAD65 (Vazquez et al., 2020; Wang et al., 2021). Nonetheless, the technique allows hundreds  
194 of thousands to millions of discrete peptide sequences to be represented in a deterministic  
195 manner, including the multiplicity of protein isoforms and variants that are known to exist *in vivo*.  
196 In this sense, PhIP-seq is complementary to mass spectrometry and other techniques that  
197 leverage fully native proteins. Given that many forms of autoimmunity exhibit significant

198 phenotypic heterogeneity, the true number of patients with shared disease-associated  
199 autoantibodies may be low (Ferre et al., 2016). Therefore, the screening of large cohorts is an  
200 essential step for identifying shared antigens and would benefit from a scaled PhIP-seq approach  
201 for high throughput testing.

202         Beyond the benefits of detecting low-frequency or low-sensitivity antigens, a scaled  
203 approach to PhIP-Seq would also facilitate increased size of healthy control cohorts. Recently,  
204 PhIP-Seq has been deployed to explore emerging forms of autoimmune and inflammatory  
205 disease, including COVID19-associated Multisystem Inflammatory Syndrome in Children (MIS-  
206 C) (Gruber et al., 2020). However, these studies suffer from a relative paucity of control samples,  
207 resulting in low confidence in the disease-specificity of the suggested autoantigens. Questions of  
208 disease-specificity, rare antigens, and antigen overlap can be addressed in larger, scaled  
209 experiments.

210         Here, we develop a high-throughput PhIP-Seq protocol. We demonstrate the utility of this  
211 protocol in the context of an expanded, multi-cohort study, including APS1, patients with Immune  
212 dysregulation, polyendocrinopathy, X-linked (IPEX) or RAG1/2 deficiency with autoimmune  
213 features, a KD patient cohort, and emerging COVID19 patient phenotypes with possible  
214 autoimmune underpinnings. In the future, scaled PhIP-Seq cohort studies could be used across  
215 additional syndromic and sporadic autoimmune diseases to develop an atlas of linear B cell  
216 autoantigens.

217

## 218 RESULTS & DISCUSSION

219

### 220 **Design and Implementation of a scaled PhIP-Seq protocol**

221 The ability to process large numbers of patient samples for PhIP-Seq in a highly uniform  
222 manner has several important benefits, including reduction of batch effects between samples from  
223 the same cohorts as well as between disease and control cohorts, detection of lower-frequency  
224 autoantigens, and the ability to simultaneously include large numbers of control samples.

225 In creating a scaled protocol, we searched for a method that would allow 600-800 samples  
226 to be run fully in parallel to reduce any batch or plate-to-plate variability. Thus, each wash or  
227 transfer step needed to be performed in rapid succession for all plates. We also prioritized  
228 reduction of any well-to-well contamination, particularly given that small, early contamination can  
229 amplify across subsequent rounds of immunoprecipitation. Finally, we required our protocol to  
230 minimize consumable waste and maximize benchtop accessibility without robotics or other  
231 specialized equipment.

232 A benchtop vacuum-based protocol (rapid, consistent wash times) in deep-well 96-well  
233 filter plates with single-use foil seals (no well-to-well contamination) met our requirements (see  
234 schematic in Figure 1A). The data for APS1 samples on our moderate-throughput manual  
235 multichannel protocols were closely correlated with vacuum-based output, including identification  
236 of previously validated antigens within each sample (Figure 1B). Additional protocol  
237 improvements included: 3-D printing template for vacuum plate adaptors (for easy centrifugation  
238 and incubation steps); direct addition of protein A/G beads to *E. coli* lysis without a preceding  
239 elution step; shortened lysis step by using square-well plates with semi-permeable membrane for  
240 aeration; and options for smaller volume and decreased reagent library preparation in both 96-  
241 well and 384-well formats. A detailed protocol, including custom part designs, for both high-  
242 throughput vacuum-based and moderate throughput multichannel-based protocols is available at  
243 [protocols.io](https://protocols.io).

244

## 245 **Large control cohorts are critical for identifying disease-specific autoantibodies**

246       Some assays for autoantigen discovery, such as protein arrays, are often used as a  
247 quantitative measure of how autoreactive an individual serum sample may be. In contrast, PhIP-  
248 Seq is an enrichment-based assay in which binders are serially enriched and amplified. A practical  
249 limitation of this technique is that non-specific phage may also be amplified, in addition to a wide  
250 array of autoreactive, but non-disease related peptides. We tested whether we could detect global  
251 differences between case and control cohorts as a measure of autoreactivity. As each APS1  
252 patient is known to have multiple, high-affinity antibodies to self-proteins (Fishman et al., 2017;  
253 Landegren et al., 2016; Meyer et al., 2016; Vazquez et al., 2020) we reasoned that this would be  
254 an ideal cohort to determine whether a global autoreactive state was discernible. As expected,  
255 each individual sample exhibits a spectrum of enriched genes, regardless of disease status  
256 (Figure 2A), indicating that measures of simple enrichment are inadequate for discrimination of  
257 cases from controls.

258       We and others have shown that PhIP-Seq can robustly detect disease-associated  
259 antigens by comparing antigen-specific signal between disease and control cohorts (Larman et  
260 al., 2011; Mandel-Brehm et al., 2019; O'Donovan et al., 2020; Vazquez et al., 2020). In this  
261 dataset, encompassing 186 control samples and 128 APS1 samples, we further evaluated the  
262 importance of control cohort size. We iteratively down sampled the number of healthy control  
263 samples in our dataset to 5, 10, 25, 50, 100, or 150 (out of n=186 total control samples). The  
264 number of apparent hits was determined in each condition, where a gene-level hit was called  
265 when the following criteria were met: 1) at least 10% of APS1 samples and less than 2 control  
266 samples with a Z-score > 10, 2) no control sample exhibiting higher signal than the highest patient  
267 signal, and 3) a minimum of one strong positive patient sample (50-fold enrichment over mock-  
268 IP). Genes that failed to meet these conditions were considered non-specific. Using these  
269 conservative criteria, a control set of 10 samples resulted in (on average) 404 apparent hits, while

270 increasing the control set to 50 samples removed an additional 388 non-specific hits, leaving 16  
271 apparent hits (Figure 2B). Further increasing the number of control samples to 150 samples had  
272 diminishing returns, although 4-5 more autoantigen candidates were removed as being non-  
273 specific, reducing the frequency of false positives, and ultimately leaving only ~1% of the original  
274 candidate list for further investigation. In sum, to improve downstream analysis aimed at detecting  
275 disease-associated hits, PhIP-Seq experimental design should include a large and appropriate  
276 number of non-disease controls.

277

### 278 **Scaled PhIP-Seq replicates and expands autoantigen repertoires across multiple** 279 **independent cohorts of APS1**

280 We previously identified and validated PhIP-Seq hits based on shared positivity of a given  
281 hit between 3 (out of 39) or more patients with APS1 (Vazquez et al., 2020). While this enabled  
282 us to robustly detect frequently shared antigens within a small disease cohort, antigens with lower  
283 frequencies – or with low detection sensitivity – would likely fall below this conservative detection  
284 threshold.

285 To improve both sensitivity and specificity, we utilized scaled PhIP-Seq to explore  
286 expanded cohorts of disease, including a much larger (n=128) APS1 cohort spanning 2 North  
287 American cohorts and 1 Swedish cohort. All hits present in at least 10% of APS1 patients also  
288 spanned all 3 cohorts, thus further validating broad prevalence of antigens that were previously  
289 described by us (RFX6, ACPT, TRIM50, CROCC2, GIP, NKX6-3, KHDC3L) and others (SOX10,  
290 LCN1) (Figure 2C) (Fishman et al., 2017; Hedstrand et al., 2001; Vazquez et al., 2020). At the  
291 gene level, we detected 39 candidate hits that were present in  $\frac{36}{128}$  (>4%) of APS1 and in 2 or  
292 fewer controls ( $\frac{2}{186}$ , 1%) (Figure 3A & 3B). As expected, the larger cohort yielded new  
293 candidate antigens that had not been detected previously. For example, prodynorphin (PDYN) is  
294 a secreted opioid peptide thought to be involved in regulation of addiction-related behaviors  
295 (*reviewed in* (Fricker et al., 2020)). PDYN was enriched by  $\frac{6}{133}$  (4.5%) of patient samples and

296 subsequently was validated by RLBA (Figure 3C). Indeed, this validated antigen was present in  
297 our previous investigations (Vazquez et al., 2020), however, it was enriched in too few samples  
298 to qualify for follow up.

299 Other notable hits with relevant tissue-restricted expression were also observed. For  
300 example, SPAG17 is closely related to the known APS1 autoantigen SPAG16 and is expressed  
301 primarily in male germ cells and in the lung, with murine genetic mutations resulting in ciliary  
302 dyskinesia with pulmonary phenotypes (Fishman et al., 2017; Teves et al., 2013). Also potentially  
303 related to ciliary and/or pulmonary autoimmunity is CROCC2/Rootletin, a protein expressed in  
304 ciliated cells, which we previously observed, and now recognize at a high frequency across  
305 multiple cohorts (Uhlen et al., 2015; Yang et al., 2002). Similarly, GAS2L2 is a ciliary protein  
306 expressed in the airway, with genetic loss of function in mice resulting in impaired mucociliary  
307 clearance, and clustered closely with CROCC2 (Bustamante-Marin et al., 2019; Uhlen et al.,  
308 2015) in this dataset. These novel putative antigens together hint at potential mucociliary airway  
309 autoreactivity. CT45A10 and GPR64 are both proteins with expression restricted primarily to male  
310 gonadal tissues (Uhlen et al., 2015). GABRR1 is a GABA receptor expressed primarily in the  
311 central nervous system as well as on platelets (Ge et al., 2006; Zhu et al., 2019) and TRIM2 is  
312 implicated in genetic disorders of demyelination within the peripheral nervous system, and  
313 therefore may be of interest to the chronic inflammatory demyelinating polyneuropathy (CIDP)  
314 phenotype that can be seen in some patients with APS1 (Li et al., 2020; Valenzise et al., 2017).  
315 In addition to our previously described intestinally expressed autoantigens RFX6, NKX6-3, and  
316 GIP, we also identify CDHR5, a transmembrane cadherin-family protein expressed on the  
317 enterocyte cell surface, as a putative autoantigen in APS1 (Crawley et al., 2014; Uhlen et al.,  
318 2015).

319

320 **APS1 disease prediction by machine learning**

321 APS1 is a clinically heterogeneous disease, and it is also heterogeneous with respect to  
322 autoantibodies (Ferre et al., 2016; Fishman et al., 2017; Landegren et al., 2016; Meyer et al.,  
323 2016; Vazquez et al., 2020). Because PhIP-Seq simultaneously interrogates autoreactivity to  
324 hundreds of thousands of peptides, we hypothesized that unsupervised machine learning  
325 techniques could be used to create a classifier that would distinguish APS1 cases from healthy  
326 controls. We applied a simple logistic regression classifier to our full gene-level APS1 (n=128)  
327 and control (n=186) datasets, resulting in excellent prediction of disease status (AUC = 0.95,  
328 Figure 4A) using 5-fold cross validation. Moreover, we found that the classification model was  
329 driven strongly by many of the previously identified autoantigens, including RFX6, KHDC3L, and  
330 others (Figure 4A), in addition to some targets that had not been previously examined (Vazquez  
331 et al., 2020). These results demonstrate that PhIP-Seq autoreactive antigen enrichment profiles  
332 are amenable to machine learning techniques, and further suggest that such data could be used  
333 to derive diagnostic signatures with strong clinical predictive value.

334

### 335 **Autoantibody discovery in IPEX**

336 IPEX syndrome is characterized by defective peripheral immune tolerance secondary to  
337 impaired T regulatory cell (Treg) function. In IPEX, peripheral tolerance rather than central  
338 tolerance is impaired, resulting in a phenotypic constellation of autoimmunity that partially  
339 overlaps with APS1 (Bacchetta et al., 2006; Powell et al., 1982). Notably, the majority of IPEX  
340 patients exhibit severe enteropathy, with early-onset severe diarrhea and failure to thrive, with  
341 many of these children harboring anti-enterocyte antibodies detected by indirect  
342 immunofluorescence (Bacchetta et al., 2006; Gambineri et al., 2018; Powell et al., 1982). We  
343 hypothesized that the same PhIP-Seq approach that was successful for APS1 would also yield  
344 informative hits for IPEX. A total of 27 patient samples were analyzed using scaled PhIP-Seq and  
345 the data processed in the same manner as for APS1.

346 A handful of IPEX autoantibodies targeting antigens expressed in the intestine have been  
347 described, including harmonin (USH1C) and ANKS4B (Eriksson et al., 2019; Kobayashi et al.,  
348 2011). In our data, enrichment of USH1C was observed in 2 IPEX patients, and this signal was  
349 fully correlated with anti-ANKS5B as previously described (Figure 5 – Figure Supplement 1)  
350 (Eriksson et al., 2019).

351 Several novel putative autoantigens were observed that were shared between 3 or more  
352 IPEX patients (Figure 5A). Among these were several with expression restricted to the intestine,  
353 including BEST4, a protein expressed by a specific subset of enterocytes, BTNL8, a butyrophilin-  
354 like molecule highly expressed in the gut epithelium, ST6GALNAC1, and ITGA4 (Figure 5A)  
355 (Mayassi et al., 2019; Schaum et al., 2018; Uhlen et al., 2015). BEST4 and BTNL8 were selected  
356 for validation by whole protein immunoprecipitation. A total of 4/26 (15%) of IPEX patients were  
357 positive for anti-BEST4 autoantibodies (Figure 5B). In the case of BTNL8, orthogonal validation  
358 identified 11/26 (42%) of IPEX patients who were positive for anti-BTNL8 antibodies (Figure 5B).  
359 Of note, all patients with anti-BTNL8 and/or BEST4 antibodies also had clinical evidence of  
360 enteropathy (Supplemental Table 1). Taken together, these results suggest that anti-BEST4 and  
361 anti-BTNL8 are associated with IPEX enteropathy.

362

### 363 **Overlap of intestinal autoantigen BEST4 in the setting of hypomorphic RAG1/2 mutations**

364 Hypomorphic *RAG1/2* mutations represent an additional and notoriously phenotypically  
365 heterogeneous form of monogenic immune dysregulation. Absent RAG complex activity leads to  
366 lack of peripheral T and B cells, therefore causing severe combined immunodeficiency (SCID).  
367 However, patients with hypomorphic *RAG1/2* have residual capacity to generate T and B cells.  
368 Depending on the severity of the defect, these patients can present with Omenn Syndrome (OS),  
369 Atypical SCID (AS), or Combined Immune Deficiency with Granulomas and Autoimmunity (CID-  
370 G/AI) (Delmonte et al., 2018, 2020). Autoimmune manifestations are particularly common in  
371 patients with AS and CID-G/AI. Cytopenias are the most frequent form of autoimmunity in patients

372 with RAG deficiency, but cutaneous, neuromuscular and intestinal manifestations have been also  
373 reported. While anti-cytokine antibodies have been described, other disease-associated  
374 autoantibody targets remain to be identified (Delmonte et al., 2020).

375         62 patients with hypomorphic RAG1/2 mutations were screened by PhIP-seq to assess  
376 for overlap with APS1 and IPEX antigens, as well as for novel autoantigen specificities. Minimal  
377 overlap was observed between RAG1/2 deficiency and APS1 and IPEX. However, two samples  
378 from RAG1/2 patients indicated the presence of anti-BEST4 antibodies (Figure 5C), which were  
379 confirmed through orthogonal validation using whole protein (Figure 5D). Both positive patients  
380 had CID-G/AI, indicating the presence of autoimmune features. Remarkably – given that  
381 enteropathy in the setting of hypomorphic RAG1/2 deficiency is rare – one of the two individuals  
382 harboring anti-BEST4 antibodies also had very early onset inflammatory bowel disease (VEO-  
383 IBD).

384         Several other putative antigens amongst the larger RAG1/2 deficiency cohort were  
385 revealed. Nearly half of the cohort sera were enriched for peptides derived from ZNF365 (Suppl.  
386 Figure 2B). ZNF365 is a protein associated with multiple autoimmune diseases, as evidenced by  
387 GWAS studies showing that variants in ZNF365 are associated with Crohn's disease and  
388 autoimmune uveitis (Haritunians et al., 2011; Hou et al., 2020). Many patients also had evidence  
389 of autoantibodies targeting REL2, a TNF receptor binding partner, and CEACAM3, a phagocyte  
390 receptor that recognizes human specific pathogens and is important for opsonin-independent  
391 phagocytosis of bacteria (Bonsignore et al., 2020; Moua et al., 2017). Autoantibodies targeting  
392 these antigens could potentially play a role in the autoinflammation seen in certain cases of  
393 hypomorphic *RAG1/2* and/or increased susceptibility to particular infections and will require  
394 additional future follow-up.

395

396 **PhIP-Seq identifies rare, shared candidate autoantigens in MIS-C**

397 MIS-C leads to critical illness in ~70% of affected children and shares some common  
398 clinical features with KD, the most common cause of acquired pediatric heart disease in the US.  
399 Despite hints for a role of abnormal adaptive immunity and autoantibodies in the pathogenesis of  
400 KD and MIS-C, the etiologies of both diseases remain enigmatic (Feldstein et al., 2020;  
401 Newburger et al., 2016). Recently, PhIP-Seq has been deployed to explore COVID19-associated  
402 MIS-C (Gruber et al., 2020). However, this study included only 4 healthy controls and 9 MIS-C  
403 patients, and as our results have shown, removal of false-positive PhIP-seq hits requires the use  
404 of substantial numbers of unaffected controls (Figure 2C). Furthermore, these previously  
405 published hits lacked orthogonal validation. Therefore, we sought to examine a MIS-C cohort in  
406 light of these results, as well as to explore for possible autoantibody overlap between KD and  
407 MIS-C.

408 First, 20 MIS-C subjects, 20 pediatric febrile controls, and 20 COVID-19 controls were  
409 examined by PhIP-Seq, each of which were compared to a cohort of adult healthy controls (n=87).  
410 No evidence for specific enrichment was observed for any of the previously reported candidate  
411 antigens that overlapped with our PhIP-Seq library (Figure 6A). Methodologic and sample  
412 differences could, in part, account for differences in our results; however, these results suggest  
413 that PhIP-Seq hits should be subjected to external replication and/or validation. Additionally, MIS-  
414 C patients are treated with intravenous immunoglobulin (IVIG), which has abundant low affinity,  
415 low avidity autoantibodies, so caution must be exercised in the timing of sample collection  
416 (Burbelo et al., 2021; Sacco et al.). While it remains unclear whether high-affinity, novel  
417 autoantibodies are likely to be present in IVIG, the majority of our MIS-C samples are confirmed  
418 to be collected pre-IVIG. Though several of our samples are of unknown IVIG status, each of the  
419 candidate autoantibodies discussed below are present in at least one sample known to be IVIG-  
420 free.

421 Analysis of our MIS-C cohort for shared candidate hits yielded only 3 candidate hits, each  
422 in 2/20 patient sera. These were CD34, RPS6KB1, and CAPZB (Figure 6B). While these targets

423 may be of interest, disease-association remains uncertain. These results suggest that a much  
424 larger MIS-C cohort, controlled by an equally large set of healthy controls, will be required to  
425 detect rare, shared antigens with confidence by PhIP-Seq.

426

#### 427 **PhIP-Seq screen of a cohort of KD patients**

428 To screen for possible KD-specific autoantibodies, we analyzed a large cohort of 70 KD  
429 subjects by PhIP-Seq. KD patients are also often treated with IVIG, so care was taken to ensure  
430 that each of these samples were collected prior to IVIG-administration. Using the same hit  
431 selection criteria as previously, we detected 25 shared hits among 3 or more of the 70 KD  
432 samples, which were specific to KD relative to adult healthy controls (Figure 6C). Of these 25  
433 shared KD hits, 17 were absent from additional control groups including the febrile pediatric  
434 patients. Each of these hits was present in only a small subset of KD samples, suggesting  
435 significant heterogeneity among samples.

436 Some of the candidate antigens have possible connections to the systemic inflammation  
437 seen in KD, including SPATA2 (6/70, 8.6%), and ALOX5B (4/70, 5.7%). SPATA2 is a protein  
438 known to regulate the TNF receptor response, with murine knockout of SPATA2 resulting in  
439 increased activation of NFkB and MAPK (Schlicher and Maurer, 2016). Similarly, ALOX15 family  
440 of lipoxygenases is known to be responsive to Th2-induced anti-inflammatory cytokine IL-4 and  
441 IL-13 in human macrophages and thereby likely plays a role in suppressing inflammatory  
442 responses, and polymorphisms have been linked to the development of coronary artery disease  
443 (Snodgrass and Brüne, 2019; Wuest et al., 2014).

444 Other KD candidate autoantigens exhibited tissue expression patterns that suggest  
445 possible relationships to sub-phenotypes of KD. For example, pancreatitis and psoriasis have  
446 been reported as rare complications of KD (Asano et al., 2005; Haddock et al., 2016). We found  
447 that 7/70 of the KD patients (10%) have increased signal for autoantibodies targeting FBXL19, a

448 protein with associations with both acute pancreatitis (Ma et al., 2020) and psoriasis (Stuart et al.,  
449 2010); and 10/70 for pancreas-expressed protein RESP18 (Zhang et al., 2007).

450 Taken together, our dataset did not uncover the presence of common autoantibodies in  
451 KD or MIS-C. Nonetheless, our findings leave open the possibility of lower frequency disease-  
452 associated autoantibodies, and future studies with increased cohort scaling, diverse  
453 representation of clinical subphenotypes, and high-sensitivity follow-up assays may shed further  
454 light on the role of autoantibodies in KD.

455

#### 456 **Autoantigen overlap between KD and MIS-C**

457 Given the partial clinical overlap of KD with MIS-C, we also searched for the low frequency,  
458 shared KD hits within MIS-C and found that 6 of the 17 KD hits were present in one or more MIS-  
459 C samples (Figure 6D). Of these, CGNL1 was of particular interest given the very high enrichment  
460 values in patients and absence of signal from all controls, as well as its anatomic expression  
461 pattern. CGNL1 is an endothelial junction protein and is highly expressed in the cardiac  
462 endothelium (Chrifi et al., 2017; Schaum et al., 2018). We confirmed anti-CGNL1 autoantibodies  
463 using a radioligand binding assay with whole CGNL1 protein (Figure 6D). We also identified an  
464 additional positive KD patient that had not been detected by PhIP-Seq. These data suggest that  
465 anti-CGNL1 antibodies, while rare, may be associated with KD and/or MIS-C.

466

#### 467 **Application of scaled PhIP-Seq to severe COVID-19**

468 Recently, it was reported that over 10% of severe COVID-19 pneumonia is characterized  
469 by the presence of anti-Type I IFN autoantibodies, a specificity that overlaps with APS1 (Bastard  
470 et al., 2020, 2021a, 2021b; Meager et al., 2006; Meyer et al., 2016; Wijst et al., 2021). We  
471 therefore looked for possible overlap of additional antigens between APS1 and COVID-19. As  
472 expected, we had low sensitivity for the known anti-type I IFN autoantibodies by PhIP-Seq, likely  
473 due to the conformational nature of these antigens; however, this cohort had been previously

474 assessed for anti-Type I IFN antibodies by other techniques (Bastard et al., 2020, 2021c). We did  
475 not detect substantial overlap of any of the other antigens that were found in 5% or more of APS1  
476 samples, suggesting that autoantibody commonalities between APS1 and severe COVID-19 may  
477 be limited (Figure 7A).

478 PhIP-Seq was then used to investigate patients with severe to critical COVID-19  
479 pneumonia (n=100) relative to patients with mild to moderate COVID19 (n=55) and non-COVID-  
480 19 healthy controls (n=125) (Figure 7A). A small number of putative autoantigens were identified  
481 in 4 or more of the severe COVID-19 group (>4%), but not within our control group, including  
482 MCAM, CHRM5, and EEA1. Of these, only EEA1, an early endosomal protein, had a frequency  
483 of >10% (Mu et al., 1995). Notably, the set of anti-EEA1 positive patients was almost fully distinct  
484 from the anti-Type I IFN antibody positive group, with only one patient shared between the groups  
485 (Figure 7B). Given the importance of the finding of autoantibodies to type 1 IFNs in >15% of critical  
486 COVID-19 pneumonia, our data suggest that anti-EEA1 antibodies may also have predictive  
487 potential worth further investigation.

488

## 489 **DISCUSSION**

490

491           PhIP-Seq is a powerful tool for antigen discovery due to its throughput and scalability.  
492 Continually declining costs in sequencing paired with scaled protocols such as the ones presented  
493 here result in a low per-sample cost and experimental time, and declining costs of custom  
494 oligonucleotide based-libraries allow for extensive adaptation (Román-Meléndez et al., 2021;  
495 Vogl et al., 2021). Yet, as the technology is relatively new, increased discussion around best  
496 practices in experimental design, methodology, and data interpretation would be beneficial. In this  
497 work, we contribute to these efforts by presenting an accessible, carefully documented scaled lab  
498 protocol. We anticipate that scaling and low cost will facilitate cross-platform comparisons of  
499 autoimmune sera, enabling a more systematic understanding of each platform's abilities and  
500 shortcomings, as well as the best approaches to combine platforms for comprehensive  
501 autoantibody profiling, including fixed protein arrays, yeast display (REAP), and PhIP-Seq (Wang  
502 et al., 2021).

503           Our scaled data reinforces the importance of including large control cohorts to improve  
504 specificity and orthogonal validations to evaluate sensitivity. The absence of appropriately sized  
505 control cohorts can result in false-positive associations between enriched protein sequences and  
506 disease which can lead to misinterpretation. Furthermore, PhIP-Seq is optimized for linear  
507 antigens and thus is inherently less robust for detection of conformational or modified epitopes.  
508 For example, our previous work highlights that known anti-IFN and anti-GAD65 antibodies can be  
509 detected only in a handful of APS1 samples by PhIP-Seq, while orthogonal assays using whole  
510 conformation protein demonstrate increased sensitivity (Vazquez et al., 2020). Similarly, the  
511 increased sensitivity of whole protein validation relative to PhIP-Seq for BTNL8 autoantibodies  
512 highlights the need for rapid and sensitive secondary assays to confirm or even increase the  
513 importance of a given candidate antigen. These principles may be extended to other related PhIP-  
514 Seq modalities (Mina et al., 2019; Román-Meléndez et al., 2021; Vogl et al., 2021).

515           Several novel APS1 antigens were identified in this work by virtue of the large cohort size  
516 and large control set, enabling the detection of low-frequency hits such as PDYN. PDYN is a  
517 secreted opioid precursor of the central nervous system, and while PDYN-knockout mice are  
518 largely phenotypically normal, they do experience hyperalgesia and altered anxiety-related  
519 behaviors relative to wildtype mice (McLaughlin et al., 2003; Sharifi et al., 2001; Wang et al.,  
520 2001). Future studies will be required to determine if anti-PDYN antibodies may themselves  
521 mediate these phenotypes. In addition to uncovering lower frequency autoantigens, antigen  
522 overlap between APS1 and other syndromic autoimmune diseases, including IPEX and RAG1/2  
523 deficiency, were evaluated. Detection of common APS1 antigens, such as RFX6 and KHDCL3L,  
524 was rare among other disease contexts, suggesting APS1 displays limited sharing with respect  
525 to other multiorgan autoimmune syndromes, including IPEX and Rag deficiency.

526           In contrast, antigen overlap between IPEX and RAG-hypomorphic patients was detected  
527 in the form of anti-BEST4 antibodies. BEST4 is a well-characterized, cell-surface intestinal ion  
528 channel (Qu and Hartzell, 2008). Recently BEST4 has become a standard and specific marker  
529 for a subset of enterocytes in the duodenum and colon, including CFTR+ enterocytes, which are  
530 involved in fluid homeostasis (Busslinger et al., 2021; Elmentaite et al., 2021; Smillie et al., 2019).  
531 The presence of an IPEX antigen BEST4 within CID-G/Al suggests a possible etiologic link  
532 between the two diseases, possibly relating to Treg dysfunction. Furthermore, given that BEST4  
533 autoantibodies were found across 2 distinct etiologies of IBD, in addition to the intestinal  
534 localization of BEST4 expression, future experiments specifically searching for the presence of  
535 anti-BEST4 antibodies in IBD and other forms of autoimmune enteropathy are warranted.

536           Another novel IPEX antigen, BTNL8, is also of particular interest given its high frequency  
537 in IPEX (11/26) as well as its biological functions. BTN and BTNL-family members belong to the  
538 family of B7 co-stimulatory receptors known to modulate T-cell responses, with structural similarity  
539 to CD80 and PD-L1 (Chapoval et al., 2013; Rhodes et al., 2015). Broadly, BTNL-family members  
540 are thought to participate primarily in regulation of gamma-delta T cells, with BTNL8/BTNL3

541 implicated in gut epithelial immune homeostasis (Barros et al., 2016; Chapoval et al., 2013). Given  
542 the cell-surface expression pattern of BTNL8, it is conceivable that antibodies to these proteins  
543 could play a functional role in immune checkpoints, rather than only the bystander role implicated  
544 for most autoantibodies to intracellular antigens. Interestingly, recent studies in patients with  
545 celiac disease have demonstrated a reduction in BTNL3/BTNL8 expression following acute  
546 episodes of inflammation, with an associated loss of the physiological normal gamma/delta T-cell  
547 subset of gut intraepithelial lymphocytes (Mayassi et al., 2019).

548         While some disease cohorts yield strongly enriched common antigens, such as BTNL8  
549 and BEST4, other cohorts appear heterogeneous. Such was the case for MIS-C and KD, where  
550 our results suggested only rare, shared putative antigens. Nonetheless, a number of observations  
551 point to a possible role for abnormal adaptive immunity and autoantibodies (reviewed in (Sakurai,  
552 2019)). Both diseases respond to varying degrees to IVIG therapy, and genetic studies suggest  
553 some children with polymorphisms in genes involving B-cell and antibody function can be  
554 predisposed to developing KD (reviewed in (Onouchi, 2018)). Autoantibodies targeting clinically  
555 relevant tissues such as the heart or endothelium have been detected in KD, though their role in  
556 disease pathogenesis is uncertain (Cunningham et al., 1999; Fujieda et al., 1997; Grunebaum et  
557 al., 2002). Although anti-CGNL1 antibodies were only found at low frequency, it is possible that  
558 these antibodies may represent a subset of specificities within anti-endothelial cell antibodies,  
559 given the endothelial expression of CGNL1 as well as its implications in cardiovascular disease  
560 (Chrifi et al., 2017). Particularly in the setting of MIS-C, expanded cohorts both to further validate  
561 CGNL1 as well as for as-of-yet undescribed antigen specificities may add additional information  
562 to these otherwise poorly understood diseases.

563         There is increasing evidence for a role for autoantibodies in other acquired disease states  
564 that have not been classified *per se* as autoimmune diseases, including severe to critical COVID-  
565 19 pneumonia (Bastard et al., 2020, 2021c; Wijst et al., 2021). Peptides derived from EEA1 were  
566 enriched in 11% of patients with severe COVID19 pneumonia, but not in in patients with mild

567 COVID19. Interestingly, similarly to anti-IFN antibodies, anti-EEA1 antibodies have also  
568 previously been reported in the setting of systemic lupus erythematosus (SLE) (Selak et al., 2000;  
569 Stinton et al., 2004; Waite et al., 1998). While intriguing, future studies will be needed to determine  
570 whether the specificity of EEA1 autoantibodies to severe-critical COVID-19 can be replicated in  
571 independent cohorts, as well as in orthogonal assays.

572 This work suggests that PhIP-Seq data may have predictive or diagnostic value when the  
573 cohorts are sufficiently large. Implementation of a logistic regression machine learning model with  
574 cross validation successfully discriminates APS1 samples from healthy control samples with high  
575 sensitivity and specificity (Figure 4A). Thus, scaled PhIP-Seq represents an avenue to not only  
576 discover and compare novel autoantigens within and across cohorts of autoimmune disease, but  
577 also suggests that machine learning techniques will be effective tools for determining, without  
578 supervision, the underlying proteins that drive successful classification using this data type. This,  
579 in turn, may have diagnostic or prognostic value in a wide spectrum of immune dysregulatory  
580 contexts.

581 Finally, we emphasize that to broadly understand autoantibody profiles, full PhIP-Seq data  
582 availability is critical for enabling expanded analysis and comparison across datasets, cohorts,  
583 and research groups. Importantly, groups with expertise in specific disease areas may add value  
584 by re-analyzing and evaluating candidate hits using additional metrics for hit prioritization,  
585 including increased weighting of orthogonal expression and/or genetic data. Full PhIP-Seq data  
586 for all cohorts presented here will be linked to this publication and will be available for download  
587 at Dryad.

588  
589  
590

591 MATERIALS & METHODS

592

593 **PhIP-Seq protocols**

594 All PhIP-Seq protocols described in detail are available at protocols.io, located at the links below.

595

596 Vacuum-based scaled:

597 <https://www.protocols.io/view/scaled-high-throughput-vacuum-hip-protocol-ewov1459kvr2/v1>

598

599 Multichannel-based scaled:

600 <https://www.protocols.io/view/scaled-moderate-throughput-multichannel-hip-protocol-8epv5zp6dv1b/v1>

602

603 Library Preparation:

604 <https://www.protocols.io/view/phage-display-library-prep-method-rm7vz3945gx1/v1>

605

606 **PhIP-Seq data alignment and normalization**

607 Fastq files were aligned at the level of amino acids using RAPSearch2 (Zhao et al., 2012).

608 For gene level analysis, all peptide counts mapping to the same gene were summed. 0.5 reads

609 were added to all genes, and raw reads were normalized by converting to percentage of total

610 reads per sample. Fold change over mock-IP (FC) was calculated on a gene-by-gene basis by

611 dividing sample read percentage by mean read percentage in corresponding AG bead-only

612 samples. Z-scores were calculated using FC values; for each disease sample by using all

613 corresponding healthy controls, and for each healthy control samples by using all other healthy

614 controls. The positive threshold used for detection of shared candidate antigens was a Z-score

615  $\geq 10$ . Shared hits were then determined by positive rate in the specified percentage of patient

616 samples and under a specified percentage ( $< 2\%$  unless otherwise specified) in controls. In

617 addition, at least one positive sample was required to have a minimum FC of 50 or above. Finally,

618 no candidate antigens were allowed where any positive control samples signal fell above the

619 highest patient sample.

620

621 **Radioligand Binding Assay**

622 Radioligand binding assay was performed as described in (Vazquez et al., 2020) for each

623 gene of interest (GOI). Briefly, GOI-myc-flag constructs was used to *in vitro* transcribe and

624 translate [<sup>35</sup>S]-methionine labeled protein, which was subsequently column purified,

625 immunoprecipitated with patient serum on Sephadex protein A/G beads, and counts per million

626 (cpm) were read out on a 96-well liquid scintillation reader (MicroBeta Trilux, Perkin Elmer).

627 Constructs: PDYN (Origene, #RC205331), BEST4 (Origene, #RC211033), BTNL8(Origene,

628 #RC215370), CGNL1 (Origene, #RC223121). Anti-MYC (Cell Signaling Technologies, #2272S)

629 and/or anti-FLAG (Cell Signaling Technologies, #1493S) antibodies were used as positive

630 controls for immunoprecipitations.

631

632 **Statistics**

633 Statistics for Radioligand binding assay were performed as described in (Vazquez et al.,

634 2020). Briefly, the antibody index for each sample was calculated as: (sample value – mean blank

635 value) / (positive control antibody value – mean blank value).

636 We applied a logistic regression classifier on log-transformed PhIP-Seq RPK values

637 from APS1 patients (n = 128) versus healthy controls (n = 186) using the scikit-learn package

638 (Pedregosa et al 2011) with a liblinear solver and L1 regularization. The model was evaluated

639 with five-fold cross-validation.

640

641  
642 **ETHICS/Human Subjects research**

643 Available clinical metadata varies across cohorts; all available clinical data is included in  
644 Supplemental Table 1.

645 APS1

646 **North America - 1:** All patient cohort data was collected and evaluated at the NIH, and  
647 all APECED/ APS1 patients were enrolled in a research study protocol approved by the  
648 NIH Institutional Review Board Committee and provided with written informed consent  
649 for study participation (protocol #11-I-0187, NCT01386437). All NIH patients gave  
650 consent for passive use of their medical record for research purposes. The majority of  
651 this human cohort data was previously published by (Ferre et al., 2016; Ferré et al.,  
652 2019).

653 **Sweden:** Serum samples were obtained from Finnish and Swedish patients with APS1.  
654 All individuals met the clinical diagnostic criteria for APS1, requiring two of the hallmark  
655 components: chronic mucocutaneous candidiasis, hypoparathyroidism, and adrenal  
656 insufficiency, or at least one of the hallmark components in siblings or children of APS1  
657 patients. The project was approved by local ethical boards and was performed in  
658 accordance with the declarations of Helsinki. All patients and healthy subjects had given  
659 their informed consent for participation.

660 **North America - 2:** All patients underwent informed consent with research study  
661 protocols approved by the UCSF Human Research Protection Program (IRB# 10-  
662 02467).

663 RAG1/2 deficiency

664 Patients with RAG1/2 deficiency were enrolled in research study protocols approved by  
665 the NIAID, NIH Institutional Review Board (protocols 05-I0213, 06-I-0015,  
666 NCT03394053, and NCT03610802). Peripheral blood samples were obtained upon  
667 written informed consent.

668 IPEX

669 All patient cohort data was collected and evaluated at Seattle Children's Hospital under  
670 an IRB-approved protocol. Clinical data on this human cohort was previously published  
671 by Gambineri E et al. 2018.

672 KD/MISC/Febrile Controls

673  
674 **UCSD cohort** (MIS-C/KD/febrile controls): The study was reviewed and approved by the  
675 institutional review board at the University of California, San Diego. Written informed  
676 consent from the parents or legal guardians and assent from patients were obtained as  
677 appropriate.

678 **Rockefeller cohort** (MIS-C): All individuals were recruited according to protocols  
679 approved by local Institutional Review Boards.

680 Severe COVID19 (Casanova lab, Lynch lab)

681 All COVID19 (non-MIS-C) patients were collected between 03/01/2020 and 7/21/2020  
682 and had positive results by SARS-CoV-2 RT-PCR in nasopharyngeal swabs. ZSFG  
683 remnant specimens (institutional review board [IRB] number 20-30387) were approved  
684 by the IRB of the University of California, San Francisco. The committee judged that  
685 written consent was not required for use of remnant specimens.

686 Adult healthy controls

687 **New York Blood Center & Vitalant Research Institute:** Healthy, pre-COVID control  
688 plasma were obtained as deidentified samples. These samples were part of retention  
689 tubes collected at the time of blood donations from volunteer donors who provided  
690 informed consent for their samples to be used for research. **Rockefeller cohort:** All  
691 individuals were recruited according to protocols approved by local Institutional Review  
692 Boards.

693

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695

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702 **COMPETING INTERESTS**  
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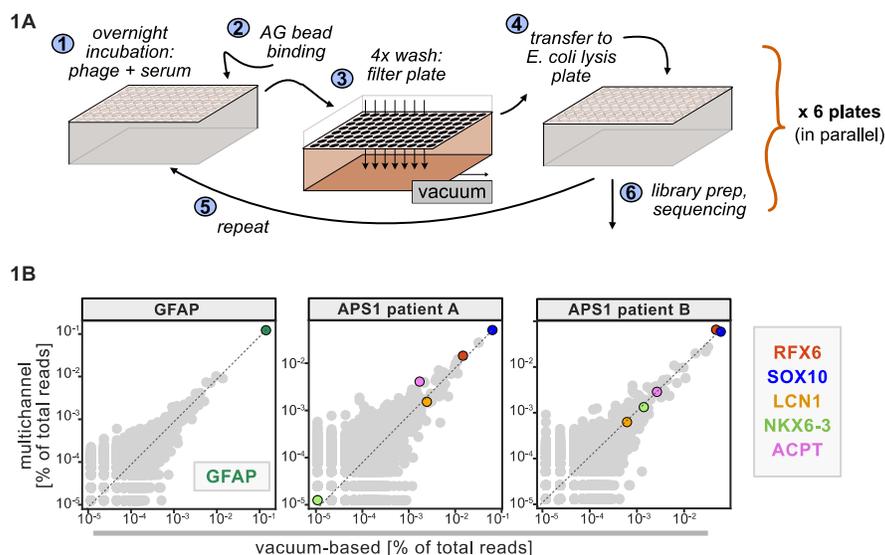
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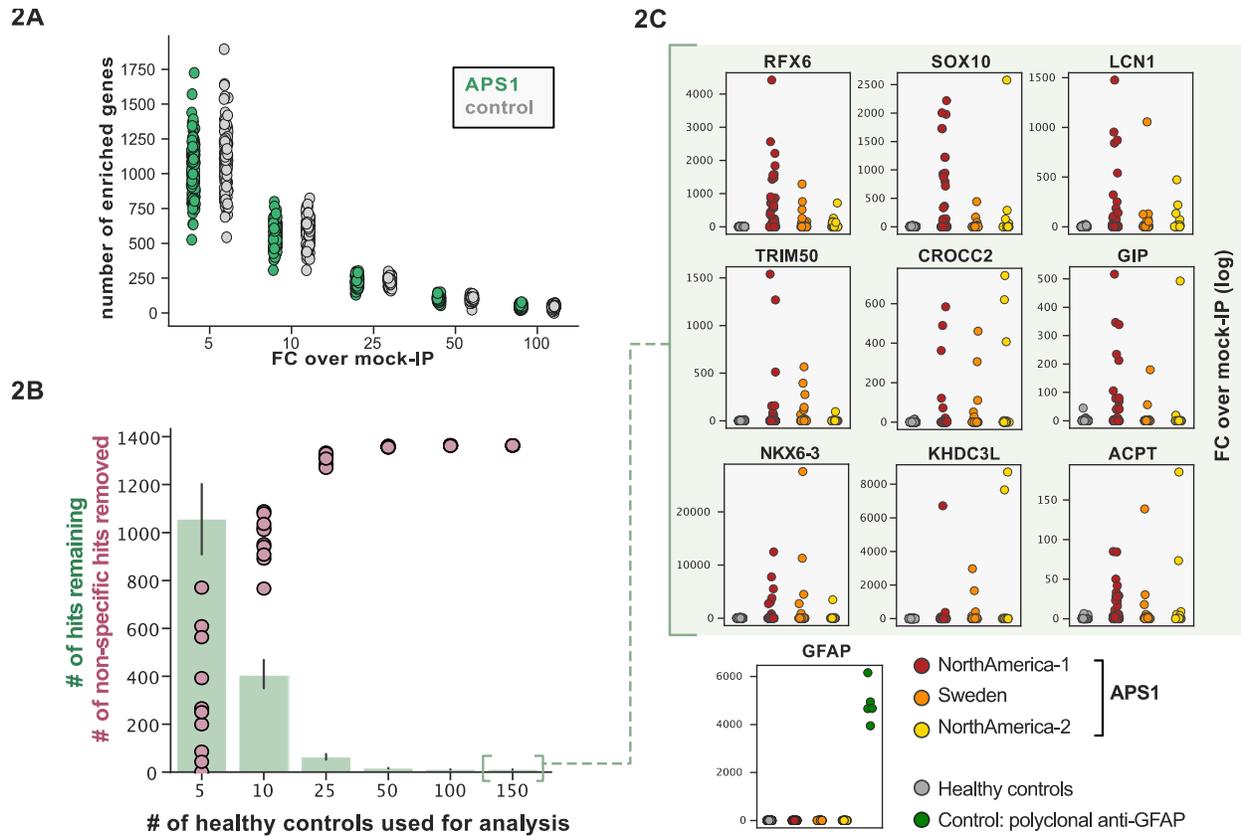
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728  
729

730 **FIGURES & FIGURE LEGENDS**



731  
732 **Figure 1. Advantages of and considerations motivating scaled PhIP-Seq.** A) Schematic of  
733 vacuum-based scaled PhIP-Seq protocol, allowing for parallelized batches of 600-800 samples.  
734 B) Comparison of moderate-throughput multichannel protocol data to high-throughput vacuum-  
735 based protocol data, with axes showing normalized read percentages. Controls include a  
736 commercial polyclonal anti-GFAP antibody (left), APS1 patient A with known and validated  
737 autoantibodies RFX6, SOX10, ACPT and LCN1 (center), and APS1 patient B with the same  
738 known and validated autoantibodies as well as NKX6-3.  
739

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744 **Figure 2. Application of scaled PhIP-Seq to expanded APS1 and healthy control cohorts.**

745 **A)** Number of hits per sample reaching 5, 10, 25, 50, and 100-fold enrichment relative to mock-

746 IP samples. Each dot represents a single APS1 patient (green) or non-APS1 control (grey). **B)**

747 When looking for disease-specific hits, increasing the number of healthy controls results in fewer

748 apparent hits and is therefore critical. Shared hits are defined as gene-level signal (>10-fold

749 change over mock-IP) which is shared among 10% of APS1 samples (n=128), present in fewer

750 than 2% of healthy controls, and with at least 1 APS1 sample with a high signal (FC of 50<).

751 Random downsampling was performed 10 times for each healthy control bin. **C)** 9 gene-level hits

752 are present in 10%< of a combined 3-group APS1 cohort. North-America-1, n = 62; Sweden, n =

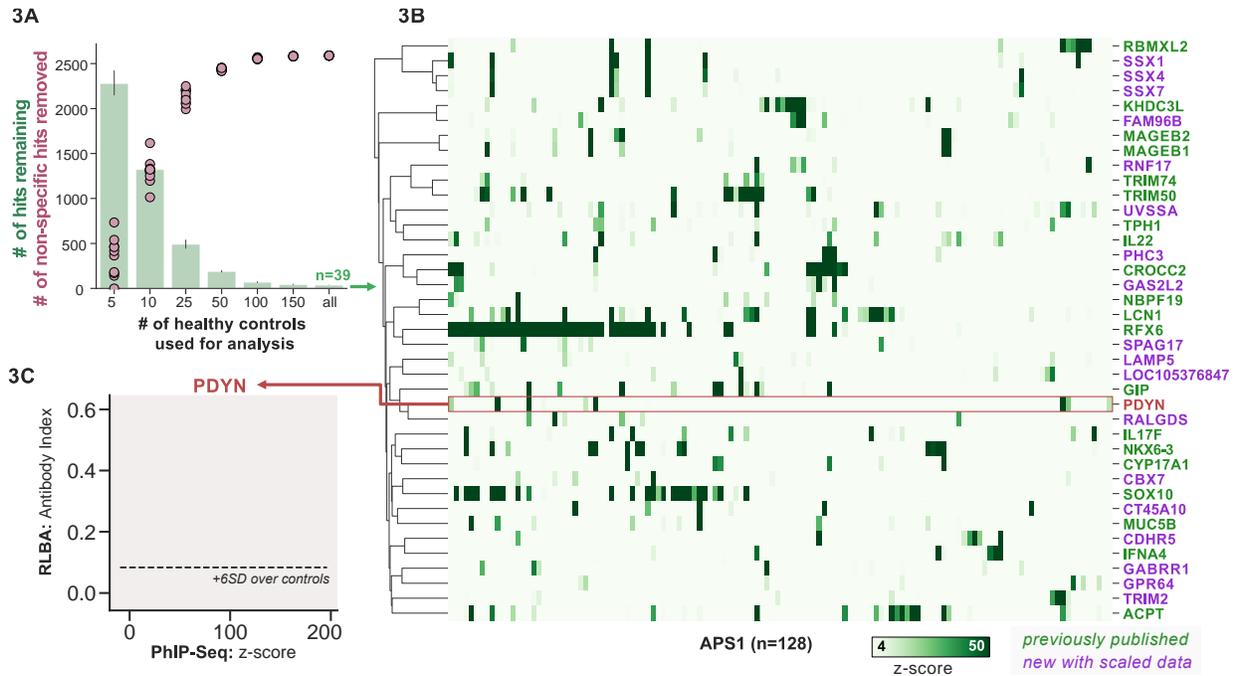
753 40; North-America-2, n = 26. Anti-GFAP control antibody (n=5) indicates that results are

754 consistent across plates and exhibit no well-to-well contamination.

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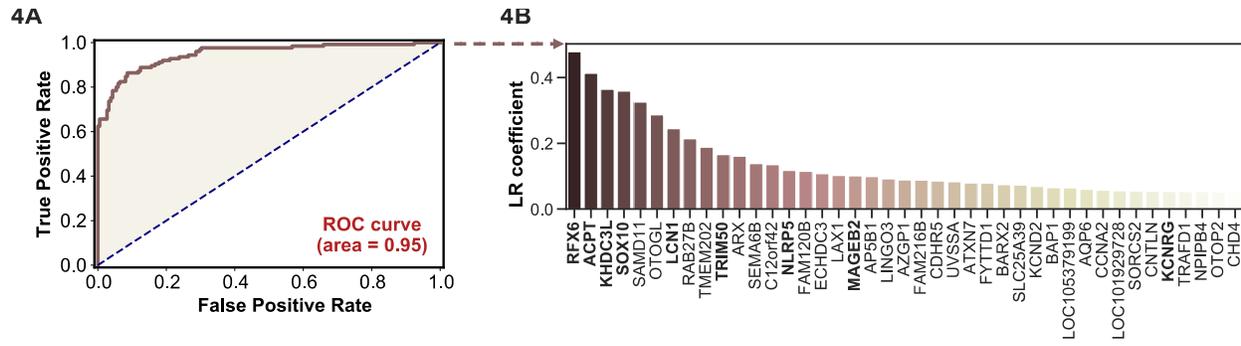
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**Figure 3. Replication and expansion of APS1 autoantigens across multiple cohorts using scaled PhIP-Seq. A)** Increasing the number of healthy controls results in fewer apparent hits and is therefore critical. Shared hits are defined as gene-level signal (>10-fold change over mock-IP) which is shared among 4%< of APS1 samples (n=128), present in fewer than 2% of healthy controls, and with at least 1 APS1 sample with a high signal (FC of 50<). Random downsampling was performed 10 times for each healthy control bin. **B)** 39 candidate hits present in 4%< of the APS1 cohort. **C)** Rare, novel anti-PDYN autoantibodies validate at whole-protein level, with PhIP-Seq and whole-protein RLBA data showing good concordance.

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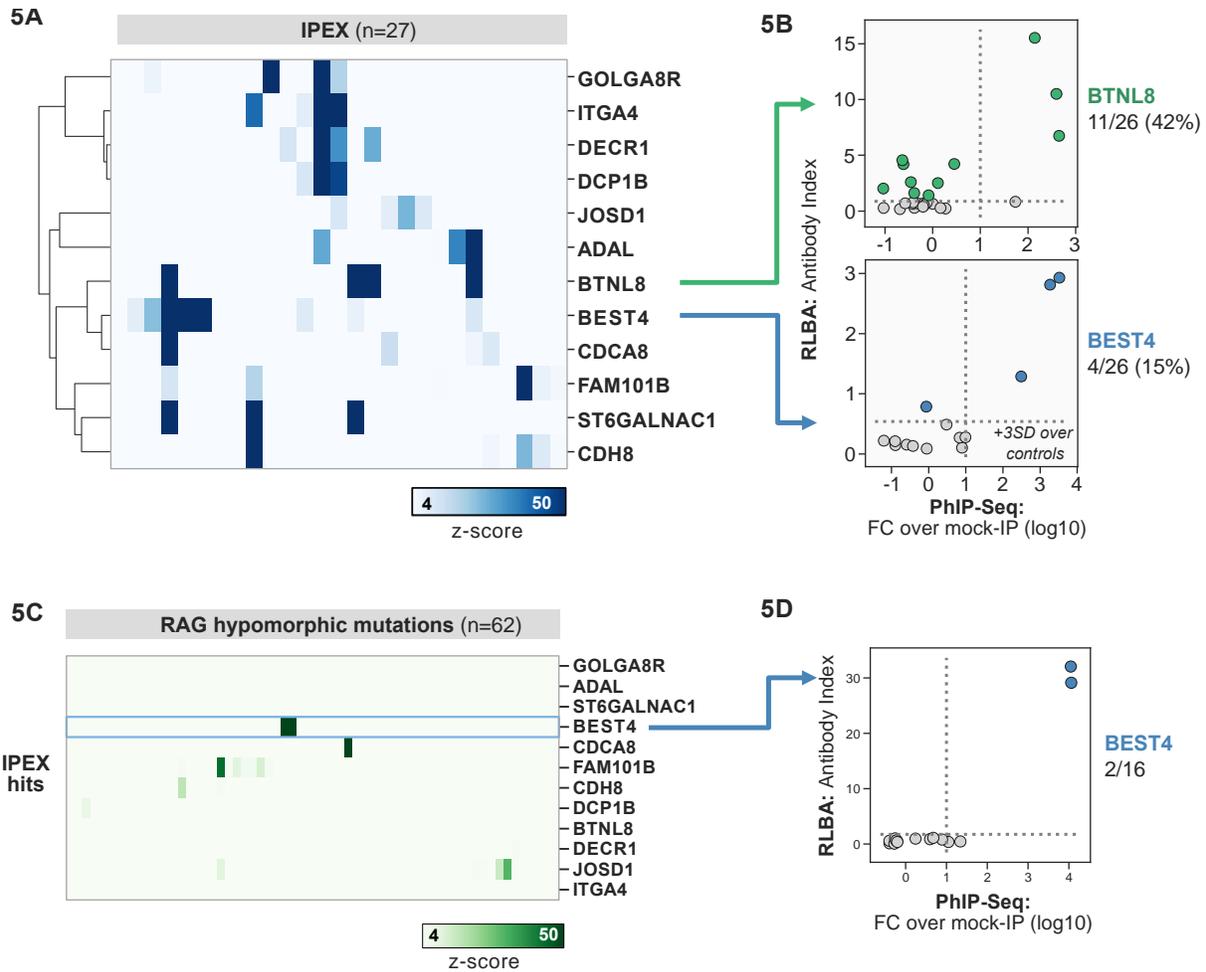
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**Figure 4. Logistic regression of PhIP-Seq data enables APS1 disease prediction. A)** ROC curve for prediction of APS1 versus control disease status. **B)** The highest logistic regression (LR) coefficients include known antigens RFX6, KHDC3L, and others.

777



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781 **Figure 5. PhIP-Seq screening in IPEX and RAG1/2 deficiency reveals novel, intestinally**

782 **expressed autoantigens BEST4 and BTNL8. A)** PhIP-Seq heatmap of most frequent shared

783 antigens among IPEX, with color indicating z-score relative to a cohort of non-IPEX controls. **B)**

784 Radioligand binding assay for BTNL8 reveals additional anti-BTNL8 positive IPEX patients (top).

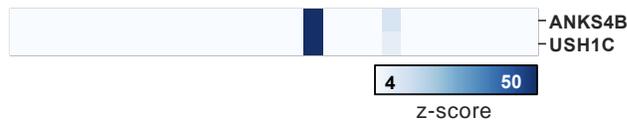
785 Radioligand binding assay for BEST4 autoantibodies correlates well with PhIP-Seq data (bottom).

786 **C)** PhIP-Seq screen of patients with hypomorphic mutations in *RAG1/2* reveals 2 patients with

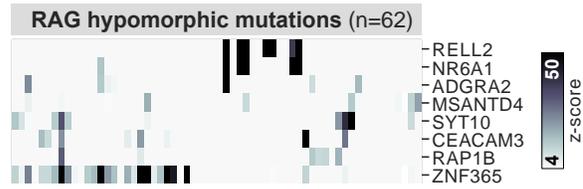
787 anti-BEST4 signal. **D)** Orthogonal radioligand binding assay validation of anti-BEST4 antibodies

788 in both PhIP-Seq anti-BEST4 positive patients.

Suppl. 1A

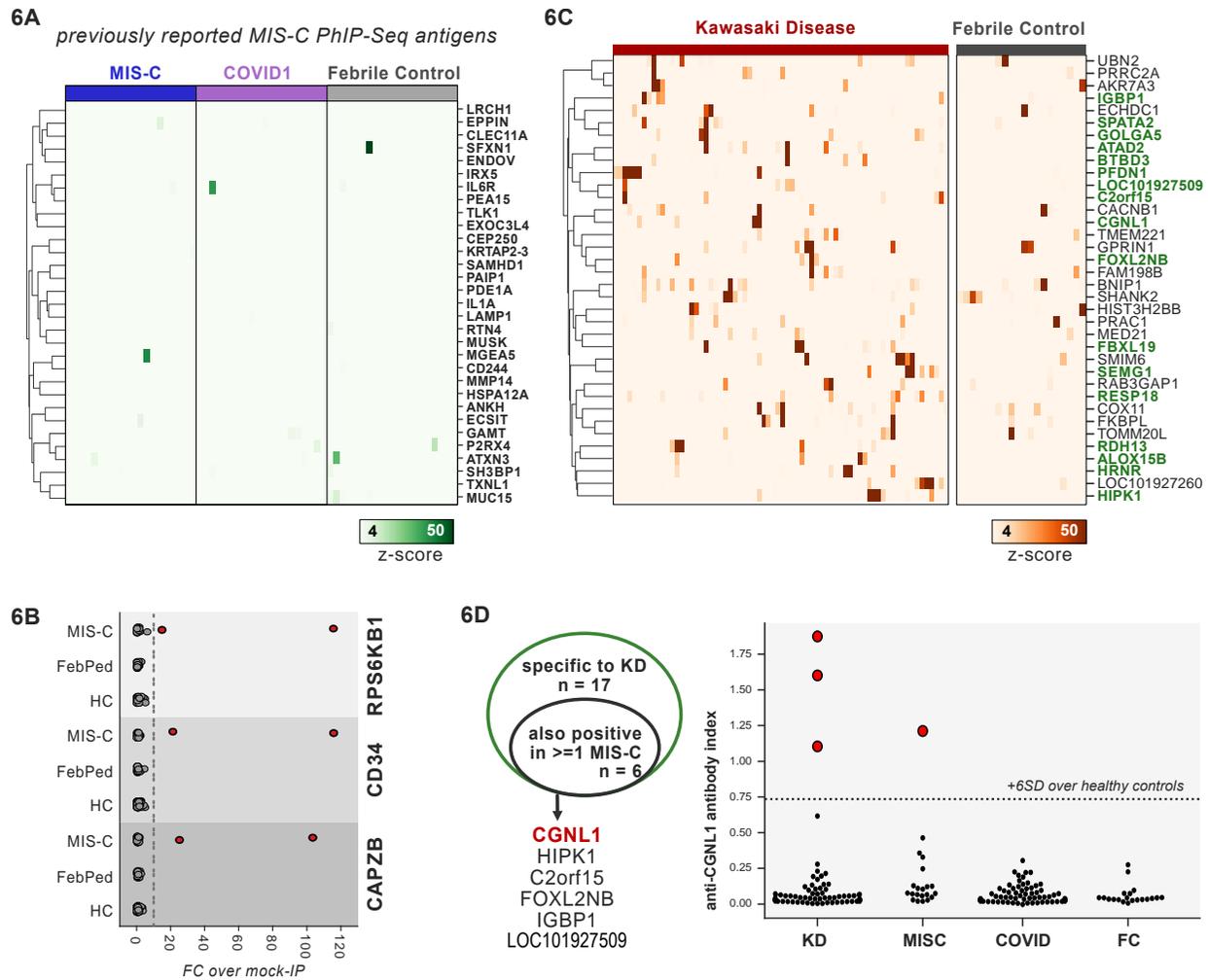


Suppl. 1B



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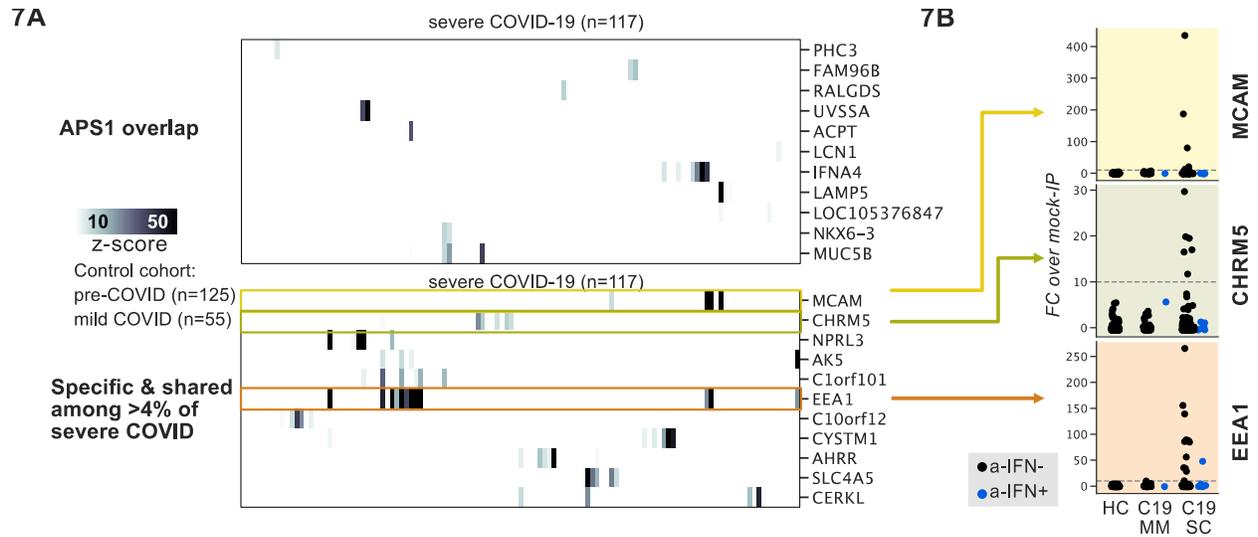
**Figure 5 – Figure Supplement 1. A)** PhIP-Seq has low detection sensitivity for known antigens USH1C and ANKS4B, but those patients with positive signal exhibit the previously reported coupled signal for both antigens. **B)** Additional, shared putative antigens within the cohort of RAG1/2-deficient patients (n=62).



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**Figure 6. PhIP-Seq screening of MIS-C and KD cohorts. A)** Heatmap of signal for putative hits from *Gruber et al. 2020*, among MIS-C, adult COVID19 controls, and pediatric febrile controls (each n=20). **B)** Only rare, shared PhIP-Seq signals were found among n=20 MISC patients. **C)** Heatmap of putative antigens in a cohort of n = 70 KD patients. Hits that are specific to KD, and are not found among n=20 febrile controls, are highlighted in green. **D)** A small number of rare putative antigens are shared between KD and MISC (left), with radioligand binding assay confirmation of antibody reactivity to whole protein form of CGNL1 in 3 KD patients and 1 MISC patient (right).

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**Figure 7. PhIP-Seq screening in severe forms of COVID-19, MIS-C and KD reveals putative novel autoantigens, including EEA1. A)** Screening of patients with severe COVID19 pneumonia shows little overlap with APS1, but enables discovery of possible novel disease associated autoantigens including EEA1. **B)** Putative novel antigens EEA1, CHR5, and MCAM are primarily found in anti-IFN-negative patients, suggesting the possibility of other frequent, independent disease-associated antibodies in severe COVID19.

821 **SUPPLEMENTAL TABLES**

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823 **Supplemental Table 1. Clinical metadata**

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