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Probing of a human proteome microarray with a recombinant pathogen protein reveals a novel mechanism by which hookworms suppress B cell receptor signaling

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Probing of a human proteome microarray with a recombinant pathogen protein reveals a novel mechanism by which hookworms suppress B cell receptor signaling

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ABSTRACT

***Na*-ASP-2 is an efficacious hookworm vaccine antigen; however, despite elucidation of its crystal structure and studies addressing its immunobiology, the function of *Na*-ASP-2 has remained elusive. We probed a 9,000 protein human proteome microarray with *Na*-ASP-2 and showed binding to CD79A, a component of the B cell antigen receptor complex. *Na*-ASP-2 bound to human B lymphocytes ex vivo and down-regulated the transcription of ~1,000 B cell mRNAs, while only ~100 mRNAs were up-regulated compared to control-treated cells. The expression of a range of molecules was affected by *Na*-ASP-2, including factors involved in leukocyte transendothelial migration pathways and the B cell signaling receptor pathway. Of note was the down-regulated transcription of *lyn and pi3k*, molecules that are known to interact with CD79A and control B cell receptor signaling processes. Together, these results highlight a previously unknown interaction between a hookworm-secreted protein and B cells, which has implications for helminth-driven immunomodulation and vaccine development. Further, the novel use of human protein microarrays to identify host-pathogen interactions, coupled to ex vivo binding studies and subsequent analyses of global gene expression in human host cells, demonstrates a new pipeline by which to explore the molecular basis of infectious diseases.**

Keywords. Hookworm, *Necator americanus*, *Na*-ASP-2, SCP/TAPS, CD79, B cell, antigen receptor, protein microarray, host-pathogen interaction

INTRODUCTION

The hookworm *Necator americanus* is one of the most prevalent pathogens of humans, infecting between 576-740 million people worldwide [1]. Hookworms can persist for many years within their hosts [2], having evolved a suite of strategies to evade immune attack, from the point of percutaneous entry and pulmonary migration by third-stage larvae (L3), through to their final site of residence as adult worms in the gut. While hookworm infection can be treated with anthelmintic drugs, reinfection rapidly occurs [3], precipitating an urgent need for development of vaccines to limit the global burden of human hookworm infections [4].

Efforts to develop vaccines against hookworm infection have focused on discovery of antigens secreted by the L3 stage. Serum antibodies from dogs vaccinated with irradiated hookworm L3 [5] and people from hookworm-endemic areas with high antibody titres but low egg counts [6] predominantly recognise members of the activation-associated protein (ASP) family, notably *Na*-ASP-2. In a phase 1a vaccine trial in hookworm naïve subjects from the U.S., recombinant *Na*-ASP-2 was well-tolerated and immunogenic [7], however, a phase 1b trial in hookworm-exposed individuals in Brazil was halted due the development of urticarial reactions in people with pre-existing IgE against *Na*-ASP-2 [8]. Despite the safety concerns, there remains interest in *Na*-ASP-2 as a vaccine candidate, particularly if used as a pediatric vaccine prior to the development of anti-hookworm IgE responses, or if it is modified to reduce its allergenic capacity [9]; which has proven effective for peanut, dust mite and wasp allergens [10-12].

Despite the relatively long history of research into hookworm ASPs as vaccines, relatively little is known about their biological roles. ASP-2 is one of the most abundant excretory/secretory (ES) proteins produced by L3 upon exposure to host serum [13, 14], however its expression is diminished as the parasite migrates through the lung, provoking the hypothesis that ASP-2 may be involved in immune regulation while the parasite resides in the circulatory system. Administration

of recombinant *Na*-ASP-2 to rodents elicits a type 2 cytokine response, robust antibody production and neutrophil recruitment [5, 15], although direct interactions between *Na*-ASP-2 and a defined cell type were not elucidated. The hookworm ASP protein family forms 3 distinct structural groups based on X-ray crystallographic studies [16, 17]. The crystal structure of *Na*-ASP-2 indicates the presence of a putative electronegative binding cavity flanked by conserved His and Glu residues [17], suggesting that an unknown ligand or binding partner exists. Another hookworm-derived ASP-like protein - Neutrophil Inhibitory Factor - has been shown to directly interact with host CD11b, thereby inhibiting neutrophil function [18], hence it remains likely that *Na*-ASP-2 binds to a cell surface receptor from its human host to exert its effector function(s).

Herein we describe the first probing of a human proteome microarray with a pathogen-derived protein to reveal a selective interaction between *Na*-ASP-2 and CD79A, a component of the B-cell antigen receptor complex. We confirm the interaction by showing that *Na*-ASP-2 binds to human B cells ex vivo and triggers changes in B cell-intrinsic gene expression, notably key signaling molecules of the antigen receptor pathway.

METHODS

Ethics

Healthy Caucasian volunteers (aged 30-45) from Cairns, Australia, a region that is not endemic for human hookworm infection, were used as a source of peripheral blood under informed consent using a protocol (H4385) approved by the James Cook University Human Research Ethics Committee.

Probing of a human protein microarray with recombinant *Na*-ASP-2

Purified, full-length recombinant *Na*-ASP-2 was a kind gift from Dr. Bin Zhan (Baylor College of Medicine, USA). The protein was expressed in *Pichia pastoris* and purified via two ion exchange chromatography steps followed by a final desalting step as described elsewhere [19]. *Na*-ASP-2 was biotinylated with NHS-LC-biotin (Thermo-Fisher) and used to probe a Human V5 ProtoArray (Invitrogen) at a final concentration of 50 $\mu g.ml^{-1}$. The ProtoArray contains over 9,000 human proteins printed in duplicate, and includes more than 2,600 membrane proteins. Binding of biotinylated *Na*-ASP-2 to proteins on the array was detected with streptavidin Alexafluor 647 at a 1:1000 dilution in Protoarray blocking buffer (Invitrogen). The array was scanned using a GeneArray 4000B scanner (Molecular Devices) at 635 nm. Results were saved as a multi-TIFF file and analyzed using Genepix Prospector software, version 7.

Molecular Modelling

Comparative modelling was applied to generate a three-dimensional atomic model of human CD79A, using the crystal structure of human CD79B (PDB accession code 3kg5) as a template. A secondary structure-based amino acid sequence alignment of CD79A and CD79B was prepared with SBAL [20] and used to guide the comparative modelling calculations. Twenty independent models were calculated with MODELLER [21], and the one with the lowest energy was selected and superimposed onto one of the monomers in the CD79B homodimer structure (rigid body modelling). The secondary structure topology and amino acid sequence of CD79A is compatible with the intermolecular interactions observed in the CD79 homodimer structure [22]. A minor manual adjustment was made to enable inter-molecular disulphide bond formation (CysA119-CysB136) in the resulting heterodimer. Both cysteine residues were in prime position for this bond, which is also observed in the CD79B homodimer. The crystal structure of *Na*-ASP-2 was docked to the CD79 heterodimer model, using a rigid body approach based on a multi-dimensional spherical polar Fourier correlation with shape and electrostatics expansion as implemented in Hex [23]. The rigid body docking calculations were carried out without preferred contacts to enable an unbiased

complex formation. From the generated 100 independent models the highest scoring one (energy score: -873, next highest -720) was chosen. The figures were generated with PyMOL [24].

Blood lymphocyte isolation and culture

Human peripheral blood mononuclear cells (PBMCs) were isolated by density centrifugation (Lymphoprep^{TM}). Cells (1×10^6 cells.ml^{-1}) were incubated for 4 or 24 h at 37°C in either 96-well plates (for flow cytometry) or 24-well plates (for sort purification) with *Na*-ASP-2 in DMEM with 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 U/mL streptomycin and 25mM HEPES (media). B-cell activation was induced by 24 h of stimulation with 10 μg/ml of goat F(ab')2 anti-human IgM (μ chain, Sigma).

Flow cytometry and cell sorting

Cells were stained with fluorophore-conjugated antibodies against CD19 (HIB19), CD20 (2H7), CD3 (OKT3), CD4 (OKT4), CD14 (61D3) CD69 (FN50), HLA-DR (LN3), CD80 (2D10.4), CD86 (IT2.2) (all from eBioscience). In experiments using biotinylated *Na*-ASP-2, streptavidin-FITC was included in the staining panel. Cells were analysed using a BD-FACSCantoII flow cytometer. Alternatively, or CD3$^-$ CD14$^-$ CD19$^+$ CD20$^+$ cells were sort-purified using a BD-FACSAria III cell sorter.

RNA isolation

RNA was extracted from sort-purified CD19$^+$ CD20$^+$ B cells for high-throughput RNA-Seq at BGI (Hong Kong) or real-time quantitative PCR (qPCR) analysis. Pelleted cells were lysed in RLT buffer (QIAGEN) + beta-mercaptoethanol and stored at -80°C until RNA was extracted using the RNAeasy kit with DNAse purification (QIAGEN). The concentration and integrity of each RNA preparation were verified on a 2100 Bioanalyzer (Agilent) and each preparation was stored in RNAstable tubes (Biomatrica) at room temperature until subsequent use.

Illumina sequencing

RNA was reverse-transcribed to cDNA using the Illumina TruSeq™ sample preparation kit. Briefly, 1.0 μg of total RNA from each cell preparation was used for poly-A mRNA selection using streptavidin-coated magnetic beads. Two rounds of poly-A mRNA enrichment were performed, followed by thermal fragmentation of the mRNA to a length of 100-500 bp. Each fragmented RNA sample was then reverse transcribed to cDNA using random primers, end-repaired and adaptor-ligated (Illumina). Ligated products were PCR-amplified (15 cycles) and cleaned using a MinElute column (QIAGEN). Amplicons were paired-end sequenced on an Illumina HiSeq 2500 (Illumina).

Bioinformatic analyses

Ninety bp single-reads were screened for the presence of adapter sequences and sequences with >10% unknown bases and sequences with >50% low-quality bases. The remaining reads were splice-aligned to the human genome reference assembly GRCh37 (NCBI build 37.1) using TopHat, which incorporates the Bowtie v0.11.3 algorithm [25]; subsequently, individual aligned read files were assembled into transcripts using Cufflinks [26]. Relative levels of transcription were measured using the Reads Per Kilobase per Million reads algorithm (RPKM) [27]. Differences in levels of gene transcription were determined as previously described [28]. The false discovery rate method (FDR) [29] was used to correct the errors associated with multiple pairwise comparisons (FDR $\leq$ 0.001) and a $|\log_2 \text{ratio}| \geq 1$ was set as the threshold for the significance of gene expression changes. Differentially expressed genes (DEGs) were mapped to Gene Ontology terms and Kyoto Encyclopedia of Genes and Genomes (KEGG) biological pathways available in the GO and KEGG databases, respectively (www.geneontology.org; www.genome.jp/kegg/). A hypergeometric test was subsequently used to identify significantly enriched GO terms and KEGG pathways in DEGs compared to the transcriptome background using the GOTerm::Finder software [30]. The calculated *P*-values were subjected to Bonferroni correction using the corrected *P*-value of 0.05 as a threshold.

Raw sequence data have been deposited in the Sequence Read Archive (SRA) of NCBI under accession number SRAXXXXXX.

Real-Time quantitative PCR

Total RNA was extracted from sort-purified B cells from four hookworm-naïve Caucasian human volunteers that were cultured for 24 h in the presence or absence of *Na*-ASP-2. cDNAs were prepared using Superscript III reverse transcriptase (Invitrogen). SYBR Green mastermix (QIAGEN) was used with a Rotor-Gene q thermal cycler (QIAGEN). The oligonucleotides were designed manually and synthesized (Integrated DNA Technologies). Data were analyzed using the ΔΔCT method whereby actin served as the endogenous gene and samples were normalized to media controls. Primer sequences were as follows: *β-actin*- forward ATTGGCAATGAGCGGTTC and reverse GGATGCCACAGGACTCCAT; *lyn* forward GAGACTCGGGGAGCCATTG and reverse ACGCTGGGGATGTTATACTCT; *pi3kr5* (23533) forward CTTCCACGCTACGTGTTGTG and reverse TGAAGTTTGAAGAACCGTGTGAG.

Statistical Analyses

Statistical analyses of qPCR data were conducted using a paired *t* test where ΔΔCT values for each gene of interest were compared between test (*Na*-ASP-2 exposed) vs control (media exposed) cells. *P* values of <0.05 were deemed statistically significant.

RESULTS

Interaction between *Na*-ASP-2 and human CD79A revealed by probing of a human proteome microarray

Probing of the human proteome array with biotinylated *Na*-ASP-2 revealed interactions between *Na*-ASP-2 and five human proteins. The strongest interaction was with propionyl-CoA carboxylase

(PCCA), an anticipated interaction because PCCA is a biotin-binding enzyme and would therefore be expected to bind to any biotinylated recombinant protein used to probe the array (**Figure 1, Supplementary Table 1)**. Indeed, we have noted consistent binding of other (unrelated) biotinylated recombinant proteins to PCCA (unpublished). The second most intense association was with CD79A, which together with CD79B forms a hetero-dimer and a subunit of the B-cell antigen receptor. The third and fifth strongest hits were also proteins involved in biotin metabolism (**Supplementary Table 1**). The fourth strongest association was with cortactin, which is involved in actin remodeling and was of sufficiently low signal intensity (2.4 fold lower than CD79A) that we deemed it unworthy of further pursuit **(Supplementary Table 1)**. Probing of a second array using identical conditions resulted in consistent binding between *Na*-ASP-2 and the same five human proteins on the array.

Modelling of *Na*-ASP-2 interactions with CD79

We recently panned a random 12-mer peptide phage display library with *Na*-ASP-2 and showed that most of the bound peptides were enriched for glutamine and histidine residues [31]. Interestingly, the extracellular domain of CD79A contains a HXQXXH motif at residues 51-56. This peptide forms part of a large surface of CD79A which has previously been implicated in binding of the B cell antigen receptor [22]. Rigid body docking of the crystal structure of *Na*-ASP-2 and a model of heterodimeric CD79A/CD79B suggests that, based on shape and electrostatics, *Na*-ASP-2 may bind to the CD79 heterodimer **(Figure 2)**. Furthermore, this model indicates that *Na*-ASP-2 mainly interacts with the CD79A surface harboring the HXQXXH motif, but a protruding loop of CD79B (residues 79-86) may also engage in interactions with the hookworm protein.

***Na*-ASP-2 binds selectively to human B cells.**

CD79A is expressed exclusively on B lymphocytes. To verify the interaction between *Na*-ASP-2 and CD79A, we cultured human PBMCs from healthy, hookworm-naïve human volunteers ex vivo

for 4 or 24 h with biotinylated *Na*-ASP-2 and performed flow cytometry. Cells were stained for B cell markers (CD19, CD20), T cell marker CD3, monocyte marker CD14, and streptavidin-FITC for detection of surface-bound *Na*-ASP-2. This experiment was performed twice, with two different blood donors. As expected, we detected negligible frequencies of $FITC^+$ cells following treatment with media or non-biotinylated *Na*-ASP-2, however treatment with increasing amounts of biotinylated *Na*-ASP-2 resulted in a dose-dependent increase in the frequency of *Na*-ASP-2/$FITC^+$ cells (**Figure 3A**). Gated $FITC^+$ cells were analyzed for various leukocyte surface markers. While only 8% of $FITC^+$ cells expressed CD3 or CD14, the majority (72%) of $FITC^+$ cells co-expressed CD20 and CD19, a phenotype consistent with human B lymphocytes (**Figure 3B**). Modifying culture time, temperature and cell permeabilization state did not enhance the detection of $FITC^+$ cells (data not shown), but in each case, B cells represented the dominant *Na*-ASP-2/$FITC^+$ population.

***Na*-ASP-2 induces substantial changes in B cell gene expression.**

To examine the biological effect of *Na*-ASP-2 on human B cells, we compared the expression of cell activation markers following polyclonal stimulation of the B cell receptor (BCR). PBMCs were treated for 4 h with 2 μg/mL *Na*-ASP-2, or bovine serum albumin (BSA) control and stimulated for 24 h with either media, or anti-human IgM. While we did not observe appreciable differences in the expression levels of CD69, CD86, HLA-DR and CD80 on B cells exposed to *Na*-ASP-2 following anti-IgM treatment, we did observe slight but consistent reductions in expression of these markers when cells underwent no exogenous stimulation (**Supplementary Figure 1**). These data suggest that B cells can still be activated in the presence of *Na*-ASP-2, but in the absence of polyclonal stimulation *Na*-ASP-2 may limit the activation state of human B cells.

In order to investigate the cascade of molecular events that follow binding of *Na*-ASP-2 to human B cells, we sort-purified $CD19^+$ $CD20^+$ B cells from one volunteer's whole PBMC culture that was

treated with either *Na*-ASP-2 or media control and performed RNA-Seq and bioinformatics analyses. B cells that were cultured with *Na*-ASP-2 for 4 h underwent minimal changes in gene expression (37 up-regulated genes, 178 down-regulated genes compared to media control, **Figure 4A**). After 24 h of culture, however, there was a pronounced bias toward down-regulation of gene expression in B cells that were cultured with *Na*-ASP-2, with 1080 significantly down-regulated genes and only 95 up-regulated genes (**Figure 4B**).

***Na*-ASP-2 down-regulates expression of genes involved in multiple biological pathways.**

Genes whose expression was significantly altered following exposure to *Na*-ASP-2 were clustered according to their KEGG annotation (**Figure 5**). In particular, the majority of down-regulated genes could be mapped to the 'cytokine-cytokine receptor interaction' (ko04060) and the 'leukocyte transendothelial migration' (ko04670) pathways, whereas the majority of genes whose expression was up-regulated by *Na*-ASP-2 binding could be mapped to the 'hematopoietic cell lineage' (ko04640) and 'chemokine signaling pathway' (ko04062), respectively.

***Na*-ASP-2 down-regulates expression of genes in the Ig receptor (CD79A) signaling pathway.**

While *Na*-ASP-2 appears to affect the expression of genes involved in multiple biological pathways, we focused on potential consequences of interactions between *Na*-ASP-2 and CD79A based on our earlier protein-protein interaction studies. In particular, three genes mapping to the KEGG B-cell receptor signaling pathway, i.e. *lyn*, spleen tyrosine kinase (*syk*) and phosphatidylinositol 3-kinase (*pi3k*) displayed significant down-regulation following exposure to *Na*-ASP-2 (**Figure 6**). Each of these gene products are involved in transmitting signals from the BCR and are crucial for essential B-cell functions such as activation, development, proliferation, inhibition and cell death.

Follow-up studies involving the analysis of B cell-intrinsic gene expression induced by *Na*-ASP-2 from four hookworm-naïve blood donors were performed using qPCR. Consistent with our findings from RNA-seq, the expression levels of both *lyn* and *pi3k* were significantly reduced in B cells that were cultured for 24 h with *Na*-ASP-2 ex vivo relative to control cells (**Figure 7**).

DISCUSSION

Advances in genomics and proteomics have served to highlight how little we know about the functional interactions of parasitic helminth proteins with their respective hosts tissues [32]. Approaches aimed at developing a "portfolio" for a specific pathogen protein of unknown function have provided information on expression, localization and immunogenicity [33]. None of these approaches, however, specifically addresses biochemical function or ligand/receptor interactions. The ES proteins of helminths from diverse phyla have been characterized using proteomics. However, despite the availability of draft genomes for some of these organisms, >50% of genes encoding secreted proteins remain functionally uncharacterized. This is true for *N. americanus*, where 33% of the genome encodes for putatively secreted proteins, but greater than 30% remain un-annotated [34].

N. americanus modulates the human host's immune system to facilitate its migration from the skin to the lungs en route to the intestine [35]. Immuno-epidemiological studies from human populations have shown that protective acquired immunity to hookworm does not develop in most individuals [36]. One of the means by which hookworms modulate the host's immune system is via the production of ES proteins [37-40]. The results from our study are consistent with this theory, where we demonstrate that *Na*-ASP-2 interacts with CD79A on human B lymphocytes, suppressing expression of genes that play key roles in multiple biological pathways, including the BCR signaling pathway. While the precise biological function of *Na*-ASP-2/B cell interactions in vivo remains unclear, our in vitro data implies that *Na*-ASP-2 engages CD79A and results in suppression

of molecules that determine the activation state of B cells. Our findings neither support nor contradict those of Bower [15] who showed that *Na*-ASP-2 recruits neutrophils. While neutrophils are essential effector cells in acute infections, they also suppress B and T cell responses by competing for antigen with professional antigen presenting cells [41]. Moreover, neutrophils indirectly regulate dendritic cell and T cell interactions [42]. Therefore, *Na*-ASP-2 might serve to suppress immune responses via two distinct but convergent pathways.

In the absence of experimental three-dimensional structures of *Na*-ASP-2 in complex with host proteins, we generated a model of the *Na*-ASP-2:CD79A:CD79B hetero-trimer complex. This model is in agreement with two previously reported findings. First, the interaction interface between the hookworm protein and the CD79 hetero-dimer has a His/Gln-containing motif with similar properties to those identified by panning a random peptide library with *Na*-ASP-2 [31]. Second, the vast majority of the interaction interface of *Na*-ASP-2 and CD79 is on the α-subunit, coinciding with the interaction interface of CD79 with membrane immunoglobulin [22]. We recently identified the human SK3 channel as a putative receptor of *Na*-ASP-2 based on the presence of a His/Gln-rich peptide that interacted with *Na*-ASP-2 in vitro [31]. Since this His/Gln peptide sequence is only present in one of the isoforms of the SK3 channel (isoform 1), and the human V5 ProtoArray included isoform 2, no interactions with SK3 were observed in this study.

The human BCR consists of a 1:1 stoichiometry of membrane-bound immunoglobulin and CD79A/CD79B. The immunoglobulin domain relies on a non-covalent association with the CD79A/CD79B heterodimer complex for triggering a signaling cascade [43]. Upon binding of antigen to membrane bound immunoglobulin, the receptor complex undergoes conformational changes, resulting in recruitment of LYN, phosphorylation of the relevant CD79 tyrosines, PI3K recruitment to CD19 and adaptor proteins, and generation of the potent lipid secondary messenger phosphatidylinositol 3,4,5-trisphosphate (PIP3). How interactions between CD79A and *Na*-ASP-2

result in down-regulated expression of *lyn* and *pi3k* is unclear, but it is possible that *Na*-ASP-2 may induce an anergic-like state in B cells by preventing BCR clustering and activation of the cell upon antigen binding. Anergic B cells are characterized by impaired propagation of activating signals that are triggered by CD79 engagement [44], substantially reduced PIP3 production [45] and have a dramatically reduced lifespan. While *Na*-ASP-2 does not limit the capacity for B cells to be activated by polyclonal stimulation, the activation state of "resting" B cells appeared to be lowered. Further studies are required to determine if *Na*-ASP-2 can regulate the B cell-intrinsic responses to more physiologically relevant antigens, particularly ES proteins. Hookworms are known to induce a state of immune hypo-responsiveness in chronic infections [36, 46], and by probing a hookworm protein microarray [34] we found that the most heavily infected individuals generated lower IgG responses than did individuals with low or moderate intensity infections (S. Gaze & A. Loukas, unpublished). These findings serve to highlight the altered B cell functions in individuals with heavy hookworm infection, and offer a potential causative role for proteins such as *Na*-ASP-2, however this hypothesis requires rigorous testing, potentially in well-controlled hookworm dose-ranging studies [47].

To our knowledge this is the first report of a pathogen protein that interacts with the BCR, or modulates expression of signaling molecules that determine B-cell function. Given the interest in hookworm-secreted proteins for treating autoimmune and allergic diseases [40, 48], our findings suggest that *Na*-ASP-2 warrants testing as a novel biologic for treating B cell-mediated autoimmunity. Administration of anti-CD79 or anti-CD79B monoclonal antibodies prevent collagen-induced arthritis [49] or lupus [50], respectively in mice, offering the potential for reduced side effects whilst maintaining B cells in a transient anergic state that does not require their depletion. Thus, *Na*-ASP-2 could prove beneficial as a treatment for autoimmune B cell-mediated diseases such as Multiple Sclerosis or rheumatoid arthritis.

Our findings herein highlight the novel application of protein microarray technology to addressing host-hookworm interactions at the protein level, and provide a powerful and rapid screening process that could be applied to the study and identification of interactions between other pathogens and host proteins. By coupling the probing of the human proteome array with ex vivo confirmation of binding to live cells and subsequent NGS to determine changes in gene expression, we have demonstrated the utility of this pipeline for elucidating novel host-pathogen interactions and assigning functions to that enigmatic group of pathogen molecules that are frustratingly referred to as "proteins of unknown function".

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FIGURE LEGENDS

Figure 1. **Binding of biotinylated *Na*-ASP-2 to CD79A and biotin-binding proteins on a Human V5 ProtoArray.** The array was probed with biotinylated recombinant *Na*-ASP-2 followed by streptavidin Alexafluor 647 and scanned using a GeneArray 4000B scanner at 635 nm using laser intensity settings of 100% (**A**) and 66% (**B**). Yellow boxes denote the area of the array containing duplicate spots of CD79A and PCCA. Panel C shows a magnified view of the area contained within the yellow boxes. All other spots are positive control proteins spotted in duplicate to allow orientation on the array. Two separate arrays were probed independently, with representative results from one experiment displayed.

Figure 2. Rigid body models of the CD79A/CD79B heterodimer (A) and the CD79A/CD79B:*Na*-ASP-2 complex (B). CD79A is coloured wheat, CD79B pale green, and *Na*-ASP-2 is coloured magenta. Cysteine residues and disulphide bonds are rendered as sticks in yellow. The CD79A peptide HFQCPH (residues 51-56) is coloured blue. Surface representation of the CD79 heterodimer model (A). Cartoon rendering of the modelled ternary complex of *Na*-ASP-2 and CD79 (B).

Figure 3. *Na*-ASP-2 binds selectively to human B-cells. PBMCs from hookworm-naïve donor volunteers were cultured for 24 h in the presence of increasing concentrations of biotinylayed recombinant *Na*-ASP-2, or with unlabeled *Na*-ASP-2. (**A**) Frequencies of cells with biotinylated *Na*-ASP-2 on the surface were quantified by flow cytometry after incubation with FITC-conjugated streptavidin. (**B**) *Na*-ASP-2^{+} $FITC^{+}$ cells were gated for analysis of the immune cell-surface markers CD3, CD14, CD20 and CD19. Experiments were conducted twice, with two different blood donors; representative data are shown.

Figure 4. Changes in mRNA expression in B cells from a hookworm-naïve human volunteer after culture with *Na*-ASP-2. PBMCs were cultured for 4 or 24 h with either media or 2 μg.ml^{-1} *Na*-ASP-2; CD19^{+} CD20^{+} B cells were sort purified and isolated RNA was subjected to RNA Seq analysis. Scatter plots comparing $\log_2$ ratios of RPKM expression values for B cells from a hookworm-naïve human volunteer following ex vivo co-culture with *Na*-ASP-2 at 4 and 24 hours. Numbers indicate the numbers of up or down-regulated genes.

Figure 5. Differential gene expression following 24 h exposure to *Na*-ASP-2. Heatmap analysis of differentially expressed genes following 24 h culture of sort-purified human B cells with *Na*-ASP-2. Differentially expressed genes are clustered according to their KEGG annotation. The top KEGG pathways assigned to the majority of genes within each cluster are also reported.

Figure 6. *Na*-ASP2 causes B-cell intrinsic reductions in expression of mRNAs immediately downstream of CD79A. Schematic representation of the B cell receptor signaling pathway (modified from http://www.genome.jp/kegg-bin/show_pathway?hsa04662). Genes whose expression was down-regulated following 24 h -co-culture of B cells with *Na*-ASP-2 are indicated by green boxes.

Figure 7. Down-regulated expression of *lyn* and *pi3k* in human B cells from four hookworm-naïve human volunteers after culture with *Na*-ASP-2. PBMCs from four hookworm-naïve donor volunteers were cultured for 24 h in the presence of medium alone or 2 μg.ml^{-1} recombinant *Na*-ASP-2. qPCR analysis of *lyn* and *pi3kR5* was performed on RNA derived from sort-purified B cells. Data were analyzed using the ΔΔCT method (Relative quantification, RQ), whereby actin served as the endogenous gene and samples were normalized to media controls. *P = 0.0295; **P = 0.0004.

Supplementary Figure 1: Impact of *Na*-ASP-2 on B cell expression of activation surface markers following anti-IgM-stimulation and in the steady-state. PBMCs from hookworm-naïve volunteers were pre-incubated for 4 h with 2 μg/mL of either a control protein (BSA) or *Na*-ASP-2 prior to incubation for 24 h with 10 μg/ml goat F(ab')2 anti-mouse IgM or media alone (No treatment). Surface marker expression on gated $CD19^+$ $CD20^+$ B cells was compared by flow cytometry. Color-coded numbers in each inset represents mean fluorescense intensity for each marker. The experiment was performed twice, with a different blood donor each time; representative data are shown.

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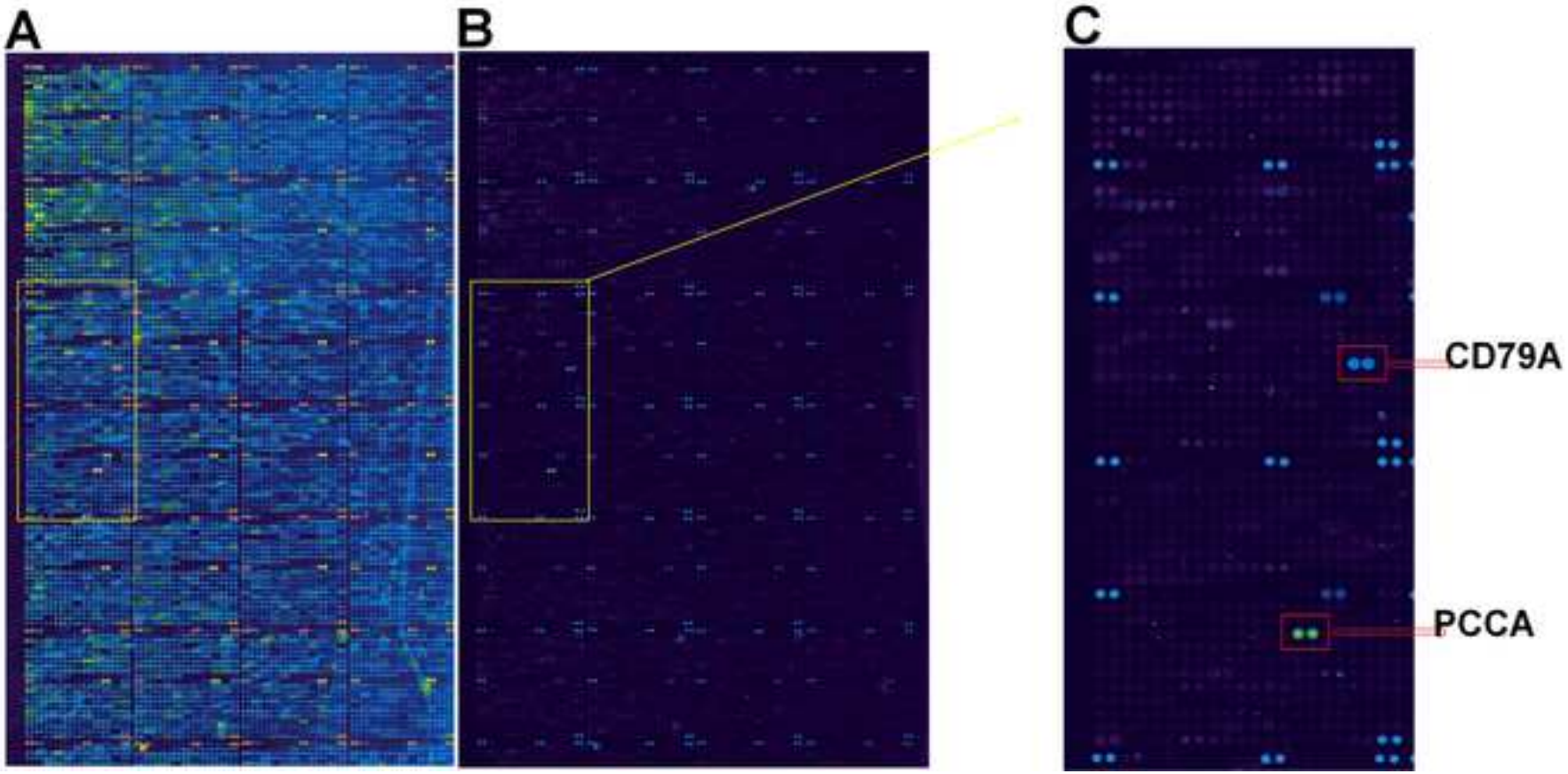

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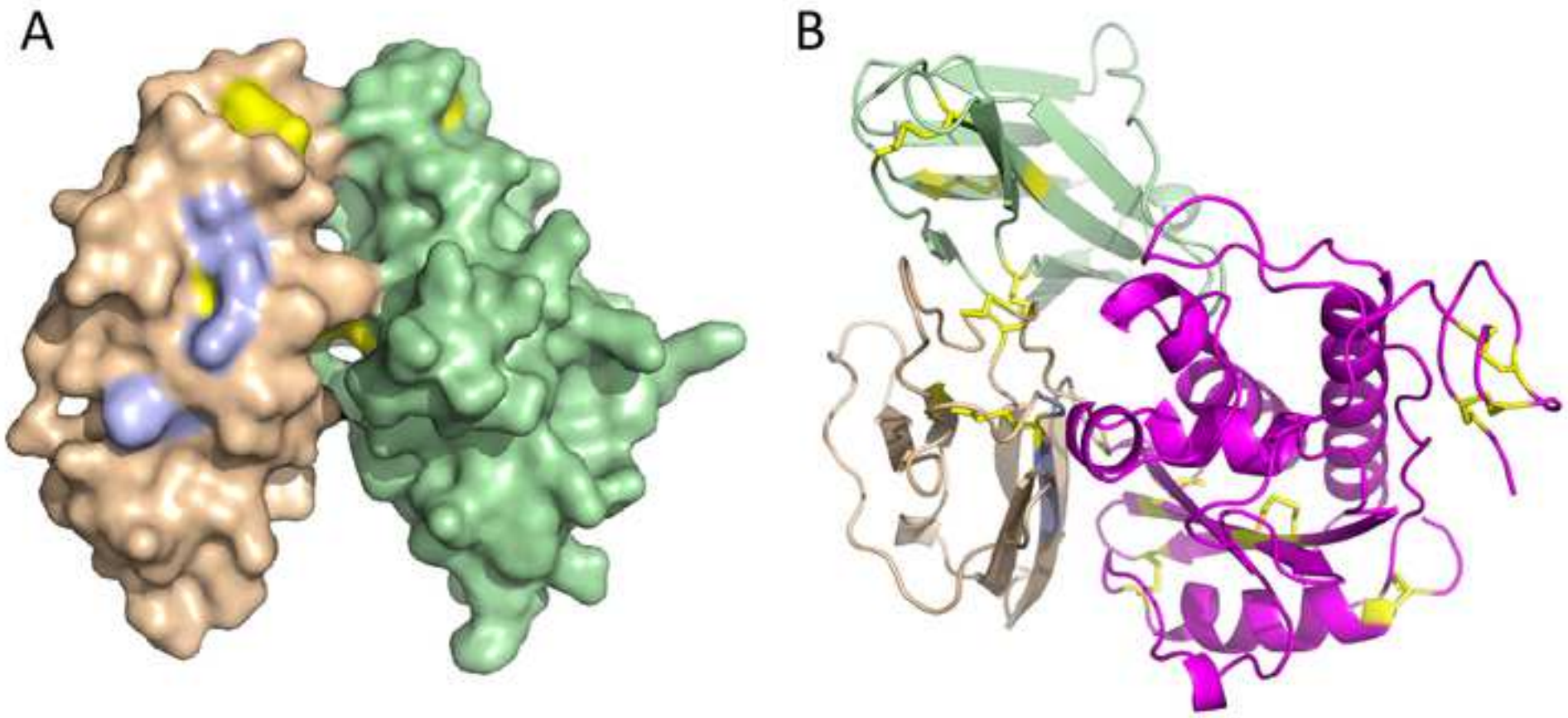

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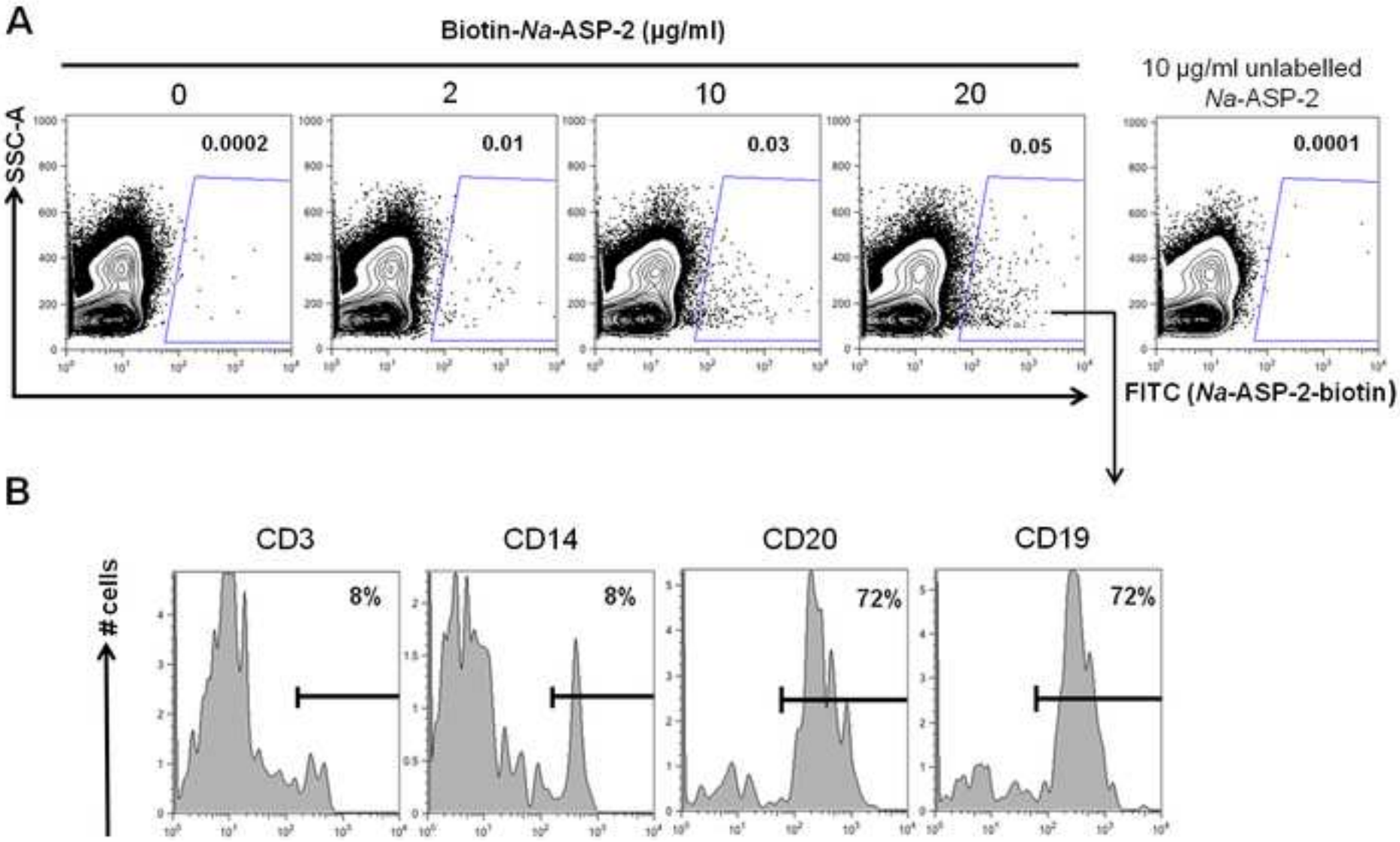

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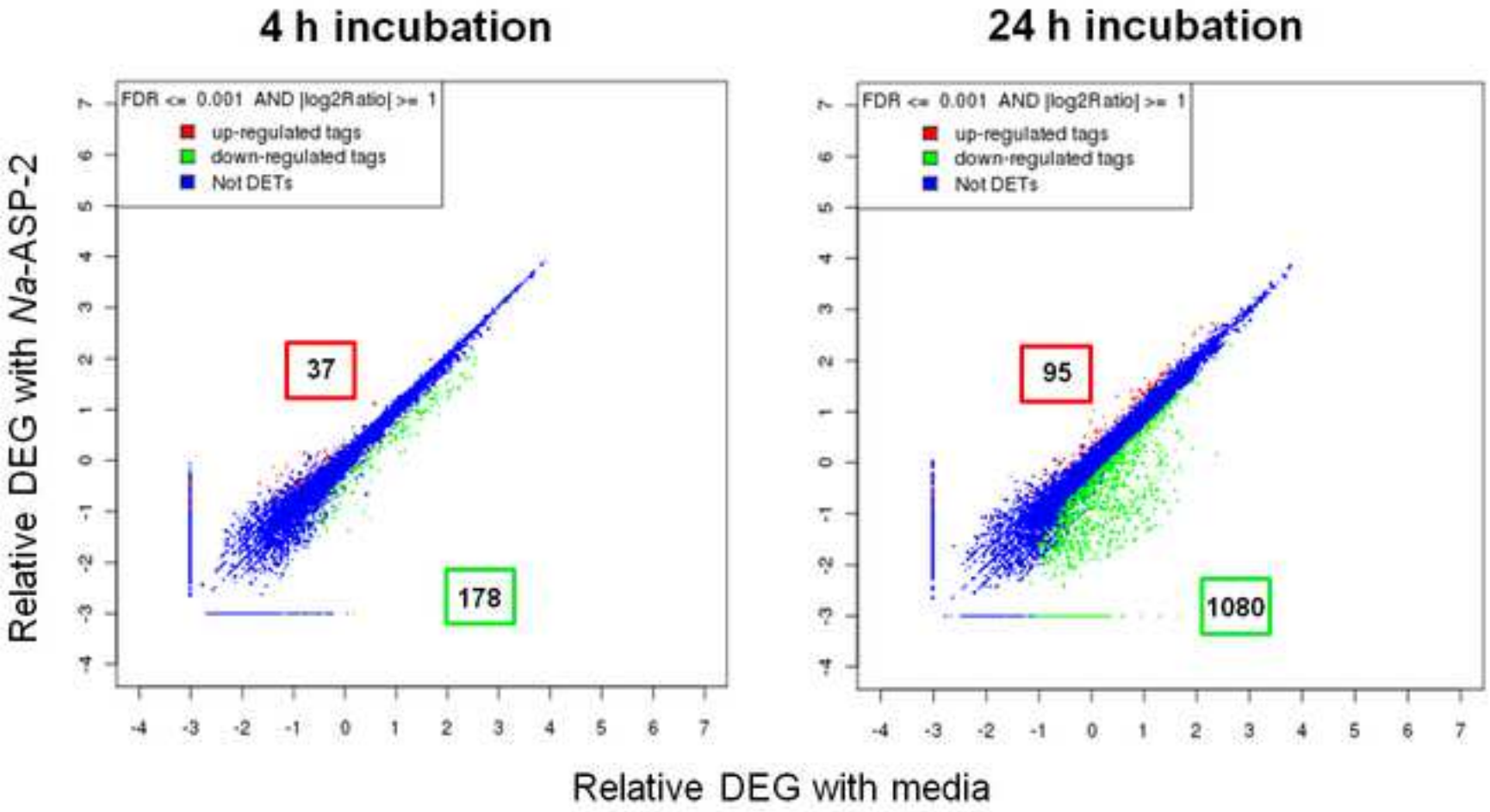

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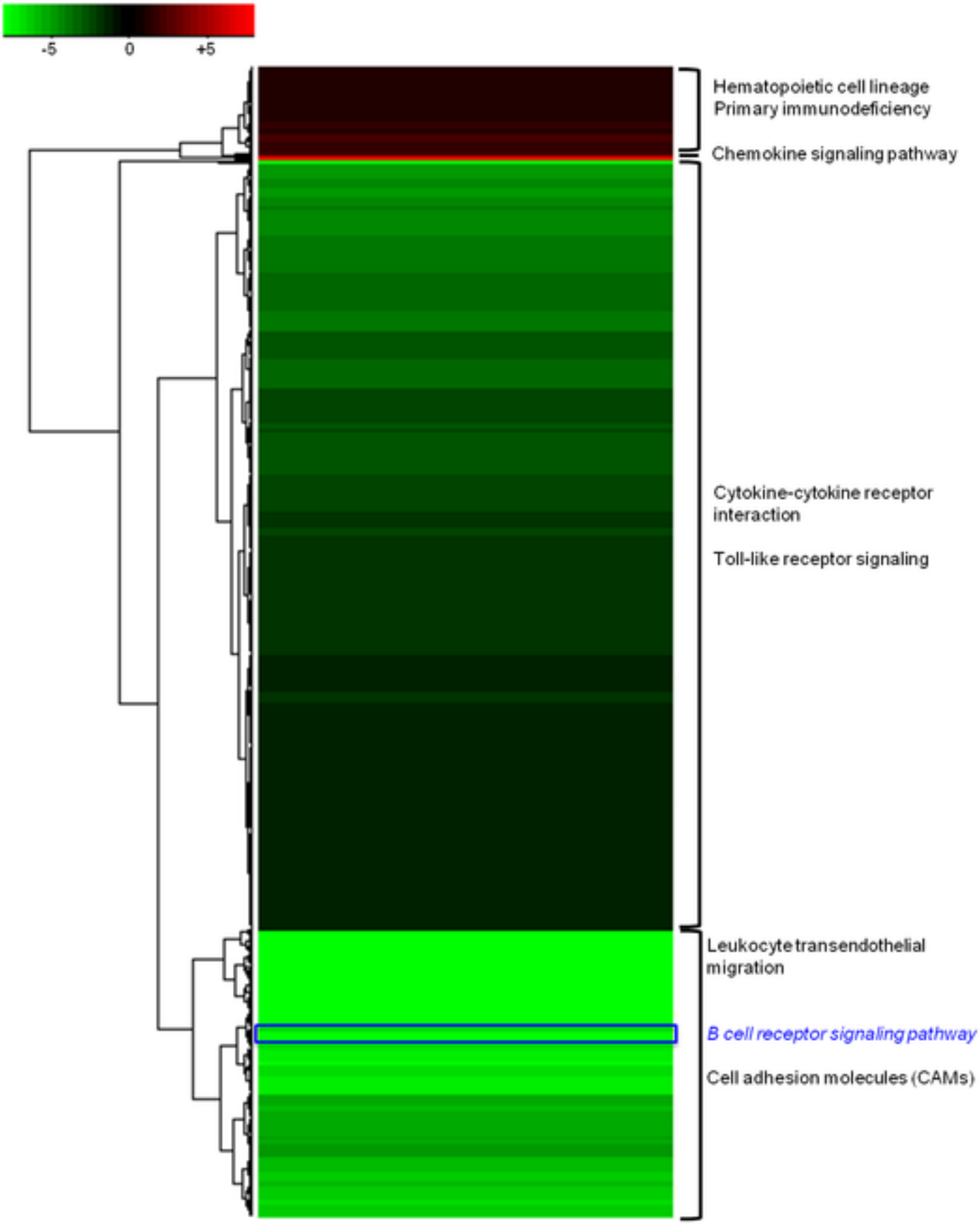

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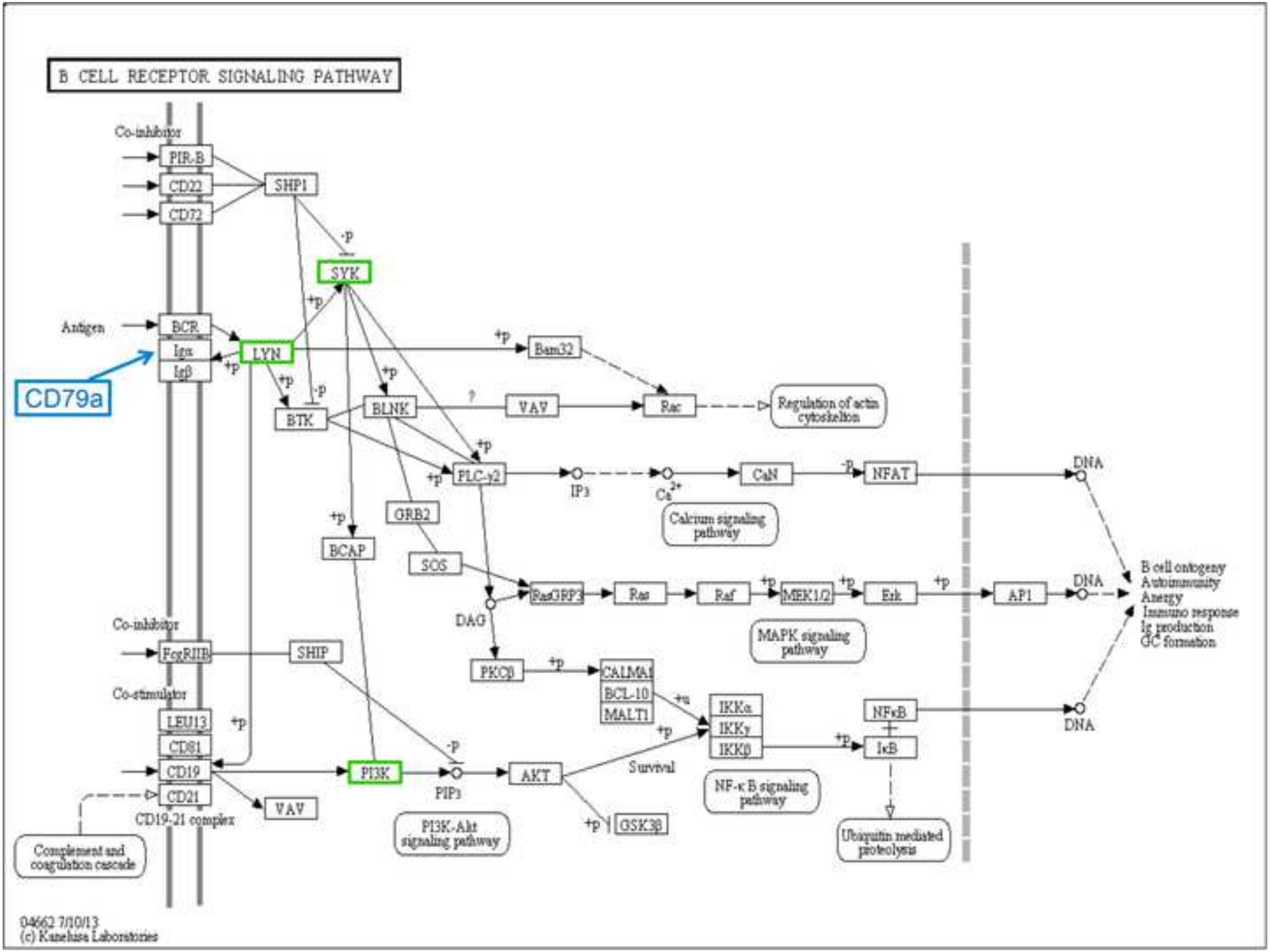

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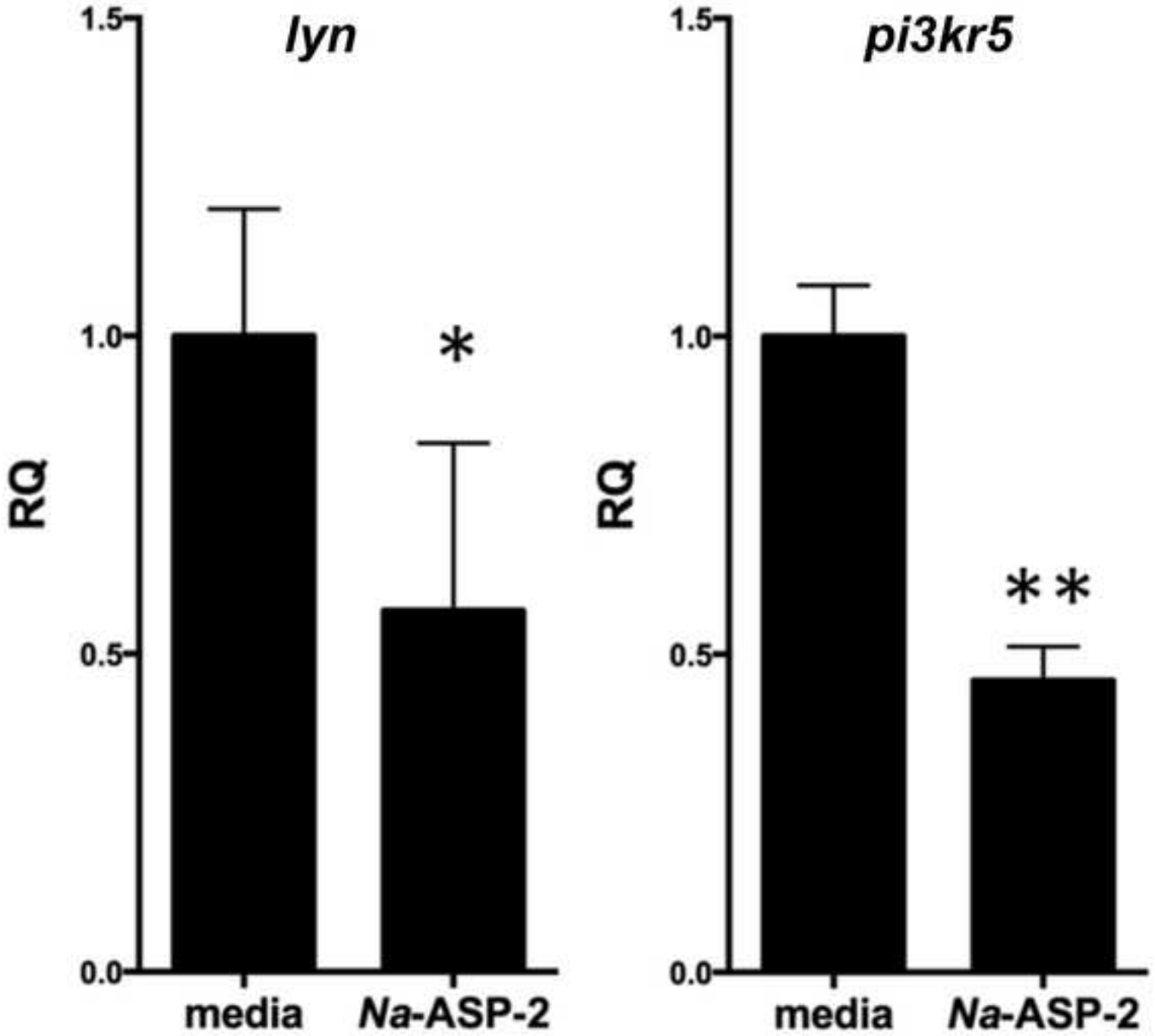

Supplementary Table 1- List of proteins on the Human V5 ProtoArray that interacted with recombinant *Na*-ASP-2.

Intensity	Protein	NCBI code	Description
1600	Propionyl coenzyme A carboxylase (PCCA)	NM_000282.1	Biotin binding enzyme
456.5	Immunoglobulin binding protein 1 (IGBP1/CD79a)	NM_001551.1	Co-receptor of B-cell receptor complex
359.5	Methylcrotonoyl-Coenzyme A carboxylase 1 (alpha) (MCCC1)	NM_020166.2	Biotin metabolism
191.5	Cortactin (CTTN), transcript variant 2, intracellular	NM_138565.1	Actin remodelling enzyme
177.5	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase (EHHADH)	BC000306.1	Biotin metabolism

Supplementary Figure 1
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