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Feature Review

Molecular disease mechanisms of human antineuronal monoclonal autoantibodies

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Autoantibodies targeting brain antigens can mediate a wide range of neurological symptoms ranging from epileptic seizures to psychosis to dementia. Although earlier experimental work indicated that autoantibodies can be directly pathogenic, detailed studies on disease mechanisms, biophysical autoantibody properties, and target interactions were hampered by the availability of human material and the paucity of monospecific disease-related autoantibodies. The emerging generation of patient-derived monoclonal autoantibodies (mAbs) provides a novel platform for the detailed characterization of immunobiology and autoantibody pathogenicity *in vitro* and in animal models. This Feature Review focuses on recent advances in mAb generation and discusses their potential as powerful scientific tools for high-resolution imaging, antigenic target identification, atomic-level structural analyses, and the development of antibody-selective immunotherapies.

Highlights

Advances in the cloning of human monoclonal autoantibodies (mAbs) and their recombinant production in theoretically unlimited amounts have sparked new opportunities to study pathogenic mechanisms *in vitro* and *in vivo*, thereby providing direct proof of autoantibody pathogenicity in autoimmune encephalopathies.

Human mAbs – in contrast to polyclonal sera or cerebrospinal fluid samples – are valuable scientific tools for novel experimental approaches ranging from super-resolution microscopy to structural determination of antibody–antigen interaction at the atomic level.

Only analyses at the monoclonal level have demonstrated that, despite binding the same molecules, mAbs group into subclasses with diverse epitopes, isotypes, and distinct functional effects.

Human mAbs can guide the development of highly selective antibody-specific immunotherapies for autoimmune encephalopathies.

Autoantibodies in neurological diseases

The discovery that autoantibodies targeting neuronal and glial proteins can be directly pathogenic has led to the definition of an entirely new entity of neurological disorders known as the autoimmune encephalitides (AEs) [1]. The rapidly expanding field of AEs is driven by the continuous identification of novel antineuronal autoantibodies (Table 1) [2]. Several of these autoantibodies confer pathogenicity by binding the exposed extracellular domains of surface-expressed neuronal antigens (Box 1) [1]. Autoantibodies can modulate electrophysiological functions and synaptic transmission by targeting ion channels [3,4] or essential synaptic proteins [5,6]. The growing awareness of these disorders brought about new concepts in several neuropsychiatric syndromes, allowing their reclassification as antibody-mediated diseases that are amenable to immunotherapy [7]. AEs constitute a heterogeneous group of disorders with diverse symptomatology including behavioral changes, cognitive impairment, memory dysfunction, psychosis, movement disorders, parasomnias, dysautonomia, and seizures [8]. The most common autoantibodies target the autoantigens aquaporin 4 (AQP4) [9] in patients with **neuromyelitis optica spectrum disorder (NMOSD)** (see Glossary), the *N*-methyl-D-aspartate receptor (NMDAR) [10], and leucine-rich glioma-inactivated 1 (LGI1) [11] in encephalitis.

Despite increasing knowledge of how autoantibodies cause neurological disease, initial functional studies were severely limited by the restricted availability of patient serum and, in particular, cerebrospinal fluid (CSF) samples. In addition, human biospecimens are a polyclonal mixture of millions of different antibodies, preventing clear-cut conclusions about whether the observed structural and functional effects are exclusively caused by monospecific antineuronal autoantibodies. The cloning and recombinant production of human mAbs is an important emerging strategy to circumvent these limitations [4–6,12]. It allows the generation of disease-specific human mAbs in theoretically unlimited amounts and has been paving the way for precise functional *in vitro* and *in vivo* characterization of autoantibodies. This also includes insights into the

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Ig repertoire of a humoral autoimmune response based on mAbs sequence information and the experimental use of precisely determined mAb concentrations, which is essential for reproducibility. Human mAbs for the first time enabled understanding of the biophysical properties of disease-driving autoantibodies and allowed high-resolution structure determination with cryogenic electron microscopy (cryo-EM) [13].

This Feature Review summarizes the most recent advances in the generation and functional characterization of human mAbs and their potential as 'tools for innovation': at a point in time where preclinical studies convincingly confirm the pathophysiological functions of mAbs, where the first structural experiments allow the mechanistic understanding of antibody–antigen interaction at atomic resolution, and – most notably – where the first tailored antibody-selective immunotherapies are emerging.

Single-cell autoantibody generation from patients

Cloning of recombinant human monoclonal antibodies

The increasingly available single-cell technology of cloning recombinant human mAbs offers rapid production of large amounts of autoantibodies for the study of antigenic specificity and effector functions [34,35]. The process begins with the collection of patient blood or CSF (Figure 1A), followed by fluorescence-activated cell sorting (FACS) to isolate single **memory B cells (MBCs)** and **antibody-secreting cells (ASCs)** into collection plates with lysis solution [3–6,35–39]. In each well, the extracted single-cell mRNA is reverse transcribed to cDNA, followed by a nested PCR approach with degenerated primers that amplifies the variable Ig gene of the Ig heavy chain (IgH) and both possible Ig light chains (IgLs), kappa and lambda. The PCR products are then sequenced and analyzed with customized bioinformatics software that automatically checks for productive rearrangements of the variable (V) regions of the Ig sequences [40]. A final PCR step specifically amplifies the productive variable genes of IgH and IgL and prepares them for subcloning into a human IgG expression vector. The expression plasmids are amplified in *Escherichia coli* and then sequenced. Plasmid DNA with 100% sequence homology with the original PCR sequence is used for antibody production by co-transfecting matching variable IgH and IgL sequences into human embryonic kidney (HEK293T) cells. HEK293T cells secrete the produced mAbs into the supernatant, from which they are collected and purified. Antigen-specific binding of the mAbs is then examined with cell-based assays (CBAs) and tissue-based assays (TBAs) (Box 2), which are diagnostic assays also employed for patient samples in clinical practice (Figure 1B). After confirmation of target binding, mAbs are used for functional *in vitro* and *in vivo* characterization.

Alternatively, mAbs can be generated with hybridoma technologies that are based on the fusion of primary human B cells with myeloma cell lines to create an antibody-secreting hybridoma. While the B cell confers antigen specificity, the myeloma cell imparts immortality, allowing indefinite cultivation of the hybridoma cell [41]. Once the hybridoma clones are established, mAb production, preserving the native pairing of the variable and constant Ig gene combination, is highly reproducible [42]. Major challenges with hybridoma technology include the time-consuming process of generating genetically stable hybridoma cell lines and the lack of suitable fusion partners, limiting the use of this methodology for mAb isolation [42]. Immortalization of B cells by infectious transformation with Epstein–Barr virus (EBV) showed some success in mAb manufacture; however, this approach has been limited by the low efficiency of B cell immortalization and low yield of mAb isolation [43].

Single-cell RNA-seq

In parallel with the rapid technical advances in mAb generation, single-cell RNA-seq methods have emerged offering high-throughput analysis of B cell diversity and Ig repertoires at the

Glossary

Affinity maturation: a process by which B cells produce antibodies with increasing affinity (strength of interaction between the antibody and its antigen) on continuous antigen exposure; involves somatic hypermutations and antigen-specific clonal selection.
Antibody-secreting cells (ASCs): plasma cells or plasmablasts that are responsible for long-term protective antibody production in health or pathogenic autoantibody production in disease.
Back-mutation: a process by which patient-derived mutated antibodies are reverted to their original unmutated germline configuration using molecular biology techniques.
Chimeric autoantibody receptor (CAAR) T cells: express the target antigen of a pathogenic autoantibody in their extracellular domains. They are re-engineered from conventional chimeric antigen receptor (CAR) T cells that incorporate a tumor-targeting antibody-derived extracellular single-chain variable fragment (scFv) instead. CAR T cells mediate an antitumor immune response and have had therapeutic success in a wide range of hematological (and increasingly solid) malignancies.
Clonal expansion: a process by which antigen-specific B cells proliferate following recognition of their cognate antigen, giving rise to a clone of identical cells with the same antigen specificity.
Fab fragment: contains the antigen-binding part of an antibody and lacks the Fc domain; thus, loses cytotoxicity effector functions and the ability of receptor internalization.
Gamma-aminobutyric acid type A receptor ($GABA_AR$) encephalitis: AE manifesting with refractory seizures. Associated autoantibodies target the $GABA_AR$, a ligand-gated chloride channel that modulates fast inhibitory synaptic transmission and suppresses neuronal activity in the adult brain.
Leucine-rich glioma-inactivated 1 (LGI1) encephalitis: limbic encephalitis presenting with memory deficits that may be preceded by episodes of faciobrachial dystonic seizures. Autoantibodies target LGI1, an extracellularly secreted neuronal protein that is involved in the formation of a trans-synaptic complex.
Memory B cells (MBCs): long-term surviving B cells that differentiate from

level of individual cells. Single-cell RNA-seq platforms based on microfluidic systems such as Drop-Seq, inDrop, or commercial 10X Genomics enable the parallelizable processing of thousands of individual cells for transcriptome and genome analysis [44–46]. Microfluidic approaches encapsulate single cells from a cell suspension in droplets containing uniquely barcoded beads that allow the isolated mRNA transcripts from each cell to be traced back to their origin with sequencing analysis. The main advantages of these bulk sequencing technologies are the relatively low cost and the ability to process a large number of cells, which enables the detection of rare cell types and Ig rearrangement with high sensitivity [46]. While single-cell RNA-seq analyses reveal insights into the clonal diversity and **affinity maturation** of a humoral immune response, the effector functions and antigenic specificity of autoantibodies cannot be characterized, emphasizing the need for mAb generation.

Understanding disease mechanisms by use of human antineuronal mAbs

Uncovering the detailed relationship between the diverse spectrum of clinical symptoms in AEs and the associated autoantibodies has just started. Patient-derived recombinant mAbs have created new opportunities for the study of pathogenic autoantibody mechanisms, which translate basic science into clinical phenotypes with relevance for immediate and near-future medical practice (see Clinician's corner). Findings from monoclonal studies highlight which extensive and detailed observations have become possible with the availability of AE-related mAbs and are reviewed in the following section.

Complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC)

CDC contributes to neuronal injuries in neuroinflammatory disorders [47]. Complement activation is caused by the binding of complement component C1q to the Fc region of autoantibodies engaging their target, leading to lysis of the target cell [48]. For example, AQP4 mAbs derived from the CSF of an NMOSD patient induced complement-mediated cell death [12]. This mechanism was specifically enhanced when C1q formed multivalent interactions with autoantibodies binding to AQP4 assembled in orthogonal arrays of particles (Figure 2A) [49–51]. Also, via recognition of the autoantibody's Fc domain, predominantly by natural killer (NK) cells, ADCC can lyse neurons as demonstrated by AQP4 mAbs (Figure 2B) [12]. *In vivo* experiments with human AQP4 mAbs confirmed the central involvement of both, CDC and ADCC, in NMOSD pathogenesis by recapitulating disease-specific histopathological lesions in rodents [12,52,53].

Similarly, preclinical studies with affinity-purified polyclonal IgG from patients with high titers of **myelin oligodendrocyte glycoprotein (MOG)** autoantibodies revealed that these autoantibodies can activate CDC and ADCC [31,32]. However, MOG autoantibodies exerted pathological changes in rodents only with intrathecal co-injection of myelin-reactive T cells [54]. Future studies with patient-derived MOG mAbs will determine their explicit contribution to disease and rodent models.

Autoantibody-mediated crosslinking and receptor internalization

Another disease mechanism is crosslinking of membrane proteins by autoantibodies, which leads to selective internalization and degradation, thus reducing the number of neuronal surface-expressed target proteins [14,33,55]. This mechanism was observed with mAbs in patients with **NMDAR encephalitis** (Figure 2C) [4,41,56,57], the most common AE in developed countries. Here, NMDAR mAbs alone were sufficient to promote receptor internalization and concentration-dependent electrophysiological changes in neurons. Cerebroventricular infusion of human NMDAR mAbs in mice resulted in decreased synaptic NMDAR density and reduced NMDAR-mediated currents and led to memory deficits, impaired hippocampal functional connectivity, and increased seizure susceptibility, mimicking key pathogenic features of NMDAR

naïve B cells after first antigen exposure with the ability to mount an immediate immune response on restimulation by the specific antigen.
Myasthenia gravis (MG): autoimmune disorder characterized by muscle weakness. Autoantibodies target postsynaptic membrane components at the neuromuscular junction, such as the nicotinic AChR or MuSK.
Myelin oligodendrocyte glycoprotein (MOG): glycoprotein expressed on oligodendrocytes that represents a target for autoantibodies in demyelinating disorders, collectively known as MOG antibody-associated disorders.
Neuromyelitis optica spectrum disorder (NMOSD): an inflammatory demyelinating disorder presenting with relapses of optic neuritis and transverse myelitis. Autoantibodies against the astrocytic water channel AQP4 are highly specific for NMOSD and allow its distinction from multiple sclerosis.
***N*-methyl-D-aspartate receptor (NMDAR) encephalitis:** most common AE, presenting with a complex neuropsychiatric syndrome, female predominance, and frequent tumor (teratoma) association, which is caused by autoantibodies against the NMDAR.
Somatic hypermutation (SHM): mechanism contributing to affinity maturation that introduces mutations into the V region of the Ig gene, which increases Ig diversity and affinity towards an antigen.
Unmutated germline ancestors: computationally or experimentally derived antibodies that represent the unmutated germline B cell receptors of the naïve B cell from which the mutated antibodies are derived.
Valency: refers to the number of antigen-binding sites of an antibody.

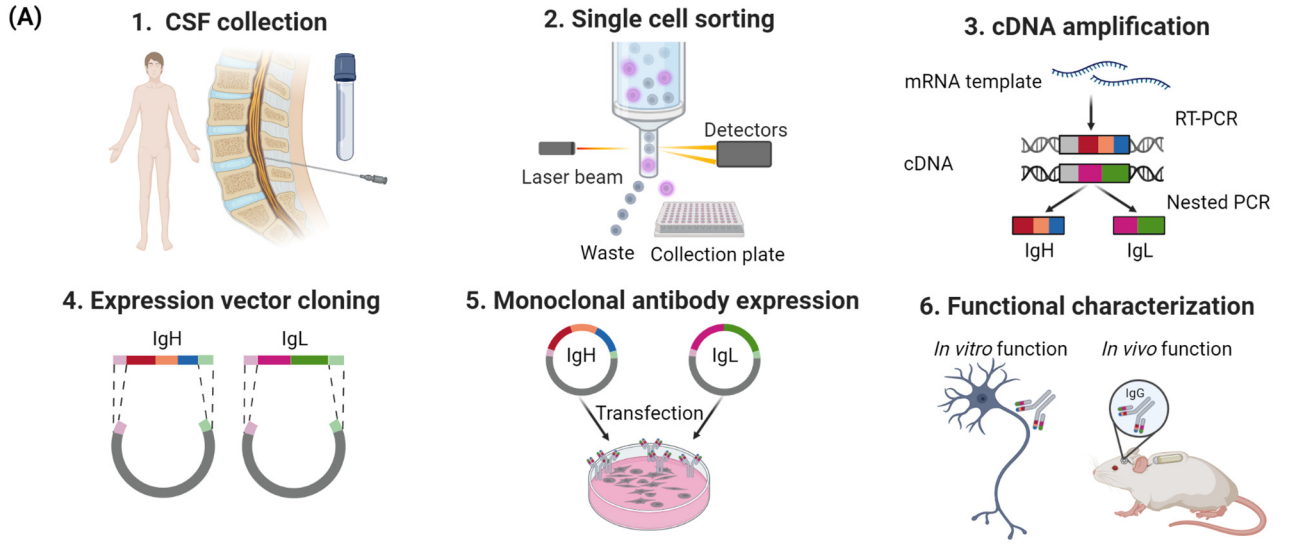
Table 1. Overview of brain-directed autoantibodies[a]

Antibody target	Main clinical symptom/syndrome	Proposed antibody pathogenicity	Availability of mAb data	Special characteristic	Refs
AMPAR	Limbic encephalitis, epileptic seizures	AMPAR internalization	No	>60% cancer association, mostly thymoma, SCLC	[14]
AQP4	NMOSD	ADCC, CDC	Yes	Relapsing clinical course	[9]
CASPR2	Limbic encephalitis, neuromyotonia, Morvan syndrome	Disruption of CASPR2–contactin-2 interactions	Yes	20–50% cancer association, mostly in patients with Morvan syndrome (thymoma)	[11,15]
$GABA_AR$	Encephalitis, refractory epileptic seizures, psychosis	$GABA_AR$ downregulation, $GABA_AR$ antagonism	Yes	MRI with cortical and subcortical T2/FLAIR hyperintensities	[16,17]
$GABA_BR$	Limbic encephalitis, epileptic seizures	$GABA_BR$ antagonism	No	~50% cancer, mostly SCLC	[18,19]
GAD65	Limbic encephalitis, epileptic seizures, stiff-person syndrome	Inhibition of GABA release	Yes	Concomitant type 1 diabetes mellitus	[20–22]
DPPX	Encephalitis, cognitive impairment, central hyperexcitability	DPPX and Kv4.2 downregulation	No	Prodromal diarrhea	[23]
GlycineR	PERM, stiff-person syndrome	GlycineR internalization, glycineR antagonism	No	<10% tumor association	[24,25]
IgLON5	Sleep disorder, movement disorder, cognitive impairment	IgLON5 internalization	No	Neurodegenerative features (hyperphosphorylated tau deposition)	[26]
LGI1	Limbic encephalitis, epileptic seizures, faciobrachial dystonic seizures	Disruption of LGI1–ADAM22/23 interactions	Yes	Hyponatremia, MRI with mesiotemporal T2/FLAIR hyperintensities	[11]
mGluR1	Cerebellar ataxia	mGluR1 antagonism, reduction of Purkinje cell activity	No	Associated with Hodgkin lymphoma	[27,28]
mGluR5	Limbic encephalitis, psychosis	mGluR5 downregulation	No	Ophelia syndrome: if associated with Hodgkin lymphoma	[29,30]
MOG	Optic neuritis, ADEM, transverse myelitis	ADCC, CDC	No	Often children and young adults	[31,32]
NMDAR	Encephalitis, psychosis, epileptic seizures, dyskinesia, dysautonomia	NMDAR internalization	Yes	Frequently associated with ovarian teratoma in young women (20–40%)	[10,33]

[a]Abbreviations: ADEM, acute disseminated encephalomyelitis; $GABA_BR$, gamma-aminobutyric acid type B receptor; GAD65, glutamic acid decarboxylase 65; GlycineR, glycine receptor; DPPX, dipeptidyl-peptidase-like protein 6; Kv4.2, voltage-dependent A-type potassium channel 4.2; mGluR1, metabotropic glutamate receptor 1; mGluR5, metabotropic glutamate receptor 5.

Box 1. Background on antibodies

Protective humoral immunity requires B cells to produce a diverse pool of antibodies (also known as Ig) for the successful elimination of different types of antigens. Given the limited number of germline genes, Ig variability is generated by two mechanisms: (i) genetic rearrangement of V, D, and J gene segments at the variable heavy (VH) and variable light (VL) chain Ig loci; and (ii) somatic hypermutations in the V segment of the Ig gene. In a misdirected immune response that may be triggered by a tumor, viral infections, or cancer immunotherapies [100], the combinatorial diversity can also give rise to the production of autoantibodies that target self-antigens of the nervous system and cause neuronal dysfunction. The pathogenicity of an antibody is determined by various molecular factors that include antibody affinity, epitope specificity, Ig isotypes, and antibody valency. Whereas a frequent mechanism of the bivalent IgG1 or IgG3 isotype is to induce crosslinking and internalization of the target antigen, IgG4 antibodies are less effective in mediating these effects [101]. IgG4 isotypes are structurally and functionally unique in the way that their heavy chains with the attached light chains can easily dissociate and randomly recombine (Fab arm exchange), rendering them bispecific and functionally monovalent with two different Fab fragments [102]. IgG4 antibodies are proposed to interfere with the normal protein–protein interactions of the target antigen, which is usually involved in cell adhesion or stabilization of receptors [101]. In most AEs, the predominant autoantibody subclass is IgG1 [103]; however, LGI1 [104], IgLON5 [61], and CASPR2 [105] autoantibodies are mainly of the IgG4 isotype. For several of these autoimmune neurological disorders, mAbs have been isolated and characterized, including those targeting NMDAR [4,56], LGI1 [5,6], CASPR2 [60], MuSK [65,66,106], AQP4 [12], $GABA_AR$ [3,63], AChR [107,108], and GlialCAM [72].



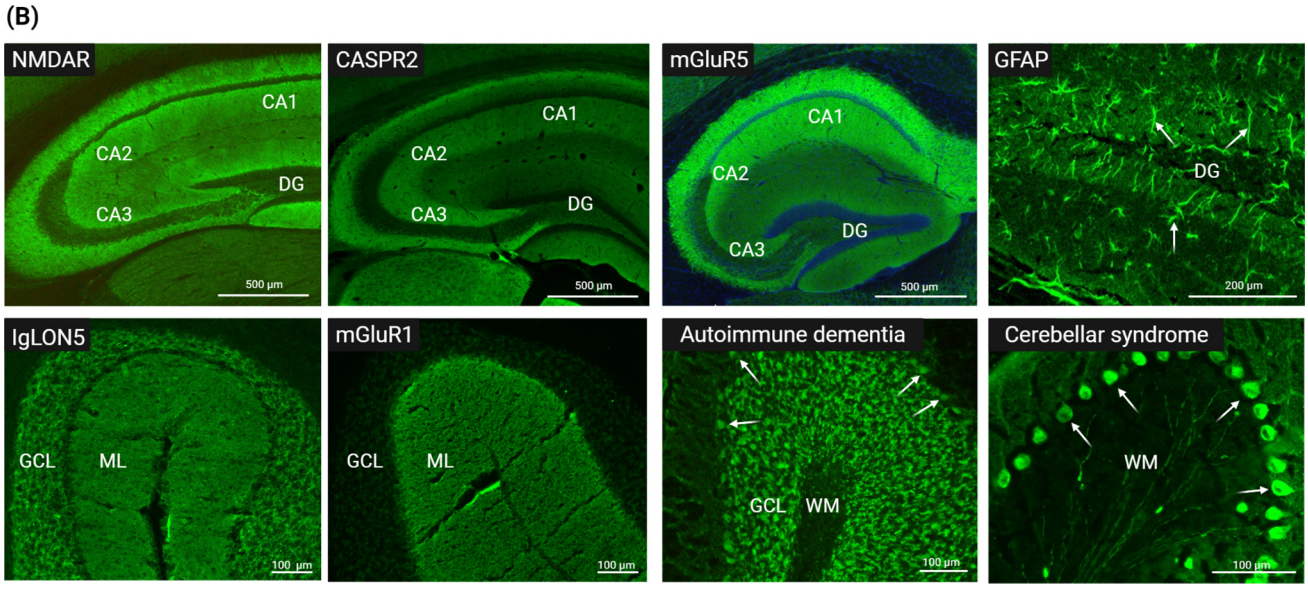


Figure 1. Recombinant production of patient-derived monoclonal autoantibodies (mAbs) and neuronal reactivity screening. (A) Following the collection of patients' cerebrospinal fluid (CSF), single B cells are isolated with fluorescence-activated cell sorting (FACS). The extracted mRNA is reverse transcribed (RT-PCR) into cDNA, followed by a nested PCR that amplifies the variable regions of the Ig heavy chain (IgH) and both Ig light chains (IgLs). Productive rearrangements are then cloned into an expression vector. Co-transfection of HEK293T cells with the expression plasmids results in mAb production for functional *in vitro* and *in vivo* characterization. (B) Immunofluorescence staining of hippocampus (top) and cerebellum (bottom) using unfixed murine brain sections. Human mAbs against the *N*-methyl-D-aspartate receptor (NMDAR), contactin-associated protein-like 2 (CASPR2), and metabotropic glutamate receptor 5 (mGluR5) show differential characteristic binding to hippocampal neuropil. Glial fibrillary acidic protein (GFAP) mAbs demonstrate distinct binding to astrocytes in the hippocampus (arrows). Human mAbs against Ig-like cell adhesion molecule 5 (IgLON5) and mGluR1 show binding to different cerebellar layers. Immunofluorescence staining of an as-yet-unknown antigen from a patient with autoimmune dementia and a not-yet-specified target from a patient with cerebellar syndrome further display strong binding to Purkinje cells (arrows). Abbreviations: CA1–CA3, hippocampal area cornu ammonis 1–3; DG, dentate gyrus; GCl, granular cell layer; ML, molecular layer; WM, white matter. Created with BioRender.com.

encephalitis (Figure 3A,D,E) [56,58]. Finally, NMDAR mAbs were used in a murine maternofetal transfer model where transient exposure during pregnancy induced lifelong behavioral and brain structural changes in the offspring (Figure 3B,C) [59]. Similar findings were demonstrated

Box 2. Diagnostic assays for autoantibody identification

TBAs with indirect immunofluorescence (IF) on rodent brain sections enable the screening of autoantibody reactivity against the CNS. Well-characterized and novel antineuronal autoantibodies show distinct staining patterns on unfixed brain sections (see Figure 1B in main text). A method complementary to TBA is to screen for autoantibodies on primary rodent neuronal cell cultures, which also has the potential to identify previously undetermined autoantibodies binding to surface-expressed proteins. Already established autoantibodies are determined with CBAs, which have become the gold standard in neuronal autoantibody detection in clinical practice [109]. In CBAs, cell lines such as HEK293T are transfected to overexpress the antigen of interest in its native conformation. The potential binding of mAbs or patient samples is detected with IF [109].

TBAs are also helpful in identifying the relatively common presence of more than one autoantibody in a human sample. For example, autoantibodies targeting NMDAR and glial fibrillary acidic protein (GFAP) are biomarkers of distinct clinical entities but frequently occur in combination (see Figure 1B in main text) [4,110,111]. Analyses at the monoclonal level demonstrated that CSF contains numerous autoantibodies beyond the disease-specific target [3–6,112]. Distinct binding patterns of different mAbs in a patient sample suggest that these mAbs harbor pathogenic capability and thus may account for the often diverse clinical phenotype in AEs. In NMOSD patients, some non-AQP4-reactive mAbs modulated the integrity of the blood–brain barrier (BBB) by targeting glucose-regulated protein 78 (GRP78) expressed on brain microvascular endothelial cells [112]. The increasing use of single-cell cloning and mAbs will markedly foster the future identification of countless antigens of brain-reactive autoantibodies, thus quantifying 'brain antibody-omics' in autoimmune neurological disorders.

with human contactin-associated protein-like 2 (CASPR2) mAbs [60] and taken together they stimulate the concept of a novel, potentially treatable neurodevelopmental congenital brain disorder contributing to lifelong neuropsychiatric morbidity. Crosslinking and receptor internalization were also proposed to occur with other autoantibodies, such as those targeting α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) [14], glycine receptors [25], Ig-like cell adhesion molecule 5 (IgLON5) [61], or gamma-aminobutyric acid type A receptor ($GABA_AR$) [55], but validation with mAbs is pending.

Inhibition of protein–protein interactions

Autoantibodies can also directly interfere with synaptic protein–protein interactions; for example, in **LGI1 encephalitis**, a severe AE with memory deficits and frequent epileptic seizures [62]. Cloning of LGI1 mAbs demonstrated the existence of at least two populations with synergistic but distinct functional effects [5,6]. LGI1 mAbs recognizing the epitempin repeat (EPTP) domain inhibited LGI1 binding to its pre- and post-synaptic receptors ADAM23 and ADAM22, while mAbs against the leucine-rich repeat (LRR) domain mediated the internalization of the ADAM22/23-bound LGI1 complex (Figure 2D). Injections of both domain-specific LGI1 mAbs into the hippocampus of mice prevented synaptic long-term potentiation induction, while only LRR-directed mAbs also impaired memory function (Figure 3F) [5]. Disruption of the trans-synaptic complex formation has been further associated with neuronal overexcitability and increased synaptic transmission, which may explain the generation of seizures in LGI1 encephalitis [6].

Competitive and allosteric receptor inhibition

A further effect of autoantibodies on neuronal function is direct interference with target receptors. In **$GABA_AR$ encephalitis**, which is characterized by severe refractory seizures and status epilepticus, detailed functional studies with polyclonal human samples faced the challenge that patients with $GABA_AR$ encephalitis frequently harbor additional pathogenic autoantibodies, such as those targeting NMDAR, LGI1, or CASPR2 [55]. Functional *in vitro* investigations revealed that CSF-derived $GABA_AR$ mAbs alone could reduce GABAergic inhibitory synaptic transmissions [3,63], and intrathecal injection of the mAb or its monovalent **Fab fragment** into mice caused spontaneous seizures [3]. The effect was independent of $GABA_AR$ downregulation [3], suggesting direct autoantibody-mediated receptor inhibition. $GABA_AR$ mAbs selectively bound to various subunit combinations where they either sterically blocked the ligand-binding site through direct antagonism (α1 subunit) or inhibited receptor functions through allosteric antagonism (α1γ2 subunit) (Figure 2E) [13].

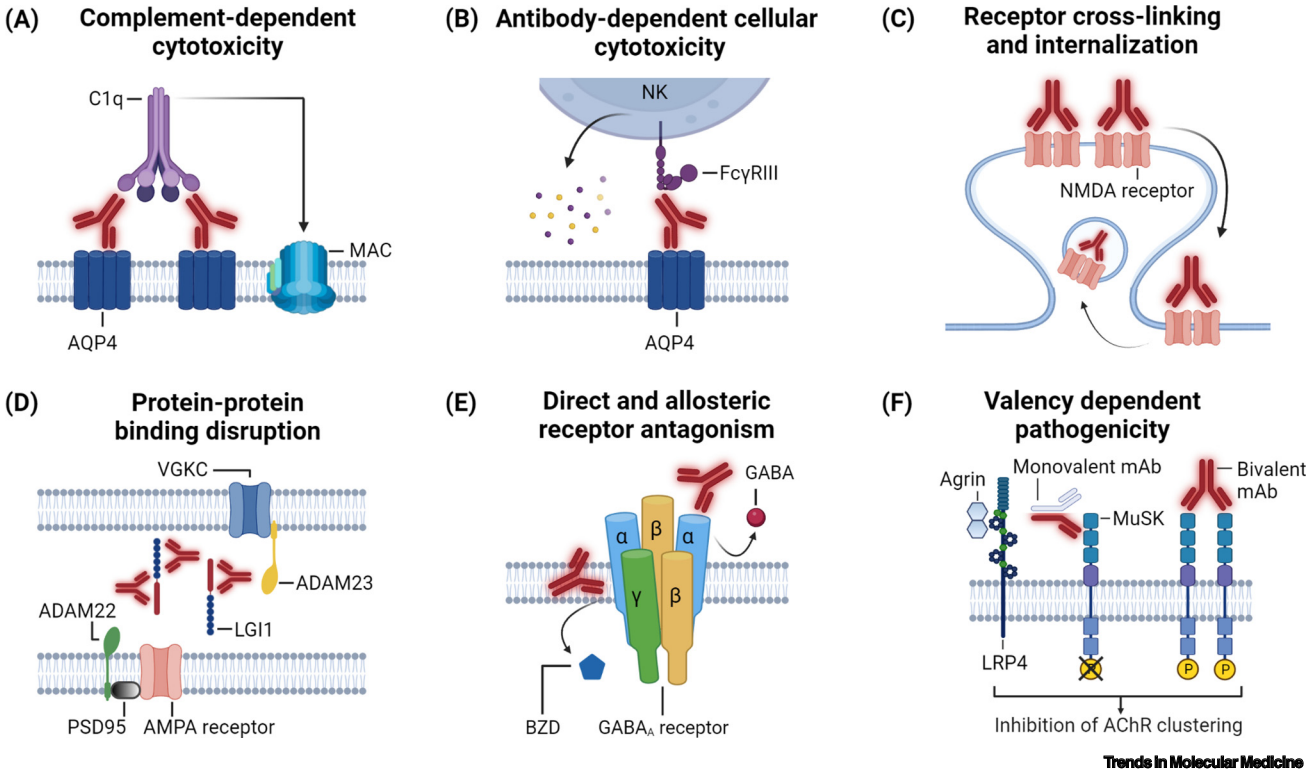


Figure 2. Disease mechanisms discovered *in vitro* by use of human antineuronal monoclonal autoantibodies (mAbs). (A) Aquaporin-4 (AQP4) mAbs bind to clustered AQP4 and form multivalent interactions with complement component C1q resulting in complement-dependent cytotoxicity with the formation of a membrane attack complex (MAC) to lyse the target cell. (B) Natural killer (NK) cells bind to the Fc domain of AQP4 mAbs with their FcγRIII receptors and induce antibody-dependent cellular cytotoxicity with the release of cytotoxic factors. (C) *N*-methyl-D-aspartate receptor (NMDAR) mAbs can crosslink NMDAR, leading to disruption of synaptic and extrasynaptic NMDAR clusters and their internalization and degradation. (D) Leucine-rich glioma-inactivated 1 (LGI1) mAbs bind to various LGI1 epitopes and disrupt the formation of a trans-synaptic complex by inhibiting the interaction of LGI1 with its pre- and postsynaptic receptors ADAM23 and ADAM22, respectively. (E) Gamma-aminobutyric acid type A receptor ($GABA_AR$) mAbs have different domain specificities and directly inhibit receptor functions via mechanisms of competitive or allosteric antagonism. (F) Monovalent muscle-associated receptor tyrosine kinase (MuSK) mAbs inhibit the interaction of MuSK with agrin-LRP4, decreasing MuSK phosphorylation, whereas bivalent MuSK mAbs crosslink MuSK, resulting in increased MuSK phosphorylation. Both valency-dependent effects eventually decrease acetylcholine receptor (AChR) clustering. Created with BioRender.com.

Autoantibody valency-dependent receptor stimulation and inhibition

The **valency** of an autoantibody can further contribute to its variable pathogenic effects. In the autoimmune neuromuscular disease **myasthenia gravis (MG)**, patients can have autoantibodies against the postsynaptic muscle-associated receptor tyrosine kinase (MuSK) [64]. Binding of patient-derived MuSK mAbs to the extracellular Ig-like 1 and 2 domains disrupted the clustering of acetylcholine receptors (AChRs) [65,66]. However, mAbs acted via two opposing mechanisms depending on the valency. While the divalent mAb crosslinked MuSK receptors and increased MuSK phosphorylation [65], the functionally monovalent autoantibody inhibited the interaction of MuSK with the argin-LRP4 complex, thereby preventing MuSK phosphorylation (Figure 2F) [65,66]. Although both mechanisms finally lead to inhibition of AChR clustering, the mAb studies demonstrate which complexity becomes visible at the monoclonal level.

Antineuronal mAbs as novel scientific tools

The reproducible and theoretically unlimited supply of antigen-specific human mAbs creates several opportunities for their use as scientific tools to understand the initial events of autoimmunity, for the standardization of diagnostic assays, and for the development of antibody-selective

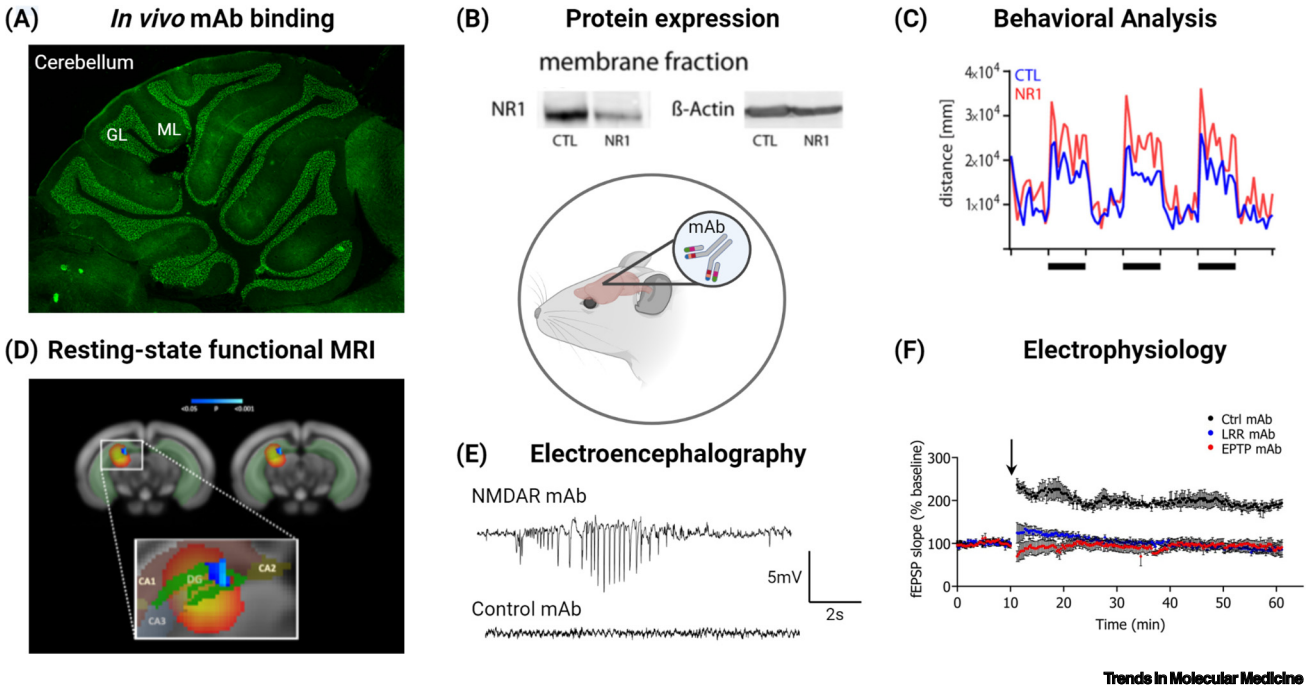


Figure 3. Understanding clinical autoimmune encephalitis (AE) symptoms from animal models receiving human antineuronal monoclonal autoantibodies (mAbs). (A) After cerebroventricular infusion of *N*-methyl-D-aspartate receptor (NMDAR) mAbs into mice, mAbs distribute in the brain and bind to NMDARs *in vivo*, as shown with immunofluorescence (IF) staining of the cerebellum. Abbreviations: GL, granular layer; ML, molecular layer. (B) Brain NMDAR binding leads to downregulation of NMDAR protein (NR1) in the neuronal membranes, as detected with western blots. Abbreviation: CTL, control. β-Actin serves as loading control. (C) Behavioral analysis reveals markedly higher locomotion during active dark phases (black bars) in the offspring after maternofetal transfer of NMDAR mAbs (NR1, red line) compared with controls (CTL, blue line). (D) Resting-state functional MRI shows reduced functional connectivity within the dentate gyrus of the right hippocampus of NMDAR mAb-treated mice. (E) *In vivo* electroencephalography (EEG) recordings reveal increased spontaneous epileptic activity in NMDAR mAb-treated mice compared with controls. (F) Field excitatory postsynaptic potential (fEPSP) recordings at hippocampal area cornu ammonis 1–3 (CA1–CA3) synapses in acute hippocampal slices demonstrate failure of long-term potentiation (LTP) induction following theta-burst stimulation (arrow) in mice treated with both domain-specific leucine-rich glioma-inactivated 1 (LGI1) mAbs – the leucine-rich repeat (LRR) mAb and the epitempin repeat (EPTP) mAb – compared with control (Ctrl). (B,C) Reproduced, with permission, from John Wiley & Sons [59]. (F) Reproduced, with permission, from Oxford University Press [5]. Created with BioRender.com.

immunotherapies. In this way, the rapidly increasing number of available patient-derived mAbs is an impactful driver of innovations in autoimmune neurological and psychiatric conditions, some of which are reviewed in this section.

Affinity maturation, somatic hypermutations, and clonal diversity

When initiating an antibody-mediated immune response (e.g., against a virus), B cells undergo affinity maturation in the germinal centers to produce antibodies with increased affinity [67]. This process involves the accumulation of extensive mutations in the V regions of Ig genes, known as **somatic hypermutations (SHMs)**, and clonal selection characterized by antigen-dependent proliferation [68].

The first studies with mAbs demonstrated that SHMs also play a role in antineuronal autoimmunity. For example, in NMOSD the intrathecal B cell repertoire arises from **clonal expansion** of AQP4-reactive B cell populations with extensive SHMs, indicating selective expansion of AQP4-reactive B cell clusters and antigen-driven affinity maturation in the CSF [12]. B cell receptor sequencing data of central nervous system (CNS)-resident and peripheral blood B cells revealed that some intrathecal AQP4 autoantibodies were exclusively produced by CSF plasma cells. The majority of AQP4 autoantibodies, however, originated from an expanded population

of AQP4-experienced peripheral blood B cells that migrate into the CSF, where they possibly undergo further antigen-specific B cell maturation and differentiation [69,70]. Likewise, recombinant mAbs derived from the CSF of patients with $GABA_AR$ encephalitis [3,63] or LGI1 encephalitis [5,6] showed high numbers of SHMs indicating affinity maturation. By contrast, patients with NMDAR encephalitis had low SHM numbers [4,56] or were even in germline configuration [37].

Unlike human polyclonal CSF samples, mAbs enable the reversion of highly mutated autoantibodies back to their **unmutated germline ancestors**. In the case of human AQP4 [71] and LGI1 [6] autoantibodies, **back-mutation** caused loss of binding to the antigen, suggesting that SHM is required for the generation of autoreactive AQP4 and LGI1 autoantibodies. By contrast, some NMDAR mAbs retained receptor binding after reverting to the germline configuration [37]. A recent study in multiple sclerosis patients discovered autoantibodies against the CNS protein GlialCAM, which lost GlialCAM binding after reversion to the germline ancestor but reacted strongly with the transcription factor EBNA1 of the MS-associated EBV [72]. Given that EBV infection is a prerequisite for the development of multiple sclerosis [73] and that NMDAR encephalitis occurs in almost one-third of patients after herpes simplex virus (HSV-1) encephalitis [74,75], back-mutated mAbs from NMDAR encephalitis patients will ultimately clarify whether similar molecular mimicry between viral and CNS antigens exists in AEs.

Autoantibody affinity

Autoantibody levels only weakly correlated with disease severity in AQP4-positive NMOSD or NMDAR encephalitis [76,77], suggesting that other biophysical autoantibody properties, such as affinity, may be a critical determinant of pathogenicity. With the availability of patient-derived mAbs, surface plasmon resonance imaging (SPRi) enables the real-time monitoring of antibody–antigen binding affinities and kinetics (Figure 4A) [78]. Affinity data may be particularly helpful in understanding the high clinical variability in the spectrum of symptoms despite similar autoantibody titers in patients. For example, high-affinity autoantibodies at low concentrations may be sufficient to cause pathogenicity [79]. However, future research with mAbs has to determine whether low-affinity autoantibodies at high concentrations or on prolonged exposure may also account for more subtle clinical symptoms such as mood changes or cognitive impairment.

Structural biology and epitope characterization

Beyond affinity, further intrinsic biophysical properties may determine how an autoantibody contributes to clinical disease. One such determinant is the target epitope, which can be analyzed by removing certain domains of a protein, by hetero-expression of various subunits of a receptor, or by specific mutation of the target proteins [3,5,6,65,66]. Such mutational analyses have, for example, shown that LGI1 autoantibodies comprise at least two separate mAb groups with distinct epitope specificities and functional effects (Figure 4B) [5,6]. Likewise, mAbs from $GABA_AR$ encephalitis patients reacted to distinct receptor subunit combinations [3], which has previously been suggested by studies with polyclonal patient samples [16,55].

For much higher resolution of epitope characterization at the atomic level, X-ray crystallography and cryo-EM come into play. Both revealed the 3D structures of glycine receptors, IgLON5, AMPAR, NMDAR, $GABA_AR$, and LGI1, which are all antigenic targets in AEs [80–85]. While X-ray crystallography has become the standard for the structure determination of crystallizable small molecules [86], the particular strength of cryo-EM is in the high-resolution-structure determination of solubilized large (transmembrane) proteins, including post-translational modifications [87].

Recently, cryo-EM has been used for the first time to study the intermolecular interaction of a neuronal receptor protein and patient-derived mAb (Figure 4C) [13]. Structural data at the atomic level

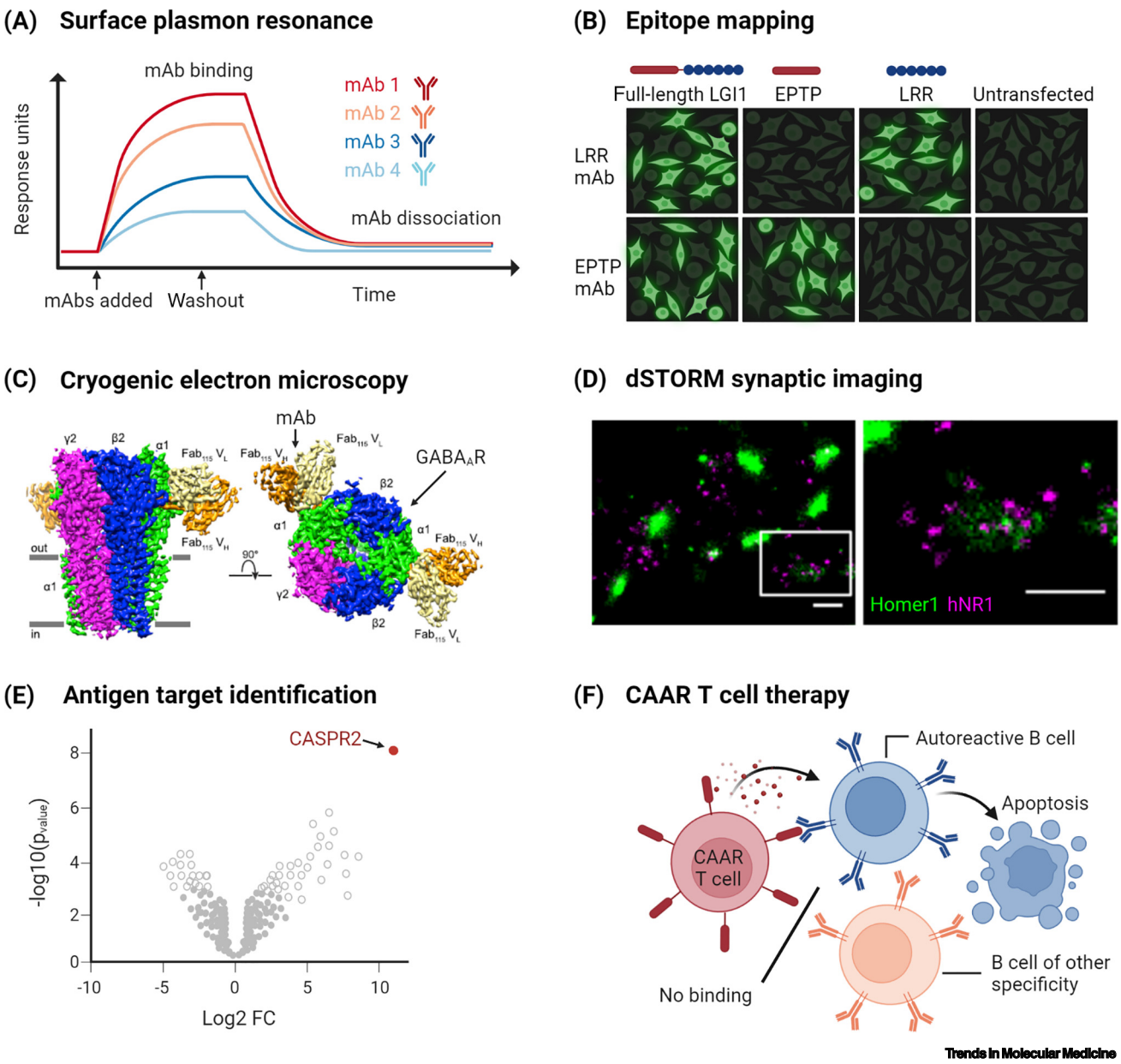


Figure 4. Monoclonal autoantibodies (mAbs) are novel scientific 'tools for discovery'. (A) Schematic representation of surface plasmon resonance measurements depicting real-time binding kinetics of various mAbs to the same target antigen. (B) Epitope mapping experiments showing immunofluorescence staining of cells overexpressing full-length leucine-rich glioma-inactivated 1 (LGI1) or its deletion constructs containing the individual epitempin repeat (EPTP) and leucine-rich repeat (LRR) subdomains. Epitope mapping reveals two distinct groups of mAbs binding to either the EPTP or the LRR domain. (C) Cryo-electron microscopy reveals the molecular interfaces at which gamma-aminobutyric acid type A receptor (GABA$_A$R) mAb fragments bind to the GABA$_A$R. (D) Direct stochastic optical reconstruction microscopy (dSTORM) of primary hippocampal mouse neurons demonstrates synaptic clusters of *N*-methyl-D-aspartate receptors (NMDAR, NR1). Postsynaptic densities are stained with Homer-1. Bar, 500 nm. (E) Exemplary volcano plot demonstrating the identification of the target antigen contactin-associated protein-like 2 (CASPR2) after immunoprecipitation/mass spectrometry with a mAb with a previously undetermined disease-specific target. (F) Schematic representation of therapeutic chimeric autoantibody receptor (CAAR) T cells that express the autoantigen (e.g., NMDAR) on their surface together with an intracellular activation domain. After binding to antigen-specific B cell receptors (= cell-specific antibody) in the membrane of an NMDAR autoantibody-producing B cell, CAAR T cells are activated and selectively kill the autoreactive B cell. (C) Reproduced, with permission, from Elsevier [13]. Created with BioRender.com.

revealed how two autoantibodies targeting $GABA_AR$ utilize distinct interfaces and modes of action to interfere with receptor function. This technology might mature into the new standard for autoantibody characterization in the field of AEs as it uncovers molecular pathomechanisms by the identification of key residues for autoantibody specificity and provides a structural basis for the development of new therapeutics.

Super-resolution microscopy

Super-resolution technologies such as direct stochastic optical reconstruction microscopy (dSTORM) or stimulated emission depletion (STED) microscopy are viable tools to study the synaptic organization of neurons at the nanoscale level [88,89]. They have already shown promise in analyzing the molecular effects of human antineuronal autoantibodies in cultured neurons. For example, dSTORM imaging on hippocampal neurons revealed that anti-amphiphysin autoantibodies, associated with paraneoplastic stiff-person syndrome, induced changes in synaptic-vesicle-associated protein levels at GABAergic synapses [89]. Patient-derived mAbs facilitate super-resolution microscopy as they allow the engineering of conformation-sensitive optical probes and histological labeling. This is exemplified by dSTORM images that can be routinely generated, showing, for example, single-molecule localization of NMDAR at the synapse (Figure 3D) [37]. The availability of many more human mAbs in the near future will also enable subunit-specific staining of neuronal antigens, as patient-derived mAbs target multiple different subunits or specific subunit combinations (e.g., $GABA_AR$ mAbs [3], LGI1 mAbs [5,6]). Such mAbs can hardly be generated by traditional means of immunization, making patient-derived mAbs exciting scientific tools.

Antigen target identification

The common finding that patient CSF samples harbor numerous antineuronal mAbs in addition to the disease-defining target creates the need to identify the underlying antigens. Likewise, diagnostic laboratories continuously recognize so-far-undefined autoantibodies in the CSF of patients with AEs (Figure 1B), and multiple new targets have been identified recently [90]. The determination of novel target antigens will foster our understanding of the autoantibodies' pathophysiological relevance alone or in combination and could support the development of diagnostic assays and treatment strategies for autoantibody-mediated AEs. Antigen target identification from polyclonal human samples, which contain innumerable different antibodies, traditionally employed immunoprecipitation (IP) followed by mass spectrometry (MS). In this approach, patients' sera or CSF with a distinct autoantibody neural staining pattern are subjected to IP with homogenized brain tissue lysates for the enrichment of antibody–antigen complexes. After tryptic digestion of these protein complexes, the peptides are submitted to MS readout for the identification of antibody–antigen interactions. CBAs are then performed for the verification of the target antigen by detection of antibody binding to HEK293T cells overexpressing the respective antigen. Using mAbs as tools for IP/MS may be superior to primary polyclonal patient samples (Figure 4E), potentially improving the characterization of novel patient autoantibodies in the future.

Standardization of diagnostic assays

Disease-defining mAbs may further be ideal reference materials for the development of highly standardized immunoassays. Providing standard samples comprising mAbs in exact concentrations will not only allow the calculation of quantitative autoantibody levels in human specimens but also enable the standardization of autoantibody titers [91], and could thus offer interlaboratory comparability and reproducibility. While currently autoantibody titers are only carefully used as disease surrogates to follow the clinical course and to monitor the effect of immunotherapy, this may change with the introduction of mAbs into routine diagnostics.

Clinician's corner

Diagnosing autoimmune encephalopathies based on underlying autoantibodies in serum or CSF has reached routine clinical practice in neurology. It has helped in the discovery of entirely new clinical entities and profoundly changed diagnostic and therapeutic concepts. CBAs using genetically modified cells overexpressing target antigens have become the gold standard in the diagnostics of antineuronal autoantibodies.

Novel pathogenic autoantibodies are continuously being discovered in patients of all ages; however, for several proof of autoantibody pathogenicity is still pending. Increased awareness of antineuronal autoantibodies has also led to the identification of autoimmune mechanisms in patients with dementia, psychosis, mood disorders, neuropathy, movement disorders, or pain.

The generation of human recombinant mAbs derived from patient CSF allowed for the first time an understanding of how monospecific autoantibodies contribute to the spectrum of clinical symptoms. Exploration of the detailed disease mechanisms using mAbs provided a rationale for the administration of intensive immunotherapies needed in many patients.

Results from laboratory experiments using mAbs can further stimulate the development of novel immunotherapies. For example, the demonstration of CDC in NMOSD mouse models reflects the clinical benefit in NMOSD patients receiving the complement inhibitor eculizumab [113].

The demonstration that a clinically well-established autoantibody, such as against LGI1, MuSK, or $GABA_AR$, includes different mAb populations with distinct modes of action can explain variabilities in patients' clinical pictures. Although this comes with challenges in the refinement of current diagnostics in clinical practice, it could lead to novel individualized treatment options in the future.

Development of autoantibody-selective immunotherapies

The perception that AEs are driven by monospecific autoantibodies has inspired research towards autoantibody-selective immunotherapies. The current armamentarium of immunotherapies for AEs is broad, comprising steroids, therapeutic apheresis, intravenous Igs, and B cell-depleting agents (e.g., the anti-CD20 monoclonal antibody rituximab) among others [7]. These existing therapies are unselective as they cannot specifically deplete pathogenic autoantibodies. Broad immunosuppression, however, carries profound risks of side effects including severe infections [92], which often require treatment discontinuation. The availability of mAbs as developmental tools is now stimulating highly selective, innovative treatment approaches.

One approach – which became possible only with the accessibility of human mAbs – aims at specifically outcompeting pathogenic autoantibodies with engineered patient-derived mAbs. Aquaporumab has high target affinity for AQP4, but is nonpathogenic as it lacks CDC or ADCC effector functions [93,94]. Evaluations at the preclinical level prevented the development of NMOSD lesions in *ex vivo* spinal cord slices and in an *in vivo* mouse model without inducing cytotoxicity [94].

Another novel approach aims to increase the clearance of pathogenic IgG autoantibodies. Seldegs ('selective degradation') constitute a recombinant antigen for the specific capture of a disease-relevant autoantibody (e.g., MOG in demyelinating disease) fused to a human Fc fragment [95–97]. They selectively bind disease-specific autoantibodies with their antigen part. Seldeg-IgG complexes are then internalized via neonatal Fc receptors (FcRns). In this way, MOG IgGs are degraded intracellularly in lysosomes while non-MOG antibodies are spared, which resulted in improvement in a mouse model of experimental AE using patient-derived MOG autoantibodies [96,97].

Other strategies aim at the selective depletion of disease-specific plasma cells using an autoantigen of interest fused to a CD138 antibody [98]. The construct binds to all plasma cells via CD138, but the antigen is recognized only if the plasma cell's own released autoantibodies are disease specific. CDC and ADCC effector mechanisms finally lead to selective depletion of these plasma cells.

Along the same lines, other options to deplete disease-driving B cells are under investigation. **Chimeric autoantibody receptor (CAAR) T cells** are genetically engineered T cells that selectively kill monospecific autoantibody-secreting B cells, which was initially explored in an autoimmune skin disease [99]. The disease antigen (e.g., an NMDAR fragment of a CAAR T cell) binds to the surface IgG (= a B cell receptor) of an NMDAR autoantibody-producing B cell and destroys it while sparing B cells with other specificities (Figure 4F). Experiments became possible only with the advent of mAb generation from patients, as mAbs allowed the generation of model B cell lines and unrestricted amounts of human autoantibodies for characterization and validation.

Concluding remarks

Together with other major advances in neurological diagnostics over the past decade, such as next-generation sequencing, multiomics, and brain imaging, the recognition that antineuronal autoantibodies are relatively common and can cause a wide spectrum of clinical symptoms has stimulated the recombinant generation of disease-driving mAbs from the CSF of affected patients. It quickly turned out that human mAbs not only provide the basis for the detailed *in vitro* and *in vivo* characterization of disease mechanisms, but will also serve the scientific community as tools for high-resolution microscopy, atomic-level structural analyses, immunology techniques, and the development of antibody-selective immunotherapies. This will be accompanied

Outstanding questions

What are the target antigens of those autoantibodies still increasingly identified in clinical practice?

How many different autoantibodies can be found in the CSF of neurological patients and healthy subjects ('brain antibody-omics')?

Can some CNS autoantibodies have neuroprotective or anti-inflammatory effects and thus be beneficial to the brain?

What are the individual functional contributions of mAbs to disease? Can they work in combination (e.g., by changing vascular and BBB properties) or do they have homeostatic potential?

How can clinical diagnostics of autoantibodies be further improved (e.g., by engineering many different antigens for detection on a chip)? Which technical improvements can help to balance costs?

Can autoantibodies induce neurodegeneration and dementia or are they a secondary phenomenon due to neuronal loss and immune presentation of neuronal antigens?

What is the role of T cells and further immune cells in antibody-mediated CNS diseases, particularly in AEs where antibodies target intracellular antigens?

Do antineuronal autoantibodies that developed in the context of viral infection show cross-reactivity with viral proteins or surfaces?

What are the immunological triggers of autoantibody generation beyond already-established mechanisms such as tumors or viral brain infections?

Can further developments in high-throughput single-cell technologies make mAb generation available for the broader scientific community?

What improvements can overcome the bottleneck of high-quality animal models in autoantibody research, given that many mAbs recognize complex conformational (3D) epitopes?

Will innovative antibody-selective treatments allow full recovery or cure in AE in the future?

by technical challenges and numerous open questions (see Outstanding questions), such as those related to the broad implementation of clinical diagnostics, the immunological cascades of triggering autoantibody formation, and the further development of high-throughput single-cell technologies. However, data generated from experiments with patient-derived mAbs have already led to the consideration of humoral autoimmunity in many diseases ranging from dementia to psychosis – a process that will continue unabated in the upcoming years.

Author contributions

S.L.D. and H.P. contributed to the conception and design of the review and drafted the manuscript. Both authors critically revised, read, and approved the final manuscript.

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Declaration of interests

The authors have no interests to declare that are relevant to the content of this Feature Review.

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