Supplementary Online Content

Dubey D, Wilson MR, Clarkson B, et al. Expanded clinical phenotype, oncological associations, and immunopathologic insights of paraneoplastic Kelch-like protein-11 encephalitis. *JAMA Neurol*. Published online August 3, 2020. doi:10.1001/jamaneurol.2020.2231

This supplementary material has been provided by the authors to give readers additional information about their work.

eMethods

Laboratory methods

Indirect immunofluorescence assay (IFA)

Patient serum and CSF and commercial antibodies were tested on a cryosectioned (4 μm) composite of adult mouse tissues: cerebellum, midbrain, cerebral cortex, hippocampus, kidney, and gut. Sections were fixed using 4% paraformaldehyde for 1 minute, then permeabilized with 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS), 0.5%, in phosphate buffered saline (PBS, for 1 minute), and then blocked for 1 hour with normal goat serum (10% in PBS). After PBS-rinse, patient specimen was applied (serum was pre-absorbed with bovine liver powder, 1:240 dilution, and CSF was non-absorbed, 1:2 dilution). After 40 minutes, and PBS wash, a species-specific secondary antibody conjugated with fluorescein isothiocyanate (FITC, 1:100) or tetramethylrhodamine (TRITC, 1:100) was applied (Southern Biotechnology Associates, Inc, Birmingham, AL, USA). Cover slips were mounted using ProLong Gold anti-fade medium (containing DAPI; Molecular Probes Thermo Fisher Scientific, USA). Fluorescence images were captured using Olympus BX51 polarizing microscope with Olympus DP73 high-performance Peltier-cooled, 17.28 megapixel camera. Patient specimens yielding positive results were titrated in doubling dilutions to determine the endpoint of autoantibody detection.

HEK293 cells overexpression Assays

HEK293 cells stably expressing GFP-tagged KLHL11 protein are seeded into black 96 well plates with clear bottoms (Greiner Bio). After 3 days, cells are fixed and exposed to patient serum (1:1000) or CSF (1:100) diluted in 10% normal goat serum for 1 hour. Cells are washed and exposed to TRITC conjugated goat anti-human IgG secondary antibody (Southern Biotech) and incubated for 45 minutes. Cells are washed and stored in PBS in the dark at 4oC until imaging. Plates are imaged at 20x using ImageXpress Micro Confocal High-Content Imaging System (Molecular Devices). Samples are scored by two blinded readers. Positive samples are reflexed to tissue immunofluorescence for confirmation.

Ma2 ELISA

Immulon 2HB Elisa plates are coated using 5ng of protein per well. Ma2 recombinant human PNMA2 from Novus Biologicals is diluted into 0.01M NaPO4 pH 7.4 and incubated one hour at 37 C. The plates are then washed three times using PBS with 0.05% Tween-20. Sera tested in duplicate are centrifuged 10K g then diluted in PBS with 0.05% Tween-20, 10% goat serum to 1/120 and incubated at 37C for one hour. The plate is washed three times then antibody two, goat anti-human IgG FC Fab2 alkaline

phosphatase labeled, is diluted in PBS with 0.05% Tween-20, 10% goat serum and incubated at 37C for one hour. Plates are washed three times then enzyme substrate is added and incubated for one hour at 37C the colorimetric reaction is stopped with the addition of 1M NaOH. The plate is read at absorbance 405nm, positives have an O.D. two times higher than the average normal sera.

Dendritic cell / T cell assay

Cryopreserved peripheral blood mononuclear cells (PBMCs, 5 $x10^6$) from KLHL11 seropositive patients or from healthy controls were thawed, washed with cold media, and re-suspended in complete RPMI 1640 containing 3% fetal bovine serum, 1% penicillin /streptomycin solution, and 0.005% Beta mercaptoethanol. To generated dendritic cells (DC) for antigen presentation, half of the cells were treated with 50 ng/mL GM-CSF, 20 ng/mL IL-4 for 72 hours. During this time the remaining cells were rested in 1 ng/mL IL-7 to promote survival and maintenance of T lymphocytes. DC and T cells were subsequently mixed (1:1) and plated at 100,000 cells per well in a 96 well round bottom plate with 10 ug/mL of KLHL11 (Origene) or vehicle only. As a positive control, wells were pre-coated with 10 ug/mL anti-CD3 (OKT3) and anti-CD28 (9.3) for 24 hours and aspirated just prior to plating cells.

Mass cytometry (CyTOF)[1-3]

Benzonase Nuclease was purchased from Sigma-Aldrich. Maxpar® reagents including water, Cell Staining Buffer (CSB), Cell Acquisition Solution (CAS), Cell-ID Intercalator-Ir, Fix and Perm Buffer, Cell-ID™ 20-Plex Pd Barcoding Kit and EQ Four Element Calibration Beads were purchased from Fluidigm. Paraformaldehyde (PFA) was purchased from EM Sciences and 10X PBS pH 7.2 was purchased from Rockland. Antibodies used for cell surface labeling and phenotyping were purchased from Fluidigm. Custom conjugated antibodies were generated in-house through the Mayo Clinic Hybridoma Core using Maxpar X8 Ab labeling kits (Fluidigm) according to the manufacturer's protocol. The following antibodies were used: 089Y-CD45, 141Pr-CD196 (CCR6), 142Nd-IL-4, 143Nd-CD5, 145Nd-CD4, 147Sm-IL-6, 148Nd-CD278 (ICOS), 149Sm-CD25 (IL-2R), 151Eu-IL-5, 152Sm-TNFa, 153Eu-CD45RA, 154Sm-TIM-3, 155Gd-PD-1, 156Gd-CD183 (CXCR3), 158Gd-CD194 (CCR4), 159Tb-CD197 (CCR7), 160Gd-CD28, 161Dy-CD274 (PD-L1), 163Dy-TGF-beta, 165Ho-CD45RO, 166Er-IL-10, 167Er-CD27, 168Er-IFNg, 169Tm-CD19, 170Er-CD3, 172Yb-IL-17a, 173Yb-Granzyme B, 174Yb-CD8a, 175Lu-Perforin, 176Yb-CD127 (IL-7R).

Immunostaining for cyTOF

After treatment with specified antigens for 72 hours, 4x106 cells were resuspended in 1 mL of CSB. Each sample was incubated for 5 minutes with 0.5 □m Cisplatin solution in PBS. Samples were then washed twice with CSB. An antibody cocktail of the entire phenotyping panel was prepared as a master mix prior to adding 50 μL of cocktail to samples resuspended in 50 μL of CSB. Samples were then incubated at room temperature for 45 minutes. After washing twice with CSB, samples were fixed with 2% PFA in PBS. After fixation and wash, samples were resuspended in 30 nM intercalation solution. Afterwards 30 μL of unique barcoding reagent was added to each sample and incubated overnight at 4°C. On the following morning cells were washed with PBS and pooled prior to resuspension in a 1:10 solution of calibration beads and CAS at a concentration of $0.5x10^6$ cells/mL. Prior to data acquisition samples were filtered).

Mass Cytometry and Data Acquisition

Samples were loaded onto a Helios CyTOF® system (Fluidigm) using an attached autosampler and were acquired at a rate of 200-400 events per second. Data were collected as .FCS files using the Cytof software (Version 6.7.1014). After acquisition intrafile signal drift was normalized to the acquired calibration bead signal using the Cytof software.

Data Analysis

Cleanup of cell debris, removal of doublets and dead cells was performed using FloJo software version 10.5.3 (Ashland, OR). Cleaned fcs files were analyzed by the R-based tool Cytofkit version 3.8 (References below). Clustering and dimensionality reduction to 10,000 events per file was performed using the Rphenograph algorhithm that included all 32 markers in the panel. Visualization of clusters was mapped onto a tSNE map. Relative marker intensities and cluster abundances per sample were visualized by a heatmap.

HLA association

HLA typing was performed on a service basis by reverse SSOP per manufacturer's protocol (LabTYPE, One Lambda Canoga Park, CA).

Statistical analysis

Fischer exact test and Mann Whitney U test were utilized to analyze nominal and continuous variables, respectively (IBM® SPSS version 25).

Assay performance characteristics

All included positive cases were tested and confirmed to be positive on both cell based assay and tissue based immunofluorescence assay. Tissue immunofluorescence (IFA) clinical specificity data was previously published.[4] All tested diseased and healthy controls were negative on tissue IFA.

Cell based assay performance characteristics

A total of 95 healthy donors were tested on the HEK293 cells overexpression assay and all samples were reported <1:1000 (serum, negative) or <1:100 (CSF, negative). In addition,141 cancer patients (including germ cell tumor [seminoma without PNS: 48; ovarian teratoma 18], thymoma 30, small cell lung cancer 45), 7 patients with Morvan's syndrome, 15 patients with Fronto-temportal dementia (FTD) or FTD and amyotrophic lateral sclerosis, 18 patients with monoclonal gammopathy of undetermined significance, 7 patients positive for anti-mitochondrial antibodies, 34 patients with systemic lupus erythematosus, 18 patients with hypergammaglobulinemia (without known underlying malignancy), 22 patients with Sjogren's syndrome, 13 Ma2 IgG seropositive patients and 122 NMDA-R IgG seropositive patients were tested (**eTable 1**). Three control cases positive on CBA were negative on tissue IFA and Phage immunoprecipitation sequencing (Phage IP Seq). Demonstrating a false positive rate 0.6% if cell based assay is utilized as the only confirmatory method. To analyze KLHL11 seropositivity use of HEK293 cells overexpression 96 well plate assays for screening, and tissue based IFA ("sparkles pattern")[4] for confirmation, provides optimal clinical specificity.

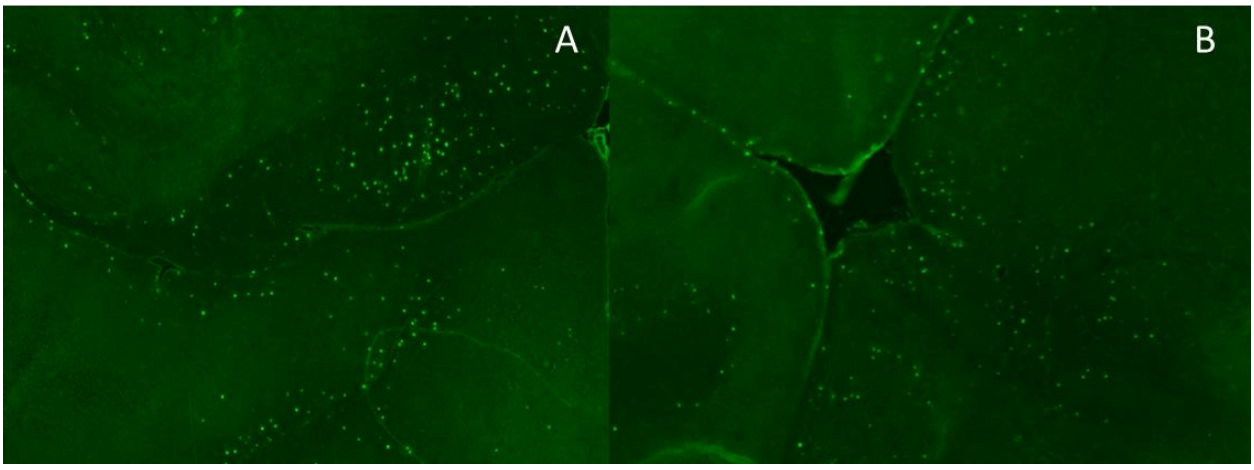
eTable 1: Healthy and disease controls tested on KLHL11 cell based assay.

Controls	Cell based assay results, positive/total	Tissue IFA and/or Phage IP Seq results (for positive cell based assay samples)
Healthy Adult	0/95 (all serums)	
Seminoma	2/48 (all serums)	Both serum samples KLHL11 IgG negative on tissue IFA and Phage IP Seq
Ovarian Teratoma	0/18 (all serums)	
Thymoma	0/30 (all serums)	
SCLC	0/45 (all serums)	
Morvan's syndrome	0/7 (serum)	
FTD or FTD and ALS [a]	0/15 (14 serums, 1 CSF)	
Anti-mitochondrial antibody positive cases	1/7 (4 serums, 3 CSFs)	Serum sample KLHL11 IgG negative on tissue IFA and Phage IP Seq
Systemic lupus erythematosus	0/34 (all serums)	
Hypergammaglobulinemia	0/18 (all serums)	
Sjogren's syndrome	0/22 (all serums)	
Ma2 postive	0/13 (8 serums, 5 CSFs)	
NMDA-R IgG positive cases	0/122 (50 serums, 72 CSFs)	

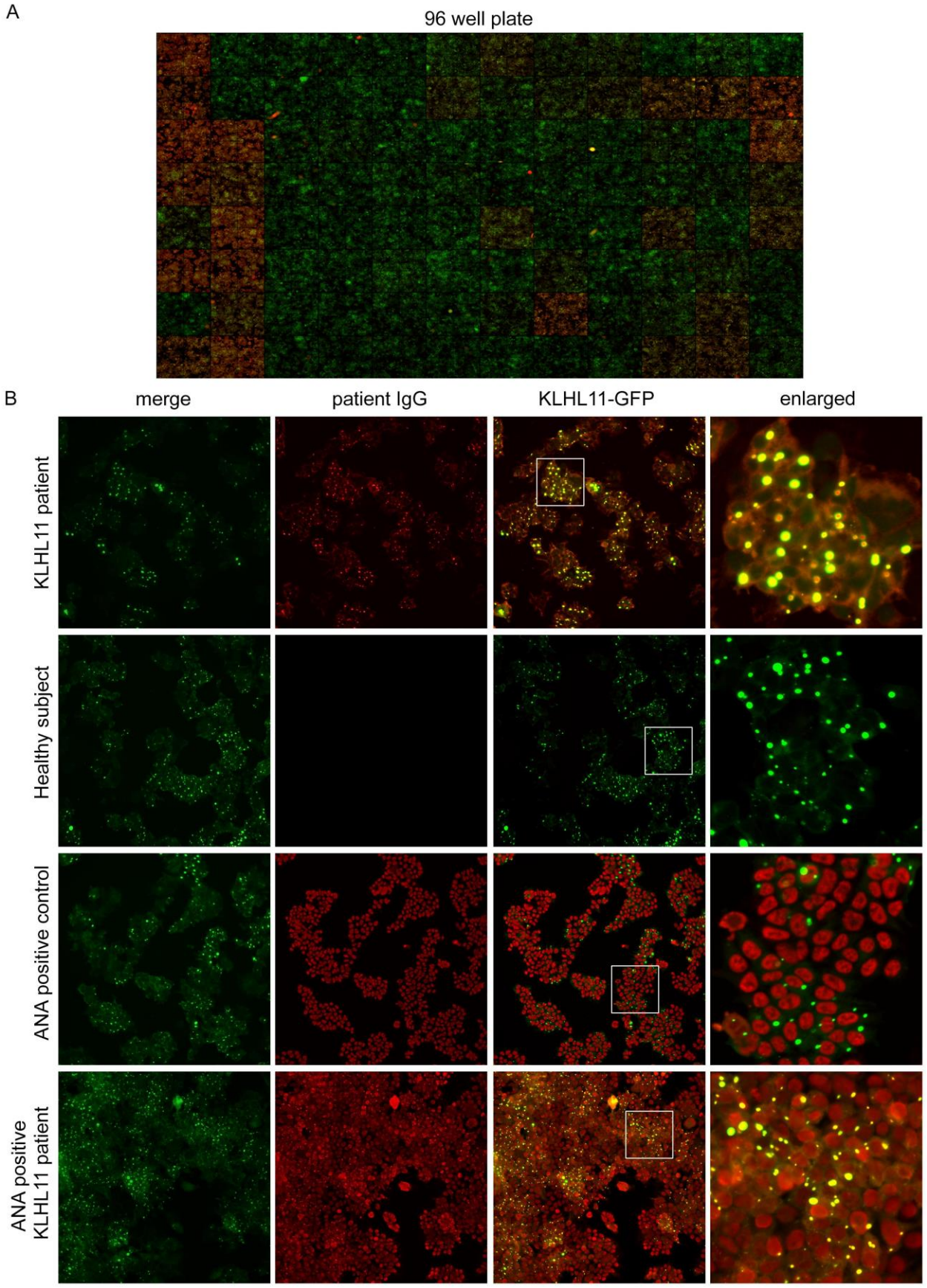
Key: IFA, Indirect immunofluorescence assay; NMDA-R, N-methyl D-aspartate receptor; KLHL11, Kelch like protein 11; Phage IP Seq, phage immunoprecipitation sequencing; SCLC, small cell lung cancer, FTD, frontotemporal dementia, ALS, amyotrophic lateral sclerosis, [a], one patient with FTD and ALS.

eFigures (1-6)

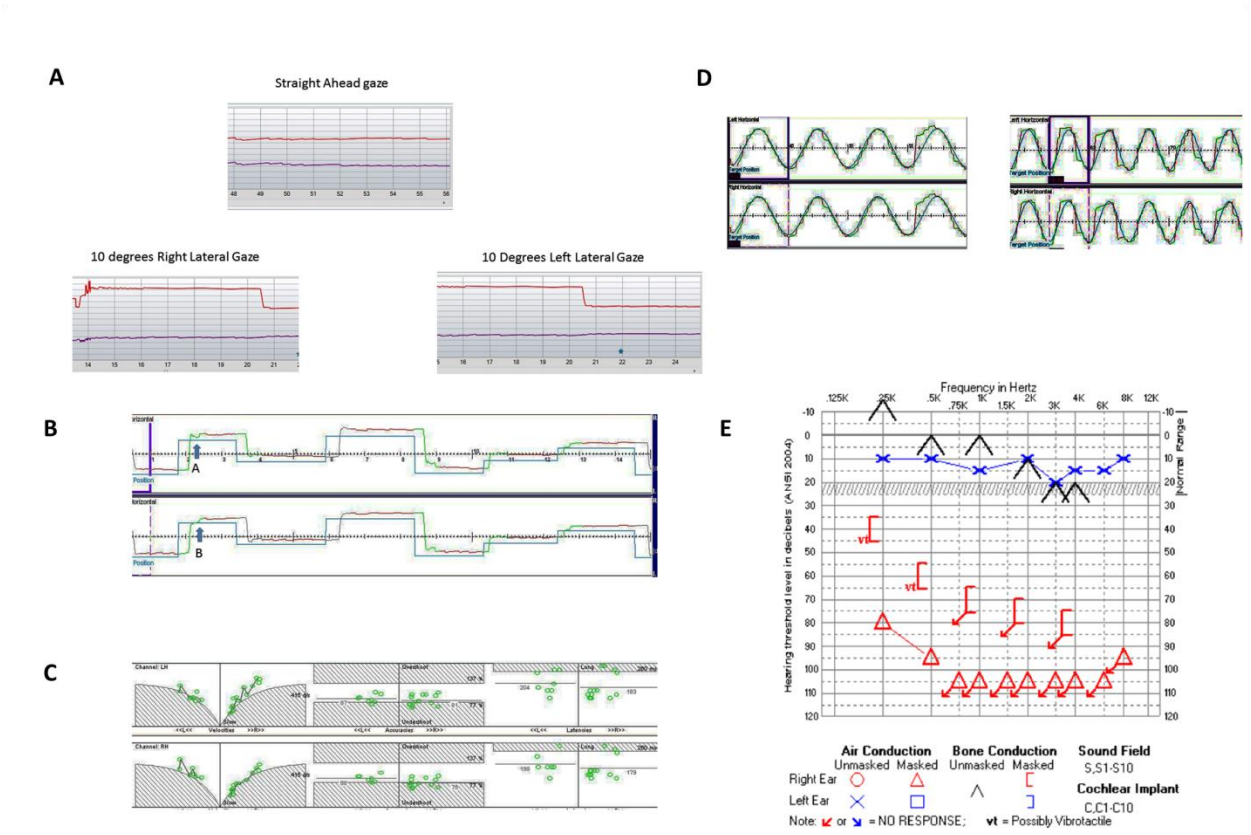
eFigure 1 (A,B) KLHL 11 patient IgG rodent brain tissue indirect immunoflouresnce assay. Punctate binding to peri-meningeal and periventricular areas (A, B) consistent with staining pattern identified as “Sparkles”.



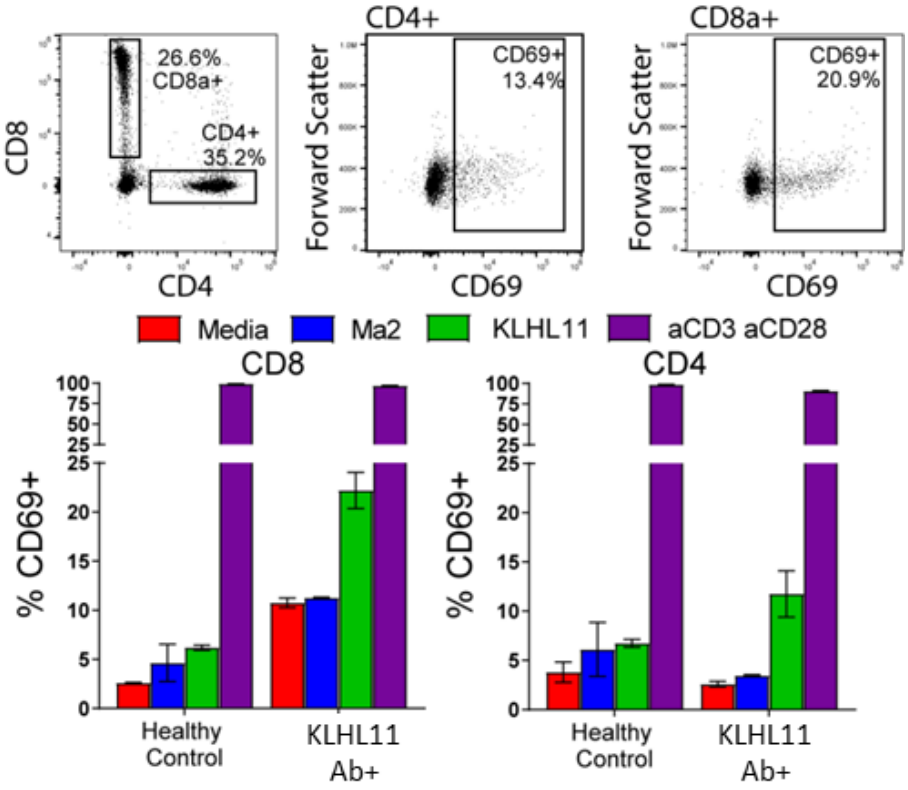
eFigure 2 (A-G): KLHL11 patient IgG binds specifically to GFP-tagged KLHL11 foci in transfected cells. View of 96 well plate scan image GFP-KLHL11 (green) and patient IgG (red) (A). Representative images of KLHL11 patient and control patient IgG binding to KLHL11 expressing cells (B). KLHL11-IgG binds specifically to GFP-positive foci (yellow in merged; top row). Healthy subject does not bind to GFP-KLHL11 protein (second row). Interfering antibody staining, such as ANA, is distinct from KLHL11-specific antibody binding (bottom two rows). Boxes indicate region enlarged in last column. Scale bars, 20 μm.



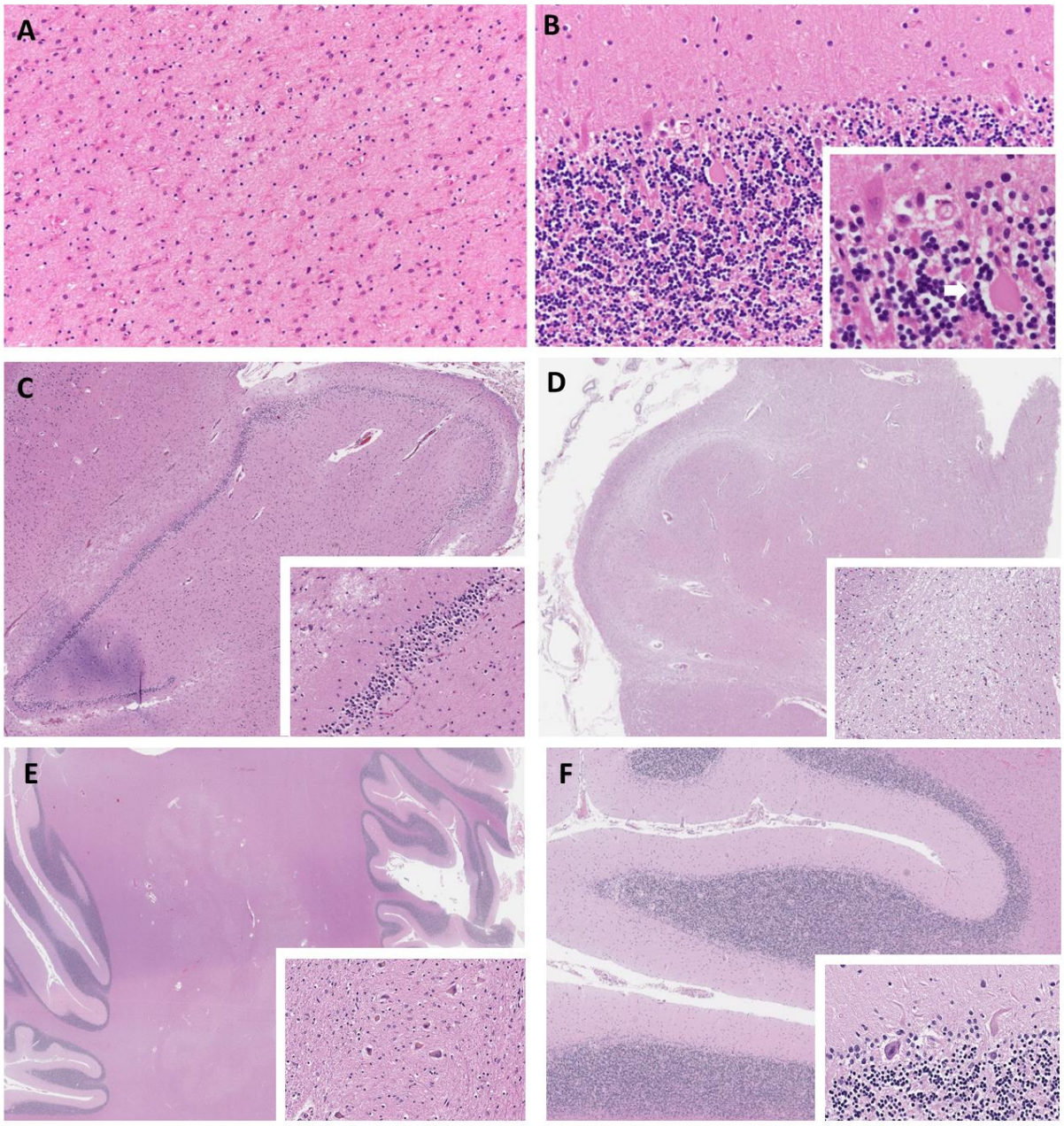
eFigure 3 (A-F): Quantification of eye movement abnormalities and sensorineural hearing loss in a patient with autoimmune KLHL11 encephalitis. Downbeat nystagmus is low amplitude in straight ahead gaze (4 deg/sec) (A, purple tracing). Head impulse test and vestibular evaluation demonstrated increased right horizontal canal gain (1.1, normal gain: 0.7 -1.0), right anterior canal gain (1.2) and reduced left anterior canal gain (0.55). Right posterior canal gain (0.98), left horizontal canal gain (0.83), and left posterior canal gain (0.98) were within normal limits (not shown). Subclinical right internuclear ophthalmoparesis based on saccade tracings (B) and velocity profile with normal latency (C). Pursuit evaluation demonstrates reduced horizontal pursuit gain (0.45, normal gain 1) to right and left (D). Audiogram depicts severe high frequency hearing loss involving the left ear (E, blue) and mild to moderate high frequency hearing loss involving the right ear (F, red).



eFigure 4: Autoantigen specific T-cell activation. PBMCs from healthy volunteers, KLHL11 IgG (KLHL11 Ab+) or Ma2 IgG seropositive (Ma2 Ab+) patient were differentiated into dendritic cells for 3 days a recombined with autologous PBMC-derived T cell cultures. DC-T cell cocultures of healthy volunteers and KLHL11 IgG+ were treated with media, full length KLHL11 and PNMA2 protein antigen for 72 hours. Flow cytometric analysis demonstrated that CD8+ and CD4+ T cells from KLHL11 Ab+ patients did not exhibit increased frequency of CD69 expressing in response to treatment with PNMA2 protein antigen compared to healthy controls, but did exhibit increased frequency of CD69 expressing CD8+ and CD4+ T cells in response to treatment with KLHL11 protein ($P < 0.001$; Tukey One-way ANOVA).



eFigure 5 (A-F): A**utopsy of two patients (Case A-B, Case C-F) with chronic cerebellar ataxia and thalamic lesions.** Autopsy of patient 1 (A,B) demonstrating severe neuronal loss and gliosis in the right thalamus (A) and loss of Purkinje cells (B). The inset in Panel B shows a torpedo (arrow), a Purkinje cell axonal swelling typical of Purkinje cell degeneration. Autopsy of patient 2 (C-F) demonstrating hippocampal sclerosis (C), inferior olivary degeneration (D), dentate nuclei degeneration (E) and Purkinje cell loss (F). The insets in the Panels C-E demonstrate respective pathology at a higher magnification.



eFigure 6 (A, B): Immunohistochemical staining of lung adenocarcinoma from KLHL11 encephalitis patient. H&E (A) and KLHL-IgG (B) staining of a lung adenocarcinoma from a patient with autoimmune KLHL11 encephalitis demonstrates cytoplasmic expression of KLHL11in the tumor cells.