

Bispecific chimeric autoantibody receptor T cells eliminate acetylcholine receptor-specific B cells in myasthenia gravis models

Niels von Wardenburg^{1,2,3, #, *}, Gregorio Spagni^{2,4, #}, S. Momsen Reincke^{1,2,3, #}, Stephanie Wernick², Hans-Christian Kornau^{2,5}, Viktoria Zinnow², Amelya Keles Slevogt^{1,2,3}, Lucie Y. Li^{1,3}, Marie A. Homeyer^{1,2,3}, Sonja Blumenau^{1,2}, Maria Stecklum⁶, Dietmar Schmitz^{2,5}, Andreas Pelz¹, Valentina Damato⁴, Nicholas Sanderson⁷, Tobias Derfuss⁷, Minh C. Pham⁸, Kevin C. O'Connor^{8,9}, Andreas Meisel^{1,10,11}, Harald Prüss^{1,2, *}

¹ Department of Neurology with Experimental Neurology, Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

² German Center for Neurodegenerative Diseases (DZNE) Berlin, Berlin, Germany

³ Berlin Institute of Health at Charité – Universitätsmedizin Berlin, Berlin, Germany

⁴ Department of Neurosciences, Psychology, Drug Research and Child Health (NEUROFARBA), University of Florence, Florence, Italy

⁵ Neuroscience Research Center (NWFZ), Charité – Universitätsmedizin Berlin, corporate member of Freie Universität Berlin and Humboldt-Universität zu Berlin, Berlin, Germany

⁶ Experimental Pharmacology and Oncology Berlin-Buch GmbH, Berlin, Germany

⁷ Neurologic Clinic and Policlinic and Research Center for Clinical Neuroimmunology and Neuroscience, Departments of Medicine, Biomedicine, and Clinical Research, University Hospital Basel, University of Basel, Basel, Switzerland

⁸ Department of Immunobiology, Yale University School of Medicine, New Haven, CT 06511, USA

⁹ Department of Neurology, Yale University School of Medicine, New Haven, CT 06511, USA

¹⁰ Neuroscience Clinical Research Center, Berlin, Germany

¹¹ Center for Stroke Research, Berlin, Germany

These authors contributed equally

* Correspondence: niels.von-wardenburg@charite.de; harald.pruess@dzne.de

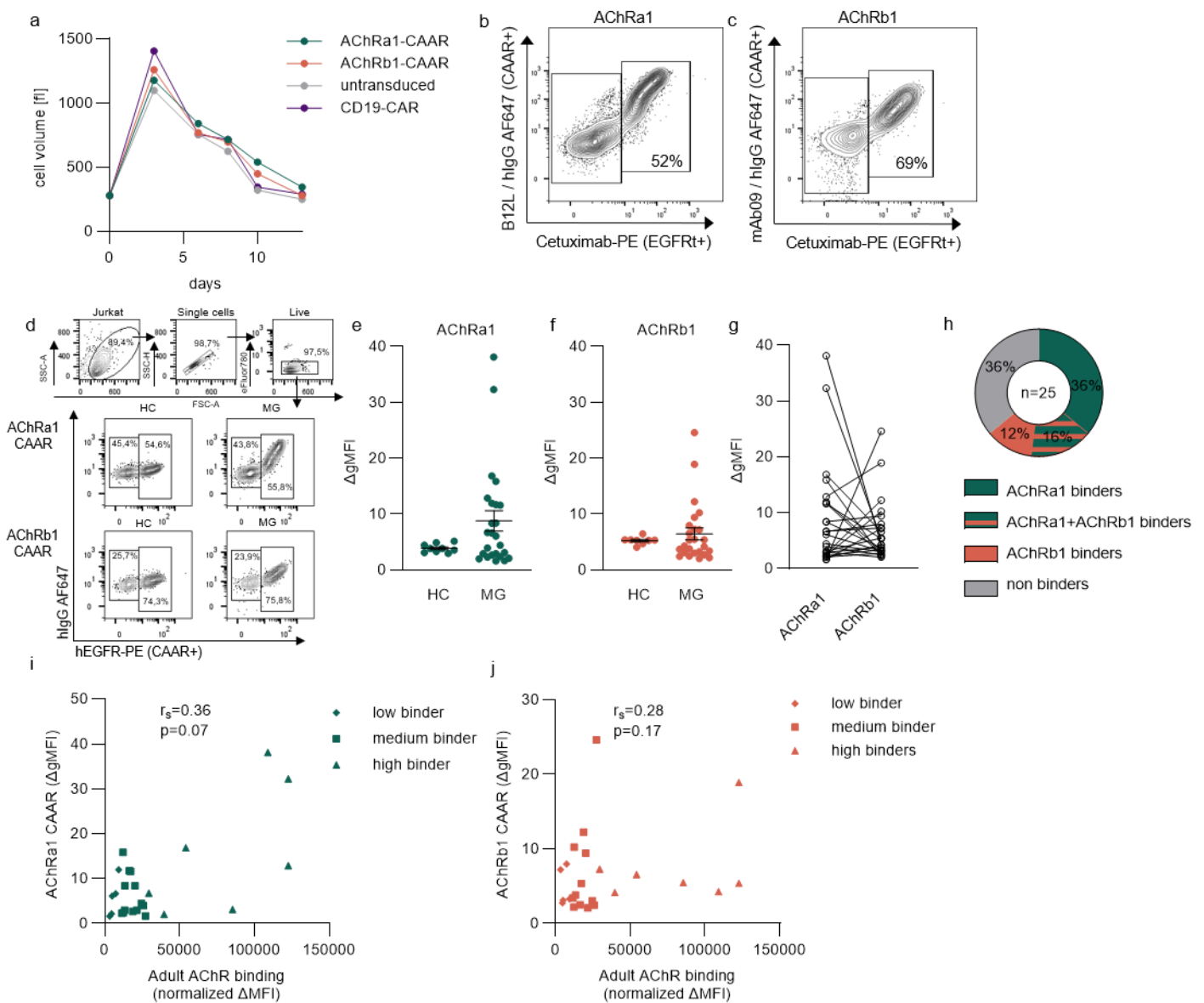
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Supplementary Fig. 1. Validation of CAAR expression by Jurkat cells and MG serum binding, related to Figure 1

(a) Changes in CAAR T cell size during expansion. T cell volume was determined by an automated cell counter.

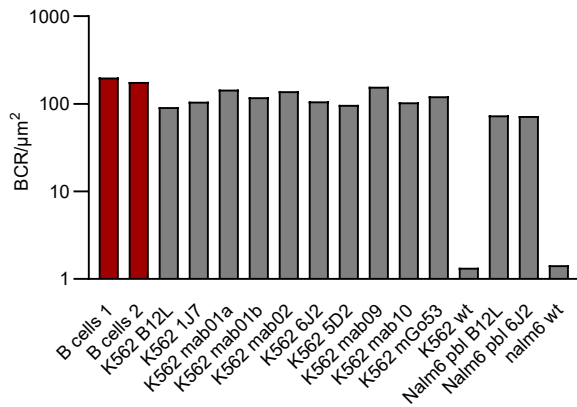
(b and c) Density plots showing EGFRt and CAAR expression in Jurkat cells. AChRa1 CAAR (b) and AChRb1 CAAR (c) expression was tested via flow cytometry by B12L (anti-AChR α 1) and mab09 (anti-AChR β 1) binding, respectively.

(d-f) IgG binding of MG and HC serum on AChR CAAR T cells. (d) indicates the gating strategy. Binding to AChRa1 (e) or AChRb1 (f) CAAR expressed on Jurkat cells was assessed by flow cytometry. Scatter plots indicate geometric mean fluorescence intensity (gMFI) of EGFRt+ cells minus gMFI of EGFRt- cells (Δ gMFI). Error bars represents mean + standard error of the mean. Data are from a single experiment.

(g) Scatter plots indicate Δ gMFI of MG serum as in (e) and (f). Connecting lines illustrate data points from individual patients. Data are from a single experiment.

(h) Percentage of binders to AChRa1 CAAR (green), AChRb1 CAAR (red), AChRa1 CAAR and AChRb1 CAAR (red and green) or non-binders (gray) are shown in a donut chart. Cut-off for binding was defined based on the mean Δ gMFI of control samples + 3 SDs.

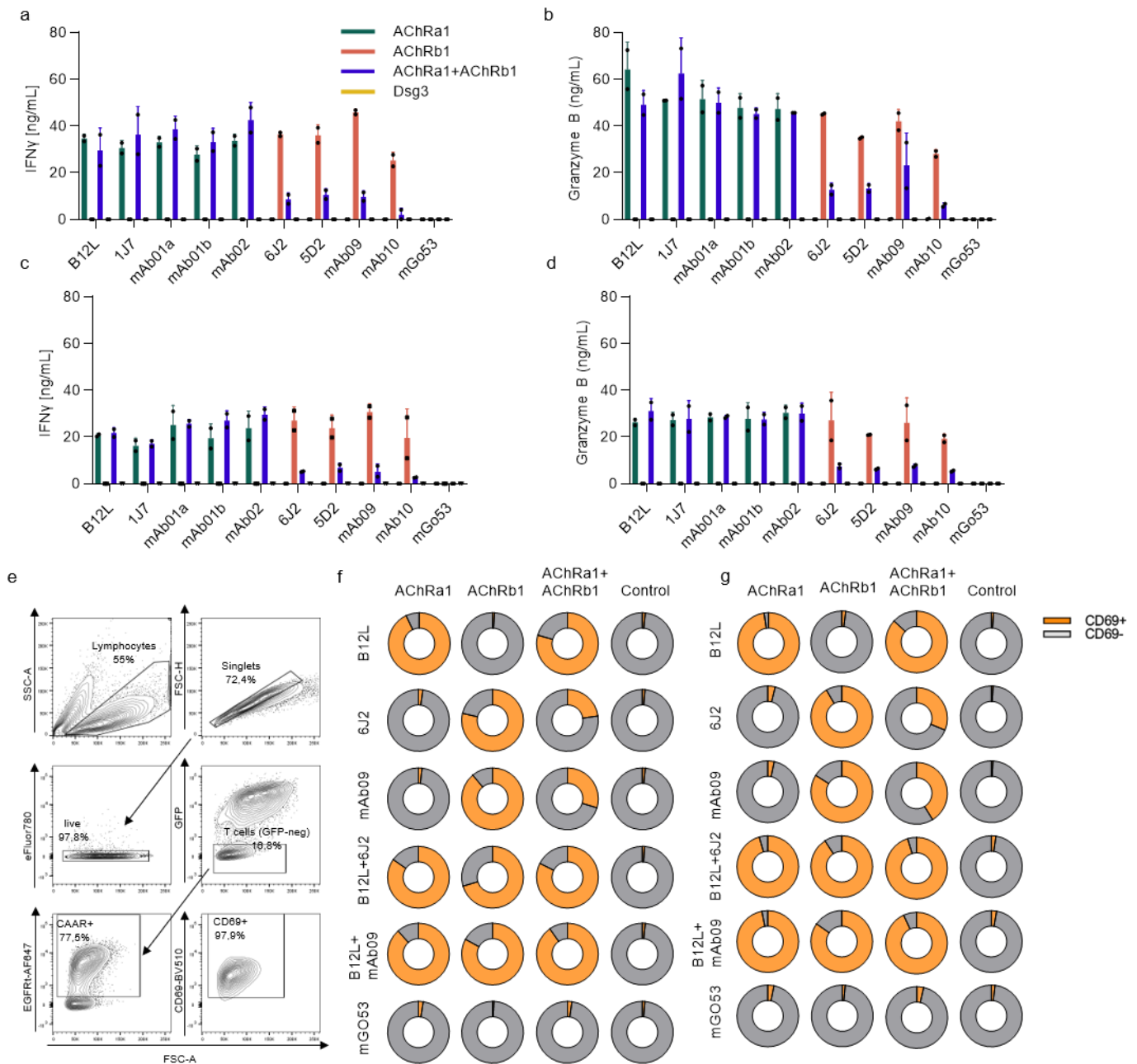
(i and j) Correlation of serum binding to AChRa1 CAAR (i) and AChRb1 CAAR (j) with binding to full AChR receptor as measured by RIA. Statistical analysis was performed by two-tailed Spearman's correlation. Data are from a single experiment. Gating strategy for these analyses is shown in Supplementary Fig. 7c



Supplementary Fig. 2. Validation of BCR expression by target cells, related to Figure 2

Bar diagram showing B cell receptor density based on monoclonal anti-human-IgG antibody binding as determined by flow cytometry. Quantification was performed using APC quantification beads. Cell surface was determined by an automated cell counter. Data are from a single experiment. Gating strategy is shown in Supplementary Fig. 2d.

APC Allophycocyanin; BCR: B cell receptor; wt: Wild type



Supplementary Fig. 3. Target cell-induced secretion of effector molecules and expression of activation marker, related to Fig. 2

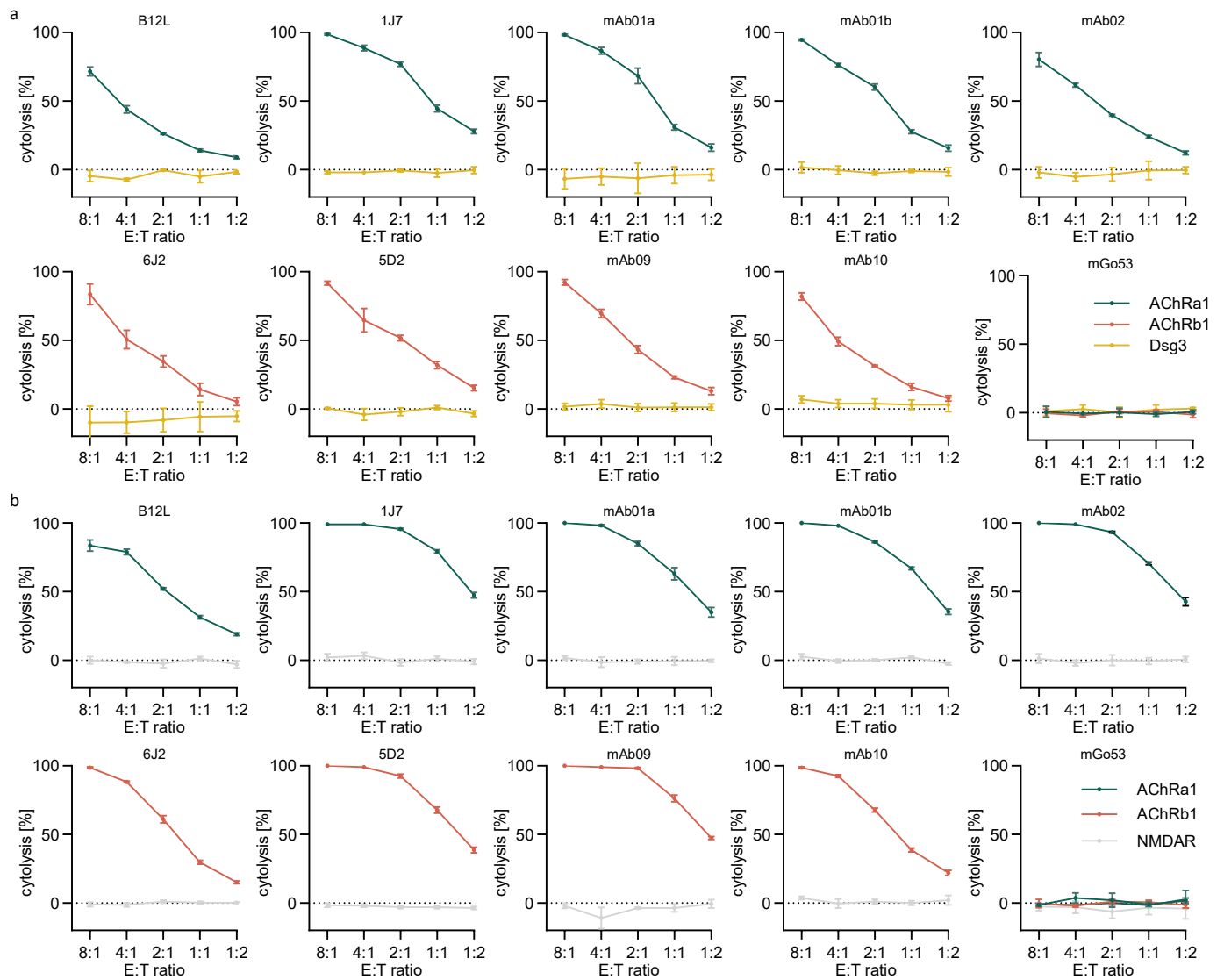
Donor-derived CAAR T cells expressing AChRa1, AChRb1, or co-transduced AChRa1+AChRb1 were co-cultured with K562 cells engineered to express AChRa1- or AChRb1-specific autoantibodies as surface B cell receptors (BCRs), to assess T cell activation and proliferation.

(a-d) After 24 hours of co-culture of CAAR T cells from two different donors, supernatants were collected and analyzed by ELISA for IFN γ (a, c) and GrB (b, d) levels. Bars represent mean $\pm$ SD of triplicate cultures.

(e) Gating strategy used to determine percentage of CD69+ CAAR T cells.

(f, g) Pie charts showing the percentage of CAAR T cells of two different donors expressing the early activation marker CD69 after 20 hours of co-culture, as determined by flow cytometry.

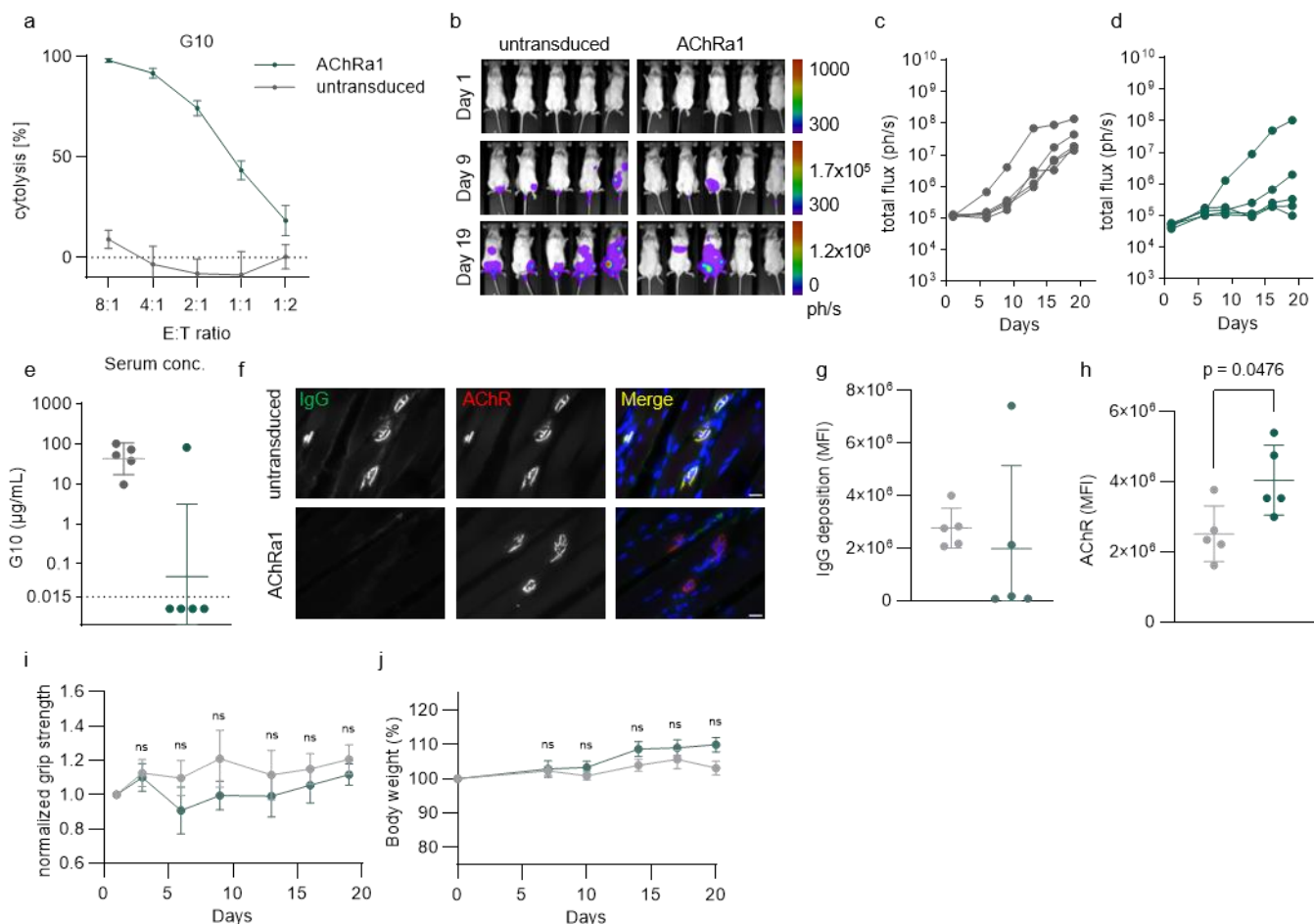
AChRa1/b1: Acetylcholine Receptor $\alpha 1$ / $\beta 1$ subunit; CAAR: Chimeric autoantibody receptor; CD: Cluster of differentiation; Dsg3: Desmoglein-3; EGFRt: truncated epidermal growth factor receptor; ELISA: Enzyme-linked immunosorbent assay; FSC-A: Forward scatter (area); FSC-H: Forward scatter (height); GFP: Green fluorescent protein; GrB: Granzyme B; IFN γ : Interferon gamma; SD: Standard deviation; SSC-A: Side scatter (area)



Supplementary Fig. 4. AChR CAAR T cells eliminate target cells in-vitro, related to Fig. 3

(a, b) AChR CAAR T cells from two different donors were incubated with ffluc-expressing target cell lines for 16-20 hours, followed by measurement of specific cytolysis via luciferase activity. Mean values $\pm$ SD of triplicate cultures are shown. Dsg3 or NMDAR CAAR T cells were included as negative controls.

AChR α 1/ β 1: Acetylcholine receptor α 1/ β 1 subunit; CAAR: Chimeric autoantibody receptor; Dsg3: Desmoglein 3; E:T ratio: Effector-to-target cell ratio; ffluc: Firefly luciferase; NMDAR: N-methyl-D-aspartate receptor; SD: Standard deviation.



Supplementary Fig. 5. AChRa1 CAAR T cells eliminate G10 hybridoma cells and prevent AChR downregulation, related to Fig. 5

(a) AChRa1 CAAR T cells were incubated with fluc-expressing and AChR α 1-antibody secreting G10 hybridoma cells for 16-20 hours, followed by measurement of specific cytolysis via luciferase activity. Mean values $\pm$ SD of triplicate cultures are shown. Untransduced T cells were included as negative controls. Data are from a single experiment. Gating strategy is shown in Supplementary Fig. 7e

(b-h) G10 hybridoma cells were engrafted into immunodeficient NOG mice, followed by treatment with AChRa1 CAAR T cells. Target cell burden was monitored by in vivo bioluminescence imaging throughout the study and immunohistological analysis of gastrocnemius muscle was performed postmortem.

(b) Representative bioluminescence images on days 1, 9, and 20 after target cell injection.

(c, d) Serial quantification of G10 hybridoma cell burden by bioluminescence imaging. Total flux of individual mice treated with untransduced T cells (c) or AChRa1 CAAR T cells (d).

(e) Serum levels (day 24) of G10 antibody as quantified by ELISA. Scatter plots show values of individual animals and geometric mean $\pm$ geometric SD. No error bars are shown for groups with values below the measurable range. A 2-tailed Mann-Whitney test was used.

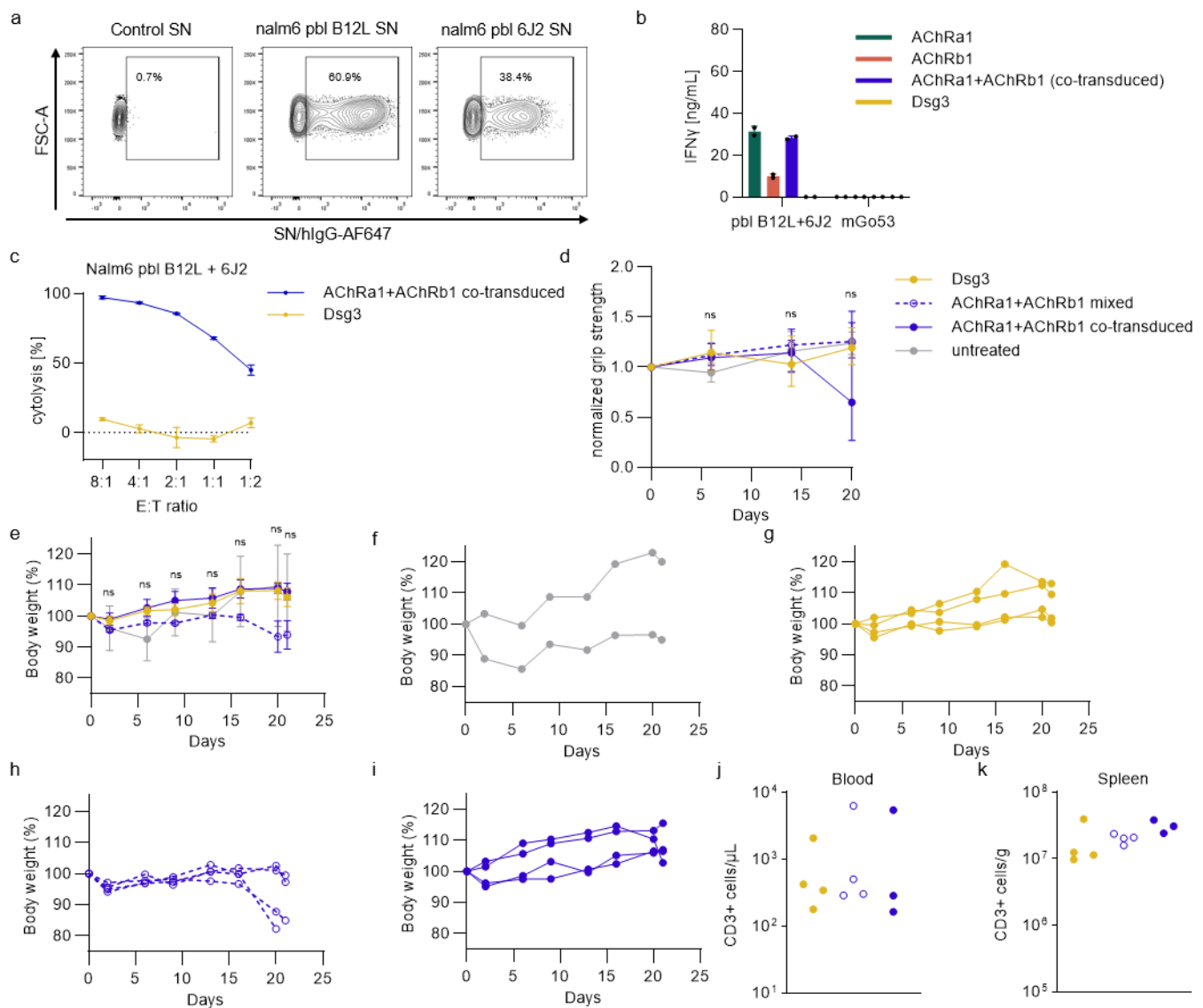
(f) Representative immunohistochemical images of gastrocnemius muscle stained. Images show IgG deposition (left column), AChR (middle column) and the merged composite of mice treated with untransduced T cells (top) or AChRa1 CAAR T cells (bottom). AChR was labeled by α -bungarotoxin. Scale bar indicates 20 μ m.

(g, h) Scatter plots showing mean MFI per animal of IgG deposition (G) or AChR (H) $\pm$ SD. Lines indicate median. A 2-tailed Mann-Whitney test was used.

(i) Mean grip strength $\pm$ SEM of individual animals are shown. For each animal, values were normalized to grip strength determined on the day of target cell injection. A two-way ANOVA mixed model with Tukey correction for multiple comparisons was used.

(j) Mean body weights $\pm$ SEM of individual animals are shown. For each animal, values were normalized to bodyweight determined the day before target cell injection. A two-way ANOVA mixed model with Tukey correction for multiple comparisons was used.

AChRa1: Acetylcholine receptor α 1 subunit; CAAR: Chimeric autoantibody receptor; ELISA: Enzyme-linked immunosorbent assay; E:T ratio: Effector-to-target cell ratio; fluc: Firefly luciferase; IgG: Immunoglobulin G; MFI: Mean fluorescence intensity; NOG: NOD.Cg-Prkdcscid Il2rgtm1Sug/JicTac; ns: Not significant; ph/s: Photons per second; SD: Standard deviation; SEM: Standard error of the mean



Supplementary Fig. 6. Antibody-secreting target cells and clinical evaluation of animals treated with AChR CAAR T cells, related to Fig. 5

(a-c) Nalm6 slg B12L and slg 6J2 were transduced to also secrete the respective antibody. These cells were named Nalm6 pbl B12L and Nalm6 pbl 6J2.

(a) Antibody secretion was validated by flow cytometry assessment of cell culture supernatant of nalm6 pbl B12L and nalm6 pbl 6J2 on Jurkat cells co-expressing AChRa1 CAAR and AChRb1 CAAR.

(b) AChR CAAR T were co-cultured with a 1:1 mixture of Nalm6 pbl B12L and Nalm6 pbl 6J2 cells. After 24 hours of co-culture, supernatants were collected and analyzed by ELISA for IFN γ . Bars represent mean $\pm$ SD of triplicate cultures. Data are from a single experiment.

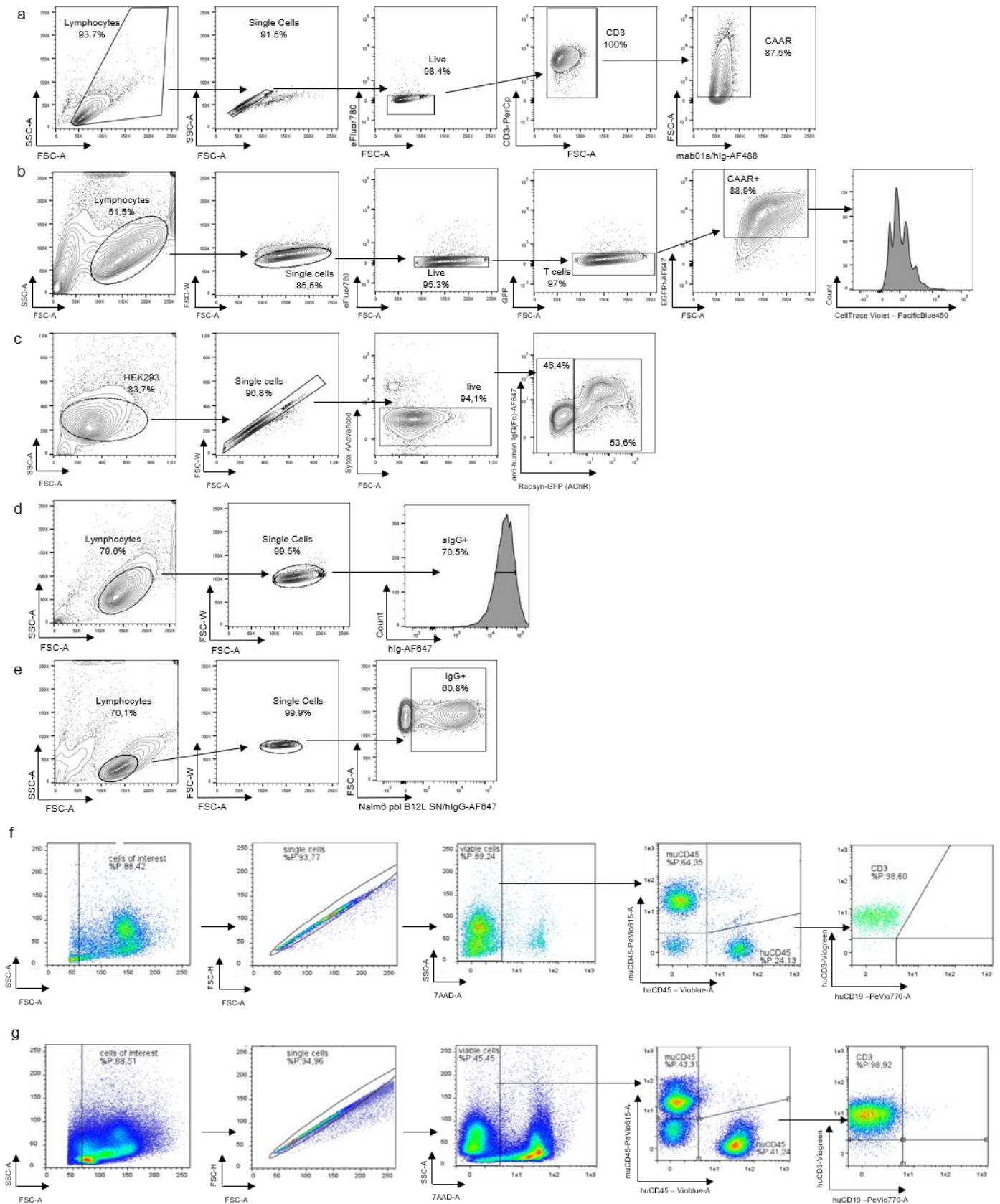
(c) AChR CAAR T cells were incubated with a 1:1 mixture of Nalm6 pbl B12L and Nalm6 pbl 6J2 cells for 16-20 hours, followed by measurement of specific cytotoxicity via luciferase activity. Mean values $\pm$ SD of triplicate cultures are shown. Data are from a single experiment.

(d) Grip strength was measured four times during the study period. Mean body weights $\pm$ SD of individual animals are reported. For each animal, values were normalized to grip strength determined the day before target cell injection. A two-way ANOVA mixed model with Tukey correction for multiple comparisons was used.

(e) Body weight was measured on days of bioluminescence measurements. Mean body weights $\pm$ SD of individual animals are reported. For each animal, values were normalized to bodyweight determined the day before target cell injection. A two-way ANOVA mixed model with Tukey correction for multiple comparisons was used.

(f-i) Body weight of individual (f) untreated animals, animals treated with (g) Dsg3 CAAR T cells, (h) mixed AChRa1 and AChRb1 CAAR T cells, or (i) AChRa1 and AChRb1 co-transduced CAAR T cells.

(j, k) Scatter plots showing CD3+ T cell counts in peripheral blood and in spleen as assessed by flow cytometry. Gating strategy is shown in Supplementary Fig. 7f (j, blood) and 7g (k, spleen).



Supplementary Fig. 7. Gating strategies

- (a) Gating strategy for the chimeric autoantibody receptor expression assays shown in Figure 1c-e.
- (b) Gating strategy for the proliferation assay shown in Figure 2e and 4d.
- (c) Gating strategy for serum AChR antibody binding to the full human AChR (adult isoform) shown in Supplementary Fig. 1i and 1j
- (d) Gating strategy for the B cell receptor density assay shown in Supplementary Fig. 2
- (e) Gating strategy for the assay shown in Supplementary Fig. 6a.
- (f) Gating strategy for analysis shown in Supplementary Fig. 6j
- (g) Gating strategy for analysis shown in Supplementary Fig. 6k

A: area; H: height; W: width; FSC= forward scatter area; SSC: side scatter; CD: cluster of differentiation; hu: human; 7-AAD: 7-amino-actinomycin-D; mu: murine; pbl: plasmablast-like; IgG: immunoglobulin G; hlgG: anti-human immunoglobulin G; AF647: Alexa Fluor 647; SN: supernatant; slgG: surface IgG; GFP: green fluorescent protein; AChR: acetylcholine receptor; Fc: fragment crystallizable; EGFRt: truncated epidermal growth factor receptor.

Supplementary Table 1. Characteristics of recombinant AChR autoantibodies. Related to all Figures.

mAb ID	Patient characteristics	Source	Specificity and isotype	Antigenic modulation	Complement activation	Direct blocking	In vivo pathogenicity
B12L	34y, M; gMG, MGFA= IVb	MBC ²	α 1, IgG1	yes	n.a. ¹	no	Yes (rat passive transfer MG model)
1J7	39y, F; gMG	CD19+ B cell ³	α 1, IgG1	yes ⁴	yes	no	Yes, when combined with 6J2 (rat passive transfer MG model)
6J2	63y, M; oMG	CD19+ B cell ³	β 1, IgG4 ⁵	yes ⁴	yes, in combination with B12L	no	Yes, when combined with 1J7 (rat passive transfer MG model)
5D2		CD19+ B cell ³	β 1, IgG4	n.a.	yes, in combination with B12L	no	n.a.
3I3	35y, M; gMG, thymoma	CD19+ B cell ³	β 1, IgG3	n.a.	yes	no	n.a.
mAb01a	21-25y, F; EOMG	B cells ⁶	α 1	yes	yes	yes ⁷	n.a.
mAb01b		B cells ⁶	α 1	yes	yes	yes	n.a.
mAb02	36-40y, F; EOMG	B cells ⁶	α 1	yes	yes	yes	n.a.
mAb09	86-90y, F; LOMG; MGFA=IVb	B cells ⁶	β 1	yes	yes	yes	n.a.
mAb10	46-50y, F; EOMG, MGFA=IIIb	B cells ⁶	β 1	yes	yes	no	n.a.

gMG = generalized myasthenia gravis; MBC = memory B cell; n.a.= not assessed; oMG= ocular myasthenia gravis;

¹ not assessed in vitro; evidence of C3 deposition colocalizing with α -BTX labelled nAChR at the NMJs at the histopathological analysis of the rat passive transfer model. ² sorted after α 1-ECD-PE bait from the CD19⁺⁺, IgG⁺⁺, antigen⁺⁺ gate. ³ antigen-specific B cells isolated with the membrane-antigen-capture activated cell sorting (MACACS) technique, after magnetic enrichment of B cells from PBMC. ⁴ nAChR reduction at the NMJ histopathological analysis (α BTX staining) in the passive transfer rat model. ⁵ cloned into an IgG1 backbone and expressed as IgG1 for in vitro and in vivo functional studies. ⁶ magnetic beads-enriched B cells cultured and differentiated in vitro into antibody-secreting cells. ⁷ as suggested by competition with α BTX, direct receptor blocking not demonstrated with electrophysiological studies.

AChR: Acetylcholine Receptor; α 1: Alpha 1 subunit (of AChR); β 1: Beta 1 subunit (of AChR); EOMG: Early-onset myasthenia gravis; F: Female; gMG: Generalised myasthenia gravis; IgG: Immunoglobulin G; LOMG: Late-onset myasthenia gravis; M: Male; mAb: Monoclonal antibody; MBC: Memory B cell; MGFA: Myasthenia Gravis Foundation of America (clinical classification system); n.a.: Not available; oMG: Ocular myasthenia gravis; y: Years

Supplementary Table 2. Clinical and demographic characteristics of MG patients included in the study. Related to Figure 1 and Supplementary Figure 1

Cohort 1 – Patients with high AChR autoantibody titers	
N. of patients	16
Median age at MG onset (IQR), years	52 (27.5-62.5)
N. of female patients (%)	9 (56%)
N. of patients with thymoma (%)	8 (50%)
Maximum MGFA grade, n. (%)	
I	1 (6%)
II	4 (25%)
III	2 (13%)
IV	4 (25%)
V	5 (31%)
Immunotherapy at sampling, n. (%)	
none	6 (38%)
prednisone	3 (19%)
prednisone + azathioprine	4 (25%)
azathioprine	2 (13%)
prednisone, azathioprine, eculizumab	1 (6%)
MGFA post-intervention status (PIS) at sampling, n. (%)	
Minimal manifestations-or-better	6 (37%)
Symptomatic (MGFA-PIS= improved, unchanged, worse)	10 (63%)
Cohort 2 – Patients with mixed AChR autoantibody titers	
N. of patients	25
Median age at MG onset (IQR), years	63 (49.5-70.5)
N. of female patients (%)	8 (32%)
N. of patients with thymoma (%)	10 (40%)
Maximum MGFA grade, n. (%)	
I	8 (32%)
II	10 (40%)
III	7 (28%)
IV	0
V	0
Immunotherapy at sampling, n. (%)	
none	8 (32%)
prednisone	11 (44%)
prednisone + azathioprine	3 (12%)
prednisone + mycophenolate mofetil	2 (8%)
MGFA post-intervention status (PIS) at sampling, n. (%)	
Minimal manifestations-or-better	14 (56%)
Symptomatic (MGFA-PIS= I, U, W)	11 (44%)

AChR: Acetylcholine Receptor; I: Improved; IQR: Interquartile range; MG: Myasthenia gravis; MGFA: Myasthenia Gravis Foundation of America (clinical grading system); MGFA-PIS: MGFA Post-Intervention Status; n.: Number; U: Unchanged; W: Worse.