CHARACTERIZATION OF GAD-REACTIVE B CELLS AND THEIR B-CELL RECEPTOR REPERTOIRE IN THE PERIPHERY AND CENTRAL NERVOUS SYSTEM

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Dissertation der
Graduate School of Systemic Neurosciences der
Ludwig-Maximilians-Universität München

April 11th, 2022

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Date of Submission: 11.04.2022
Date of Defense: 22.07.2022

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III. LIST OF ABBREVIATIONS

aa	amino acid
ab	antibody
AE	autoimmune epilepsy
ag	antigen
AID	activation-induced cytidine deaminase
ANA	anti-nuclear abs
ANNA	anti-neuronal nuclear auto-abs
BCR	B cell receptor
bm	bone marrow
C	carboxy
CA	cerebellar ataxia
CBA	cell-based assay
CD	cluster of differentiation
CDR	complementary determining region
CNS	central nervous system
CSF	cerebrospinal fluid
CSR	class switch recombination
D	diversity
ELISA	enzyme-linked-immunosorbent-assay
ELS	ectopic lymphoid structures
Fab	ag-binding fragment
Fc	constant fragment
FR	framework region
GAD-AIND	GAD65-ab-associated autoimmune neurological disorders
GABA	γ-aminobutyric acid
GAD	glutamic acid decarboxylase
GAD-LE	Anti-GAD ab mediated autoimmune limbic encephalitis
gc	germinal center
HCs	heavy chains
HSC	hematopoietic stem cell

Ig	immunoglobulin
IL	interleukin
J	joining
kDa	kilodalton
LCs	light chains
LE	limbic encephalitis
LGI1	leucine-rich glioma inactivated 1
mab	monoclonal antibody
MRI	magnetic resonance imaging
N	amino
NMDAR	N-methyl-D-aspartate receptor
OCBs	oligoclonal bands
PLP	pyridoxal-5´-phosphate
RIA	radioimmunoassay
SHM	somatic hypermutation
SPS	stiff person syndrome
T1DM	type 1 diabetes mellitus
TLE	temporal lobe epilepsy
TLR	toll-like receptors
UCA	unmutated common ancestor
V	variable
WB	western blot

1. ABSTRACT

Antibodies (abs) against the intracellular cytoplasmic protein glutamic acid decarboxylase 65 (GAD65) occur in different neurological disorders. Hereby, patients with anti-GAD65 ab associated autoimmune neurological disorders (GAD-AIND) exhibit high levels of anti-GAD65 abs in the serum as well as in the cerebrospinal fluid (CSF). However, the sites of immunological dysregulation and the triggers for the production of auto-abs against GAD65 are unclear. Furthermore, investigations on the pathogenic relevance of anti-GAD65 abs and their producing cells have yielded conflicting results. In consequence, the treatment of patients with GAD-AIND remains challenging. Therefore, investigating the functional properties of GAD-reactive B cells and anti-GAD65 abs could help to gain insights into the pathophysiology of GAD-AIND and assist the development of new treatment strategies.

To this end, we analyzed GAD-reactive B cells in peripheral blood of patients with GAD-AIND as well as bone marrow (bm) cells of one patient. As a next step, we generated monoclonal patient-derived abs from peripheral blood (PB) and CSF cells and characterized their reactivity to GAD in different assays. Moreover, we analyzed the auto-abs for sequence specificities, e.g., subclass distribution, and somatic hypermutations (SHMs).

In the first study, we could detect anti-GAD65 ab producing cells derived from GAD-reactive B cells in the peripheral blood (PB) of fifteen patients with GAD-AIND. Compared to our healthy control group consisting of nineteen donors, cells producing anti-GAD65 abs were highly elevated. Furthermore, we could identify bm plasma cells as an additional source of anti-GAD65 abs. The frequency of GAD-reactive B cells was comparable to B cells reactive for common recall antigens (ags) e.g. tetanus toxoid. In a second study, we included six patients with GAD-AIND and were able to generate 30 monoclonal abs (mabs) with 25 mabs derived from CSF cells and 5 mabs derived from PB cells. Mabs could be generated in patients with a short disease duration at the time of sampling. Thereby, we identified 10 GAD65-reactive mabs (CSF: 6/25; PB: 4/5) with enzyme-linked-immunosorbent-assay (ELISA). Furthermore, we could demonstrate that

the intrathecal anti-GAD65 response is polyclonal and that the mabs had undergone affinity maturation when being compared to non-GAD65-reactive mabs. By reverting the SHMs of three GAD-reactive mabs to their germline sequences, we could show that affinity maturation is essential for the reactivity towards GAD65.

Taken together, our data characterize the B cell response in the CSF of patients with GAD-AIND, showing that the anti-GAD65 response is polyclonal and affinity maturation is necessary for the recognition of GAD65. Additionally, we could for the first time proof that anti-GAD65-ab-producing cells are present in the central nervous system. In addition, our data support the clinical observation that a B-cell depleting therapy might not be suitable for the treatment of long-standing GAD-AIND.

2. INTRODUCTION

2.1. Autoimmunity – B cells and autoantibodies

The immune system consists of innate and adaptive immune mechanisms and constitutes the body's line of defense against pathogens and foreign substances [1–3]. Thereby, the innate immune system assures a fast and unspecific immune response against pathogens, whereas specific defenses are mediated via the adaptive immune system [1,3,4]. Components of the innate immune system include mechanical barriers such as the skin, the complement system and phagocytosis. Important players of the innate immune system are highly conserved pattern recognition receptors that are expressed by numerous immune cells and recognize pathogen- and damage-associated molecular patterns. Toll-like receptors (TLRs) are one class of pattern-recognition receptors that sense bacterial proteins e.g. membrane components, viral double stranded RNA and microbial DNA. The activation of these receptors initiates inflammatory responses via the production of cytokines, chemokines and interferons, which in turn activates adaptive immune responses. The adaptive immune response hereupon facilitates specific immune cell responses through T and B lymphocytes. Thereby, the interplay between innate and adaptive immunity is necessary for an adequate immune response that is efficient enough to combat infections without harming the host [1,3–5]. Especially T and B lymphocytes are effector cells with a strong immunoregulatory potential to discriminate between self and non-self to avoid immune responses directed against host tissue[1,6,7]. To prevent the development of autoimmunity, our immune system has evolved mechanisms to gain self-tolerance. On the contrary, autoimmunity is caused by imbalanced or diminished self-tolerance that can lead to a broad spectrum of autoimmune diseases [1].

2.1.1. B lymphocytes and their role as antibody-producing cells

B lymphocytes are an essential part of the adaptive immune system and develop in the bone marrow (bm). To assure that naïve B cells are capable to discriminate between self and non-self-derived patterns (antigens [ags]), they express a highly specific B cell receptor (BCR) on their cell surface that enables them to recognize their respective ags [1,8–11].

Structurally, these immunoglobulin (Ig) BCRs with a size of ~150 kilodalton (kDa) are composed of two identical heavy chains (HCs) encoded on chromosome 14 and two identical light chains (LCs) [1,8]. LCs can be distinguished into κ LCs encoded by the κ locus on chromosome 2 and λ LCs encoded by the λ locus on chromosome 22 [8,12]. In this complex, each of the HCs has a molecular weight of 50kDa whereas each LC has a molecular weight of 25kDa. Every chain is composed of a constant regions comprising the carboxyl terminal portions and a variable region comprising the amino terminal portions. The latter are designated as either V_H and V_κ or V_λ and assure the uniqueness towards an ag since they are involved in the formation of the ag-binding fragment (Fab) [1,13,14]. Based on their sequential and structural similarity, encoded variable regions of Igs can be grouped into families. HCs consist of eight families, whereas κ LCs consist of six families and λ LCs consisting of four such families [8,12].

Additionally, the variable domain of an Ig molecule is functionally divided into the complementary determining regions (CDR) 1 to 3 that are flanked by stable sequence regions, namely the framework regions (FR) 1 to 4 [12]. The hypervariable ag-binding pocket is mainly formed by the two CDR3 segments of each V_H and V_κ or V_λ, resulting in two identical ag binding sites for each Ig known as paratops (**FIGURE 1**) [1,12,15,16]. In contrast to the Fab domain the constant fragment (Fc) domain of abs is formed by the constant regions of the heavy chains and is preserved within each subclass [1,12,14]. The HC gene contains exons that encode 9 different constant regions to synthesize different classes of Ig molecules [1].

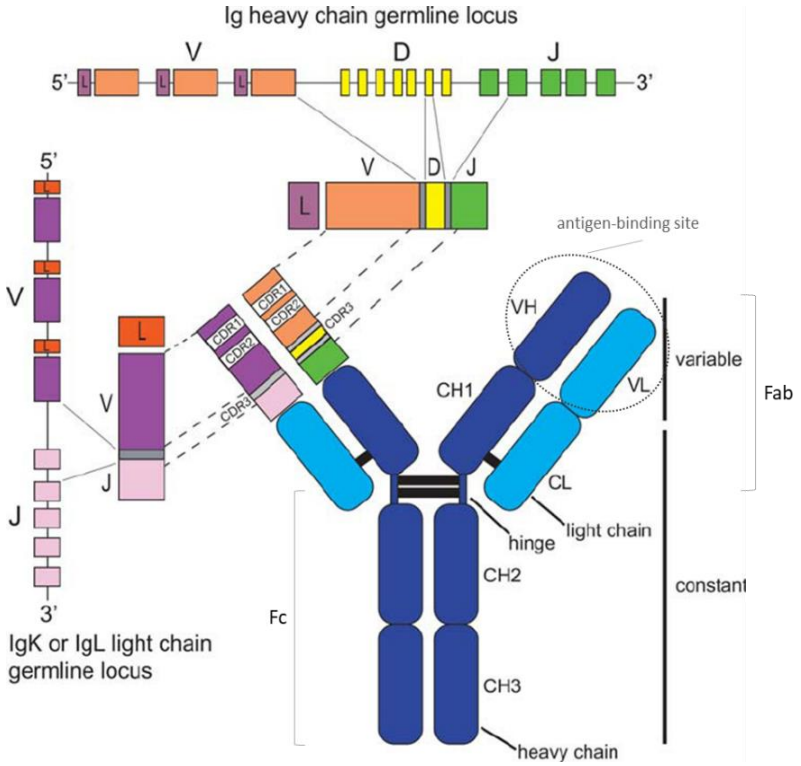

FIGURE 1: AB STRUCTURE AND V(D)J ENCODING.

The unarranged Ig HCs and LCs (κ=K or λ=L) germline loci are depicted on the top and left-hand side of the figure. Both undergo stepwise rearrangements to generate highly diverse variable V(D)J junctional regions. These regions in turn encode the ag-binding site that sets the ag specificity. CH1-CH3 and CL are the constant region of HCs or LCs and are encoded by downstream exons. The black line segments represent the disulfide bridges that connect the single protein chains. Modified from Boyd, S.D. and Joshi, S.A [17]. ASM authorizes an advanced degree candidate to republish the requested material in his/her doctoral thesis or dissertation free of charge, provided that proper credit is given to the original ASM publication: journals@asmusa.org.

In order to respond to the high diversity of existing pathogen-derived ags, a multiplicity of different BCRs have to be generated. One mechanism accounting for the high variability of lymphocyte precursor BCRs, is the so-called V(D)J recombination (combinatorial diversity) in the bm. It describes a stepwise recombination of variable (V), diversity (D) and joining (J) gene segments encoding the variable regions of the Ig complex (**FIGURE 1**) [1,8–11,18]. Thereby variable regions of LCs arise from joined recombined V and J gene segments and the variable regions of HCs arise from V, D, and J recombined gene segments [8,12]. Since, the rearrangement of the HC locus includes D elements, HCs are considered to be more diverse (6000 possible combinations) and show a much higher combinatorial diversity when being compared to LC loci (320 possible

combinations). Specifically, 40 V_H, 25 D_H and 6 J_H elements can be recombined to generate the variable region of HCs whereas only 40 V_κ, 5 J_κ, and 30 V_λ, 4 J_λ display the repertoire of the κC and λC loci, respectively. The rearrangement of LC gene segments usually takes places after HC rearrangements [18]. Another mechanism accounting for the diversity of Ig molecules is known as junctional diversity. Junctional diversity describes a process where random nucleotides are inserted between recombining genes in addition to an exonuclease trimming of these genes [18,19]. At the end, this rearrangement process of combinatorial and junctional diversity results in a naïve ab repertoire of at least 10^{12} unique Ig molecules [19,20].

Lymphoid precursors that produce a functional, non-auto-reactive BCR differentiate into mature naïve cluster of differentiation (CD)19^+ and $CD27^-$ B cells and recirculate throughout the body and/or persist in lymphoid organs until encountering their ag [21–23]. Until the first contact of B cells with their ag, most BCRs express the germline sequence with relatively low affinity [24]. After antigenic stimulation, the mature naïve B cell migrates to the follicles of secondary lymphoid organs where it interacts with follicular T cells and follicular dendritic cells, undergoing germinal center (gc) reactions [6,22,25]. These reactions include the clonal expansion of B cells as well as Ig heavy-chain class switch recombination (CSR) and random somatic hypermutations (SHMs), resulting in the formation of high affinity abs (**FIGURE 2**) [16,26,27]. Mature and naïve B cells express the low affinity Ig receptor as membrane-bound IgM and IgD molecule on their surface (**FIGURE 2**) [6,28]. Once they are stimulated, during CSR, enzymes like the RNA-editing enzyme, activation-induced cytidine deaminase (AID), lead to DNA rearrangements, i.e. the previously mentioned rearranged V(D)J gene segments of the heavy chains are moved into other positions upstream [1,10]. Due to this recombination process, B cells can generate abs of different isotypes without losing their specificity that is ensured by the variable domains. The secretion of different interleukins (IL) by other immune cells such as T cells can cause switching to other high affinity isotype classes like IgA and IgE or to other IgG subclasses like IgG1, IgG2, IgG3 or IgG4 [1,15]. Among them, IgG, or, and more precisely, IgG1 is the most abundant isotype subclass and has the longest serum half-life compared to the others [12,15]. Especially the isotypes IgM and IgG are key

activators of the complement system via the classical pathway. The activation of the complement system induces factors of the innate immune system that involve a proteolytic cascade leading to the opsonization and lysis of pathogens. Furthermore, the activation of the complement system can induce adaptive immune responses due to proinflammatory processes [29,30]. To conclude, Ig molecules fulfill two important tasks: they assure the recognition of ags, triggering cell activation by signaling as membrane-bound receptors and they assure the neutralization of ags as shedded soluble molecules [12,15,31].

Finally, the introduction of SHMs results in random mutations in the variable regions of HCs and LCs, resulting either in increased or decreased affinity for the ag. The latter leads to cell death caused by reduced receptor-mediated growth signals. In case of increased affinity, the antibody (ab)-producing B cells proliferate [1,16]. Importantly, CSR only occurs in the HC locus, whereas SHMs affect the HC and LC loci [16].

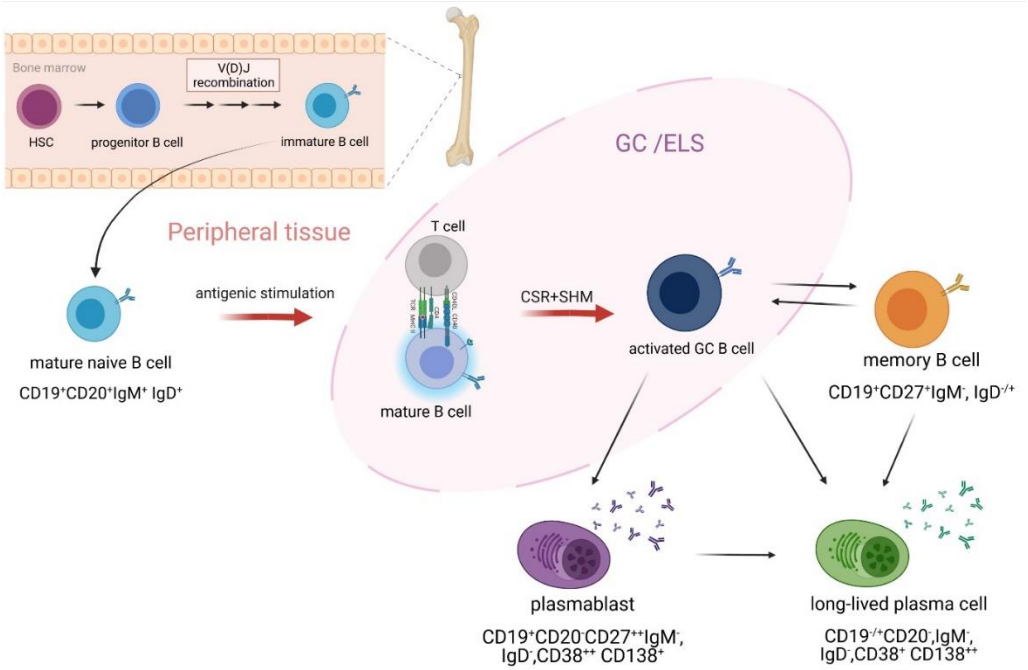

FIGURE 2: DIFFERENTIATION OF AB PRODUCING B CELLS IN GC REACTIONS.

In the bone marrow, hematopoietic stem cells (HSC) differentiate into progenitor B cells and further into immature B cells by V(D)J recombination. Mature naïve B cells that leave the bone marrow can be stimulated by antigens and migrate to germinal centers. With the help of T cells, mature B cells undergo class switch recombination and somatic hypermutation reactions and differentiate into activated GC B cells that can further differentiate into highly ag-specific ab-producing cells or memory B cells. Memory B cells as well as plasmablasts can further differentiate into long-lived plasma cells. Created in BioRender.com.

After undergoing gc reactions, clonally expanded and activated B cells can differentiate either into ab-secreting $CD19^{low}CD20^{-}CD27^{high}CD38^{high}CD138^{-/+}$ plasmablasts or into long-lived $CD19^{low}CD20^{-}CD27^{+}CD38^{+}CD138^{high}$ plasma cells that persist in niches in the bm or to memory B cells that constitute another important hallmark of our immune system [21,32]. Memory B cells are long-lived $CD19^{+}CD20^{-}CD27^{+}CD38^{-/+}$ cells that remain in an inactive state until they reencounter their specific ag. After antigenic stimulation, they proliferate and differentiate into plasma cells, thereby providing a fast, enhanced, and improved immune reaction through the production of specific abs (**FIGURE 2**) [23,32].

Taken together, the occurrence of SHMs in functional Ig genes together with V(D)J recombination and rearrangement of constant regions leads to an enormous diversity of the ab repertoire that provides protective immunity under physiological conditions [9,11,13,24]. In contrast, the generation and production of B cell clones expressing auto-abs can occur and mediate immune responses directed against self-ags [9]. Hereby self-reactive abs produced by auto-reactive B cell subsets may attack host tissue and cause pathology [6,7].

2.1.2. Generation of auto-abs

It is widely accepted that B cells play a major role in the development of autoimmunity and consequently autoimmune diseases like anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis and systemic lupus erythematosus [33,34]. These autoimmune diseases are associated with the occurrence of self-reactive abs accompanied by tissue disruption and systemic inflammation [35].

To avoid the generation of auto-abs, our body has implemented mechanisms to achieve self-tolerance: central and peripheral checkpoints. During the development of the BCR, central tolerance mechanism preselect naïve B in the bm [9,11]. One of the contributing mechanisms is the affinity-dependent selection where - depending on the BCR binding to presented self-ags - the cells become either apoptotic, anergic or ignorant [8,9,11,36]. The latter two induce unresponsiveness towards ags and entail the release of naïve B cells that cannot

be activated upon ag-stimulation. In contrast, the BCR-induced apoptosis is known as clonal deletion that is used to remove self-reactive immature B cells before leaving the bm [37]. Another mechanism to decrease self-reactivity is the editing of BCRs (receptor editing) by secondary gene rearrangements, especially of the Ig LCs. Receptor editing can rescue a significant proportion of auto-reactive B cells from cell death [38]. Following this affinity-dependent selection, presumably around 20 - 50 % of all peripheral naïve B cells have undergone receptor editing [9,39]. However, studies using healthy patient-derived B cells could show that a high number of auto-reactive naïve B cells is still released after undergoing bm pre-selection [40]. Therefore, additional tolerance checkpoints in the periphery (peripheral tolerance) help to decrease the number of mature auto-reactive ag-activated B cells and plasma cells [31,36,39–41]. Defects of peripheral tolerance can result in the generation of auto-abs as non-auto-reactive precursors can differentiate into auto-reactive affinity maturated B cells producing auto-abs. Thereby, the produced abs are often class-switched and highly mutated [41]. Several studies showed that, the reversion of these SHMs to the germline sequence can lead to a decreased affinity or loss of affinity towards the ag [42,43]. In many autoimmune diseases, auto-reactive B cells emerge from gc reactions, suggesting defects of peripheral tolerance [41,44].

Furthermore, ectopic lymphoid structures (ELS) can provide a gc-like environment in which CSRs and SHMs of BCRs can take place [45]. ELS were described in the organs that are affected by the autoimmune disease, such as the meninges in multiple sclerosis or in the synovial tissue in rheumatoid arthritis, and mostly develop in the chronic state of the disease [45–47]. ELS are characterized by aggregates consisting of T and B cells and may help to maintain disease processes by supporting the affinity maturation and differentiation of auto-reactive B cells [41,45–47].

Another hypothesis of how the generation of self-reactive B cells is triggered is molecular mimicry between self-ags and viral, bacterial or tumor ags. Thereby, in gc reactions, T cells recognizing the epitope of foreign ags interact and activate B cells that are not only specific for this particular epitope but also specific for another epitope of a self-ag [41,48,49]. Hence, it is possible that abs

produced by activated B cells can simultaneously target against viral ags and self-ags. Similarly, an antibody can be directed against multiple self-ags. [41,50,51].

Auto-abs can be detected in many autoimmune diseases including neurological disorders. In some cases the pathogenic potential of the auto-abs is well demonstrated, whereas in other cases clear evidence for their pathogenicity is still lacking [52–55]. In the central nervous system (CNS), several auto-abs are known to target membrane proteins that are located on the surface of cells such as leucine-rich glioma inactivated 1 (LGI1) or NMDAR. On the other hand, there are neuronal auto-abs targeting intracellular proteins or nuclear molecules, e.g., anti-neuronal nuclear auto-abs (ANNA) such as anti-Hu abs [41,50,56]. Whereas the pathogenic role of abs against proteins on the neuronal surface was demonstrated in elaborate studies [33,57–59], it remains a matter of debate whether abs against intracellular proteins can also mediate pathogenicity and how they could reach their target ag [41,52,55]. One hypothesis of how auto-abs can access intracellular ags is cell death, in particular, apoptosis. If a cell undergoes programmed cell death, intracellular molecules are released into the extracellular space. To prevent an immune reaction, phagocytes generally clear these released molecules. However, due to genetic defects or an excessive release of molecules caused by inflammation, these molecules might remain in the extracellular space [41,54,55].

2.2. Glutamic acid decarboxylase

2.2.1. Structure and localization

The enzyme glutamic acid decarboxylase (GAD) was first described in the 1950s. Located at inhibitory synapses in the CNS, it is an intracellular cytoplasmic protein existing in two isoforms, namely GAD65 and GAD67, which are 65kDa and 67kDa, respectively [52,53,60–63]. They not only differ in their molecular size but also in their antigenicity, cellular location, enzymatic activity and amino acid (aa) sequence, since separate genes encode them [61,63–65]. While GAD67 is encoded by *GAD1* on the chromosome 2q31.1, GAD65 is encoded by *GAD2* on the chromosome 10p12 [61,64–67]. Both are highly conserved among vertebrates and found in synaptic vesicles [62,66]. GAD65 is postnatally expressed and mainly located at axonal terminals [61,63,68]. On the contrary, GAD67 is already expressed during early embryogenesis and is more commonly found in the cell bodies and dendrites [61,68,69]. Also, the expression of GAD65 and GAD67 in the brain differs among regions [61]. GAD67 has been reported to be less abundant in the cortex than GAD65, which accounts for 70 % of all GAD protein [68], however both are mainly expressed in regions enriched with neuro-inhibitory cells such as the brainstem, hippocampus and cerebellum [70–72]. Thus, the reactive synaptic staining pattern of a GAD65 auto-ab in the cerebellum is more prominent in the granular layer compared to the molecular layer [73].

Both isoforms consist of three functional domains according to their linear sequences. First the amino (N)-terminal domain (GAD65: aa 1-188, GAD67: aa 1-197) followed by the catalytic center domain, namely, PLP-binding domain (GAD65: aa 189-464, GAD67: aa 198-473) and the carboxy (C)-terminal domain (GAD65: aa 465-585, GAD67: aa 474-594) [72,74,75]. In particular, the middle and last domain share a high similarity between the two isoforms with around 74 % overlap. The N-terminal domain, on the other hand, is less similar with only 25 % identity (**FIGURE 3**) [69,72,75]. Overall, the aa sequences of the two isoforms show a similarity of around 65 % [62,67,74].

Furthermore, GAD is not only expressed widely in the CNS but also in pancreatic β-cells, and to a lesser extent in epithelial cells of the fallopian tube and the spermatozoa of the testes [62,76].

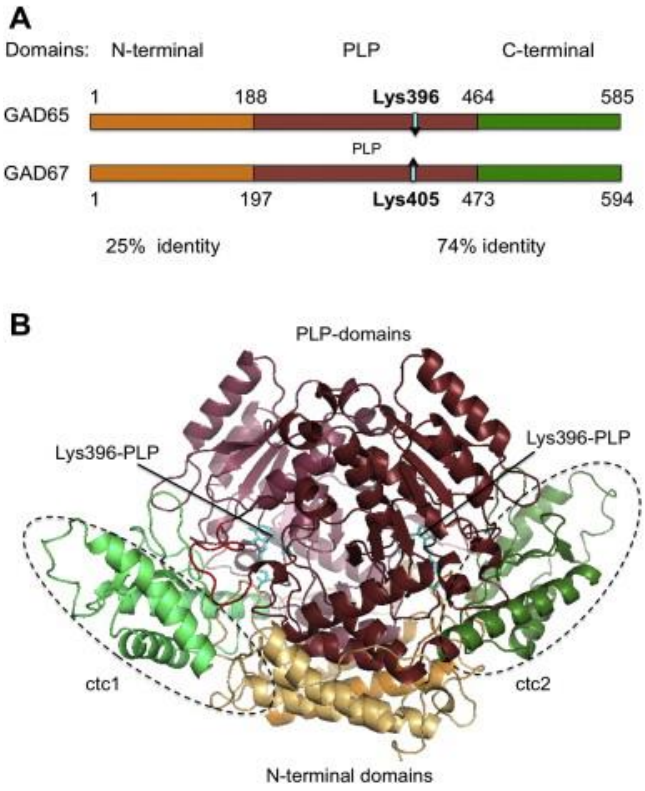

FIGURE 3: STRUCTURE AND DOMAINS OF THE GAD MONOMERS GAD65 AND GAD67.

(A) GAD can be divided into three domains: the N-terminal domain (orange), the catalytic PLP-domain (red) including the PLP-binding sites represented by Lys396 and Lys405 and the C-terminal domain (green). **(B)** depicted is the dimeric structure of GAD65 with one monomer in lighter colors. License Number: 5076990051711 from Elsevier - Journal of Autoimmunity [75].

2.2.2. Functions of GAD

GAD is the rate-limiting enzyme in the synthesis of the inhibitory neurotransmitter γ-aminobutyric acid (GABA) [61,77,78]. Together with its cofactor pyridoxal-5´-phosphate (PLP), GAD converts L-glutamate to GABA and carbon dioxide by irreversible alpha-decarboxylation [61,65,68,70,76]. The main function of GABA is the reduction of neuronal excitability in the brain and through this, it also indirectly regulates the muscle tone [79]. The 65kDa membrane-associated isoform of GAD mainly regulates the production of GABA required for neurotransmission and

ensures a fast synthesis if required [61,65,66,68]. In contrast, the soluble, constantly-active 67kDa isoform regulates almost 90 % of the basal levels of GABA (**FIGURE 4**). Further, GAD67 plays an important role in synaptogenesis and with that in the development of neuronal tissues [80]. Besides other neurotransmitter, Glutamate is known to be the main excitatory neurotransmitter and makes up more than half of the total amount of neurotransmitters in the brain, whilst 40 % are GABA [66,74,80]. As a result, a dysfunction or general synthesis problem of either GABA or Glutamate leads to an imbalance of excitation and inhibition in the brain and may cause disease [66,80]. Underlining this, it could be shown that GAD65 knockout mice are susceptible to seizures [80].

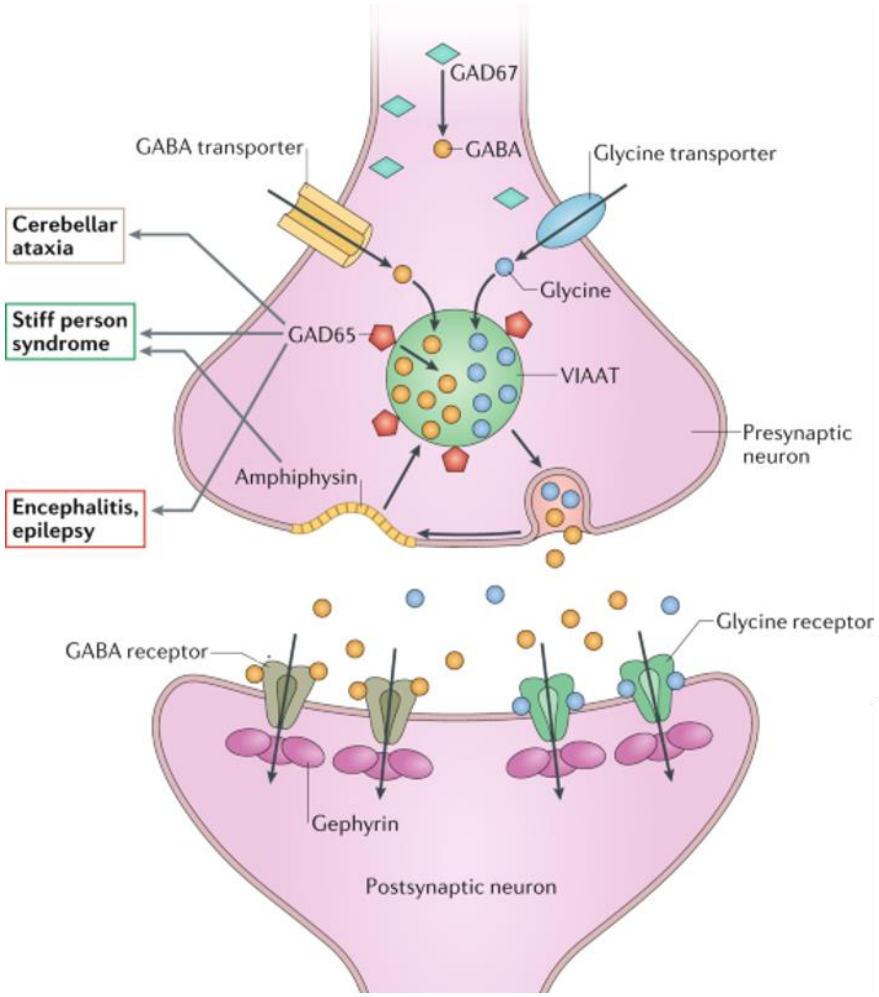

FIGURE 4: GAD65 AND GAD67 ARE LOCATED AT THE PRESYNAPTIC NEURON.

GAD65 and its isoform GAD67 catalyze the synthesis of the inhibitory neurotransmitter GABA. Neurological disorders with abs against the GABA synthetizing enzyme GAD65 are depicted in squares on the left-hand side. VIAAT = vesicular inhibitory amino acid transporter. Slightly modified from F. Graus et al. with the License Number: 5076990153818 from Springer Nature - Nature Reviews Neurology [81].

2.3. GAD-ab-associated neurological disorders

Several auto-abs targeting synaptic proteins such as GAD65, first described in 1988, have been associated with the occurrence of neurological disorders [50,56,64,82]. The spectrum of GAD65-ab-associated neurological disorders (GAD-AIND) includes stiff person syndrome (SPS), cerebellar ataxia (CA), limbic encephalitis (LE) and autoimmune epilepsy (AE) (**FIGURE 4**) [77,78]. Around 94 % of patients suffering from GAD-AIND exhibit high serum levels of anti-GAD abs above a threshold of 10,000 IU/mL as measured by ELISA [69].

2.3.1. Stiff person syndrome

SPS, which was first described in 1956, is an extremely rare neurological disorder with a prevalence of one in a million. It is characterized by constant muscle contractures leading to stiffness and rigidity [67,83,84]. Thereby, an increased stimulus-sensitivity leads to painful spasms especially in paraspinal, abdominal and lower extremity muscles [56,67,85,86]. The symptoms range from troubles in turning and bending to progressive muscle rigidity mainly in the leg muscles, resulting in a "freezing"-like appearance, especially during the chronic phase of the disease [69,84,87,88]. These contractures can be triggered by movement, emotional and sensory stimuli or even appear spontaneously [67,83,86]. At later stages, patients struggle with simple tasks in daily life and require support to manage their household [87]. The consequence of an untreated SPS can be total disability [85]. Often, SPS is misdiagnosed or underrecognized as, e.g., Parkinson´s disease, multiple sclerosis, encephalomyelitis or other spasm-related diseases [86,88]. More women than men (70 %) are affected with an average age of onset of 30-35 years [69,73,87]. One-third of patients with SPS suffer from additional autoimmune diseases. The most common is type-1 diabetes mellitus (T1DM) [67]. Generally, patients suffering from SPS have normal brain and spinal magnetic resonance imaging (MRI)s [56,67]. Patients with SPS show a disrupted GABAergic neurotransmission [89], with a continuous activity of motor units leading to the co-contractions of agonist and antagonist muscles [85]. The reduced GABA titers cause

a continuous discharge of gamma motor neurons resulting in a decrease of inhibitory signals, leading to a state of constant rigidity [69,84,85,89]. Therefore, symptoms improve when treating with GABA agonists, e.g., benzodiazepines like diazepam and during sleep due to the lack of sensory stimuli [56,84]. One of the characteristics of SPS are high anti-GAD ab titers [85,90]. However, the occurrence is not mandatory for the disease determination as only around 60 % of SPS patients exhibit abs against GAD [83,91]. Auto-abs against other inhibitory synaptic proteins can occur, e.g., anti-amphiphysin, anti-gephyrin [92].

2.3.2. Cerebellar ataxia

Immune-mediated CA as the second-most frequent GAD-AIND, is characterized by cerebellar dysfunction leading to an inability to coordinate balance, gait and eye movements [71,93,94]. The cerebellar damage does not only impair motor coordination but also cognitive abilities[95]. Furthermore, patients suffer from nystagmus, dysphagia and severe dysarthria [71,94,96]. The characteristic cerebellar atrophy, analogous to other immune-mediated CA, develops over time with increasing frequency [64]. CA mostly affects women in their 60s and the diagnosis of an anti-GAD65 ab-associated CA is confirmed by intrathecal anti-GAD ab production [71]. Moreover, when analyzing the brain tissue of patients with CA postmortem, a selective loss of Purkinje cells can be detected. Hereby, it was suggested that this could be caused by an overload of glutamate through excitotoxicity [71,94]. In addition, oligoclonal bands (OCBs) in the CSF exist [69,71]. Generally, the subacute type without cerebellar atrophy shows a better prognosis than the chronic type of CA [71,97].

2.3.3. Limbic encephalitis and autoimmune epilepsy

First described in 1960, LE was considered to be of paraneoplastic origin [60], whilst nowadays it is known that LE can also occur as a non-paraneoplastic disease [98]. Anti-GAD ab mediated autoimmune LE (GAD-LE) is a rare disease with a prevalence of 1.9/100,000, affecting more women than men. Furthermore, LE patients exhibit MRI and CSF findings consistent with inflammation of the temporal lobe [60]. Moreover, some of the patients have OCBs in the CSF and an intrathecal synthesis of anti-GAD abs is a diagnostic criteria for GAD-LE [99]. The symptoms of patients with LE consist of a disrupted short-term memory (59 %), seizures (97 %) or psychiatric features including depression and hallucinations [100,101]. GAD-ab associated AE was firstly associated with refractory temporal lobe epilepsy (TLE) in 1988 [50,69]. Since then, the spectrum of anti-GAD65 ab associated autoimmune LE has expanded [102]. 10 % of adults with longstanding epilepsy have high anti-GAD ab levels [103]. As for most GAD-AINDs, young female patients are predominantly affected and the disease often presents as a chronic pharmaco-resistant epilepsy [65,69,102].

2.4. Anti-GAD abs

2.4.1. Pathogenic potential

Depending on the ag-localization, three groups of auto-ab associated AE can be distinguished: encephalitis associated with abs targeting cell-surface ags, intracellular synaptic ags and intracellular cytoplasmic/nuclear ags. Anti-GAD abs, anti-amphiphysin abs, and anti-synapsin 1 abs belong to the group with abs against intracellular synaptic ags [104]. In contrast to abs directed against cell-surface ags, the pathogenic role of B cells and auto-abs is not as clearly established in GAD-ab-associated disorders and an interplay between B and T-cells is assumed [95,105]. Immunotherapy is effective in patients with auto-abs against extracellular ags (like NMDAR), whereas patients with auto-abs associated to intracellular ags (such as anti-Hu abs) show an insufficient response to immunotherapy [56,104]. Patients with GAD-abs show an intermediate response to immunotherapies like plasma exchange or intravenous immunoglobulins, indicating that B cells and auto-abs could be involved in pathogenesis (**FIGURE 5**) [90,106].

As a consequence, the pathophysiology of auto-abs against intracellular synaptic ags, such as amphiphysin and synapsin 1, has been investigated intensively [56,107–109]. It could be shown that auto-abs against amphiphysin and synapsin 1 are internalized, whereby they gain access to the ags. Anti-amphiphysin abs are internalized via an epitope-specific mechanism, whereas anti-synapsin 1 abs are internalized via clathrin-dependent endocytosis [56,109]. Hereupon, the internalization of auto-abs at the inhibitory synapse leads to a disrupted synaptic transmission [107,109].

The role of anti-GAD65 abs in several neurological disorders and their pathogenic potential have been controversially discussed [52,69,81,110].

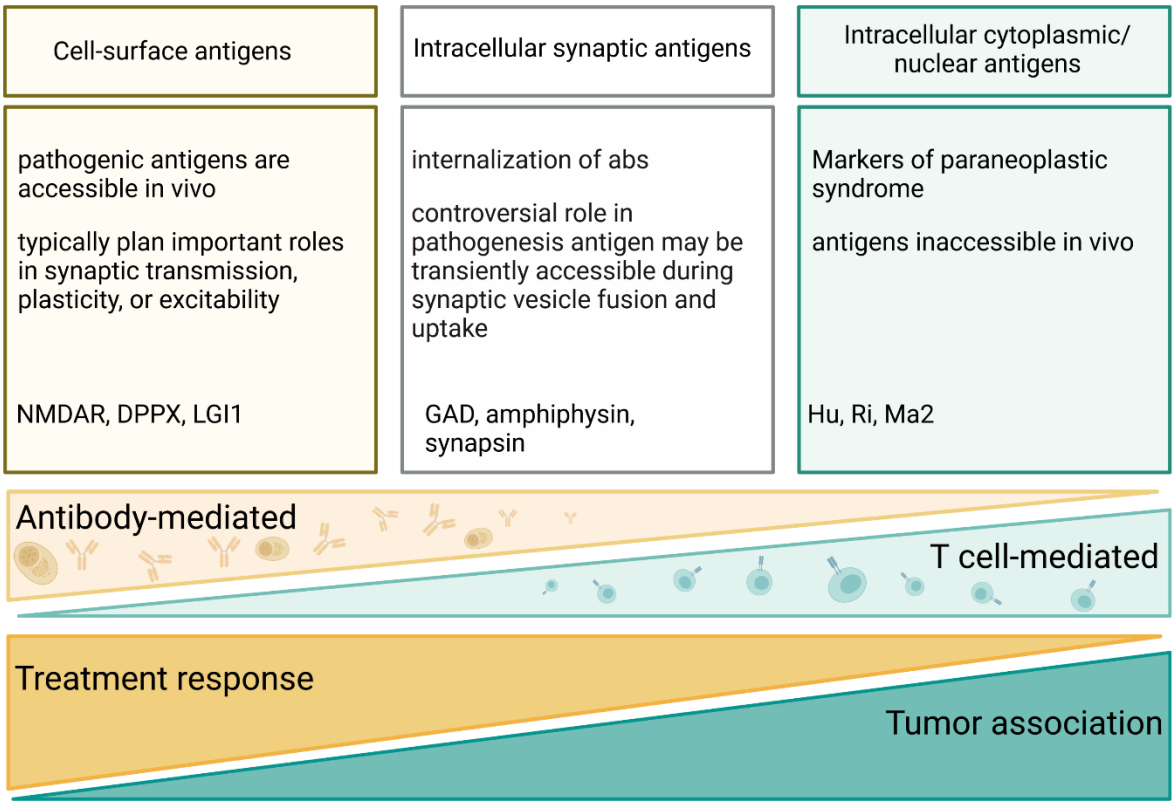

FIGURE 5: COMPARISON OF THE THREE GROUPS OF NEURONAL ABS.

Pathogenicity, accessibility, treatment response and tumor association of cell-surface ags, intracellular synaptic ags and intracellular cytoplasmic/nuclear ags. DPPX = dipeptidyl peptidase like protein 6; LGI1 = leucine rich glioma inactivated protein 1. Adapted from B. Balint et al. BRAIN, and under the terms of the Creative Commons CC BY license, Copyright © 2017, Oxford University Press, by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates [104].

The detection of anti-GAD65 abs in SPS and an intrathecal production of anti-GAD65 abs in CA and LE (TLE) were proposed as diagnostic criteria for GAD-AIND [81]. In addition, ~ 40 % of patients with GAD-AIND have OCBs in the CSF [111–113], indicating a clonal B cell activation in the CNS [77,114]. Several studies suggest an inhibition of glutamate to GABA conversion caused by anti-GAD abs [50,60,95]. As a consequence, patients with anti-GAD abs would have reduced levels of GABA followed by an enhanced neuronal excitability and a decreased seizure threshold [50,60,98]. Interestingly, CSF analysis of patients with anti-GAD ab associated SPS revealed reduced levels of GABA and the sera from patients could inhibit the enzyme activity of GAD65 *in vitro* [85,89,115]. Moreover, the passive transfer of purified Ig obtained from anti-GAD ab positive sera (SPS, CA) in *in vivo* studies leads to a partially reproduced disease manifestation in rats and mice.

This disease manifestation was characterized by persistent motor activity leading to abnormally increased reflexes [78,116]. When anti-GAD65 abs were absorbed by recombinant GAD65, the induced synaptic impairment was abolished [117]. In line with this observation, studies in which mice were immunized with GAD65 could induce the generation of anti-GAD abs and revealed a loss of GABAergic neurons [118].

However, other studies could not demonstrate a pathogenic potential of GAD-abs and the internalization of anti-GAD65 abs could not be detected in experiments using cultured rat hippocampal neurons [69,110]. Moreover, there is no real evidence that GAD is expressed on the cell surface. Not only that, high serum titers are not invariably associated with a more severe outcome of the disease [74,110]. Nevertheless, it has recently been shown that GAD65 can associate with the plasma membrane and in this way might be transiently expressed on the cell surface [64] and an auto-reactivity directed against GAD65 is more common than to GAD67 [50,62,78,119].

To conclude, GAD65 is an intracellular protein and the influence and pathogenic potential of its related abs remain to be elucidated [50,53,110]. Experimental data regarding the effect of anti-GAD abs from patients on mice and rat brains *in vivo* as well *in vitro* showed controversial results. The question whether anti-GAD abs are just markers of an aberrant immune response remains unanswered [52,69,74,93,99].

2.4.2. Autoimmunity

Interestingly, around 70 % of patients with GAD-AIND have one or more coexisting autoimmune diseases such as T1DM (30% of GAD-AIND patients have coexisting T1DM). Pernicious anemia and thyroid disease are also frequently observed (**FIGURE 6**) [69,73]. This raises the question whether a general predisposition to develop autoimmune disorders exists in patients with GAD-AIND. A genetic contribution or environmental risk factors could contribute to such a predisposition leading to a loss of self-tolerance and resulting in the co-occurrence of several autoimmune diseases [53,67,119]. In line with this theory, a

small explorative study including patients with GAD-AIND and other autoimmune diseases detected several variations in genes linked to autoimmunity or representing check-point proteins. This suggests that patients with GAD-AIND carry genetic risk factors [119].

Still, anti-GAD abs do not only occur in the context of neurological disorders and anti-GAD65 abs can be observed in healthy controls [73]. Along with this, a high percentage of people with T1DM harbor low titers of anti-GAD abs (5-2000 IU/mL as measured by ELISA) as GAD is expressed in pancreatic ß-cells (**FIGURE 6**) [69]. Thereby, anti-GAD65 abs are thought to be markers of pancreatic cell destruction since GAD is not expressed on the cell surface [65]. Besides this, only patients with typical GAD-AIND reveal very high anti-GAD ab titers that stay high over time (**FIGURE 6**) [64,69]. Additionally, supporting the argument of a neurologically induced pathogenicity, only GAD-AIND patients and not patients with T1DM patients have detectable anti-GAD abs in the CSF [64,99].

In conclusion, the relevance of the presence of anti-GAD65 abs in patients (with T1DM and other autoimmune disorders and/or GAD-AIND) has to be evaluated in the overall clinical context [61,73].

2.5. Detection methods of anti-GAD abs

Different assays are used to detect anti-GAD abs. Here, it can be distinguished between quantitative assays such as radioimmunoassay (RIA) and enzyme-linked-immunosorbent-assay (ELISA) and qualitative assays including tissue based assays with detection via immunohistochemistry or indirect immunofluorescence, cell-based assays (CBAs) and western blots (WBs)/line blots (**FIGURE 6**) [69,110,111]. ELISAs and RIAs have been implemented to detect low titers of anti-GAD abs as seen, for instance, in T1DM patients and to distinguish between anti-GAD67 and anti-GAD65 abs (**FIGURE 6**) [69,110,111,120]. The RIA assay uses human recombinant Iodine-linked GAD65 to detect anti-GAD65 abs. Thereby, the patient sample is precipitated with this radio-labelled GAD65 and analyzed with the gamma counter. [64,120]. However, the necessity of a radioisotope can sometimes be inconvenient [120]. A study that compared RIA and ELISA sensitivities observed that RIAs have a higher sensitivity when measuring low levels of anti-GAD abs [121]. On the contrary, qualitative assays can detect conformational epitopes recognized by anti-GAD abs and can distinguish unspecific bindings, thereby decreasing the number of false positive results [69,110]. Hereby, in tissue-based assays a typical GAD65 staining in the cerebellum is characterized by an irregular staining of the granular layer [64].

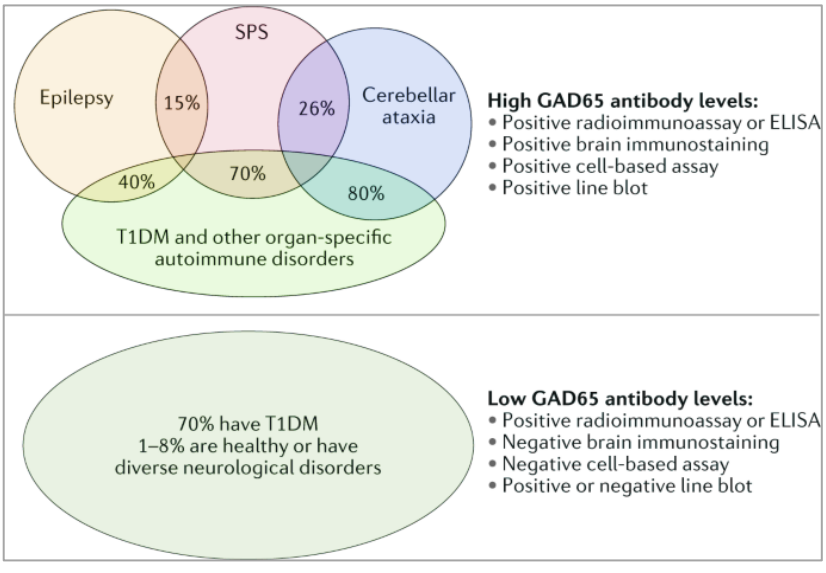

FIGURE 6: DETECTION OF ANTI-GAD65 ABS IN GAD-AIND AND OTHER ACCOMPANIED AUTOIMMUNE DISORDERS.

High anti-GAD65 ab titers are associated with GAD-AIND are also detectable in type 1 diabetes mellitus. In patients with type 1 diabetes mellitus low anti-GAD65 ab titers are detected, whereas in GAD-AIND high levels of anti-GAD65 abs that are detectable using different assays are found. License Number: 5076990153818 from Springer Nature - Nature Reviews Neurology [81].

Thus, the use of different detection assays in clinical practice with varying sensitivities and detection limits can lead to difficulties when comparing patient frequencies and the occurrence of GAD-AIND between different centers [52,71,73]. As a consequence, depending on the definition of threshold values, different percentages concerning GAD-AIND in different studies are published. For example, an examination of neurological patients with the RIA showed that a large number of patients had high anti-GAD ab titers, which were above the threshold of 41,000 U/mL [52]. In contrast, another study defined patients as being positive for a GAD-AIND, inter alia, once anti-GAD ab titers were above 2,000 U/mL in RIA [110]. In order to define clinically relevant values of anti-GAD65 abs, cut-off values such >2000 U/mL in RIA and >1000 IU/mL or >20 nmol/L measured by ELISA, or a strong positive labeling at low dilutions in immunohistochemistry were proposed [64].

2.6. Treatment of anti-GAD-ab positive patients – immunotherapy

Unfortunately, most of the published data regarding immunotherapy in GAD-AIND discuss and investigate the effect of different combined drugs only in small studies or case reports. This is due to the rarity of GAD-AIND [67,96,99,100,122,123]. Furthermore, treatments often start years after disease onset, which does not allow appropriate conclusions on their effectiveness. A more structured study with a higher number of patients and better follow-up assessments would be necessary to compare outcomes of immunotherapy in different GAD-AIND [52]. Nevertheless, one can say that if the immunotherapy is effective, an improvement of symptoms is seen quite early. In conclusion, if the patient does not show an improvement after a short period of time, the therapy should be re-evaluated [94,100].

2.6.1. First-line therapy

As a first step, immunotherapy with first-line therapies such as intravenous immunoglobulins, corticosteroids and plasma exchange is applied [69]. Different treatment responses depending on the subtype of GAD-AIND were described [124]. Intravenous immunoglobulins were reported to improve symptoms in ~80 % of SPS patients [85]. In contrast, plasma exchange and corticosteroids were not associated with a clear therapy response [124]. Moreover, the therapeutic success of immunotherapy in patients with different types of chronic subtypes of CA and AE has not been proven yet [71,100]. In some studies, patients with a subacute presentation of CA responded rather well to immunotherapy [71,94]. Overall, patients with an anti-GAD ab-associated epilepsy do not respond well to seizure medication [125]. Besides immunotherapy, treatments targeting the disrupted GABAergic transmission, like benzodiazepines in SPS, are important measures to alleviate symptoms [123].

2.6.2. Second-line therapy

Second-line therapies are immunosuppressants, like methotrexate, azathioprine, cyclophosphamide, and mycophenolate mofetil[69,100,126]. Furthermore, mabs such as rituximab that transiently depletes CD20 expressing B cells is commonly used to treat GAD-AIND, especially SPS and LE [69,87,124,127]. For all of the GAD-AINDs, a fast initiation of therapy seems to prognosticate a better clinical outcome [100,102,127–129]. Supporting this, it was shown that treatment with rituximab was not effective in patients with long-standing GAD-AIND treatment [124].

3. AIMS OF THE THESIS

High titers of anti-GAD abs occur in a number of neurological diseases. This raises the question if anti-GAD65 abs mediate the associated syndromes or if the abs themselves are bystanders of disease. Furthermore, it is unclear why anti-GAD65 abs are associated with a diverse spectrum of neurological disorders, as well as how and where the immunological dysregulation in those patients is caused. Moreover, the pathogenic role of abs against GAD65 is highly controversial and the published findings are inconclusive. Additionally, the cells producing anti-GAD abs are largely unexplored and triggers for anti-GAD ab production remain elusive.

Compared to patients with auto-abs against neuronal surface ags, the treatment of patients with GAD-AIND remains challenging. Therefore, investigating the immune responses in GAD-AIND patients could help to gain pathophysiological insights and with that help to develop new treatment strategies. In addition, it could help to understand the underlying mechanisms of auto-abs targeting intracellular proteins in other autoimmune diseases.

Therefore, the aim of my thesis is to characterize the B cell response against GAD in different B cell compartments and to gain more insights into the complexity of GAD-AIND, and with that into the underlying pathomechanisms.

To achieve this, we analyzed the peripheral blood (PB)-compartment, the CSF-compartment, as well as bm cells from GAD-AIND patients. We generated monoclonal patient-derived anti-GAD65 abs from CSF and PB. Furthermore, we characterized sequence specificities of the mabs. We analyzed the anti-GAD65-reactivity of the mabs in different qualitative and quantitative assays, as well as the reactivity to a panel of viral antigens. Lastly, we generated and produced the germline-derived unmutated common ancestors (UCAs) through reversion of introduced SHMs in variable regions of selected mabs in order to evaluate their specificity towards GAD65.

3.1. Aim of the study I

The aim of the study was the characterization of anti-GAD ab producing B cells in the PB and bm cells of patients with GAD-AIND.

Abundant Glutamic Acid Decarboxylase (GAD)-Reactive B Cells in GAD-Antibody– Associated Neurological Disorders

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ANN NEUROL 2019;9999:1–7

https://doi.org/10.1002/ana.25414

For this paper, I performed half of all experiments and helped to analyze the experimental data together with Dr. med. Franziska Thaler. More specifically, I performed the experiments used for Fig. 1 (A, B, F, G, H, J) and Fig. 2 (A, B, C). I also contributed to drafting the text and preparing the figures, together with Dr. med. Franziska Thaler. The contribution of other authors is specified in the paper.

Abundant Glutamic Acid Decarboxylase (GAD)-Reactive B Cells in GAD-Antibody–Associated Neurological Disorders

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High levels of antibodies against glutamic acid decarboxylase (GAD) are observed in patients with different neurological disorders, but cells producing these autoantibodies are largely unexplored. We detect circulating GAD-reactive B cells in peripheral blood that readily differentiate into antibody-producing cells. These cells are highly elevated in most patients with GAD-antibody–associated disorders (n = 15) compared to controls (n = 19). They mainly produce GAD65 antibodies of the IgG1 and IgG4 subclasses and are as abundant as B cells reactive for common recall antigens. Bone marrow cells represent an additional source of GAD antibodies. The identification of GAD-antibody–producing cells has implications for the selection of cell-specific biologics.

ANN NEUROL 2019;9999:1–7

The cytoplasmic protein glutamic acid decarboxylase (GAD) is the rate-limiting enzyme in the synthesis of the major inhibitory neurotransmitter γ-aminobutyric acid.[1] High serum levels of antibodies (Abs) against GAD have been associated with neurological syndromes like stiff person syndrome, cerebellar ataxia, limbic encephalitis, epilepsy, or oculomotor dysfunction.[2–6] A marked intrathecal Ab response against GAD or cerebrospinal fluid oligoclonal bands supports an association of GAD Abs with neurological syndromes[7] and indicates clonal B-cell activation in the central nervous system.[8] Histologically, neuronal loss and infiltrating T cells are seen in patients with GAD-Ab–associated neurological disorders.[9] This supports the idea that immune reactions are relevant in these patients, but precise mechanisms have not been identified. GAD exists in two isoforms: GAD65 and GAD67. In patients with neurological disorders, autoantibodies are mainly directed against GAD65 and less frequently against GAD67.[10,11]

Compared to patients with neuronal surface Abs, effective treatment in GAD-Ab–positive patients remains challenging.[12,13] Therefore, determining the immune responses in these patients can help to gain pathophysiological insights facilitating therapeutic decisions. Thus, we analyzed GAD-Ab–producing B cells in peripheral blood cells and bone marrow (BM) cells of patients with GAD-Ab–associated neurological syndromes.

Subjects and Methods

Patients

Fifteen patients with neurological symptoms and GAD Abs detected in clinical routine along with 19 healthy controls were analyzed (Table). Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation. Bone marrow mononuclear cells (BMMCs) were obtained by iliac crest aspiration. BMMCs from controls included: hairy cell leukemia (n = 1), Mixed lineage leukemia (MLL)-rearranged acute lymphoblastic leukemia (n = 1), and relapsing–remitting multiple sclerosis and pancytopenia after alemtuzumab treatment (n = 1). All patient and control samples were collected following written informed consent, and the study was approved by the institutional review board of Ludwig-Maximilians University, Munich, Germany.

Cell Culture and Cell Stimulation

PBMCs were stimulated with the toll-like receptor (TLR) 7/8 ligand R848 (Sigma Aldrich, St Louis, MO; 2.5 μg/ml) and IL-2 (R&D, Wiesbaden, Germany; 1000 IU/ml) at a concentration of 600,000 cells/ml in 24-well plates for 11 days as described.[14] B-cell frequency was determined by flow cytometry using

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Received Jul 18, 2018, and in revised form Jan 9, 2019. Accepted for publication Jan 9, 2019

View this article online at wileyonlinelibrary.com. DOI: 10.1002/ana.25414.

anti–CD19-PerCP-Cy5.5 (eBioscience, Frankfurt, Germany; SJ2C1). To determine frequencies of antigen-reactive cells, PBMCs were seeded in limiting dilutions at $10,^{2}$ $10,^{3}$ $10,^{4}$ $5 \times 10,^{4}$ and 10^{5} cells per 200 μl and stimulated as described above. Frequency calculation of antigen-reactive cells was performed according to the Poisson distribution as the seeded PBMC number at which 37% of the cultures were negative. BMMCs were cultured at 3×10^{5} cells per 200 μl for 5 days without addition of stimuli.

Enzyme-Linked Immunosorbent Assays

IgG, IgM, and IgA concentrations were determined using the Human IgG, IgM, and IgA enzyme-linked immunosorbent assay (ELISA) development kit (Mabtech, Nacka Strand, Sweden). GAD Abs were quantified by human anti-GAD ELISA (IgG; Euroimmun, Lübeck, Germany). Measles virus (MV) Abs were detected by Serion ELISA Classic Measles IgG (Virion\Serion, Würzburg, Germany). Tetanus toxoid (TT)-specific Abs were determined by

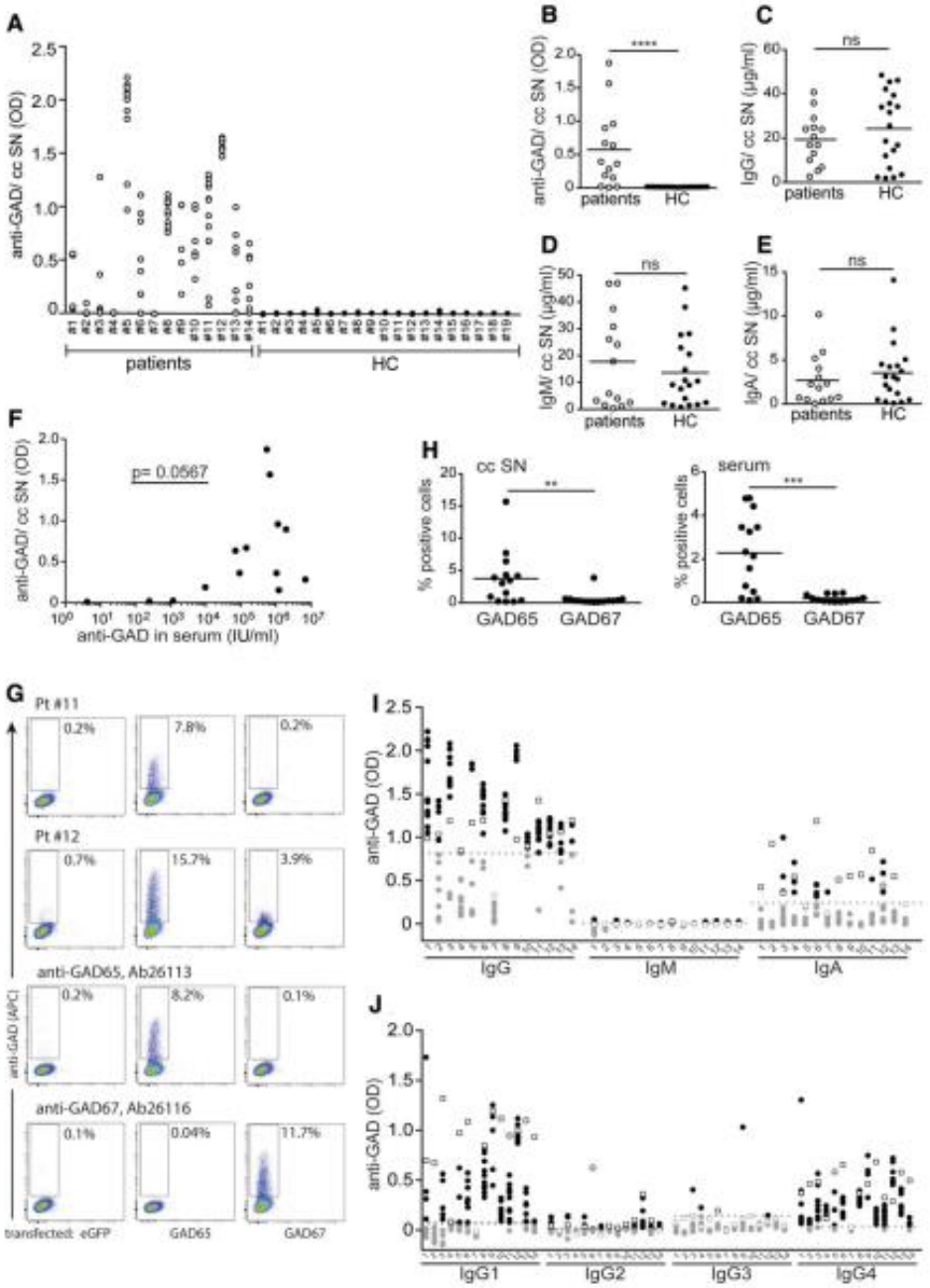

FIGURE 1: Legend on next page.

coating TT (Merck Millipore, Billerica, MA; 1 μg/ml) or bovine serum albumin and detection with anti-human Ig-HRP (Jackson ImmunoResearch, West Grove, PA; 109-036-003). GAD IgG, IgM, IgA, IgG1, IgG2, IgG3, and IgG4 were determined by coating GAD protein (Diamyd Medical, Stockholm, Sweden; 2 μg/ml) and detecting with anti-human IgG-HRP (Jackson ImmunoResearch, 109-035-098), anti-human IgA-HRP (Thermo Scientific, Waltham, MA; PA1-74395), anti-human IgM-HRP (Zymed Laboratories, South San Francisco, CA; 05-4920), anti-human IgG1-HRP (Zymed Laboratories, 05-3320), anti-human IgG2-HRP (Zymed Laboratories, 05-0520), anti-human IgG3-HRP (Zymed Laboratories, 05-3620), and anti-human IgG4-HRP (Zymed Laboratories, 05-3820).

Cell-Based Assay

Hek293T cells transfected with GAD65 in pcDNA3.1, GAD67 in pCMV6 (Origene Technologies, Rockville, MD), or enhanced green fluorescence protein in pMSCV were fixed and permeabilized and stained with sera diluted 1:50 and cell culture supernatants diluted 1:10. For detection, anti-human IgG-biotin (Jackson ImmunoResearch, 109-066-098) and Alexa Fluor 647–conjugated streptavidin (Jackson ImmunoResearch, 016-600-084) were applied. Anti-GAD65 (Abcam, Cambridge, MA; Ab26113) and anti-GAD67 (Abcam, Ab26116) were used as positive controls.

Results

Peripheral B Cells Can Be Stimulated to Produce GAD Abs

To determine the presence of GAD-Ab–producing B cells in peripheral blood, we isolated PBMCs from Patients #1 to #14 and from 19 healthy donors (see Table), and differentiated the cells in vitro into Ig-producing plasmablasts.[14] After cultivation, GAD Ab production was restricted to GAD-Ab–positive patients (Fig. 1A, B). Culture supernatants from healthy donors contained similar levels of IgG, IgM, and IgA (Fig. 1C-E) but no GAD Abs (Fig. 1A, B). Next, we compared the GAD Ab levels released by blood-derived B cells after in vitro differentiation in each patient with their respective GAD Ab level in serum (Fig. 1F). All patients with anti-GAD levels $>10^4$ IU/ml had GAD-reactive B cells in their blood, but there was no significant overall correlation suggesting different sources of GAD Abs (see also below). GAD Abs in cell culture supernatants of stimulated PBMCs and serum were mainly directed against GAD65; in only 1 patient, anti-GAD67 reactivity was observed (Fig. 1G, H). They were mainly IgG, and only to a small extent IgA (Fig. 1I). Furthermore, they were mostly composed of the subclasses IgG1 and IgG4 (Fig. 1J). This pattern was also observed in the serum of the corresponding patients (Fig. 1I, J).

GAD-Reactive B Cells in Peripheral Blood Show Comparable Frequencies as MV- and TT-Reactive B Cells

The stimulation experiments suggested high frequencies of GAD-reactive B cells ready to differentiate into GAD-Ab–producing plasma cells. We further analyzed 13 patients by limiting dilution assay and determined the frequencies of GAD-reactive B cells in comparison to B cells reactive for the common recall antigens MV and TT. After stimulation of PBMCs, supernatants were analyzed by ELISA for the presence of GAD Abs, MV Abs, and TT Abs (representative data for Patient #12 is shown in Fig 2A). Using the Poisson distribution, we determined the frequency of GAD-reactive cells, MV-reactive cells, and TT-reactive cells (representative data for Patient #12 is shown in Fig 2B). The frequencies of antigen-reactive PBMCs and antigen-reactive B cells were calculated for all analyzed

FIGURE 1: Glutamic acid decarboxylase (GAD)-reactive B cells in the blood of patients with GAD antibodies (Abs) in the serum. (A, B) Peripheral blood mononuclear cells (PBMCs) from GAD-Abs–positive patients (n = 14) and healthy controls (HC; n = 19) were cultured with R848 and IL-2 for 11 days, and GAD Ab (IgG) levels were determined by enzyme-linked immunosorbent assay (ELISA) in cell culture supernatant (cc SN). Results of the stimulation of the PBMCs from Patient #15, who was under treatment with rituximab and exhibited B-cell depletion in peripheral blood, are not depicted in Figure 1 but are presented in Figure 2D. (A) Each circle represents 1 stimulated well. The numbers of analyzed wells are as follows. Patients: #1, 11; #2, 11; #3, 10; #4, 13; #5, 12; #6, 11; #7, 23; #8, 12; #9, 5; #10, 8; #11, 13; #12, 12; #13, 10; #14, 8; HC: #1, 10; #2, 24; #3, 10; #4, 15; #5, 12; #6, 10; #7, 7; #8, 14; #9, 9; #10, 16; #11, 8; #12, 11; #13, 9; #14, 8; #15, 8; #16, 8; #17, 8; #18, 8; #19, 8. (B–E) Each circle represents the mean of all stimulated wells in 1 patient. Horizontal lines indicate the mean of all patients. (B) GAD-Ab production was significantly higher in patients than in HC (Mann–Whitney test). (C, D, E) Total IgG, IgM, and IgA levels in cc SN were determined by ELISA. IgG, IgM, and IgA production was not significantly different between the 2 groups (Mann-Whitney test for IgG and IgA, unpaired *t* test for IgM). (F) The mean anti-GAD reactivity of the stimulated PBMCs did not correlate with GAD Ab serum levels in the respective patients (Spearman correlation). (G, H) Anti-GAD65 and anti-GAD67 reactivity of cc SN of stimulated PBMCs (for each patient, the SN with the highest GAD Ab levels in ELISA was selected) and sera were compared by flow cytometry using Hek293T cells transfected with plasmids coding for GAD65, GAD67, and enhanced green fluorescence protein (eGFP) as a control. (G) Results of cc SN analysis for 2 representative patients (Patients #11 and #12) and of the control Abs are depicted. (H) The percentage of cells staining for anti-GAD65 and anti-GAD67 is shown for cc SN (left panel) and serum (right panel). Horizontal lines indicate the mean. Anti-GAD65 reactivity was significantly higher than anti-GAD67 reactivity (Mann–Whitney test for cc SN, unpaired *t* test for serum). (I, J) Anti-GAD IgG, IgM, IgA, and IgG1–4 in the cc SN and in serum were determined by ELISA. Each circle represents 1 stimulated well (positive values shown in black). Serum values are depicted with squares. The cutoff values for each isotype/subclass were calculated as the mean optical density (OD) of the healthy controls' supernatants +2 standard deviations. IE = international units; ns = not significant, APC = Allophycocyanin; ** ($p \leq 0.01$), * ($p \leq 0.001$), **** ($p \leq 0.0001$).**

TABLE. Characterization of Patients and Healthy Controls

Patient	Sex	Age at PBMC Sampling, yr	Disease Duration, yr	Diagnosis	IST at PBMC Sampling	Anti-GAD/Serum, IU/ml	B-Cell Frequency, % Living Cells
#1	F	34.3	9	SPS	sc Ig + aza	1,198,242	15.5
#2	F	75.4	4	CA	MMF	1,160	19.5
#3	F	77.2	12	CA	None	9,641	7.5
#4	F	38.6	6	LE	None	244	5.8
#5	F	61.9	0.25	CA	GCs	547,972	24.5
#6	M	59.3	0.83	CA	None	1,035,731	40.8
#7	M	34.8	0.33	LE	PLEX, iv Ig[a]	4	10.1
#8	F	18.6	1.67	LE	None, GCs[b]	1,134,949	11.1
#9	F	22.0	1.58	LE, APECED	aza	141,340	11.2
#10	F	62.4	4	CA	None	69,976	11.7
#11	F	53.6	6	SPS	None	1,931,662	5.3
#12	F	28.5	0.17	LE	None	668,244	10.8
#13	M	75.6	0.67	SPS	None	91,090	10.4
#14	F	53.0	11.25	CA	None	6,829,116	19.2
#15	F	48.9	6	LE	RTX + aza[c]	145,909	B-cell depletion
		F/M	**Mean ± SD**	**Mean ± SD**		**Mean ± SD**	**Mean ± SD**
Patients		12/3	49.6 ± 18.8	4.3 ± 3.9		920,352 ± 1,678,500	14.5 ± 9.0
HC		13/6	43.7 ± 8.9				

[a]Three weeks prior to PBMC sampling.
[b]Two months prior to PBMC sampling.
[c]Analysis of bone marrow mononuclear cells.
APECED = autoimmune polyendocrinopathy–candidiasis–ectodermal dystrophy; aza = azathioprine; CA = cerebellar ataxia; F = female; GAD = glutamic acid decarboxylase; GC = glucocorticosteroid; HC = healthy controls; IST = immunosuppressive treatment; iv = intravenous; LE = limbic encephalitis; M = male; PBMC = peripheral blood mononuclear cells; PLEX = plasma exchange; RTX = rituximab; sc = subcutaneous; SD = standard deviation; SPS = stiff person syndrome.

patients and revealed frequencies of GAD-reactive B cells in peripheral blood comparable to MV-reactive or TT-reactive cells (see Fig 2C).

GAD Abs Are Produced by Plasma Cells in the BM

As our comparison of GAD Ab levels in serum with the levels of GAD Abs produced by stimulated B cells in peripheral blood indicated different sources of GAD Abs (see Fig 1F), we aimed to analyze the BM as a possible additional source of GAD Ab production. Patient #15 (see Table) developed relapses with seizures and cognitive impairment and showed high GAD Abs in the serum despite treatment with rituximab and azathioprine. The azathioprine dosage was therefore increased, which led to severe anemia and leukopenia. Treatment with azathioprine was stopped, granulocyte colony-stimulating factor was administered once, and a BM aspiration was performed. Here, intact hematopoiesis and an absence of CD20-positive cells were observed. After stimulation of the PBMCs with R848 and IL-2, we detected very low IgG levels and no GAD Ab production, in line with depletion of B cells in the peripheral blood under treatment with rituximab (see Fig 2D). BMMCs cultured for 5 days without additional stimuli spontaneously secreted GAD Abs, which was not observed in BMMCs from the

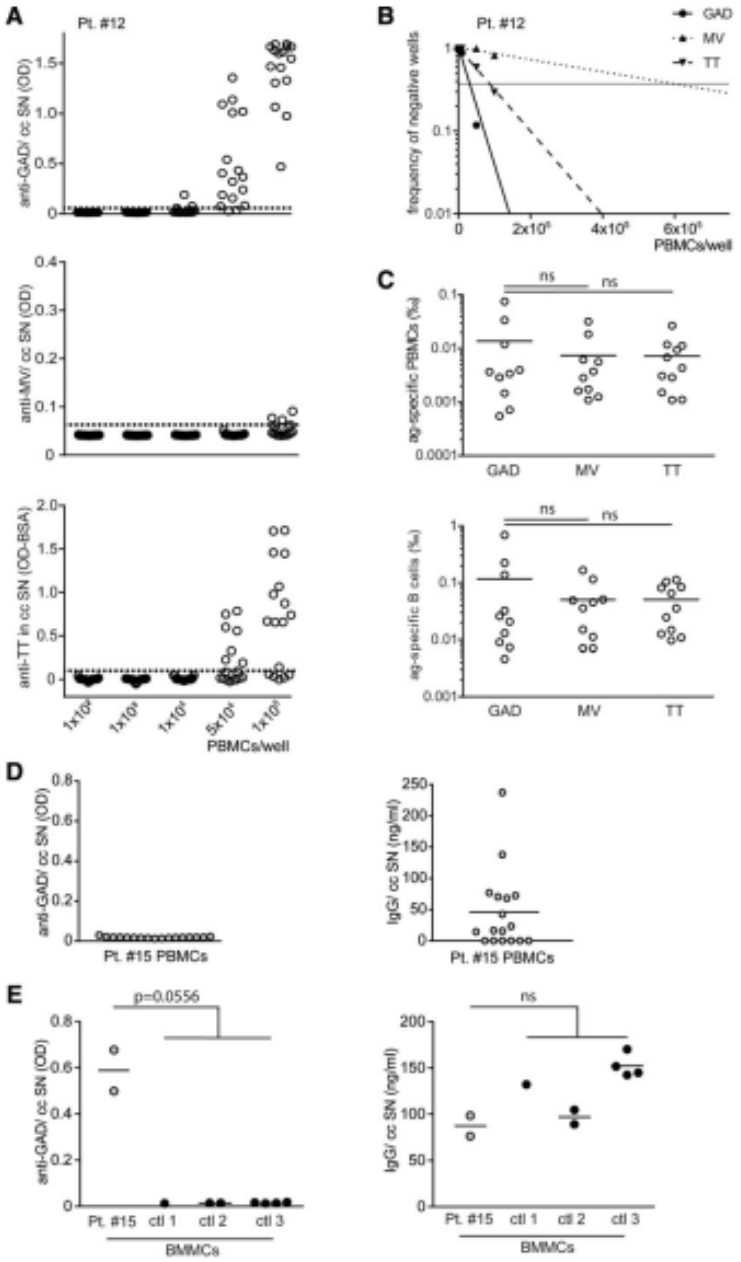

FIGURE 2: Glutamic acid decarboxylase (GAD) antibody (Ab)-producing B cells in the peripheral blood show frequencies comparable to those of measles virus (MV)- and tetanus toxoid (TT)-reactive B cells. GAD Abs are produced by bone marrow (BM) cells. (A–C) Limiting dilution analysis with peripheral blood mononuclear cells (PBMCs) from 13 patients was performed (for 1 patient, no further PBMC samples were available). PBMCs were seeded at concentrations of $10,^2$ $10,^3$ $10,^4$ $5 \times 10,^4$ and 10^5 cells/well with 17 different wells for each cell concentration and cultured for 11 days in the presence of IL-2 and R848. GAD Ab, MV Ab, and TT Ab production was determined by enzyme-linked immunosorbent assay (ELISA). (A) GAD Abs, MV Abs, and TT Abs in cell culture supernatant (cc SN) from individual wells in 1 representative patient (Patient #12). Cutoff was set as 4 × standard deviation (SD) for GAD, 1 × negative control +1/2 SD for MV, and at an optical density (OD) of 0.2 for TT; each circle represents 1 stimulated well. (B) Depiction of the calculation of the frequencies of antigen (ag)-reactive cells in 1 representative patient according to the Poisson distribution. (C) The frequencies of ag-reactive PBMCs (upper panel) and B cells (lower panel) in all patients are depicted. Calculation of ag-reactive B cells is based on total B-cell frequencies in each patient (see Table); horizontal lines indicate the mean. No significant difference between GAD-reactive, MV-reactive, and TT-reactive PBMCs and B cells was detected (Kruskal–Wallis test followed by Dunn multiple comparisons test). Calculation of ag-reactive cells was not possible in n = 3 (GAD), n = 3 (MV), and n = 2 (TT) as no positive wells were present. (D) PBMCs from Patient #15, who was under treatment with rituximab and exhibited B-cell depletion in peripheral blood at the time of analysis, were stimulated with R848 and IL-2 for 11 days. No GAD Ab production and only very low IgG Ab production (ng/ml as compared to µg/ml in patients who did not receive rituximab; see Fig 1) were determined by ELISA. (E) Bone marrow mononuclear cells (BMMCs) of Patient #15 as well as BMMCs from 3 control (ctl) patients were cultured for 5 days, and GAD Ab and IgG production was determined by ELISA. Each circle represents one cultured well; horizontal lines indicate the mean. BMMCs from Patient #15 produced GAD Abs, whereas BM cells from control patients did not; the difference was not statistically significant with a detectable trend (Mann–Whitney test; E, left panel). IgG production did not differ between Patient #15 and control patients (Mann–Whitney test; E, right panel). BSA = bovine serum albumin; ns = not significant.

3 control patients (see Fig 2E, left panel). In contrast, total IgG production by the patient's BMMCs was comparable to the control patients' BMMCs (see Fig 2E, right panel).

Discussion

Three theories concerning long-lasting Ab production and plasma cell generation exist.[15] First, persisting antigen stimulation might continuously generate short-lived plasma cells from memory B cells.[16] Second, activation via cytokines and TLR ligands might give rise to plasma cells from memory cells.[17,18] Third, plasma cells might persist in survival niches, for example, in the BM.[19] We provide evidence that aligns with the second notion; GAD-Ab–producing cells can be generated from memory B cells by cytokine receptor and TLR stimulation. GAD Ab production could be achieved under minimalized culture conditions of unfractionated PBMCs stimulated with only R848 as a TLR ligand and IL-2. This protocol was identified to efficiently and selectively activate memory B cells by directly stimulating TLR7 on memory B cells and TLR8 on myeloid cells that produce additional cytokines supporting B-cell proliferation and differentiation.[14] Similar conditions could also be encountered during infections in patients. The presence of the specific antigen GAD was not required for induction of GAD-Ab–producing cells. GAD Ab production by stimulated peripheral blood cells did not correlate with GAD Ab serum levels, suggesting an additional source of GAD Abs. In line with the third theory presented above, plasma cells in the BM are shown here to be a further source of GAD Ab production.

These findings have several important clinical implications. GAD-reactive memory B cells in peripheral blood can be targeted by antiproliferative medications and CD20-directed therapies. Consequently, these treatments decrease the pool of GAD-Ab–producing cells without completely abolishing GAD-Ab–producing cells, as GAD-Ab–producing long-lived plasma cells would not be targeted. This is in line with the observation that rituximab only moderately decreases GAD Ab levels in serum.[13,20] Currently, the therapeutic response in many GAD-Ab–positive patients is not satisfying.[12,13] Therefore, targeting both memory B cells (eg, with rituximab) and plasma cells (eg, with bortezomib) might be a potential treatment option, with the awareness that such treatment regimens come with strong potential side effects.

Together, our study shows a strikingly high frequency of GAD-reactive B cells in blood of most patients and demonstrates rituximab-resistant BM plasma cells as an additional source of GAD Abs. The identification of the GAD-Ab–producing cells is relevant to developing strategies of cell-specific therapies in patients with GAD-Ab–associated neurological disorders. The abundance of GAD-reactive B cells extends our understanding of the dysregulated self-tolerance in these patients.

Acknowledgment

This work was supported by the German Research Foundation (SFB TR128), the Munich Cluster for Systems Neurology (ExC 1010 SyNergy), and the Federal Ministry of Education and Research ("Krankheitsbezogenes Kompetenznetz Multiple Sklerose" and "Kompetenznetz Degenerative Demenzen").

We thank M. Sölch and H. Rübsamen for excellent technical assistance and Drs N. Kawakami, A. Peters, and M. C. Tuohy for critical comments on the manuscript.

Author Contributions

F.S.T., R.H., T.K., and E.M. contributed to the conception and design of the study. F.S.T., A.L.T., M.B., E.S., S.W., C.F.M., R.G., S.V., F.S., and M.S. contributed to the acquisition and analysis of data. F.S.T., A.L.T., M.B., E.S., C.F.M., S.V., R.H., T.K., and E.M. contributed to drafting the text and preparing the figures.

Potential Conflicts of Interest

Nothing to report.

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3.2. Aim of the study II

The aim of the study was the analysis of the spectrum of monoclonal patient-derived anti-GAD65-abs in the PB and CSF of GAD-AIND patients. I determined the affinity to GAD using different detection assays and characterized molecular and functional properties of patient-derived mabs.

Reconstructing the GAD65-specific B-cell repertoire reveals a critical contribution of CSF B cells during early stages of GAD65-antibody-associated neurological disorders

Michelle Biljecki, Katharina Eisenhut, Eduardo Beltrán, Stephan Winklmeier, Simone Mader, Anna Thaller, Peter Eichhorn, Philipp Steininger, Andrea Flierl-Hecht, Jan Lewerenz, Tania Kümpfel, Martin Kerschensteiner, Edgar Meinl, Franziska S. Thaler

Manuscript under peer review in BRAIN, 31.03.2022

For this manuscript, I contributed to the conception and design of the study. I performed the majority of experiments and analyzed most of the results. More specifically, the experiments used for Fig. 1, Fig. 2A-2D, Fig. 3, Fig. 4A-4B, Suppl. Fig. 1, Suppl. Fig. 2, Suppl. Fig. 3 and Suppl. Fig. 4 were done by me. I generated and produced the mabs used for the experiments in Fig. 2E, Fig. 4C and Suppl. Tab. 2. Further I designed the primers used for cloning the mabs in the IgG vector shown in Suppl. Tab. 1 and created Tab. 2, including all data besides the cell-based assay experiments. I also contributed to drafting the text and mainly prepared the figures, together with Dr. med. Franziska Thaler. The contribution of other authors is specified in the manuscript.

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Journal:	*Brain*
Manuscript ID	BRAIN-2022-00563
Manuscript Type:	Original Article
Date Submitted by the Author:	18-Mar-2022
Complete List of Authors:	Biljecki, Michelle; Ludwig-Maximilians-University Munich Institute of Clinical Neuroimmunology Eisenhut, Katharina; Ludwig-Maximilians-University Munich Institute of Clinical Neuroimmunology Beltrán, Eduardo; Ludwig-Maximilians-University Munich Institute of Clinical Neuroimmunology Winklmeier, Stephan; Ludwig-Maximilians-University Munich Institute of Clinical Neuroimmunology Mader, Simone; Ludwig-Maximilians-University Munich Institute of Clinical Neuroimmunology Thaller, Anna; Université de Paris; Ludwig-Maximilians-University Munich Institute of Clinical Neuroimmunology Eichhorn, Peter; Ludwig Maximilian University, Institute of Clinical Chemistry Steininger, Philipp; Friedrich-Alexander-Universität Erlangen-Nürnberg Flierl-Hecht, Andrea; Ludwig-Maximilians-University Munich Institute of Clinical Neuroimmunology Lewerenz, Jan; Ulm University, Department of Neurology Kümpfel, Tania; Ludwig-Maximilians-University Munich Institute of Clinical Neuroimmunology Kerschensteiner, Martin; Ludwig Maximilians University Munich, Institute of Clinical Neuroimmunology Meinl, Edgar; Ludwig-Maximilians-University, Institute of Clinical Neuroimmunology Thaler, Franziska; Ludwig-Maximilians-University Munich Institute of Clinical Neuroimmunology
Methodology:	NEUROBIOLOGY OF DISEASE
Subject area:	NEUROINFLAMMATION

Reconstructing the GAD65-specific B-cell repertoire reveals a critical contribution of CSF B cells during early stages of GAD65-antibody-associated neurological disorders

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Running title: GAD65-specific B cells in the CNS

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Character count: Title: 98, Running Title: 28; Word count: Abstract: 400, Introduction: 339, Material and Methods: 1548, Results: 1077, Discussion: 1191, Body of the Manuscript: 4161; Number of References: 44; Number of Figures: 4, Tables: 2; Number of supplementary Figures/Tables: 6.

Keywords: Glutamic acid decarboxylase, monoclonal antibodies, cerebrospinal fluid, somatic hypermutations.

Abbreviation: Abs = antibodies; ANA = anti-nuclear-antibody; CA = cerebellar ataxia; CDR = complementary determining region; CNS = central nervous system; CSF = cerebrospinal fluid; FR = framework region; GABA = γ-aminobutyric acid; GAD = glutamic acid decarboxylase; GAD65 = glutamic acid decarboxylase 65; HC = heavy chain; IIFT = indirect immunofluorescence test; LC = light chain; LE = limbic encephalitis; mAbs = monoclonal abs; MBCs = memory B cells; OCBs = oligoclonal bands; PB = peripheral blood; PDS = patient-derived sequences; SHMs = somatic hypermutations; SPS = stiff person syndrom; TLE = temporal lobe epilepsy; UCAs = unmutated common ancestors.

Abstract

Antibodies (abs) against the cytoplasmic protein glutamic acid decarboxylase 65 (GAD65) are detectable in patients with a range of neurological syndromes together referred to as GAD65-ab-associated neurological disorders. The response of some of these patients to plasmapheresis indicates that GAD65-abs could be important contributors to disease pathogenesis at least at some stages of disease. While we have previously shown that GAD65-reactive B cells are abundant in peripheral blood (PB), it is currently unclear whether GAD65-ab-producing cells are also present in the central nervous system (CNS) and if and how anti-GAD65 immune responses differ between peripheral and central compartments.

To address this question we studied six patients with high levels of GAD65-abs detectable in serum and cerebrospinal fluid (CSF) and generated monoclonal abs (mAbs) derived from single cells in CSF and PB. The sequence characteristics, the reactivity to GAD65 and to a panel of viral antigens, as well as the role of somatic hypermutations (SHMs) of the mAbs were analysed. In total, 30 mAbs could be generated (n=25 derived from CSF and originating from three different patients; n=5 derived from PB and originating from one patient). In 2/6 patients GAD65-ab-producing cells could be identified in the CSF with 75% of the mAbs being GAD-reactive (6/8 mAbs). MAbs derived from CSF-cells could only be generated in patients with a short disease duration at the time of sampling. The intrathecal anti-GAD65 response was polyclonal. No cross-reactivity of GAD65-reactive mAbs to a panel of viral antigens was observed. GAD65-abs were mostly of the IgG1 subtype and had undergone affinity maturation with higher numbers of SHMs of GAD65-reactive mAbs (mean: IGHV: 20.0, IGK/LV: 9.4) compared to non-GAD65-reactive mAbs (mean: IGHV: 12.3, IGK/LV: 5.6).

Reversion of three GAD65-reactive mAbs to their corresponding germline-encoded unmutated common ancestors abolished GAD65-reactivity.

Our data show that GAD65-specific B cells are present in the CNS and represent a sizable fraction of CSF B cells in particular early in the disease course. The anti-GAD65-response in the CSF is polyclonal and shows evidence of antigen-driven affinity maturation that is required for GAD65-recognition. Our data support the hypothesis that the accumulation of GAD65-specific B cells and plasma cells in the CSF is an important feature of early disease stages. These findings are in line with the clinical observation that B-cell depleting therapies are not effective in long-standing GAD65-ab-associated neurological disorders and have obvious implications for the timing of B-cell directed therapies in these patients.

Introduction

The cytoplasmic protein glutamic acid decarboxylase (GAD) is the rate-limiting enzyme in the synthesis of the major inhibitory neurotransmitter γ-aminobutyric acid (GABA) [1]. High serum levels of antibodies (abs) against the isoform GAD65 have been associated with neurological syndromes like stiff person syndrome (SPS), cerebellar ataxia (CA), limbic encephalitis (LE), and temporal lobe epilepsy (TLE) [2-6]. Low levels of GAD65-abs are detectable in around 1-8% of patients with diverse neurological disorders and healthy controls [7-9]. This finding complicates the evaluation of serum GAD65-abs in patients presenting with neurological symptoms and questions the relevance of these peripheral GAD65-abs in disease pathogenesis. Currently, the detection of GAD65-abs in cerebrospinal

fluid (CSF) in SPS and an intrathecal production of GAD65-abs in CA and TLE are therefore proposed as diagnostic criteria for GAD65-ab-associated neurological disorders [10] highlighting the relevance of an intrathecal anti-GAD65 response in these disorders. Still, the intrathecal B-cell repertoire and the abundance of GAD65-specific B cells in the central nervous system (CNS) are largely unknown. A more refined understanding of the immune response in these patients would however be of dire need, as an effective treatment in patients with GAD65-ab-associated neurological disorders remains challenging. We previously found that immune therapy including B-cell depletion with rituximab did not result in an improvement of outcome in patients with long-standing disease [11]. In contrast, prompt initiation of treatment was associated with good outcome in a cohort of patients with GAD65-ab-associated cerebellar ataxia [12]. Therefore, early initiation of immune therapy seems to be critical and understanding the involved immune reactions is crucial to facilitate therapeutic decisions. We now sought to analyse autoimmunity to GAD65 in the CNS by generating a panel of monoclonal antibodies (mAbs) derived from B cells in the CSF of six patients with GAD65-ab-associated neurological disorders. We could detect a sizeable fraction of GAD65-reactive B cells in the CNS at early stages of disease and provide evidence that GAD65-reactive B cells experienced antigen-driven affinity maturation. Overall, these findings support a role of CNS B-cell responses against GAD65 in early disease pathogenesis.

Material and Methods

Study approval

The study was performed according to the declaration of Helsinki after approval by the institutional review board of the Ludwig-Maximilians-Universität, Munich and the University of Ulm. All patients or their legal representatives gave written informed consent prior to study enrolment.

Patients

We included six patients with GAD-ab associated neurological disorders, all of which exhibited high GAD-ab-titers both in serum and CSF and evidence of intrathecal GAD-ab-production. Intrathecal GAD-antibody synthesis was determined by calculating the antibody index (AI) using the following formula: (CSF GAD-specific IgG) x (serum total IgG)/ [(CSF total IgG) x (serum GAD-specific IgG)] with an AI above 1.4 indicating intrathecal synthesis [13]. Clinical information as well as CSF characteristics are provided in Table 1. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient separation. Fresh CSF was centrifuged at 300g for 15 minutes at 4°C. Cell pellets were either immediately used for single cell sorting or directly frozen in 500 µl freezing medium (90% FCS and 10% DMSO), stored at -80°C for 3 days at -196°C afterwards, and used later for single cell sorting.

CSF single cell isolation

For fluorescence-activated cell sorting (FACS) CSF B cells were pre-enriched by negative selection using the following kits: human CD3 positive selection kit II (Stem Cell Technologies, #17851), human CD14 positive selection kit II (Stem Cell Technologies, #17858), human CD56 positive selection kit II (Stem Cell Technologies, #17855). Enriched B cells were stained on ice using the following abs: CD14-FITC (Biolegend, clone: 63D3, 1:40), CD56-FITC (Biolegend, clone: HCD56, 1:40), CD19-APC Fire (Biolegend, clone: HIB19,

1:40), CD27-BV605 (Biolegend, clone: O323, 1:40), CD20-PerCP (Biolegend, clone: 2H7, 1:40), CD24-APC (Biolegend, clone: ML5, 1:50), CD138-PE (Stem Cell Technologies, clone: MI15, 1:40), CD38-ef450 (Invitrogen, clone: HB7, 1:40), CD3-AF700 (Invitrogen, clone: OKT3, 1:40), Fc receptor blocking (Miltenyi Biotec, 1:50). Cells were sorted into 96-well plates using the FACS BD FACSAria Fusion Cell Sorter with wells containing 5 µl of RNA lysis buffer (0.4 Unit RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen), 0.038% Triton-X-100, 1 µM Oligo dT primer, 1 mM dNTP mix (Invitrogen)).

Immunoglobulin sequence analysis

Reverse transcriptase-polymerase chain reaction (RT-PCR) was performed by adding 5 µl RT master mix to the lysed cells containing 50 Units SuperScript™ II Reverse Transcriptase (Invitrogen), 1x superscript II first strand buffer (5x) (Invitrogen), 5 mM DTT (Invitrogen), 0.92 M Betaine, 6 mM $MgCl_2$, 10 Units RNase OUT (Invitrogen), and 1 µM TSO no. 1 (Suppl. Table 1). The following PCR conditions were used: 42°C for 90 minutes/ 10 cycles: 50°C for 2 minutes and 42°C for 2 minutes/ 70°C for 15 minutes. cDNA amplification was performed by adding 15 µl containing 0.2 µM TSO no. 2 (Suppl. Table 1), 1x KAPA Hifi Mix (2x) (Roche Applied Science). The following PCR conditions were used: 98°C for 3 minutes/ 25 cycles: 98°C for 20 seconds, 67°C for 15 seconds, 72°C for 6 minutes/ 72°C for 5 minutes. For the gene amplification of the variable immunoglobulin heavy chain (IGH) and the possible lambda (IGL) and kappa (IGK) light chains a nested PCR-strategy was applied as described previously [14]. In brief, PCRs were performed in 25 µl containing 0.4 µM gene specific primers: TSO no. 3, HG_Out, Kappa_Out, and Lambda_Out with all PCR primers together in one master mix for the outer PCR, and TSO_no. 3, HG_IN, Kappa_IN, and Lambda-IN in separate reactions for heavy chain (HC), kappa chain (KC), and lambda chain (LC) for the inner PCR. 1x Phusion buffer (5x) (Roche Applied Science), 0.2 µM dNTPs (Invitrogen), 1

µl DNA template, 0.5 µl Phusion® HF DNA Polymerase (2000 U/mL) (New England biolabs) were added to the respective primers. The following PCR conditions were used: 98°C for 30 seconds/ 30 cycles: 98°C for 20 seconds, 60°C for 20 seconds, 72°C for 50 seconds/ 72°C for 10 minutes. All products from the second PCRs were applied to agarose gel electrophoresis and bands at the correct height with matching heavy and light chains were isolated by gel purification (Qiagen Minelute kit). Purified PCR-products were sequenced with inner primers for IGH, IGK, and IGL. All primers used in the single-cell analysis are listed in Supplementary Table 1. Sequences were compared with variable (diversity) joining (V(D)J) segments in the IMGT database. The number of somatic hypermutations (SHMs) in the complementary determining regions (CDR) as well as in the framework regions (FR) were analysed and the length of the CDR3 was determined. Furthermore, IgG subclasses were determined as described previously [15].

Generation of mAbs

Immunoglobulin cloning was performed by introducing restriction sites into heavy and light chains (SalI-HF and SacII for IGH, BssHII and KasI for IGK and IGL) using individual immunoglobulin gene-specific primers (Supplementary Table 1). PCR conditions were as described above for the nested PCR approach. Digested and purified PCR-products were cloned into the pTT5 expression vector which adds a C-terminal HIS6-tag to the abs and contains the IgG1 constant domain. Ig vector pairs were transiently transfected into HEKEBNA cells cultured in Freestyle Medium 293 supplemented with 1% Pluronic F68 (Gibco) and 25 µg/ml Geneticin (Gibco) using Polyethylenimine (Polysciences). Supernatants were harvested after 4 days of culture. Antibody purification was performed by immobilized metal affinity chromatography as described previously [16].

Stimulation of memory B cells to antibody-producing cells

PBMCs were stimulated with the toll-like receptor (TLR)-7/8-ligand R848 (Sigma Aldrich, 2.5 µg/ml) and IL-2 (R&D, 1,000 IU/ml) at a concentration of 600,000 cells/ml in 24-well plates for 11 days as described [17].

Screening for GAD65-reactivity

ELISA

GAD65-abs were detected and quantified by human anti-GAD65 ELISA (IgG) (Euroimmun). **Indirect Immunofluorescence test (IIFT)**

In a tissue-based assay primate cerebellar tissue (Euroimmun, FB 1111-1010-17) was used. Patient-derived mAbs (50 µg/ml), sera (1:100) and CSF supernatant (1:1) were analysed for brain reactivity following the manufacturer´s instructions. For detection the goat anti-human IgG Alexa Fluor 488 antibody (Southern Biotech 2049-30; 5 µg/ml) was applied. Additionally, mAbs tested positive in the assays described above were tested by IIFT in a cell-based assay on GAD65-transfected HEK293 cells as well as control transfected cells (Euroimmun, FA 1022-1005-50). MAbs were used at a concentration of 50 µg/ml and in a first step diluted 1:10 followed by dilutions of 1:100/1:400/1:1600/1:6400/1:25600 if positive and applied undiluted if negative.

Generation of mAbs from PBMCs

Patient-derived memory B cells were isolated using the human B cell enrichment kit (Stemcell Technologies) and the IgG^+ memory B cell isolation kit (Miltenyi Biotec). B cells were cultured on feeder cells. For this, $1x10^5$ PBMCs/well previously irradiated with 12 Gy

and B cells in concentrations ranging from 1-500 cells/well were cultured in a 96 well plate in RPMI complete media containing 10% fetal bovine serum, 1% Penicillin/Streptomycin, 1% L-Glutamin, 1% non-essential amino acids and supplemented with 2.5 µg/ml CpG ODN 2006 (Invivogen). Epstein-Barr virus (EBV) producing B95-8 monkey cells were starved for 7 days and the EBV containing supernatant was collected and 100 µl were added to feeder cells and B cells. The EBV immortalized B cells were cultured for 2 to 3 weeks until colonies became visible. Supernatants were heat inactivated and tested for GAD65 reactivity in ELISA (Euroimmun) as described above.

To stain GAD65-ab producing cells a GAD65-tetramer was applied. To this end, GAD65 protein (Diamyd, 45-08029-01) was biotinylated with the EZ-Link NHS-PEQ4-Biotinylation Kit (Thermo Fisher Scientific, #21455). GAD65-tetramer was produced as previously described [18]. B cells were stained with 1µg/ml of GAD65-tetramer. Tetramer-positive cells were single cell sorted using the FACS Aria Fusion Cell Sorter into RNA cell lysis buffer followed by single cell RT-PCR, PCR amplification and cloning as described above.

Generation of germ-line abs

To identify SHMs that arose throughout affinity maturation we aligned the sequences of the HCs and LCs of our GAD65-reactive mAbs against the 2021-10-11 germline reference set with IMGT/HighV-Quest v.3.5.27. We selected three mabs (5D1, 7A2, 4A10) and reverted the sequences in FR1, FR2, FR3, CDR1, CDR2, and flanking regions of CDR3 to the germ-line sequence by nucleotide synthesis (GeneArt, Thermo Fisher Scientific) and performed cloning into the pTT5 expression system as described above.

Viral antigen recognition

Binding to viral antigens was determined using the following assays: Herpes simplex virus (HSV)-IgG (ELISA, Virion\Serion and Immunblot, Mikrogen), Varicella zoster virus (VZV)-IgG (ELISA, Virion\Serion), EBV-viral capsid antigen (VCA)-IgG (chemiluminescent microparticle immunoassay (CMIA), Abbott), anti-EBV-Nuclear-Antigen (EBNA) (CMIA, Abbott), cytomegalovirus (CMV)-IgG (CMIA, Abbott), human herpes virus (HHV)-6-IgG (IIFT, Scimedx Corporation), HHV-7-IgG (IIFT, BIOCELL Diagnostics), HHV-8-IgG (IIFT, in house assay, Institute of Clinical and Molecular Virology, Erlangen), hepatitis A virus (HAV)-abs (chemiluminescence immunoassay (CLIA, DiaSorin), anti-Hepatitis B surface antigen (HBs) (CMIA, Abbott), anti-Hepatitis B core antigen (HBc) (CMIA, Abbott), anti-Hepatitis B e antigen (HBe) (CLIA, DiaSorin), Hepatitis C Virus (HCV)-abs (CMIA, Abbott), Hepatitis E Virus (HEV)-IgG (Immunblot, Mikrogen), human immunodeficiency virus (HIV)-1/2-abs (CMIA, Abbott), human T-lymphotropic virus (HTLV)-1/2-abs (CMIA, Abbott), measles-IgG (ELISA, Virion\Serion), mumps-IgG (ELISA, Virion\Serion), rubella-IgG (CMIA, Abbott), parvovirus B19-IgG (Immunblot, Mikrogen), adenovirus-IgG (ELISA, Virion\Serion), enterovirus-IgG (ELISA, Virion\Serion), hantavirus-IgG (Immunblot, Mikrogen), Tick-borne encephalitis (TBE)-Virus-IgG (ELISA, Virion\Serion), west nile virus (WNV)-IgG (ELISA, Virion\Serion), severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2)-S1/S2-IgG (CLIA, DiaSorin; neutralizing antibody assay (NAB), Shenzhen YHLO Biotech).

Statistics

Statistical significance was assessed with Prism Software (GraphPad) by unpaired or paired, non-parametric or parametric t-test analysis, as appropriate. P-values of *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 were considered significant and designated accordingly.

Data availability

The data that support the findings of this study are available from the corresponding author, upon reasonable request. No de-identified patient data will be shared.

Results

Patient cohort characteristics and GAD65-reactivity

We aimed to generate mAbs derived from CSF and PB from patients with GAD65-ab-associated neurological disorders. We included six patients with GAD65-abs and an associated neurological disorder, four with LE, one patient with CA, and one patient with recurrent neuritis nervi optici (NNO). One patient received azathioprine at the time of sampling, the other patients were untreated. As described [19] autoimmune comorbidities were common. CSF-specific OCBs were present in 3/6 patients (Table 1). As a first step, we set out to characterize the anti-GAD65 response in our patient cohort in detail using different detection assays and different biomaterials. All patients exhibited GAD65-abs in ELISA with higher absolute values in serum than in CSF (Fig. 1A) and an intrathecal GAD-ab production (Table 1). Additionally, all serum and CSF samples were tested by tissue-based assay using primate cerebellar slices and four patients showed a GAD65-specific staining pattern which is characterized by a leopard-like granular layer staining (Fig. 1B). In

two patients (patients #1 and #4) we observed an anti-nuclear-antibody (ANA) staining interfering with the clear evaluation of a GAD65-specific staining pattern (Fig. 1B, Suppl. Fig. 1). Next, we determined the presence of GAD65-ab-producing B cells in PB and therefore differentiated patient-derived PBMCs *in vitro* into Ig-producing plasma blasts as previously described [17 20]. To this aim, for each patient PBMCs were divided into 11 wells, PBMCs were stimulated, and GAD65-ab production was measured. Positive wells as surrogates for GAD65-reactive B cells were identified in all patients. Patients #3 and #5 showed the highest number of circulating GAD65-reactive B cells (Fig. 1C).

GAD65-ab producing B cells are present in the CSF at early stages of the disease

Next, we aimed to analyse GAD65-reactivity on a single-cell level. We performed single-cell sorting of CSF of all six patients and produced patient-derived mAbs (Fig. 2A). We were able to generate 25 mAbs derived from CSF B cells from three different patients (17 mAbs from patient #1, 3 mAbs from patient #3, and 5 mAbs from patient #5) (Table 2). In order to compare CSF-derived mAbs to mAbs derived from PB, we applied EBV-immortalization of memory B cells (MBCs) from PB and staining of MBCs with a GAD65-specific tetramer followed by single cell sorting of tetramer-positive cells. Hereby, we generated five mAbs derived from B cells circulating in PB from one patient (patient #3) (Table 2). All generated mAbs were screened for their binding capacity to GAD65 using ELISA. Hereby, we identified five mAbs with low affinity to GAD65 in ELISA (one derived from blood and four derived from CSF) and five mAbs with high affinity to GAD65 in ELISA (three derived from blood and two derived from CSF) (Fig. 2B) (Table 2). We tested our mAbs in dilution series in order to determine their affinity and detected Kd-values ranging from 152pM to 20.4µM (Fig. 2C, D). A GAD65-specific staining pattern in tissue-based assay using primate cerebellar slices was observed for six mAbs (two derived

from blood and four derived from CSF) (Suppl. Fig 2A, Table 2). These six mAbs also tested positive in a cell-based assay using GAD65-transfected HEK293 cells (Fig. 2E, Table 2). Four mAbs revealed an ANA-staining (Suppl. Fig. 2B). Together, about 24% (6/25) of all mAbs derived from B cells in the CSF early in the disease were specific for GAD65 and in the two patients with detection of GAD65-reactive B cells in the CSF they even represented 75% (6/8) of all mAbs. In contrast, at later stages GAD65-specific B cells could no longer be found in the CSF as in patients #2, #4, and #6 with disease durations of 81, 122, and 172 months we could not generate mAbs derived from CSF B cells.

Detailed characterization of mAbs reveals an increased rate of SHMs in GAD65-reactive mAbs

We analysed sequence characteristics of all generated mAbs. Hereby, we found a higher proportion of lambda light chains (LCs) in GAD65-reactive mAbs (64%) compared to non-GAD65-reactive mAbs (41%), however the difference was not statistically significant. IgG subclass analysis revealed that all mAbs, except 7E8, belonged to the IgG1 subclass (Table 2). VH-family 3 was predominant in our mAbs (17/30) (Table 2). No clonal relationships were identified among the mAb sequences, indicating a polyclonal anti-GAD65 response (Table 2). The number of SHMs was determined in the V region of all ab-chains and was significantly higher in both the heavy chains and LCs of GAD65-reactive mAbs compared to non-GAD65-reactive mAbs (Fig 3A, left panel). When only CSF-derived abs were analysed, GAD65-reactive abs still revealed higher numbers of SHMs, however the difference was not statistically significant, presumably due to the lower number of abs (Fig. 3A, right panel). No difference between numbers of SHMs of GAD65-reactive mAbs derived from CSF and PB was detected (Suppl. Fig. 3). Within GAD65-specific mAbs, ratios of replacing to silent mutations in CDRs were higher than in FRs, which is characteristic of antigen-driven maturation (Fig. 3B).

Monoclonal GAD65-reactive abs show no cross-reactivity to viral antigens

As viral infections are discussed as potential triggers for autoimmune reactions, we analysed cross-reactivity of a fraction of our mAbs (HK3 as a control, 1C10, 4C5, 4A10, 4D1, 5D3, 5D1, 7H1, 7A2) to a panel of viral antigens. We detected no reactivity of our abs to HSV, VZV, EBV, CMV, HHV-6, HHV-7, HHV-8, HAV, HBV, HCV, HEV, HIV-1/2, HTLV-1/2, measles virus, mumps virus, rubella virus, parvovirus B19, adenovirus, enterovirus, hantavirus, TBE-Virus, and west nile virus. Mab 4C5 showed borderline reactivity to SARS-CoV-2-S1/S2 in CLIA, however this could not be confirmed by neutralizing antibody assay (Suppl. Table 2).

Affinity maturation is required for GAD65-recognition

In order to determine the role of affinity maturation we identified SHMs and reverted three monoclonal GAD65-reactive Abs (5D1, 7A2: derived from CSF-cells; 4A10: derived from PB-cells) to their corresponding unmutated common ancestors (UCAs) (Fig. 4A). Next, affinities to GAD65 of the patient-derived sequences (PDS) and their UCA counterparts were compared by dilution series in GAD65-ELISA. Here, we found that reactivity to GAD65 was completely abolished in the UCAs (Fig. 4B). In cell-based assay using GAD65-transfected HEK293 cells, GAD65-reactivity was also abolished in the UCAs (Fig. 4C). Moreover, binding to primate brain tissue as well as pancreas islet cells was abolished in the UCAs (Suppl. Fig. 4A, B). As observed for the PDS, no clear reactivity of the UCAs to viral antigens was observed; only the UCA of mAb 4A10 showed weak positivity in the WNV-IgG ELISA which we interpreted as unspecific binding (Suppl. Table 2).

Discussion

Our study provides insights into the CSF GAD65-specific B-cell receptor repertoire in patients with GAD65-ab-associated neurological disorders. An intrathecal production of GAD65-antibodies is currently considered an essential diagnostic criterion in GAD65-ab neurological disorders at least in LE/TLE and CA [10]. However, direct proof of an intrathecal production of GAD65-specific abs by CSF B cells was lacking so far. We were able to demonstrate for the first time that GAD65-ab producing cells are present in the CSF of patients with GAD65-ab-associated disorders. We hereby provide direct evidence that GAD65-abs in the CSF are not merely a result of transfer of serum abs into the CSF compartment but of a polyclonal anti-GAD65 response by B cells in the CNS.

The facts that the amount of SHMs did not differ between GAD65-specific mAbs derived from CSF and from PB and that the anti-GAD65 response in the CNS is polyclonal indicate that GAD65-reactive B cells probably originate from the periphery before migrating to the CNS. In line with this hypothesis, we previously found a strong dysregulation of the peripheral anti-GAD65-response with both memory B cells as well as plasma cells in the bone marrow as sources of GAD65-ab production [20]. The present study suggests that autoreactive GAD65-ab producing B cells can cross the blood-brain barrier. Our CSF-derived GAD65-specific mAbs showed comparable features to mAbs derived from patients with LGI1-encephalitis [21] and $GABA_A$-receptor encephalitis [22]. Here, high proportions of antibody-secreting cells expressing LGI1-abs or $GABA_AR$-abs and a high numbers of SHMs were observed. In contrast, in patients with NMDAR-encephalitis, CSF-derived NMDAR-reactive mAbs often lacked any mutation as compared to the germ-line

sequence and only a small fraction of antibody-secreting cells exhibited abs against the NMDAR [23,24]. Intriguingly, in patients with NMDAR-encephalitis teratoma-based germinal center-like structures with ongoing germinal center reactions were observed [25].

During B cell development two tolerance checkpoints with elimination of autoreactive B cells from the maturing B cell repertoire exist. The central tolerance checkpoint affects immature B cells in the bone marrow, whereas the peripheral tolerance checkpoint relates to B cells at the transition from new immigrant to mature naïve cells in the periphery [26]. Defects in B cell tolerance checkpoints have been identified as essential characteristics in the development of autoimmunity [27-32]. We sought to determine the relevance of early defects in B cell tolerance in the development of GAD65-antibodies. To this aim, we reverted the sequence of three of our GAD65-reactive mAbs (two derived from CSF and one derived from PB) to their UCAs thereby generating mAbs corresponding to the B-cell receptors expressed by naïve B cell precursors of these GAD65-ab-producing cells. Hereby, GAD65-reactivity was abolished. SHMs therefore play an important role in the generation of GAD65-reactivity. Similary, an essential role of SHMs in introducing autoantigen-specific reactivity has been described in other autoimmune disorders like for antibodies against aquaporin-4 in patients with neuromyelitis optica spectrum disorders [33], for antibodies against desmoglein-3 in pemphigus vulgaris [34], and for antibodies against double-stranded DNA or extractable nuclear antigen in systemic lupus erythematosus [35,36]. The importance of affinity maturation in the development of GAD65-reactivity is further illustrated by the fact that in our study mAbs reactive to GAD65 showed higher numbers of SHMs compared to non-GAD65-reactive mAbs.

Regarding the initiation of autoimmune processes, viral infections are discussed as probable triggers. As examples for this concept EBV infection has been linked to multiple sclerosis epidemiologically [37] and molecular mimicry between the EBV transcription factor EBNA1

and the CNS protein GlialCAM was observed [38]. In autoimmune encephalitis, it was shown that HSV-1 infection of the CNS can trigger anti-NMDAR-encephalitis [39 40,41]. Recently, brain-reactive abs were identified in patients with severe COVID-19 [42] and it was shown that SARS-CoV-2 reactive mabs react with brain tissue [43]. Therefore, molecular mimicry between viral and brain antigens could be a relevant feature in the pathogenesis of autoimmune encephalitis. To test this hypothesis, we analysed reactivity of our mAbs to a panel of viral antigens. Hereby, no cross-reactivity to viral antigens was detected. Of note, these results cannot exclude a role of molecular mimicry in the pathogenesis of GAD65-ab-associated disorders as we could only test a limited panel of putative viral candidates. Testing with an unbiased approach also including bacterial antigens could be of future interest.

In our patient cohort, we were only able to generate mAbs from patients with a short disease duration (7, 2, and 3 months versus 81, 122, and 172 months). Most mAbs were isolated from plasmablasts and plasma cells. Although our patient cohort is small, this might indicate that plasmablasts and plasma cells have escaped into survival niches in the CNS at later stages of the disease and, once there, are not accessible to treatment any longer. Alternatively, plasma blasts and plasma cells might get degraded or leave the CNS compartment as the disease progresses. Fitting to these observations, it was shown that an early initiation of immune therapy and a subacute onset in GAD65-ab-associated CA was associated with therapy response [12]. At later stages of disease, activated CD8+ T-cells in CSF and PB have been found to be elevated in patients with GAD65-ab-associated LE and numbers of activated CD8+ T-cells negatively correlate with hippocampal volume and memory function. Therefore, a role of CD8+ T-cells in mediating neurodegenerative effects at later disease stages was postulated [44]. On the contrary, numbers of B-cells and plasma cells were not altered in CSF and PB of patients with long-standing GAD65-ab-associated LE [44]. In line with these findings, we previously found no clinical response to

rituximab-treatment in long-lasting GAD65-associated neurological disorders when the modified Rankin Scale is used as outcome measure [11].

Limitations of our study are the small patient number which is attributed to the rareness of GAD65-associated neurological disorder, the requirement of direct processing of the CSF, and the elaborate procedure to generate the mAbs. Furthermore, no patients with SPS were included in our study. Therefore, our results cannot readily be translated to this patient-subgroup. In patient #1, although a high number of CSF-derived mAbs was generated, no GAD65-ab-producing cells could be detected in the CSF. Patient #1 showed a quite severe clinical phenotype with requirement of intensive care treatment which is not commonly observed in patients with GAD65-ab-associated neurological disorders. Therefore, in this patient a co-existing autoimmune process must be postulated. Our data underline the inhomogeneity of patients with GAD65-ab-associated neurological disorders and emphasize the necessity to always take the clinical picture, the results from CSF analysis, and, whenever possible, the results from two different GAD65-ab-detection assays into account when diagnosing patients with GAD65-ab-associated neurological disorders.

Together, our study indicates that GAD65-specific B cells are generated during a normal immune reaction from B cells that are not specific for GAD65. Our data underline the importance of SHMs in introducing autoantigen-specific reactivity during an ongoing immune response. Early, but not late in the disease course, GAD65-specific B cells are abundant in the CSF. The time point of treatment initiation seems to be decisive in GAD65-ab-associated neurological disorders and B-cell depleting therapy should - if used - be applied early in the disease course. Furthermore, B-cell depletion within the CNS compartment might be a therapeutic option.

Acknowledgements

We thank Heike Rübsamen for excellent technical assistance and Naoto Kawakami and Anneli Peters for critical comments on the manuscript. Part of the flow cytometry analysis was supported by Dr. L. Richter, Core Facility Flow Cytometry, Biomedical Center, Ludwig Maximilians University Munich. Confocal microscopy was conducted at the Core Facility Bioimaging, Biomedical Center, Ludwig Maximilians University Munich. We thank Prof. Dr. Wolfgang Hammerschmidt (Research Unit Gene Vectors, Helmholtz Zentrum München, German Research Center for Environmental Health and German Centre for Infection Research (DZIF)) for providing the cell line B95-8.

Funding

This work was funded by the Deutsche Forschungsgemeinschaft (DFG, German Research Foundation) under Germany's Excellence Strategy within the framework of the Munich Cluster for Systems Neurology (EXC 2145 SyNergy – ID 390857198), the Else-Kröner Fresenius Foundation, and the Gemeinnützige Hertie Stiftung.

Competing interests

The authors report no competing interests.

Supplementary material

Supplementary material is available at *Brain* online

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Figure legends:

Figure 1: **Characterization of GAD65-ab profile of the patient cohort.** (**A**) GAD65-abs in serum and cerebrospinal fluid (CSF) of patients #1-6 were determined by ELISA. (**B**) Tissue-based assay with indirect immunofluorescence on primate cerebellar slices was performed to detect anti-GAD65 specific staining patterns in serum (1:100) and CSF (1:1) of patients #1-6 as well as a healthy control (ctrl). A condition with only secondary antibody was used as an additional negative ctrl. * indicates patients with overlaying anti-nuclear antibody-staining (see also Suppl. Fig. 1). (**C**) Peripheral blood mononuclear cells (PBMCs) from patients #1-3, and patients #5, 6 were divided into 11 wells for each patient and each well was cultured with resiquimod (R848) and Interleukin-2 for 11 days and GAD65-ab levels were determined by ELISA in cell culture supernatant (ccSN); each dot represents one stimulated well; data are presented with a median violin plot; the horizontal dotted line indicates 4x OD of the negative control. For patient #4 no PBMCs were available.

Figure 2: **Evaluation of GAD65-reactivity of generated mAbs**. (**A**) Overview of the technical workflow to generate CSF-derived mAbs (created with BioRender) (**B, C, D**) Patient-derived mAbs were tested for GAD65-reactivity in ELISA. Horizontal dotted line indicates 4x OD of the negative control; the horizontal dashed line divides abs with high affinity and low affinity to GAD65. Data is shown as the mean of at least three independent experiments; error bars indicate the SEM. Kd-values were calculated using nonlinear regression analysis. (**B**) MAbs were tested at a concentration of 40µg/ml and undiluted (concentrations ranging from 0.03-3.08mg/ml). * MAbs 1A8 and 2E6 were tested in concentrations of 20µg/ml. (**C, D**) MAbs positive in the screening-ELISA were tested in different dilutions with high affinity GAD65-reactive abs presented in (**C**) and low affinity GAD65-reactive abs in (**D**). (**E**) Cell-based assay using HEK293 cells transfected with GAD65 was performed. MAbs were used at a concentration of 50 µg/ml and in a first step diluted 1:10 followed by dilutions of 1:100/1:400/1:1600/1:6400/1:25600 if positive and applied undiluted if negative. The highest dilutions that revealed a positive staining are depicted. Abbreviations: Kd, dissociation constant; na, non applicable; OD, optical density.

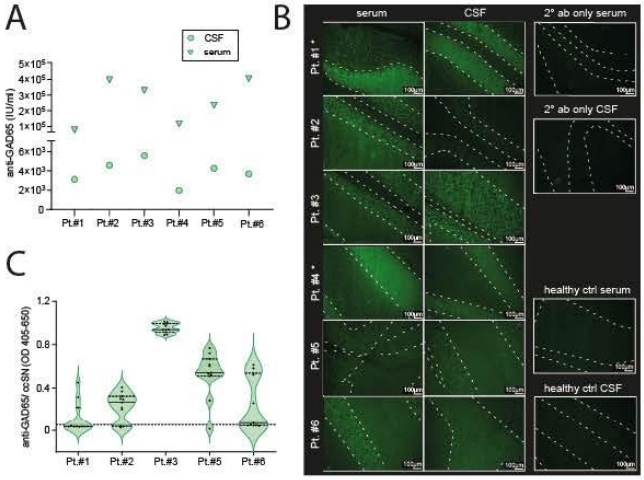
Figure 3. Comparison of GAD65-reactive and non-GAD65-reactive mAbs. (**A**) The number of somatic hypermutations (SHMs) of the heavy chain (HC) V segment and the light chain (LC) V segment was compared for GAD65+ and GAD-mAbs and is depicted for all mAbs (left panel) and all cerebrospinal fluid (CSF) mAbs (right panel). GAD65-reactivity was defined by GAD65-reactivity in ELISA (unpaired T-test, * = $p<0.05$). (**B**) The mean ratios of replacing to silent mutations (R/S ratio) was calculated for framework region (FR)1, complementarity determining region (CDR)1, FR2, CDR2, and FR3 and is depicted for all mAbs (left panel) and all CSF

mAbs (right panel). Abbreviations: IGHV, immunoglobulin heavy chain V gene segment; IGK/LV, immunoglobulin kappa or lambda light chain V gene segment.

Figure 4. Unmutated common ancestors (UCAs) of three GAD65-reactive mAbs. (**A**) The differences of the amino acid (AA) sequences of heavy and light chains between the patient derived sequences (PDS) and their corresponding UCAs are illustrated. The differing AA are depicted in white letters and the AA positions in black numbers. (**B**) GAD65-reactivity of PDS and their corresponding UCAs was compared by GAD65-ELISA. MAb 5D1 is not included due to weak reactivity in ELISA with positivity only at high concentrations (>350µg/ml); however, the corresponding UCA was not producible at such high concentration. All data points of mAb 4A10 UCA were negative and are hidden behind the data points of mAb 7A2. Horizontal dotted line indicates 4x OD of the negative control; data is shown as the mean of at least 3 independent experiments; error bars indicate the SEM. (**C**) Cell-based assay using HEK293 cells transfected with GAD65 was performed. MAbs representing PDS or UCAs were used at a concentration of 50 µg/ml and in a first step diluted 1:10 followed by dilutions of 1:100/1:400/1:1600/1:6400/1:25600 if positive and applied undiluted if negative. The highest dilutions that revealed a positive staining are depicted. Abbreviations: CDR, complementarity determining region; FR, framework region; HC, heavy chain; κC, kappa light chain; λC, lambda light chain.

Thumbnail. Study overview. After encountering their antigen, naïve B cells undergo affinity maturation in germinal centers (GC) giving rise to polyclonal GAD65-specific B cells. Circulating GAD65-specific memory B cells (MBCs) differentiate into Ig-secreting

plasma cells (PCs) and plasmablasts (PBs). GAD65-specific PBs and PCs migrate to the central nervous system (CNS) in the early phase of disease course and produce brain-reactive mAbs. GAD65-specific long-lived PCs escape into survival niches in the bone marrow contributing to the serum GAD65-ab pool [20] (created with BioRender).



Biljecki et al. Fig. 1

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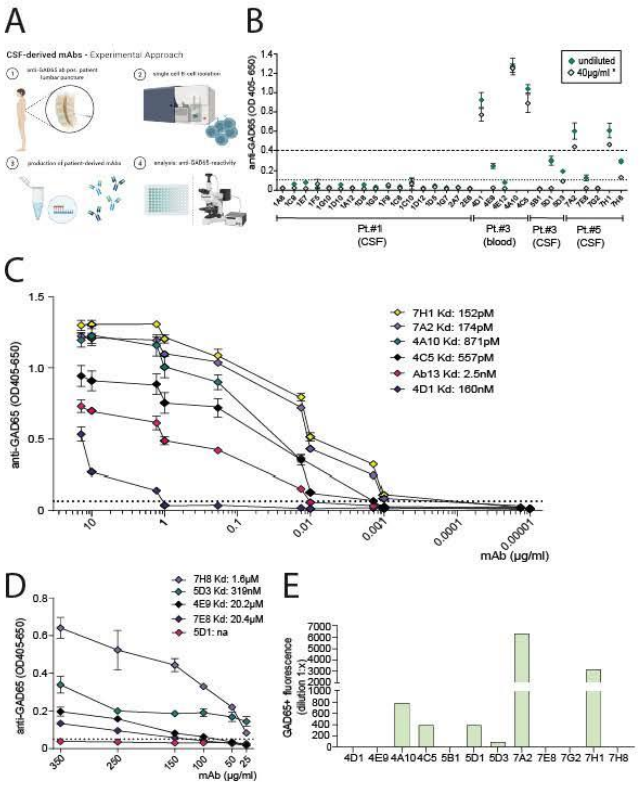

Biljecki et al. Fig. 2

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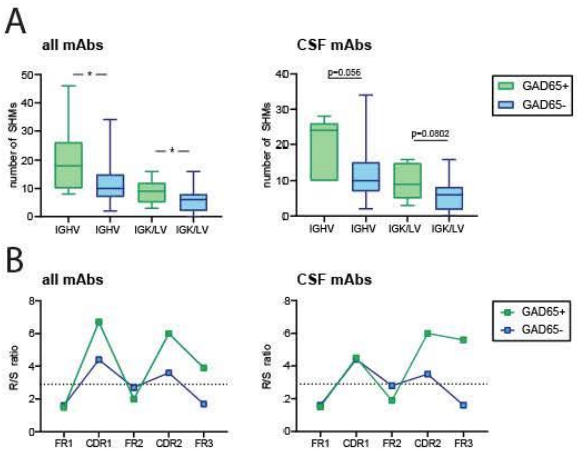

Biljecki et al. Fig. 3

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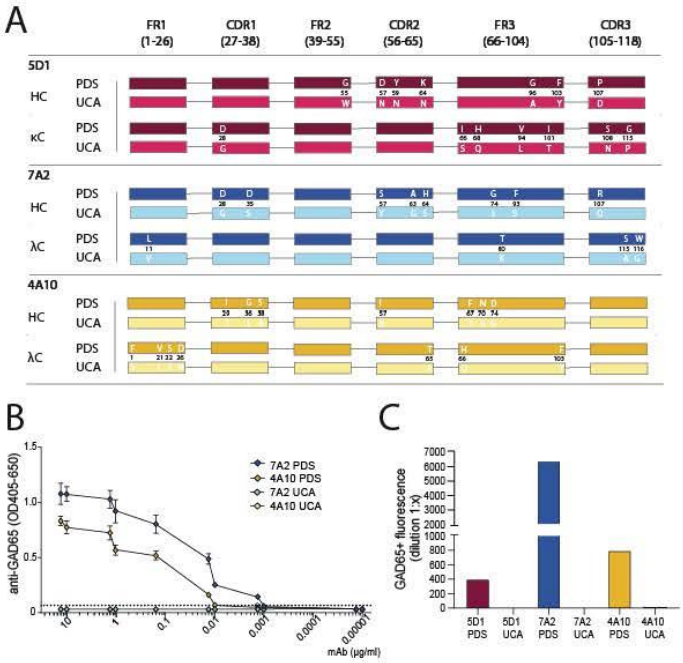

Biljecki et al. Fig. 4

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Table 1: Clinical characterization of the patient cohort

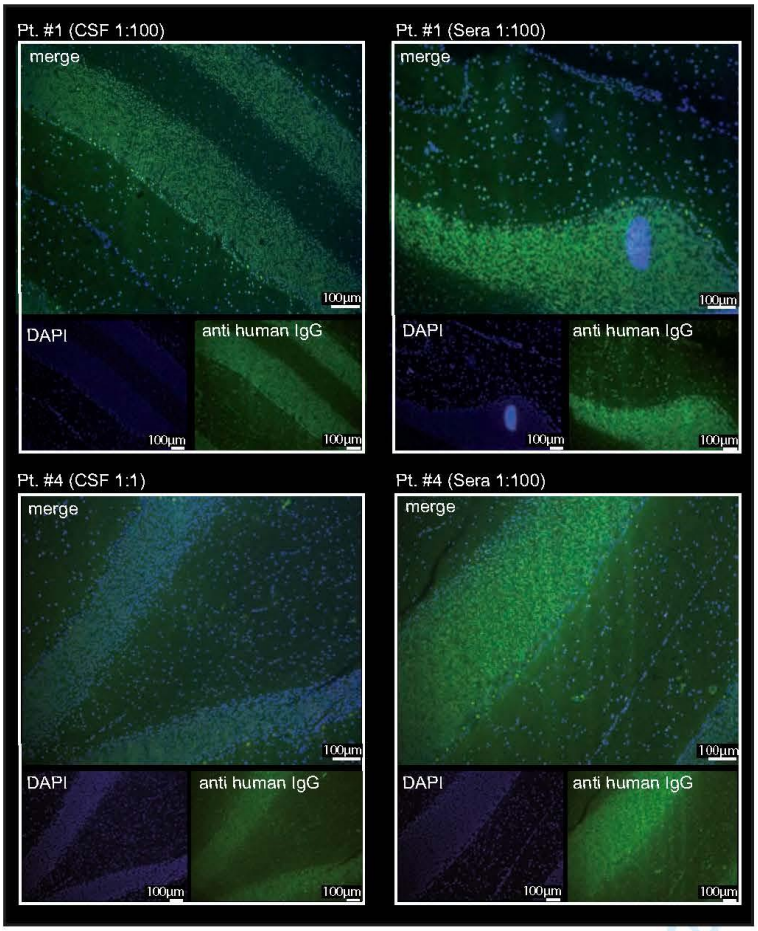
Pt. No.	Clinical presentation	Age at disease onset (y)	Disease duration (mo)	Sex	Symptom constellation	Relevant comorbidities	Treatment at sampling	Previous treatments	CSF	GAD65 -AI	mAbs *	GAD65 mAbs **
#1	LE	58	7	f	Cognitive impairment, generalized seizures, agitation, sensory symptoms, depression, tetraparesis, decreased consciousness, respiratory impairment; ICU requirement	Struma multinodosa, ANAs: 1:12800	-	IVGC, IVIG	10 cells/µl, OCBs	7.1	+	-
#2	LE	57	81	f	Focal seizures	autoimmune thyreoditis, LADA, vitiligo, type-A-gastritis	-	IVGC with oral tappering	<1 cell/µl, no OCBs	3.8	-	-
#3	LE	28	2	f	Generalized and focal seizures, cognitive impairment	-	-	-	3 cells/µl, OCBs	4.1	+	+
#4	LE	56	122	m	Recurrent status epilepticus, cognitive impairment, organic affective disorder	Type 1 diabetes, hashimoto thyroiditis, type-A-gastritis	-	IVIG	1 cell/µl, no OCBs	3.1	-	-
#5	CA	59	3	f	Limb ataxia, gait ataxia, dysarthria	CLL, hashimoto thyreoiditis	-	IVGC, IVIG	<1 cell/µl, no OCBs	5.6	+	+
#6	NNO	43	172	f	Recurrent NNO	-	AZA 150mg/die	AZA	<1 cell/µl, OCBs	3.4	-	-

* Patients in whom mAbs could be generated from CSF-cells; ** Patients in whom GAD65-reactive mAbs could be generated from CSF cells. Abbreviations: AI, antibody index; AZA, azathioprine; CA, cerebellar ataxia; CLL, chronic lymphocytic leukemia; CSF, cerebrospinal fluid; f, female; ICU, intensive care unit; IVGC, intravenous glucocorticosteroids; IVIG, intravenous immunoglobulins; LADA, latent autoimmune diabetes in adults; LE, limbic encephalitis; NNO, neuritis nervi optici; OCBs, specific oligoclonal bands in CSF; PLEX, plasma exchange; Pt, patient; SPS, stiff person syndrome.

Table 2: Characterization of monoclonal antibodies

mAb	Patient No	Biomaterial	Cell type	HC AA junction	LC AA junction	LC type	CDR3 length HC	CDR3 length LC	VH family	IgG subclass	GAD65 ELISA	TBA/ IIFT	CBA/ IIFT
1A8	1	CSF	PC	CARGGTIFVRGAVPRDYFYYYGLDVW	CMQTLQTSEYTF	Kappa	24	10	3	IgG1	neg	neg	-
1C9	1	CSF	PC	CARVRLGLDVW	CQQYYSPPLTF	Kappa	9	9	3	IgG1	neg	neg	-
1E7	1	CSF	PC	CAIDEYGDYLW	CQSADSSGTYAVF	Lambda	9	11	3	IgG1	neg	neg	-
1F5	1	CSF	PB	CAKDLRMGGQFVPGAFDYW	CQQYGTSPITF	Kappa	17	11	3	IgG1	neg	neg	-
1G10	1	CSF	PC	CARERQSDNGGYYPYWYFDLW	CHQSSGLPRTF	Kappa	19	9	4	IgG1	neg	neg	-
1D10	1	CSF	PC	CARNSFYYDSSAYHGLYYYYGMDVW	CMQALQTPTF	Kappa	23	8	1	IgG1	neg	neg	-
1A12	1	CSF	PB	CAKDWSPFVVVIAATFDYW	CSSYTSSNTLGVF	Lambda	17	11	3	IgG1	neg	neg	-
1D8	1	CSF	PC	CVKSFCGYDCYNDYW	CQNYKRAPLTF	Kappa	13	9	3	IgG1	neg	neg	-
1G5	1	CSF	PC	CARSGKDIVVVIDGMDVW	CQQANTFPWTF	Kappa	16	9	1	IgG1	neg	neg	-
1F9	1	CSF	PC	CARASGDTGWYLNHYFDSW	CHQXSSLPXXF	Kappa	17	9	4	IgG1	neg	neg	-
1C8	1	CSF	PC	CARSDIAVVVAARGFDPW	CQSYDSSLSGFWVF	Lambda	16	12	1	IgG1	neg	neg	-
1C10	1	CSF	PC	CARDPDAYYVDFRGLGRYAMDVW	CQQYNSYSRTF	Kappa	21	9	3	IgG1	neg	neg	-
1D12	1	CSF	PB	CARDFSRW	CMQGTHWPPWTF	Kappa	6	10	3	IgG1	neg	neg	-
1D5	1	CSF	PC	CGNSRSYSTYDNAWXVGIEDW	CLQHNDYPLTF	Kappa	19	9	4	IgG1	neg	neg	-
1G7	1	CSF	PC	CAKDRLTMIVVVISSFDYW	CSSYTNSNTLGVF	Lambda	17	11	3	IgG1	neg	neg	-
2A7	1	CSF	PC	CARGRYGSGFFDYW	CQSADSLAVVF	Lambda	12	9	1	IgG1	neg	neg	-
2E6	1	CSF	PC	CARALYCTGGSCYSLHYW	CMQALQTPPYTF	Kappa	16	10	3	IgG1	neg	neg	-
4D1	3	PBMCs	EBV MBC	CAKSPGADPRIRFYYGMDVW	CQVWDSSSDHWVF	Lambda	18	11	3	IgG1	+++	neg	neg
4E9	3	PBMCs	EBV MBC	CARGHYSDRSGHFWIDSW	CQSYDNSSQVF	Lambda	15	9	3	IgG1	+	neg	neg
4E12	3	PBMCs	EBV MBC	CAREIYNILTGYMGFFDYW	CQVWDSSTGVF	Lambda	17	9	3	IgG1	neg	neg	-
4A10	3	PBMCs	EBV MBC	CARAPYSNRYYYGMDVW	CQVWDSSSDHWVF	Lambda	15	11	1	IgG1	+++	pos	pos
4C5	3	PBMCs	EBV MBC	CVREGCGSNCRNRHWYFDLW	CSSYTSSSTLIF	Lambda	18	10	3	IgG1	+++	pos	pos
5B1	3	CSF	PB	CARRDDFSRSFKYW	CMQALQTPXTF	Kappa	12	9	4	IgG1	neg	neg	-
5D1	3	CSF	PB	CARPRSDLWSGYNQNWFAPW	CQQASSFPGLTF	Kappa	18	10	7	-	+	pos	pos
5D3	3	CSF	ambiguous	CASCSSTTCLTWFDPW	CQVWDSTSDHQVF	Lambda	14	11	1	IgG1	+	pos	pos
7A2	5	CSF	MBC	CARRRGYSGYDPYFDYW	CGTWDSSLSSWVF	Lambda	15	11	4	IgG1	+++	pos	pos
7E8	5	CSF	PB	CARDNSKWSWDSW	CQHLHGLPPAF	Kappa	11	9	3	IgG2	+	neg	neg
7G2	5	CSF	PC	CTRGRVPPLGGFYNQFDYW	CLLSYGGARVAF	Lambda	17	10	3	IgG1	neg	neg	neg
7H1	5	CSF	MBC	CARHQNTIYSYYGMDVW	CMQALETSITF	Kappa	15	9	5	IgG1	+++	pos	pos
7H8	5	CSF	MBC	CARASWFGDLTVDNW	CLQHSNWGPTF	Kappa	13	9	3	IgG1	++	neg	neg

Abbreviations: CBA, cell based assay; CDR3, complementarity determining region 3; CSF, cerebrospinal fluid; EBV, Epstein-Barr Virus; HC, heavy chain; IIFT, indirect immunofluorescence test; LC, light chain; mAb, monoclonal antibody; MBC, memory B cell; No, Number; PBMCs, peripheral blood mononuclear cells; PB, plasma blast; PC, plasma cell; TBA, tissue based assay. The IgG subclass of mab 5D1 could not be determined.



Biljecki et al. Suppl. Fig. 1

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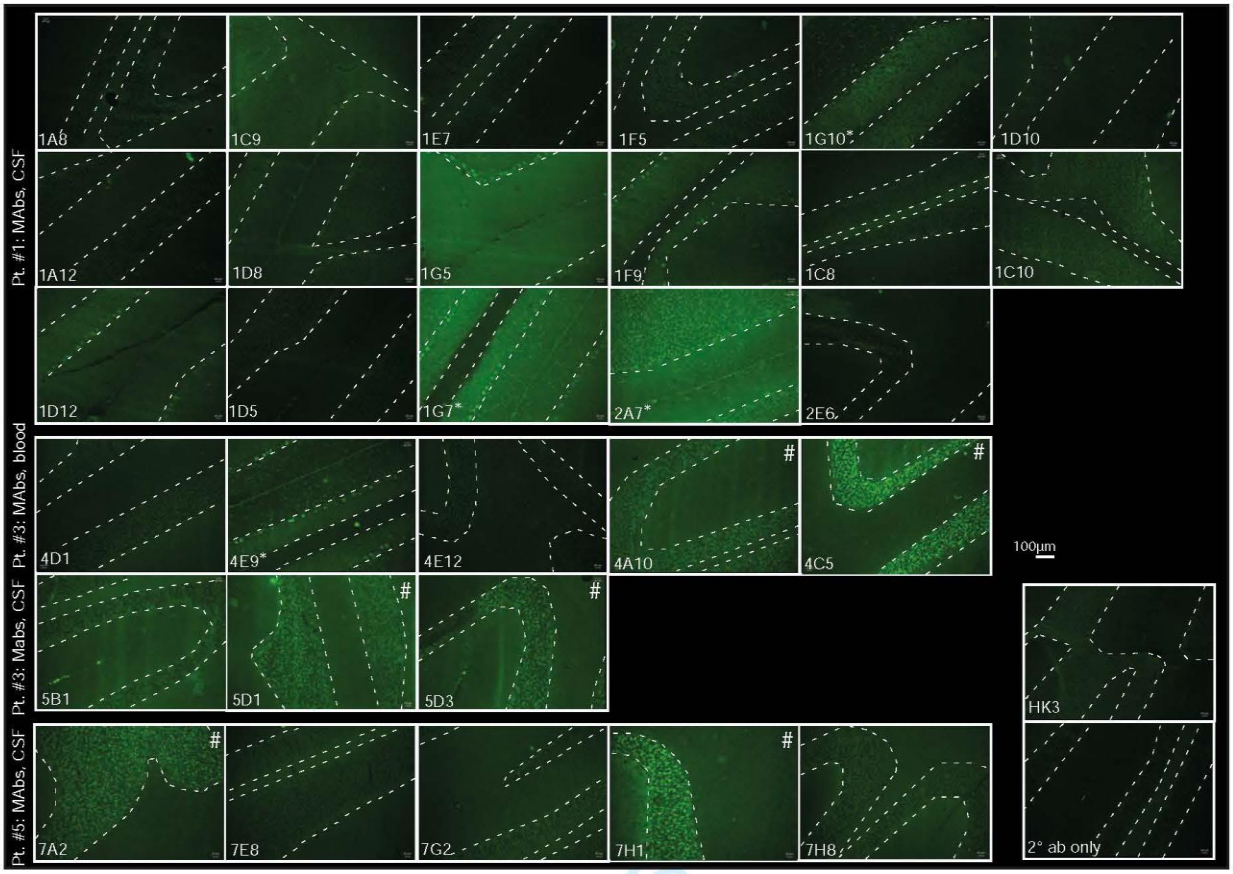

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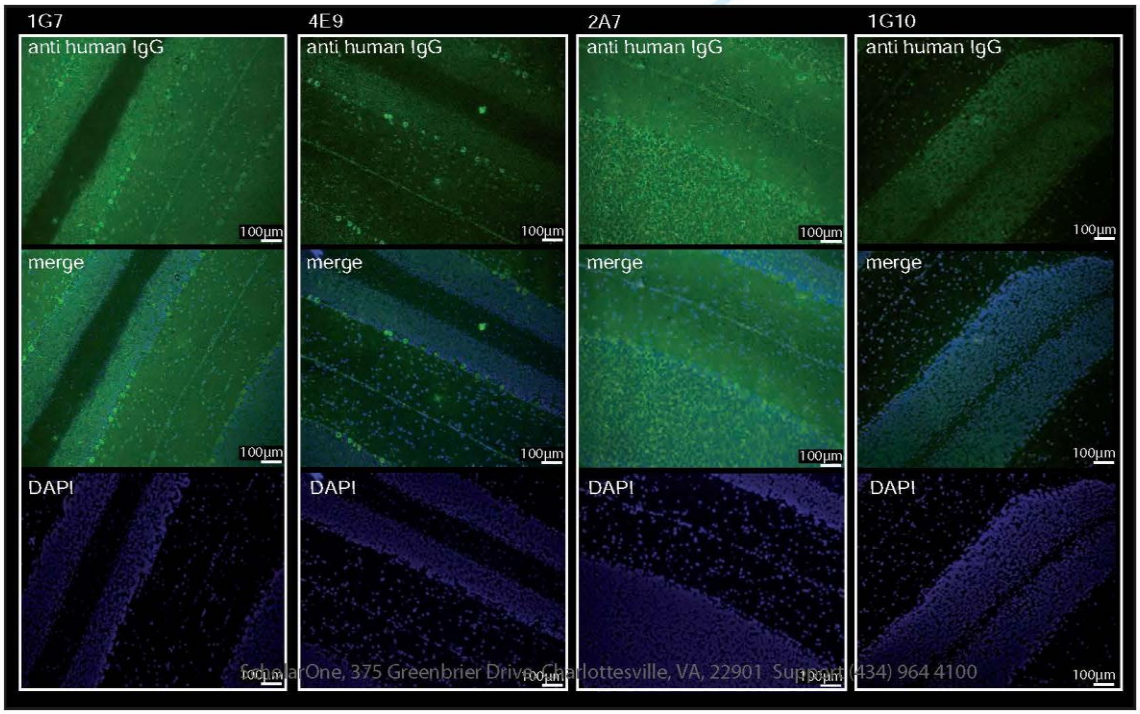
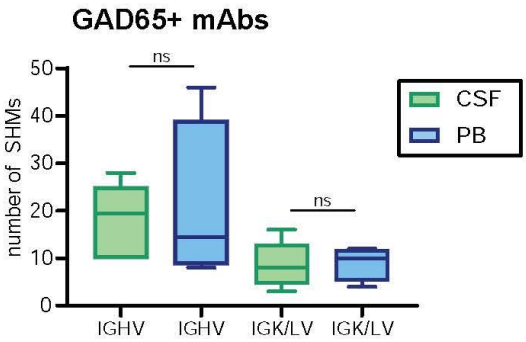
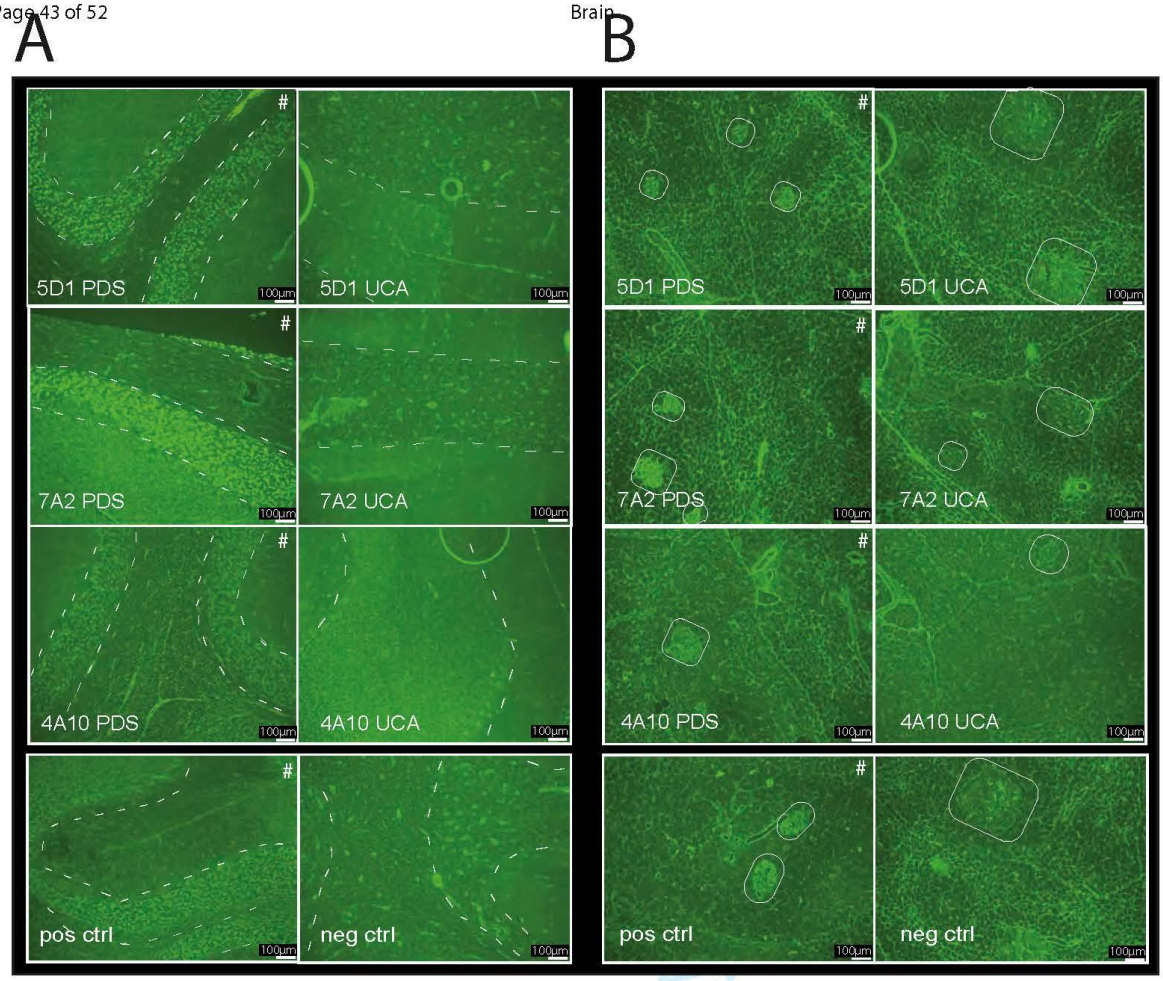


Biljecki et al. Suppl. Fig. 2





Biljecki et al. Suppl. Fig. 4

Supplementary Figure 1. Anti-nuclear antibody (ANA)-staining of CSF and serum. Tissue-based assay with indirect immunofluorescence on primate cerebellar slices was performed using CSF or serum. Furthermore, slices were stained with 4′,6-Diamidin-2-phenylindol (DAPI) to visualize cell nuclei and the merge of the stainings is depicted. Results from patient #1 and patient #4 are shown to illustrate the ANA-staining in these two patients. Slices were stained with serum (1:100) and cerebrospinal fluid (CSF) (1:1) for patient #4 and with serum (1:100) and CSF (1:100) for patient #1.

Supplementary Figure 2. Tissue-based assay using patient-derived mAbs. (**A**) Tissue-based assay with indirect immunofluorescence on primate cerebellar slices was performed to detect anti-GAD65 specific staining patterns using the patient-derived mAbs (50µg/ml) and mAb 1A8 at a concentration of 30µg/ml. The mAb HK3 [1] was used as a negative control (ctrl). A condition with only secondary antibody was used as an additional negative ctrl. # marks mabs that show a GAD65-specific staining pattern characterized by a leopard-like granular layer staining; * marks mAbs with an anti-nuclear antibody (ANA)-staining (see also **B**); the dotted line confines the cerebellar granular layer. (**B**) DAPI staining of cerebellar slices that were additionally stained with the mAbs 1G7, 4E9, 2A7, and 1G10 is depicted to illustrate the ANA-staining of those mAbs.

Supplementary Figure 3. Comparison of somatic hypermutations (SHMs) in GAD65-reactive mAbs (GAD65+) from cerebrospinal fluid (CSF) and peripheral blood (PB). The number of SHMs of the heavy chain (HC) V segment and the light chain (LC) V segment was compared for GAD+ mAbs in CSF and PB. GAD65-reactivity was defined by GAD65-reactivity in ELISA (unpaired T-test).

Supplementary Figure 4. Comparison of binding to primate cerebellar and pancreas tissue of patient-derived sequences (PDS) and unmutated common ancestors (UCAs). (A, B) For the mAbs 5D1, 7A2, and 4A10 binding of the PDS and UCAs to primate cerebellar tissue and pancreas tissue was compared by indirect immunofluorescence with concentrations of 50 µg/ml of the mAbs. (**A**) Binding to primate cerebellar tissue is illustrated. The dashed white lines confine the leopard-like staining pattern of the granular layer that is specific for GAD65 abs. GAD65-specific staining pattern is marked with #. (**B**) Binding to primate pancreas islets is illustrated. Pancreas islet cells are marked with a white circle. GAD65-specific staining pattern is marked with #. ctrl = control, pos = positive, neg = negative.

1. Brandle SM, Cerina M, Weber S, *et al*. Cross-reactivity of a pathogenic autoantibody to a tumor antigen in GABAA receptor encephalitis. *Proc Natl Acad Sci U S A*. Mar 2 2021;118(9)doi:10.1073/pnas.1916337118

Supplementary methods.

Tissue-based assay using patient-derived mAbs

In a tissue-based assay, primate cerebellar tissue (Euroimmun, FB 1111-1010-17) was used to evaluate binding of patient-derived mAbs by indirect immunofluorescence. Additionally, primate cerebellar brain tissue as well as pancreas tissue were used to compare binding of patient-derived sequences and unmutated common ancestrors (Euroimmun, FA 1020-3). MAbs were used at a concentration of 50 µg/ml.

Anti-nuclear antibody staining

To control for nuclear staining 4',6-Diamidin-2-phenylindol (DAPI) (Thermo Scientific 815-968-0747; 0.04 µg/ml) was applied.

Supplementary Table 1. Primers used for single-cell analysis.

Description	Name	Sequence
RT TSO	TSO no. 1	AAGCAGTGGTATCAACGCAGAGTGAATrGrG +G -
genome amplification TSO	TSO no. 2	AAGCAGTGGTATCAACGCAGAGT
inner and outer PCR		
template switch oligo for smart scribe	TSO no. 3	AAG CAG TGG TAT CAA CGC AG
human IgG heavy chain	HG_OUT	AGT TCC ACG ACA CCG TCA C
kappa chain Ig	Kappa_OUT	CAC ACA ACA GAG GCA GTT CC
lambda chain Ig	Lambda_OUT	CAC CAG TGT GGC CTT GTT GG
human IgG heavy chain	HG_IN	AGA GGT GCT CTT GGA GGA G
kappa chain Ig	Kappa_IN	GAA GAT GAA GAC AGA TGG TGC
lambda chain Ig	Lambda_IN	GCT TGG AGC TCC TCA GAG G
cloning mAbs in pTT5 IgG		
mon Ab always the same for Heavy chain reverse for cloning into pTT5	Heavy chain reverse primer	GTGCCCCCAGAGGTcgaCTTGG
mon Ab always the same for kappa chain reverse for cloning into pTT5	kappa rev primer	AGACAGATGGcGCcGCCACAG
	4D1 silent mutation	GGGAGCCGACCCACGGATACGCTTTTA
	4D1 heavy chain fwd	GTTTCCGCGGTGGGTCCTGTCC**GAGGTGCA GCTGGTGGAGTCTGGGGG**
	4D1 Lamda fwd primer	AACGGGCGCGCGATGT**TCCTATGTGCTGACT CAGCC**
	4D1 Lamda rev primer	AGATGGcGCcGCCACAGTTCG**TAGGACGGTC AGCTTGG**
	4A10 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTCCAG CTTGTGCAGTCTGGGGC
	1C8 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTTCAA CTGGTGCAGTCTGG
	1C8 lambda fwd primer	AACGGGCGCGCGATGT**CAGTCTGTGCTGAC GCAGCCGCCC**
	1C8 lambda rev primer	AGATGGcGCcGCCACAGTTCG**TAGGACGGTC AGTTTGGTCCC**
	1C9 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCGAGGTGCAG CTGGTGGAGTCTGGGG
	1C9 kappa fwd primer	AACGGGCGCGCGATGT**GACATCGTGATGAC CCAG**
	1G10 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAG CTGCAGGAGTCGGGCCC
	1G10 kappa fwd primer	AACGGGCGCGCGATGT**GAAATTGTTCTGAC TCAGTCTCC**
	4E9 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCGAGGTGGA GCTGGTGGAGTCTGGGGG
	4E9 lambda fwd primer	AACGGGCGCGCGATGT**AATTTTATGATGACT CAGCCCC**
	4E9 lambda rev primer	AGATGGcGCcGCCACAGTTCG**TAGGACGGTC AGCTTGG**
	1A8 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCGAGGTGCAG CTGGTGCAGTCTGGGGG
	1A8 kappa fwd primer	AACGGGCGCGCGATGT**GATATTGTGATGA**

	1C10 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCGAGGTGCAG GTGGTGGAG
	1C10 kappa fwd primer	AACGGGCGCGCGATGT**GACATCCAGATGAC CC**
	1D5 silent mutation in heavy	GAGCTCTGTGACCGCCACGGACACGGC
	1D5 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAG CTACAGCTGTGGGGCG
	1D5 kappa fwd primer	AACGGGCGCGCGATGT**GACATCCAGATGAC CC**
	1D12 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCGAGGTGCAG CTGGTGGAGTCTGGGGG
	1D12 kappa fwd primer	AACGGGCGCGCGATGT**GATGTTGTGATGAC TCAGTCTCC**
	1F5 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCGAAGTGCAG CTGGTGGAGTCTGGGGG
	1F5 kappa fwd primer	AACGGGCGCGCGATGT**GAAATTGTGTTGAC GCAGTCTCC**
	1F9 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAG CTGCAGGAGTCGGGCCC
	1F9 kappa fwd primer	AACGGGCGCGCGATGT**GAAGTTGTACTGAC TCAGTCTCC**
	1G5 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTTCAG CTGGTGCAGTCTGGAGC
	1G5 kappa fwd primer	AACGGGCGCGCGATGT**GACATCCAGATGAC CCAG**
	2E6 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCgaggtgcagctg gtggagtctgggga
	2E6 kappa fwd primer	AACGGGCGCGCGATGT**GATATTGTGATGAC TCAGTCTCC**
	1D10 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTTCAG CTGGTGCAGTC
	1D10 kappa fwd primer	AACGGGCGCGCGATGT**GATATTGTGATGAC TCAGTCTCC**
	1D8 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTACAG CTGGTAGAGTCTGGGGG
	1D8 kappa fwd primer	AACGGGCGCGCGATGT**GACGTCCAGCTGAC CCAGTCTCC**
	1A12 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAA CTGGTGGAGTCTGGGGG
	1A12 lambda fwd primer	AACGGGCGCGCGATGT**CAGTCTGCCCTGACT CAGCCTGCCTCCG**
	1A12 lambda rev primer	AGATGGcGCcGCCACAGTTCG**TAGGACGG**
	1E7 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCGAAGTGCAG CTGGTGGAG
	1E7 lambda fwd primer	AACGGGCGCGCGATGT**TCCTATGAGCTGAC ACAGCCACCCTCGG**
	1E7 lambda rev primer	AGATGGcGCcGCCACAGTTCG**TAGGACGGTC A**
	1G7 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAT CTGGTGGAGTCTGGGGG
	1G7 lambda fwd primer	AACGGGCGCGCGATGT**CAGTCTGCCCTGACT CAGCCTGCC**
	1G7 lambda rev primer	AGATGGcGCcGCCACAGTTCG**CAGGACGGTC AGCTTGG**

	4E12 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAG CTGGTGGAGTCTGGGGG
	4E12 lambda fwd primer	AACGGGCGCGCGATGT**TCCTATGAGCTGACT CAGCC**
	4E12 lambda rev primer	AGATGGcGCcGCCACAGTTCG**TAGGACGGTC AGCTTGGTCCC**
	2A7 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAG CTGGTGCAGTCTGGGGC
	2A7 lambda fwd primer	AACGGGCGCGCGATGTTCCTTTGAGCTGACA CAGCCACCC
	2A7 lambda rev primer	AGATGGcGCcGCCACAGTTCG**TAGGACGGTC AGCTTGG**
	5D1 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAG CTGGTGCAATCTGGG
	5D1 kappa fwd primer	AACGGGCGCGCGATGT**GACATCCAGATGAC CCAGTCTCC**
	5B1 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAG CTACAGCAGTGGGGC
	5B1 silent mutation in heavy fwd	CCCTGAAATTGAGGTCTGTGACTGCCACGGA CG
	5B1 kappa fwd primer	AACGGGCGCGCGATGT**GATATTGTGATGAC TCAGTCTCC**
	5D3 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTTCAG CTGGTGCAGTCTGG
	5D3 lambda fwd primer	AACGGGCGCGCGATGT**TCCTATGTGCTGACT CAGCCACCC**
	5D3 lambda rev primer	AGATGGcGCcGCCACAGTTCG**TAGGACGGTC AGCTTGGTCCC**
	7H8 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAG CTGGTGGAGTCTGGGGG
	7H8 kappa fwd primer	AACGGGCGCGCGATGT**GAAGTTGTGTTGAC ACAGTCTCC**
	7H1 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCCCAGGTGCAG CTGGTGCAATCTGGG
	7H1 kappa fwd primer	AACGGGCGCGCGATGT**AATATTGTGATGAC TCAGTTTCC**
	7G2 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCC**GAGGTGCA GCTGTTGGAGTCTGGGGG**
	7G2 lambda fwd primer	AACGGGCGCGCGATGT**CAGGCTGTGGTGAC TCAGGAGCCC**
	7G2 lambda rev primer	AGATGGcGCcGCCACAGTTCG**TAGGACGGTC AACTTGGTCCC**
	7E8 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCC**GAGGCGCA GCTGGTGG**
	7E8 kappa fwd primer	AACGGGCGCGCGATGT**GACATCCACTTGAC CCAGTCTCC**
	7A2 heavy fwd primer	GTTTCCGCGGTGGGTCCTGTCC**CAGCTGCAG CTGCAGGAGTCGGGCCC**
	7A2 lambda fwd primer	AACGGGCGCGCGATGT**CAGTCTGTGTTGAC GCAGCCGCCC**
	7A2 lambda rev primer	AGATGGcGCcGCCACAGTTCG**TAGGACGGTC AGCTTGGTCCC**
IgG subclass determination		
	VH1L for IGHVH1/7	CCATGGACTGGACCTGGA

	VH1F for IGHVH1/7	CCATGGACTGGACCTGGA
	VH3L for IGHVH3	CCATGGACTGGACCTGGA
	VH3F for IGHVH3	CCATGGACTGGACCTGGA
	VH4L for IGHVH4	CCATGGACTGGACCTGGA
	VH4F for IGHVH4	CCATGGACTGGACCTGGA
	VH5L for IGHVH5	CCATGGACTGGACCTGGA
	VH5F for IGHVH5	CCATGGACTGGACCTGGA
	VH6L for IGHVH6	CCATGGACTGGACCTGGA
	VH6F for IGHVH6	CCATGGACTGGACCTGGA
	IGREV : universal reverse primer for VH family	CCATGGACTGGACCTGGA

Abbrevations: fwd = forward, rev = reverse.

Supplementary Table 2. Analysis of reactivity of mAbs to viral antigens.

analyt	method	manufacturer	result
HSV-IgG	ELISA	Virion\Serion	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
HSV-IgG	Immunblot	Mikrogen	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
VZV-IgG	ELISA	Virion\Serion	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
EBV-VCA-IgG	CMIA	Abbott	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
anti-EBNA	CMIA	Abbott	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
CMV-IgG	ELISA	Virion\Serion	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
HHV-6-IgG	IIFT	Scimedx Corporation	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
HHV-7-IgG	IIFT	BIOCELL Diagnostics	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
HHV-8-IgG	IIFT	in-house	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
HAV-ab	CLIA	DiaSorin	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
anti-HBs	CMIA	Abbott	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
anti-HBc	CMIA	Abbott	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
anti-HBe	CLIA	DiaSorin	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
HCV-ab	CMIA	Abbott	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
HEV-IgG	Immunblot	Mikrogen	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
HIV-1/2-ab	CMIA	Abbott	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
HTLV-1/2-ab	CMIA	Abbott	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
Measles-IgG	ELISA	Virion\Serion	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
Mumps-IgG	ELISA	Virion\Serion	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
Rubella-IgG	CMIA	Abbott	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
Parvovirus B19-IgG	Immunblot	Mikrogen	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
Adenovirus-IgG	ELISA	Virion\Serion	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
Enterovirus-IgG	ELISA	Virion\Serion	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
Hantavirus-IgG	Immunblot	Mikrogen	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
TBE-Virus-IgG	ELISA	Virion\Serion	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
WNV-IgG	ELISA	Virion\Serion	neg (1C10, 4C5, 4A10, 4D1, 5D3, HK3)
SARS-CoV-2-S1/S2-IgG	CLIA	DiaSorin	4C5 borderline pos (14,2 rep. 13,5 AU/ml) neg (1C10, 4A10, 4D1, 5D3, HK3)
SARS-CoV-2-S1/S2-IgG	NAB	Shenzhen YHLO Biotech	neg (4C5)

Abbrevations: CLIA = chemiluminescence immunoassay, CMIA = chemiluminescent microparticle immunoassay, CMV = Cytomegalovirus, EBNA = -EBV-Nuclear-Antigen, EBV = Epstein-Barr virus, HAV = Hepatitis A Virus, HBc = -Hepatitis B core antigen, HBe = -Hepatitis B e antigen, HBs = Hepatitis B surface antigen, HCV = Hepatitis C Virus, HEV = Hepatitis E Virus, HHV = Human Herpes Virus, HIV = human immunodeficiency virus, HSV = Herpes Simplex Virus, HTLV = Human T-lymphotropic Virus, IIFT = Indirect Immunofluorescence test, mabs = monoclonal antibodies, NAB = neutralizing antibody assay, neg = negative, pos = positive, rep = repetition, SARS-CoV-2 = Severe acute respiratory syndrome coronavirus 2, TBE = Tick-borne encephalitis, WNV = West Nile Virus, VCA = EBV-viral capsid antigen, VZV = Varicella-Zoster Virus.

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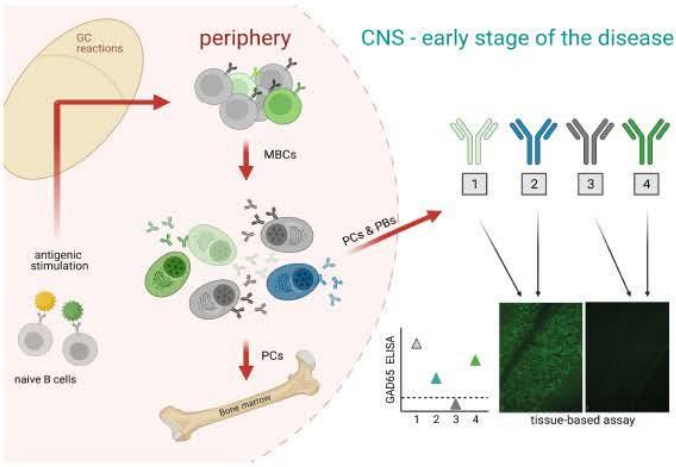

Biljecki et al. Thumbnail

Study overview. After encountering their antigen, naïve B cells undergo affinity maturation in germinal centers (GC) giving rise to polyclonal GAD65-specific B cells. Circulating GAD65-specific memory B cells (MBCs) differentiate into Ig-secreting plasma cells (PCs) and plasmablasts (PBs). GAD65-specific PBs and PCs migrate to the central nervous system (CNS) in the early phase of disease course and produce brain-reactive mAbs. GAD65-specific long-lived PCs escape into survival niches in the bone marrow contributing to the serum GAD65-ab pool 20 (created with BioRender).

201x291mm (72 x 72 DPI)

STROBE statement: Reporting guidelines checklist for cohort, case-control and cross-sectional studies

SECTION	ITEM NUMBER	CHECKLIST ITEM	REPORTED ON PAGE NUMBER:
TITLE AND ABSTRACT			
	1a	Indicate the study's design with a commonly used term in the title or the abstract	3-4
	1b	Provide in the abstract an informative and balanced summary of what was done and what was found	3-4
INTRODUCTION			
Background and objectives	2	Explain the scientific background and rationale for the investigation being reported	4-5
	3	State specific objectives, including any pre-specified hypotheses	4-5
METHODS			
Study design	4	Present key elements of study design early in the paper	5
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	6
Participants	6a	Cohort study—Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up Case-control study—Give the eligibility criteria, and the sources and methods of case ascertainment and control selection. Give the rationale for the choice of cases and controls Cross-sectional study—Give the eligibility criteria, and the sources and methods of selection of participants	6
	6b	Cohort study—For matched studies, give matching criteria and number of exposed and unexposed Case-control study—For matched studies, give matching criteria and the number of controls per case Variables	na
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	6-11

SECTION	ITEM NUMBER	CHECKLIST ITEM	REPORTED ON PAGE NUMBER:
Data sources/measurements	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group.	6-11
Bias	9	Describe any efforts to address potential sources of bias.	6-11
Study size	10	Explain how the study size was arrived at	6
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	na
Statistical methods	12a	Describe all statistical methods, including those used to control for confounding	12
	12b	Describe any methods used to examine subgroups and interactions	na
	12c	Explain how missing data were addressed	na
	12d	Cohort study—If applicable, explain how loss to follow-up was addressed Case-control study—If applicable, explain how matching of cases and controls was addressed Cross-sectional study—If applicable, describe analytical methods taking account of sampling strategy	na
	12e	Describe any sensitivity analyses	na
RESULTS			
Participants	13a	Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	12
	13b	Give reasons for non-participation at each stage	na
	13c	Consider use of a flow diagram	na
Descriptive Data	14a	Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	**Table 1**
	14b	Indicate number of participants with missing data for each variable of interest	Figure legend 1
	14c	Cohort study—Summarise follow-up time (eg, average and total amount)	na
Outcome Data	15*	Cohort study—Report numbers of outcome events or summary measures over time	12

SECTION	ITEM NUMBER	CHECKLIST ITEM	REPORTED ON PAGE NUMBER:
		Case-control study—Report numbers in each exposure category, or summary measures of exposure Cross-sectional study—Report numbers of outcome events or summary measures	
Main Results	16a	Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (e.g. 95% confidence interval). Make clear which confounders were adjusted for and why they were included	12-15
	16b	Report category boundaries when continuous variables were categorized	na
	16c	If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	na
	16d	Report results of any adjustments for multiple comparisons	na
Other Analyses	17a	Report other analyses done—e.g. analyses of subgroups and interactions, and sensitivity analyses	na
	17b	If numerous genetic exposures (genetic variants) were examined, summarize results from all analyses undertaken	na
	17c	If detailed results are available elsewhere, state how they can be accessed	na
DISCUSSION			
Key Results	18	Summarise key results with reference to study objectives	16
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	19
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	19
Generalisability	21	Discuss the generalisability (external validity) of the study results Other information	19
FUNDING			
	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	20

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies.

4. DISCUSSION

4.1. Long-lasting ab production and intrathecal synthesis

Patients with GAD-AIND typically reveal very high anti-GAD ab titers that stay high over time [64,69]. In other words, anti-GAD ab titers do not necessarily decline with therapy [77]. All together, this raises the question, how a persistent ab production is facilitated? Three theories exist regarding plasma cell generation and a subsequent long-lasting ab production [130]. The first argues with a continuous ag stimulation of memory B cells that might lead to the generation of short-lived plasma cells [131]. The second theory confers to a stimulation of memory cells, which gives rise to plasma cells, due to the activation via cytokines and TLR ligands [132,133]. Lastly, plasma cells persisting in the bm that functions as a survival niche can be the source of long-lasting ab-production [134].

In Thaler et al., we provided evidence for circulating GAD65-reactive memory B cells that can be stimulated through cytokines and TLR ligands. We were able to differentiate anti-GAD ab harboring memory B cells into anti-GAD ab producing plasmablasts derived from peripheral blood mononuclear cells (**FIGURE 7**). To this aim, we cultured patient-derived peripheral blood mononuclear cells and stimulated them with Resiquimod as a TLR ligand and IL-2 [77]. Pinna et al. showed that this protocol is able to efficiently and selectively activate memory B cells and induce differentiation into plasmablasts [26]. Remarkably, no additional antigenic stimulus, e.g., GAD protein, was required. Surprisingly, the anti-GAD ab production did not correlate with anti-GAD ab levels in the serum of patients.

With this, our findings did point toward an additional source of anti-GAD abs. To investigate the third theory, whether long-lived plasma cells in the bm produce anti-GAD abs, we cultured bm mononuclear cells from a rituximab-treated patient without any further stimulation. After 5 days, we could detect a spontaneous anti-GAD ab secretion, which was not detectable in bone marrow cells from control patients. Furthermore, in order to exclude any other sources of anti-GAD abs (B cells derived from the periphery), we cultured and stimulated the

peripheral blood mononuclear cells of this patient as described above. No anti-GAD ab production could be detected since the patient was treated with rituximab that transiently depletes CD20 expressing B cells. To summarize, in Thaler et al., we could confirm that both circulating memory B cells as well as bm-derived plasma cells are sources of anti-GAD abs (**FIGURE 7**) [77].

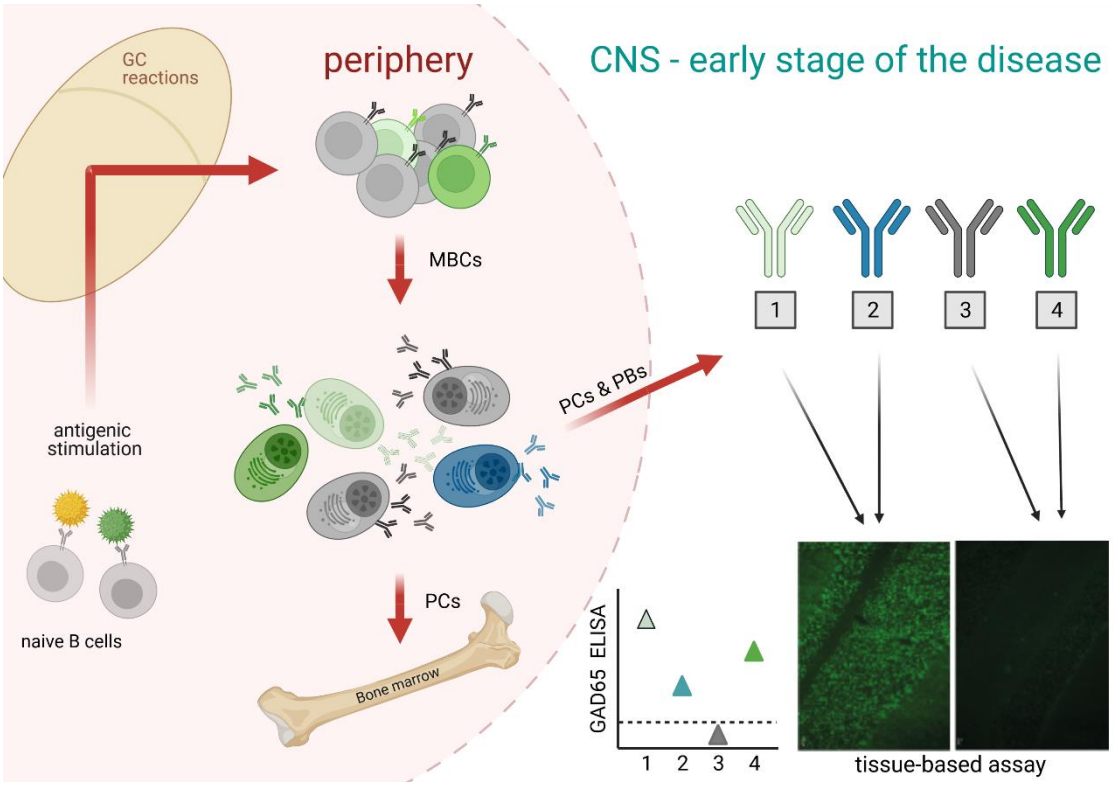

FIGURE 7: IG-SECRETING PLASMA CELLS AND PLASMABLASTS ARE PRESENT IN THE CNS OF GAD-AIND PATIENTS.

After encountering their ag, naïve B cells undergo affinity maturation in gc reactions, giving rise to circulating polyclonal GAD65-specific memory B cells. Memory B cells further differentiate to Ig-secreting plasma cells and plasmablasts. Long-lived plasma cells escape into survival niches, e.g., the bm, whereas another proportion of plasma cells and plasmablasts migrate to the CNS in the early phase of disease course, producing brain-reactive mabs. We were able to generate mabs derived from Ig-secreting B cells of peripheral blood cells and CSF cells of patients suffering from GAD-AIND. MBC = memory B cells, PCs = plasma cells, PBs = plasmablasts. Adapted from Biljecki et al., by BioRender.com (2022). Retrieved from https://app.biorender.com/biorender-templates.

The intrathecal synthesis of anti-GAD abs is very important for the diagnosis of GAD-AIND. In line with this, Graus et al. even suggested to consider an intrathecal anti-GAD ab production as an essential diagnostic criterion in LE/TLE and CA [81]. However, a direct proof of CSF B cells producing anti-GAD abs

was missing, even though there is a strong evidence for an intrathecal anti-GAD ab production in patients with GAD-AIND [69].

In Biljecki at al., we studied 6 patients with GAD-AIND and were able to generate GAD65-reactive mabs derived from the CSF in 2 patients. Herewith, we could provide direct evidence that anti-GAD65 ab producing cells are present in the CSF. Moreover, we could show that the anti-GAD response of B cells in the CNS is polyclonal (**FIGURE 7**).

With this, we could confirm the suggestion of Gresa-Arribas et al. who implied that the presence of anti-GAD65 abs in the CSF is not merely a result of a passive ab transfer of serum into the CSF compartment but even more, part of an active immune response. They showed that 74 % of analyzed patient-derived CSF samples were able to recognize all domains of GAD65, pointing towards a broader CSF immune response against GAD65 when being compared with the peripheral immune response (22 % of sera samples) in those patients [110].

Nevertheless, it remains to be investigated whether GAD-specific B cells are re-stimulated in the CNS, triggering anti-GAD ab production, and whether affinity maturation and differentiation of plasmablasts and plasma cells mostly take place in the periphery.

4.2. Triggers of autoimmunity and affinity maturation

In Thaler et al., we found a strikingly high frequency of GAD-reactive B cells in the PB of almost all patients. The abundance of GAD65-reactive B cells was even comparable to the frequency of B cells reactive for common recall ags (tetanus toxoid and measles virus), suggesting a dysregulation of self-tolerance in these patients [77].

The multiple-hit theory of autoimmunity addresses, inter alia, the initiation of autoimmune processes due to an initial breakdown of tolerance. Strengthening this, it is known that most auto-abs are present years before disease onset and herewith not only as a consequence of inflammation. The initial breakdown of tolerance (first hit) that favors the production of auto-abs is triggered by genetic and environmental factors. Next, the so called second hit enables the auto-abs to expend pathogenic effects and with that to preserve the autoimmune response. Here, an example is myositis, where auto-abs target regenerating myocytes which preserves the cycle of injury and regeneration [41]. A model describing the pathogenicity of anti-GAD65 abs in CA could be that auto-abs generated in the first hit are able to reduce the release of GABA (due to a diminished GAD65 enzyme activity), leading to a hyperexcitability of cells followed by an increase of neuronal loss [69]. This in turn would lead to a higher proportion of GAD65 protein that is released from neurons and thereby accessible in the extracellular space, providing further antigenic stimuli.

Still, considering anti-GAD abs, it is unknown when the production starts. Supporting the multiple-hit theory and particularly the initial breakdown of tolerance, Akman et al. previously mentioned, that it is unclear whether the production of anti-GAD abs is related to neuronal injuries, a disruption of the blood-brain barrier after trauma, or viral or bacterial infections that are related to a predisposing underlying immune deficiency [135]. Intriguingly, it is known that some bacteria of the human gut microbiota have the capability to produce or consume GABA. Hereby, some of the GABA producers use GAD to facilitate their synthesis [136]. Interestingly, a recently published *in silico* analysis gave evidence of similarities between bacterial GAD and human GAD65, implying the possibility

of bacterial-derived GAD as ag stimuli for auto-reactivity towards human GAD65 [137].

Moreover, microbial triggers as infectious agents are already well-established in some autoimmune encephalitis cases [51]. As an example an untreated infection with Group A *Streptococcus* (*S. pyogenes*) is known to cause autoimmune sequela in target tissues and manifest as either a rheumatic fever (target tissue: heart) or Sydenham's chorea (target tissue: CNS) [51]. Other examples of the initial breakdown of tolerance are viral infections that trigger the production of auto-abs [138–140]. It is known that an infection with the *Herpes Simplex Virus* Typ 1 can be followed by relapsing symptoms in the form of an encephalitis. It was observed that the encephalitis is not a true viral relapse but rather another immune-mediated disorder, namely NMDAR-encephalitis. Hereby, it was shown that infections with the *Herpes Simplex Virus* Typ 1 can induce the synthesis of anti-NDMAR abs [139]. Moreover, it is known that abs generated as a first line of defense in an acute infection carry a low number of SHMs (near germline abs - UCA). This bears the risk of abs being simultaneously self-reactive, as it was shown for human-derived *severe acute respiratory syndrome coronavirus* type 2 reactive mabs that were able to bind to murine brain tissue [141].

Taken together, molecular mimicry between bacterial or viral ags and brain ags could be a relevant component in the pathogenesis of autoimmune encephalitis. As a consequence, in Biljecki at al., we investigated the hypothesis of viral ags being triggers for anti-GAD autoimmunity. In order to do so, we tested the reactivity of several GAD-reactive mabs to a panel of viral ags. Hereby, we could not observe any cross-reactivity to the viral ags being tested. Nonetheless, we cannot exclude that molecular mimicry contribute to the pathogenesis of GAD-AINDs since only few viral ags and mabs could be tested in our study.

As indicated earlier, genetic changes might play an important role in the pathogenesis of auto-ab-associated neurological disorders. Considering that, it is common that patients or their relatives suffer from other systemic autoimmune diseases, arguing for a shared predisposition that can cause a loss of self-tolerance [53,67]. Patients with GAD-AIND often have co-occurring autoimmune diseases [53,119]. Moreover, it was suggested that they carry genetic risk factors [119].

Generally, but not necessarily, this suggests deficiencies of central tolerance checkpoints in patients with autoimmune diseases. In line with this, the treatment of a patient, suffering from recurring melanoma, with nivolumab (immune-checkpoint inhibitor) subsequently lead to the occurrence of GAD-AIND. The patient presented with epilepsy, LE, CA and SPS together with anti-GAD65 abs in the serum and CSF, indicating that an impaired central tolerance (due to impaired immune-checkpoints) can lead to the development of anti-GAD autoimmunity [142]. Tolerance checkpoints in gc compartments could have an important role concerning the induction of auto-reactivity. In gc compartements, mature naïve B cells that experienced an antigenic stimulus, clonally expand. Here, they undergo affinity maturation (SHMs) and class switching before differentiating into memory B cells or plasma cells. In particular, SHMs can give rise to auto-reactive abs or even enhance the affinity of existing auto-reactive B cells. Moreover, most auto-abs are present years before disease onset. For instance, it was reported that affinity maturation and isotype switching of mabs occur before clinical symptoms start for anti-citrullinated protein abs occuring in patients with rheumatoid arthritis. Thereby, every change can be associated with an increased pathogenic potential of the anti-citrullinated protein abs in rheumatoid arthritis [41].

Hence, in Biljecki at al., we aimed to analyze the importance of affinity maturation, and in particular, SHMs for the reactivity of our mabs towards GAD65. To do so, we reverted SHMs occurring in the variable domains (FR1-FR3 and CDR1-CDR3) of three CSF-derived mabs to their UCAs. Hereby, we could show that affinity maturation is required for the GAD-reactivity, as the UCAs had a strongly reduced ability to bind to GAD65. Beyond that, in our study anti-GAD65 abs carried higher numbers of SHMs compared to non-GAD-reactive mabs. Furthermore, we compared our CSF-derived anti-GAD65 abs with other published CSF-derived auto-abs. Here, we could show that our anti-GAD65 abs exhibit features that can be compared with abs derived from patients with LGI1-encephalitis [57] and GABAA-receptor encephalitis [59]. More precisely, in our study we could observe a high proportion of ab-secreting cells expressing anti-GAD65 abs that carry a high number of SHMs. CSF-derived LGI1 abs or GABAA-receptor abs show similar features. On the contrary, CSF-derived anti-NMDAR abs from patients with NMDAR-encephalitis do not show this features. Here, only a small

fraction of ab-secreting cells harbors abs against the NMDAR. Besides, anti-NMDAR abs often lack any mutation as compared to the germline sequence [33,58]. In another step, we compared the amount of SHMs occurring in anti-GAD65 abs derived from the CSF and from the PB. Intriguingly, we could not detect a significant difference, which could indicates that affinity maturation of GAD-reactive B cells takes place in the periphery before the cells migrate to the CNS. This highlights the relevance of negative selection by peripheral tolerance checkpoints and indicates that GAD-reactive B cells might have escaped at this point [143].

Summarizing our findings from Biljecki et al., we were able to provide insights into the GAD-specific B cell receptor repertoire in GAD-AIND, demonstrating that the anti-GAD65 response is polyclonal and affinity maturation is required for the specificity towards GAD65. Moreover, we did not find any cross-reactivity of GAD65-reactive mabs to a panel of viral ags.

4.3. Detection of anti-GAD abs

Currently, different detection assays for anti-GAD abs are being used in different centers. In our study Biljecki et al., we determined the reactivity of patient-derived mabs applying different detection assays. Here, we analyzed ten GAD-reactive mabs with ELISA. However, only six mabs were GAD-reactive in quantitative assays, including immunohistochemistry and CBA.

As reviewed by Tsiortou et al., high anti-GAD ab titer (>10,000 IU/ml) are associated with an autoimmune neurological disease in 94 % of patients exhibiting anti-GAD abs. Therefore, the detection of high anti-GAD ab titers is a useful diagnostic tool to distinguish between GAD-AIND and atypical or non-specific neurological disorders. Hereby, high titers in the serum confer to measurable anti-GAD abs in the CSF [69].

Further, we were able to generate a high proportion of GAD-reactive CSF-derived mabs in two patients. Paradoxically, we could not generate GAD-reactive mabs from patient #1 although a high number of CSF-derived mabs could be generated, even though all of our patients exhibited high anti-GAD ab titers measured by ELISA with being >77,500 IU/ml in the serum and >1,900 IU/ml in the CSF. Intriguingly, patient #1 harbored high titers of anti-nuclear abs (ANAs), pointing toward a possible false-positive result of the ELISA due to the interfering ANAs or to a co-existing autoimmune disorder with a B cell response in the CNS targeting another ag.

With my contributions in Biljecki et al., we strengthen the concept by applying two different detection systems to screen for anti-GAD abs. Hereby, it could be of benefit to use one quantitative (displaying linear epitopes of GAD) and one qualitative (displaying conformational epitopes of GAD) assay in order to avoid false-positive results. Further, the occurrence of ANAs should be taken into consideration when interpreting positive anti-GAD ab results.

4.4. Implications for treatment

Currently, the response of patients with GAD-AIND to immunotherapy is not satisfying [94,144], although patients with GAD-AIND associated-SPS showed a systematic improvement due to intravenous immunoglobulins [85]. Moreover, one-third of AE patients and 52 % of anti-GAD65 ab associated CA patients responded well to immunotherapy [73], indicating that GAD-AIND are at least partially responsive to immunotherapy. Furthermore, the improvement of disease correlated with a decrease in the ab titers in some patients [71,135]. Still, anti-GAD ab titers rarely decline due to therapy [64,69] and, unfortunately, a long-lasting and aggressive immunotherapy to treat patients with GAD-AIND carries a high risk for severe side effects. Thus, a faster diagnosis and more suitable therapies are desperately needed [104]. Hence, it could help to investigate the pathogenic potential of anti-GAD abs to adjust the diagnosis and treatment of GAD-AIND at an early stage of the disease. Therefore, the knowledge that anti-GAD abs facilitate pathogenicity could justify an early and intensively usage of a B-cell depleting therapy.

Currently, rituximab is commonly prescribed as immunotherapy for patients with GAD-AIND and effectively abolishes CD20 expressing memory B cells in the periphery without having effects on plasma cells and plasmablasts. However, we could demonstrate in Thaler et al. and Biljecki et al., that GAD65-reactive long-lived plasma cells producing anti-GAD abs are present in the bm and, likewise plasma cells and plasmablasts in the CSF. This implicates that only the pool of GAD-reactive memory B cells in the PB is decreased and with that only a partial effect using rituximab can be achieved. Taken together, our findings support the observation that rituximab barely decreases anti-GAD ab level in the serum [90,144]. Furthermore, it could be shown that patients with long-lasting GAD-AIND do not show a response to rituximab-treatment [124]. Hence, a potential treatment option might be a dual immunotherapy targeting memory B cells with rituximab and plasma cells with e.g. bortezomib. However, the side effects of such a combinatorial treatment should be taken into account.

Furthermore, we could indirectly provide evidence that auto-reactive anti-GAD ab producing B cells might cross the blood-brain barrier in Biljecki et al. In other words, we did not detect any difference when analyzing the amount of SHMs in GAD-reactive mabs derived from the CSF and PB. Therefore, it could be of benefit to block the transmigration of GAD-reactive lymphocytes into the CNS.

Interestingly, Arino et al. found that an early initiation of immunotherapy and a subacute onset in GAD-ab associated CA was associated with therapy response [94]. A possible explanation would be, that the anti-GAD abs might be pathogenic solely at disease onset or shortly after. In contrast, they would become indolent serological markers at later stage of the disease [74]. In line with this, Dik et al. found activated $CD8^+$ T cells to be elevated in the CSF and PB at later stages of the disease when analyzing patients with GAD-LE. Moreover, the numbers of these cells negatively correlated with the hippocampal volume together with memory function. This implicates a putative role of $CD8^+$ T cells in neurodegeneration at later stages of the disease [145].

Supporting the assumption of different stages being characterized by different cell-mediated processes (B cells/T cells) in the disease course of GAD-AIND, we were solely able to generate mabs from patients with a short disease duration in Biljecki et al. Here, most mabs originated from plasma cells and plasmablasts, indicating that at later stages of the disease, these cells might have escaped into survival niches in the CNS, left the CNS compartment, or got degraded as the disease progressed. However, our patient cohort was small; thus, it would require a bigger study cohort to confirm this assumption.

Taken together, our observations support the idea that B-cell depleting therapy may not be a convenient treatment-option in long-standing GAD-AIND, whilst a plasma cell-directed therapy with, e.g., bortezomib or daratumumab at disease onset might be advantageous. Nonetheless, only small studies and case reports addressing the treatment of GAD-AIND are available and randomized prospective studies are urgently needed.

4.5. Closing remarks

To this day, anti-GAD ab producing cells are largely unexplored and the link between antigenicity and pathogenicity of anti-GAD65 abs is heavily discussed. Although, we could already give insights into GAD-reactive B cells and their receptor repertoire in the periphery and CNS, more studies investigating these cells and their abs in detail are needed. Thus, it would be important to perform larger binding studies with mabs derived from patients with GAD-AIND and elaborate panels of bacterial and viral ags. Beyond that, it could help to analyze additional CSF- and PB-derived mabs from a bigger patient cohort to confirm and/or extend our findings concerning the B cell receptor repertoire. Regarding the mabs, an extensive study of SHMs could help to give an insight on which mutation/mutations facilitate the affinity towards GAD65. Here, one possible approach would be to revert single or combinations of SHMs to the germ-line sequence followed by binding studies and affinity determination. In addition, more functional *in vitro* and *in vivo* studies using mabs are needed to investigate the pathogenic potential of anti-GAD abs, which would help to provide insights for future drug development.

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6. LIST OF PUBLICATIONS

- Stetter MG, Zeitler L, Steinhaus A, Kroener K, **Biljecki M** and Schmid KJ (2016). **Crossing Methods and Cultivation Conditions for Rapid Production of Segregating Populations in Three Grain Amaranth Species**. Front. Plant Sci. 7:816. doi: 10.3389/fpls.2016.00816.

- FS Thaler, AL Thaller, **M Biljecki**, et al. (2019). **Abundant GAD-reactive B cells in GAD-antibody-associated neurological disorders**, Annals of Neurology. doi.org/10.1002/ana.25414.

- Richardson JR, Armbruster NS, Günter M, **Biljecki M**, Klenk J, Heumos S and Autenrieth SE (2019). **PSM Peptides From Community Associated Methicillin-Resistant Staphylococcus aureus Impair the Adaptive Immune Response via Modulation of Dendritic Cell Subsets in vivo**.Front. Immunol. 10:995. doi:10.3389/fimmu.2019.00995.

- F.S. Thaler, B. Bangol, **M. Biljecki**, et al. (2020). **Possible link of genetic variants to autoimmunity in GAD-antibody-associated neurological disorders**, Journal of the Neurological Sciences, doi.org/10.1016/j.jns.2020.116860.

7. CURRICULUM VITAE

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11/18 – ongoing	**PhD, Department of Clinical Neuroimmunology, Biomedical Center Munich, LMU** PhD project: B cell mediated immune responses in GAD-antibody-associated neurological disorders Supervisor: Dr. med. Franziska Thaler
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8. AFFIDAVIT

Hiermit versichere ich an Eides statt, dass ich die vorliegende Dissertation

"CHARACTERIZATION OF GAD-REACTIVE B CELLS AND THEIR B-CELL RECEPTOR REPERTOIRE IN THE PERIPHERY AND CENTRAL NERVOUS SYSTEM" selbstständig angefertigt habe, mich außer der angegebenen keiner weiteren Hilfsmittel bedient und alle Erkenntnisse, die aus dem Schrifttum ganz oder annähernd übernommen sind, als solche kenntlich gemacht und nach ihrer Herkunft unter Bezeichnung der Fundstelle einzeln nachgewiesen habe.

I hereby confirm that the dissertation "*CHARACTERIZATION OF GAD-REACTIVE B CELLS AND THEIR B-CELL RECEPTOR REPERTOIRE IN THE PERIPHERY AND CENTRAL NERVOUS SYSTEM*" is the result of my own work and that I have only used sources or materials listed and specified in the dissertation.

München, den 11.04.2022 Michelle Biljecki

Munich, date Unterschrift/signature

9. DECLARATION OF AUTHOR CONTRIBUTIONS

- **Abundant Glutamic Acid Decarboxylase (GAD)-Reactive B Cells in GAD-Antibody– Associated Neurological Disorders, ANNALS of Neurology, 2019**

Franziska S. Thaler, Anna L. Thaller, **Michelle Biljecki,** Elisabeth Schuh, Stephan Winklmeier, Christoph F. Mahler, Ramona Gerhards, Stefanie Völk, Frauke Schnorfeil, Marion Subklewe, Reinhard Hohlfeld, Tania Kümpfel, and Edgar Meinl

The contribution of authors is as follows:

F.S.T., R.H., T.K., and E.M. contributed to the conception and design of the study. F.S.T., A.L.T., M.B., E.S., S.W., C.F.M., R.G., S.V., F.S., and M.S. contributed to the acquisition and analysis of data. F.S.T., A.L.T., M.B., E.S., C.F.M., S.V., R.H., T.K., and E.M. contributed to drafting the text and preparing the figures

My contribution to this work:

For this paper, I performed half of all experiments and helped to analyze the experimental data together with Dr. med. Franziska Thaler. More specifically, I performed the experiments used for Fig. 1 (A, B, F, G, H, J) and Fig. 2 (A, B, C). I also contributed to drafting the text and preparing the figures, together with Dr. med. Franziska Thaler. The contribution of other authors is specified in the paper.

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- **Reconstructing the GAD65-specific B-cell repertoire reveals a critical contribution of CSF B cells during early stages of GAD65-antibody-associated neurological disorders, Manuscript under peer review in BRAIN, 31.03.2022**

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The contribution of authors is as follows:

MB, TK, MK, EM, and FST contributed to the conception and design of the study. MB, EB, SW, AT, PE, and PS contributed to the acquisition and analysis of data. KE, AF-H, and JL contributed to collect the samples and data that is associated with it. SM contributed with critical comments in regard to experiments and results. MB and FST contributed to drafting the text and preparing the figures. All authors critically commented on the manuscript.

My contribution to this work:

For this manuscript I contributed to the conception and design of the study. I performed the majority of experiments and analyzed most of the results. More specifically, the experiments used for Fig. 1, Fig. 2A – 2D, Fig. 3, Fig. 4A-4B, Suppl. Fig. 1, Suppl. Fig. 2, Suppl. Fig. 3, Suppl. Fig. 4 were done by me. I generated and produced the mabs used for the experiments in Fig. 2E, Fig. 4C and Suppl. Tab. 2. Further I designed the primers used for cloning the mabs in the IgG vector shown in Suppl. Tab. 1. Further, I produced all the data shown in Tab. 2, besides the cell-based assay experiments. I also contributed to drafting the text and mainly prepared the figures, together with Dr. med. Franziska Thaler.

Dr. med. Franziska Thaler Michelle Biljecki

10. ACKNOWLEDGEMENTS

Zuallererst möchte ich mich herzlich bei Dr. Franziska Thaler für das Ermöglichen meiner Arbeit sowie die Betreuung während meines PhDs bedanken. Hierbei konnte ich zu jeder Zeit Fragen stellen oder Probleme erörtern, ohne je abgewiesen zu werden. Vielen Dank für deine Geduld mit mir.

Des Weiteren bin ich dankbar für das tolle Arbeitsklima innerhalb der AG Meinl, AG Thaler, AG Kawakami und AG Mader. Durch unsere gemeinsamen Mittags- und Kaffeepausen waren stressige Labortage nicht schlimm. Selbst während der Corona-Pandemie ist das Gemeinschaftsgefühl nie verloren gegangen. Ein besonderer Dank geht an Heike Rübsamen. Ich konnte immer auf deine Hilfe und Unterstützung zählen! Ein herzlicher Dank gilt vor allem an alle meine Laborkollegen (Simone, Stephan, Ramona, Kathi, Katie, Sam, Cate) für die jahrelange Unterstützung und das Aufbauen nach deprimierenden Tagen. Ebenso bin ich sehr dankbar für die Arbeitsmoral und die Hilfsbereitschaft in unserem Team, durch welche jeder dem anderen unter die Arme greift.

Weiter möchte ich mich bei meinem TAC Komitee: Dr. Franziska Thaler, Prof. Dr. Martin Kerschensteiner, Prof. Dr. Arthur Liesz und Prof. Dr. Edgar Meinl bedanken. Für alle anregenden Kommentare, aber auch für alle kritischen Fragen in den TAC-Meetings, welche das Projekt deutlich vorangebracht haben.

Ebenso möchte ich mich bei der Graduate School of Systemic Neurosciences bedanken für die Möglichkeit meinen PhD in einem internationalen Netzwerk zu bewältigen, in welchem man durch viele Kurse und soft skills die Gelegenheit bekommen hat über den Tellerrand zu schauen.

Nicht zu vergessen, möchte ich mich bei all meinen Freunden (daheim und auch im Lab) bedanken die mir immer ein offenes Ohr geschenkt haben! Besonders bei: Julia, Alina, Cate, Katrin, Bella, Miri, Ramona und die Kuba-Gruppe. Ihr habt mir durchweg emotional und aber auch professionell beigestanden, egal wie oft ich mich wegen Kleinigkeiten geärgert oder verrückt gemacht habe. Dabei konnte ich immer auf wundervolle ablenkende Unternehmungen (sei es Tischkickern) hoffen, sowohl im Lab als auch in unserer Freizeit. Ein besonderer Dank geht an Hannah aus Tübingen und die dunkle Seite :-P. Ihr seid die besten und tollsten

Freunde die man sich wünschen kann und ich bin unfassbar dankbar euch kennen gelernt zu haben! Dankbar für all unsere geteilten Emotionen, ob Freude oder Frustration. Ich hoffe wir werden uns nie aus den Augen verlieren. Auf viele weitere Dark-moments. Ihr habt die Zeit unvergesslich gemacht.

Zu guter Letzt möchte ich meiner Familie danken. Ihr habt mich durch das ganze Studium hindurch großartig unterstützt, ermutigt und ausgehalten. Ich weiß es war oft sehr anstrengend (vor allem daheim – ich entschuldige mich für die schlaflosen Nächte). Trotzdem wart ihr immer da, habt mir geduldig zugehört und mir meine Ängste genommen. Vor allem meinen Schwestern Mia und Madleen, sowie meinem Kletterbuddy David (always forever) möchte ich für ihr da sein danken. Es hat oft geholfen zu wissen, dass jemand da ist der einem den Rücken stärkt. Ihr seid mir das Wichtigste und unersetzbar!