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The effect of immunosuppression or thymectomy on the response to tetanus revaccination in myasthenia gravis

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ABSTRACT

Objective: To determine the effect of tetanus toxoid (TT) revaccination on circulating B-, T- and NK-cell compartments in myasthenia gravis (MG) patients.
Methods: Lymphocyte (sub)populations and differentiation stages were assessed by flow cytometry in 50 TT revaccinated MG patients. TT-specific proliferative responses were explored in PBMC cultures.
Results: In patients treated with azathioprine B- and NK cell numbers were strongly decreased. Lymphocyte (sub) populations remained unaffected upon TT revaccination. t All patients showed a significant TT-induced proliferative response.
Conclusion: TT revaccination is effective in MG patients with stable disease irrespective of their thymectomy status and medication and does not alter the composition of the lymphocyte compartment.

1. Introduction

Safety and efficacy of vaccinations has recently been heavily debated. For patients with autoimmune myasthenia gravis (MG) vaccination might be vital to prevent infections (Gilhus and Verschuuren, 2015). Pathogenic antibodies of the IgG isotype, a strong association with HLA-B8DR3 (Giraud et al., 2008; Janer et al., 1999), T-cell proliferation upon *in vitro* AChR stimulation(Lewis et al., 1995; Sisely et al., 1989), the presence of thymic abnormalities and effect of thymectomy (Wolfe et al., 2016) support a role for T cells in MG. Regulatory T cells (Treg) are believed to play an important role, by suppressing (autoimmune) inflammatory responses, but results of studies on frequency of Treg in MG patients, compared to healthy controls (HC), are contradicting (Huang et al., 2015; Jakubikova et al., 2015). Some studies report low frequency of these cells in MG patients while others report no difference (Huang et al., 2015; Thiruppathi et al., 2012a), or less functionality of Tregs (Balandina et al., 2005; Thiruppathi et al., 2012a; Thiruppathi et al., 2012b). Furthermore, little data exists on the effect of immunosuppressive medication (IM) and these cells. A large part of MG patients need long term IM, affecting the composition of the B- and T-cell compartments (Crosti et al., 1994; Han et al., 1999; Yilmaz et al., 2015). For example a lower frequency of CD4+ T-cells (Crosti et al., 1994; Han et al., 1999) and a higher frequency of CD19+ and CD27+ B-cells in IM treated patients (Yilmaz et al., 2015) has been reported. Whether altered composition of the B- and T cell compartments influence the response to vaccination in MG patients is not known. Vice versa it is not known how vaccination affects cellular immunity in (immunosuppressed) MG patients.

Recently, we reported that tetanus revaccination in MG is safe and induces a protective antibody response (Strijbos et al., 2017). The humoral response was not affected by thymectomy, but IM was associated with lower antibody titers (Strijbos et al., 2017). Here we investigated the effect of tetanus revaccination on the subsets of the B- and T-cells and NK-cells as well as the *in vitro* tetanus-specific T cell responsiveness in the same MG cohort, with a focus on the effect of IM and of a preceding thymectomy.

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2. Materials and methods

2.1. Standard protocol approvals, registrations, and patient consents

The study was approved by the Local Committee on Medical Ethics of the Leiden University Medical Centre. Subjects provided written informed consent for participation in the study and received reimbursement of travel costs. The tetanus trial is listed on clinicaltrialsregister.eu under 2014-004344-35.

2.2. Study protocol

This single-centre, prospective study was performed at the Leiden University Medical Centre. About 80 consecutive patients from our outpatient clinic were screened based on information containing the in- and exclusion criteria. A group of 51 patients with AChR MG and stable disease, meeting the inclusion and exclusion criteria indicated below, entered the study and was revaccinated with tetanus toxoid.

At the day of, but prior to, revaccination and 4 weeks thereafter a blood sample was obtained. One of the AChR MG patients was excluded from analysis because of receiving other vaccines (diphtheria/tetanus/polio (DTP) and typhoid), before the control time point 4 weeks after tetanus toxoid (TT) revaccination, resulting in a study cohort of 50 patients. A group of 28 historical healthy age- and gender -matched individuals without MG was used as a control cohort for comparison of the pre vaccination lymphocyte subsets; 15 of these healthy individuals were also investigated in the proliferation assays.

Inclusion criteria for patients were a confirmed diagnosis of MG, age between 18 and 65 years and stable disease during at least 3 months before participation. Diagnosis of MG was based on clinical signs or symptoms suggestive of MG and a positive serological test for AChR antibodies. Stable disease was defined as an unchanged dosage of IM in the 3 months before revaccination till at least 4 weeks after tetanus revaccination. A maximum daily dose of 30 mg of prednisolone (+/− 5 mg, in the 3 months before participation) was allowed as well as the use of other immunosuppressive medication (IM, see Table 1). Patients continued their medication during the study.

The exclusion criteria were instable disease, evidenced by a change in immunosuppressive medication, a Myasthenia gravis Foundation America (MGFA) classification of 4 or 5, presence of a thymoma, other relevant immunosuppressive/secondary immunodeficiency conditions, pregnancy, no previous tetanus vaccination or a tetanus revaccination in the past year. Healthy controls had neither immunosuppressive medication nor any auto-immune disease.

2.3. Tetanus vaccine

A commercially available tetanus vaccine was used, manufactured by Bilthoven Biologicals (tetanus vaccine, RVG 17639)(2013) (Tetanus vaccine SPC, 2013). One dose of 0.5 mL contains ≥40 IU tetanus toxoid (TT), 1.5 mg aluminium phosphate and 0.05 mg thimerosal. Administration was intramuscularly, as a bolus, in the non-dominant upper arm.

2.4. Patient samples

Leukocyte and lymphocyte counts were determined on a hematology analyser. Peripheral blood mononuclear cells (PBMC) were isolated by Ficoll density gradient centrifugation and stored in liquid nitrogen until further use.

PBMC were thawed in AIM-V (Invitrogen, Thermofisher Scientific, Waltham, MA, USA) supplemented with penicillin/streptomycin (PS) and 20% heat inactivated foetal calf serum (FCS, Greiner, Kremsmünster, Austria) and incubated for 5 min at 37 °C with DNase (1600 IU/mL; Sigma, St. Louis, MI, USA) and washed twice. Next PBMC were incubated for one hour at 37 °C to let viable cells recover, and subsequently live cells were counted and used in lymphocyte proliferation assays and/or flowcytometry.

2.5. Lymphocyte proliferation

Triplicate cultures of 1×10^5 PBMC per well were performed in 96 well round-bottom microtiter plates (Corning, Corning, NY, USA) in a final volume of 200 μL RPMI 1640 glutamax 1 (Invitrogen) supplemented with 10% heat-inactivated pooled human AB serum (Sanquin Bloodbank, Amsterdam, the Netherlands) and penicillin/streptomycin (PS; 100 U/mL /100 μg/mL; MBL, Woburn,MA, USA). Cells were cultured in medium only or stimulated with TT (0.2 or 20 Lf/mL; NIBSC, Potters Bar, Hertfordshire, United Kingdom) for 5 days at 37 °C and 5% CO2. Subsequently, 1 μCi/well ^{3}H-thymidine (Perkin Elmer, Waltham, MA, USA) was added 18 h before harvesting. ^{3}H-thymidine uptake of cultured PBMC was measured as counts per minute (cpm) of triplicate cultures by liquid scintillation counter (Perkin Elmer) and expressed as a stimulation index (SI) (ratio geometric mean cpm antigen-stimulated / geometric mean cpm medium control). SI ≥3.0 was considered as

Table 1
Characteristics of the AChR MG cohort and the controls.

	IM-	IM+	*p-value*	Total AChR MG	HC
Number of patients	27	23		50	28
Gender, female (%)	21 (78)	16 (70)	0.54	37 (74)	20 (71)
Age at entering the study, median years (range)	54 (21–65)	57 (22–65)	0.79	56 (21–65)	57 (24–67)
Duration of disease, median years (range)	7.0 (0.3–39)	14.0 (2–47)	0.044	9.5 (0.3–47)	–
MGFA* classification			0.12	4 (8)	–
Remission, N (%)	4 (15)	0		4 (8)	
1, N (%)	2 (7)	2 (8)		40 (80)	
2, N (%)	21 (78)	19 (83)		2 (4)	
3, N (%)	0	2 (9)			
Prednisolone, N (%)	–	13 (57)		13 (26)	–
Mean daily dose, mg (range)	–	10.4 (5–15)			–
Azathioprine, N (%)	–	15 (65)		15 (30)	–
Mean daily dose, mg (range)	–	108.3 (25–200)			–
Mycophenolic acid, N (%)	–	2 (9)		2 (4)	–
Mean daily dose, mg (range)	–	1250 (500–2000)			–
Cyclosporine, N (%)	–	3 (13)		3 (6)	–
Mean daily dose, mg(range)	–	141.7 (75–200)			–
Combination of immunosuppressive medication, N (%)	–	18 (78)		18 (36)	–
Thymectomy (>1 year ago), N (%)	12 (44)	17 (74)	0.05	29 (58)	–
Last tetanus vaccination, years ago, median (range)	24 (2–57))	20 (2–57)	0.65	22.5 (2–57)	–

* MGFA classification: Myasthenia gravis foundation America classification, IM-: without immunosuppressive medication, IM+: with immunosuppressive medication, HC: healthy controls.

evidence of antigen-induced proliferation(Brinkman et al., 2003), the clinical relevance of this threshold is unknown. However, this cut-off is relatively strict and we expect that this does indicate a robust antigen specific response, which is likely to be clinically meaningful. As positive control, the proliferative capacity of the PBMC was tested after mitogenic or polyclonal stimulation, using phytohemagglutinin (PHA, 5 μg/mL Murex, Dartford, England) and IL-2 (100 IU/mL, Chiron, Emeryville, CA, USA) or CD3 (coat 1 μg/mL OKT3, muromab; Janssen-Cilag, Beerse, Belgium), followed by ^{3}H- thymidine incorporation at day 4.

2.6. Flow cytometry

For lymphocyte subset determination and investigation of B-cell differentiation, PBMC were stained for 30 min on ice with a mixture of antibodies (see Table e-1) For analysis of T-cell differentiation PBMC were stained for 15 min at 37 °C with a mixture of antibodies. Samples for investigation of functional T helper cells were preincubated for 20 min at 37 °C with FcR blocking reagent and subsequently stained for 15 min at 37 °C with a mixture of antibodies (see Table e-2). After staining, the PBMC were washed twice before analysis.

T regulatory cells (Treg) were first stained with fixable vitality dye UV on ice and washed twice, subsequently preincubated for 20 min at 37 °C with FcR blocking reagent and next stained for 15 min at 37 °C with a mixture of antibodies staining surface membrane markers. Cells were fixed and permeabilized with FoxP3 bufferkit according to the manufacturer's protocol. After permeabilization cells were preincubated for 20 min at 37 °C with FcR blocking reagent, FoxP3 was stained intracellularly and next the samples were washed three times before analysis.

Samples were measured on a BD LSRII flowcytometer (BD Biosciences, San Jose, CA, USA) and data were analyzed using Kaluza software (Beckman Coulter, Brea, CA, USA).

2.7. Statistical analysis and power

The aim of this study is to investigate the effect of tetanus revaccination on the subsets of the B- and T-cells and NK-cells as well as the *in vitro* tetanus-specific T cell responsiveness. The study is powered for an expected humoral response rate of 75% with a 95%-confidence interval of 63–87%. Therefore, this study provides class III evidence for the primary research question. Lymphocyte proliferative responses are expressed as stimulation index (SI), geometric mean ± 95% confidence interval. Lymphocyte subsets and differentiation stages, determined using flowcytometry data, are expressed as absolute cell counts, geometric mean with 95% confidence interval. Unpaired *t*-test was used to compare two groups for numerical data and chi-squared test for categorical data. Comparisons between 3 groups were conducted using one-way ANOVA test. Correction for multiple testing is done in a one-way ANOVA with the Tukey multi comparison test and thereafter/and overall with a Bonferroni correction for the number of figures.

All analyses were performed using GraphPad Prism 7 (GraphPad Software, Inc., USA). In all tests a two-tailed $p < 0.05$ after correction for multiple testing was considered statistically significant. All data were log transformed. Within the AChR MG cohort, responses were compared between patients with (IM+) and those without (IM-) immunosuppressive medication and, within the IM-group, between patients with and those without thymectomy. These analyses were pre-planned. The patients with immunosuppression were also further divided in IM containing AZA and IM without AZA (IM+ other), based on our findings.

3. Results

3.1. Patient characteristics

Fifty AChR MG patients (74% female, median age 56 years, range 21–65 years) with a median disease duration of 9.5 years were included and revaccinated with TT. Fifty-eight percent of this cohort underwent a thymectomy in the past, which was always >1 year before entering the study. IM was given to 23 (46%) of the patients and included azathioprine (AZA) in 15 (65%) of them. Between patients with and without immunosuppressive medication (IM- versus IM+) there were no significant differences in baseline characteristics, except for the duration of disease since onset, this was significantly longer in the IM+ group ($p = 0.044$).

The age- and gender matched healthy controls (HC, $n = 28$) had a median age of 57 years, range 24–67 years. Baseline characteristics of the AChR MG cohort and the healthy controls are given in Table 1.

3.2. Lymphocyte proliferation in response to TT

All PBMC samples showed a proliferative response to PHA, indicating cell viability. To allow for an effect of antigen dose in the proliferation assay, we used two concentrations of TT for stimulation, *i.e.,* 0.2 and 20 Lf/mL. Upon *in vitro* TT stimulation with either concentration, a significant increase of the stimulation index (SI) was observed when comparing the post vaccination to the pre vaccination SI in patients with and without IM (Fig. 1A and 1B). The IM+ group had significantly ($p = 0.0174$) lower post vaccination SI than the patients who received no IM, but proliferation was still significantly increased after vaccination. (Fig. 1A and 1B). The post vaccination SI was comparable to that of cells from healthy controls, of whom the date of the most recent vaccination was unknown.

AZA is known to affect the B- and T-cell compartments. We investigated whether AZA was the cause for the above described differences in SI between the IM- and IM+ groups. By dividing the IM+ group into patients with and without AZA (AZA/IM+ other), it became apparent that AZA use did not selectively account for these reduced lymphocyte responses (Fig. 1C and 1D) because SI's were comparable between use of AZA and other IM. The SI pre and post vaccination per individual also showed a significant increase ($p = 0.0085$) of SI in the AZA group (Fig. 2). The thymus is essential for the development of T-cells. We investigated whether thymectomy affected the proliferative response. Our results show that a preceding thymectomy in patients without IM (to exclude an effect of medication) was not associated with a lower SI before revaccination, and the increase in SI after revaccination was similar in patients with and without thymectomy (Fig. 1E and 1F). The time since the previous tetanus revaccination before inclusion in the study or since thymectomy had no influence on the magnitude of the tetanus-specific proliferative response (Figure e-6).

3.3. Lymphocyte populations and B- and T-cell differentiation stages

Because we found lower SI in patients treated with IM, we investigated the absolute numbers of cells in subsets of the B- and T-cell compartments. AZA treated patients had slightly lower geomean counts of lymphocytes, T cells and T-cell subsets, whereas NK cell and B cell counts were significantly lower in this group compared to the HC, IM- and IM+ not receiving AZA (IM+ other) groups (Fig. 3). Geomean counts of leucocytes, lymphocytes, T cells, CD4+ and CD8+ T-cell subsets, TCRγδ+ T cells NK cells and B cells in the IM- group and in the IM+ other group were within the range of healthy adult controls. TT revaccination by itself had no effect on cell numbers (Fig. 3). Thymectomy status in patients without immunosuppression had no significant impact on the composition of the lymphocyte subpopulations (Figure e-1).

Analysis of the differentiation stages of the B-cells indicated that AZA treated patients had significantly lower counts in all stages analysed (Fig. 4 and Figure e-2). Because of the trend of lower counts of T-cells, CD4+ and CD8+ T-cell subsets in AZA treated patients, we also analysed the differentiation stages in the T-cell subsets. In the CD4+ T-cell subsets no significant differences between AZA treated patients compared to the patients without IM (IM-) or with IM not containing AZA (IM+ other)

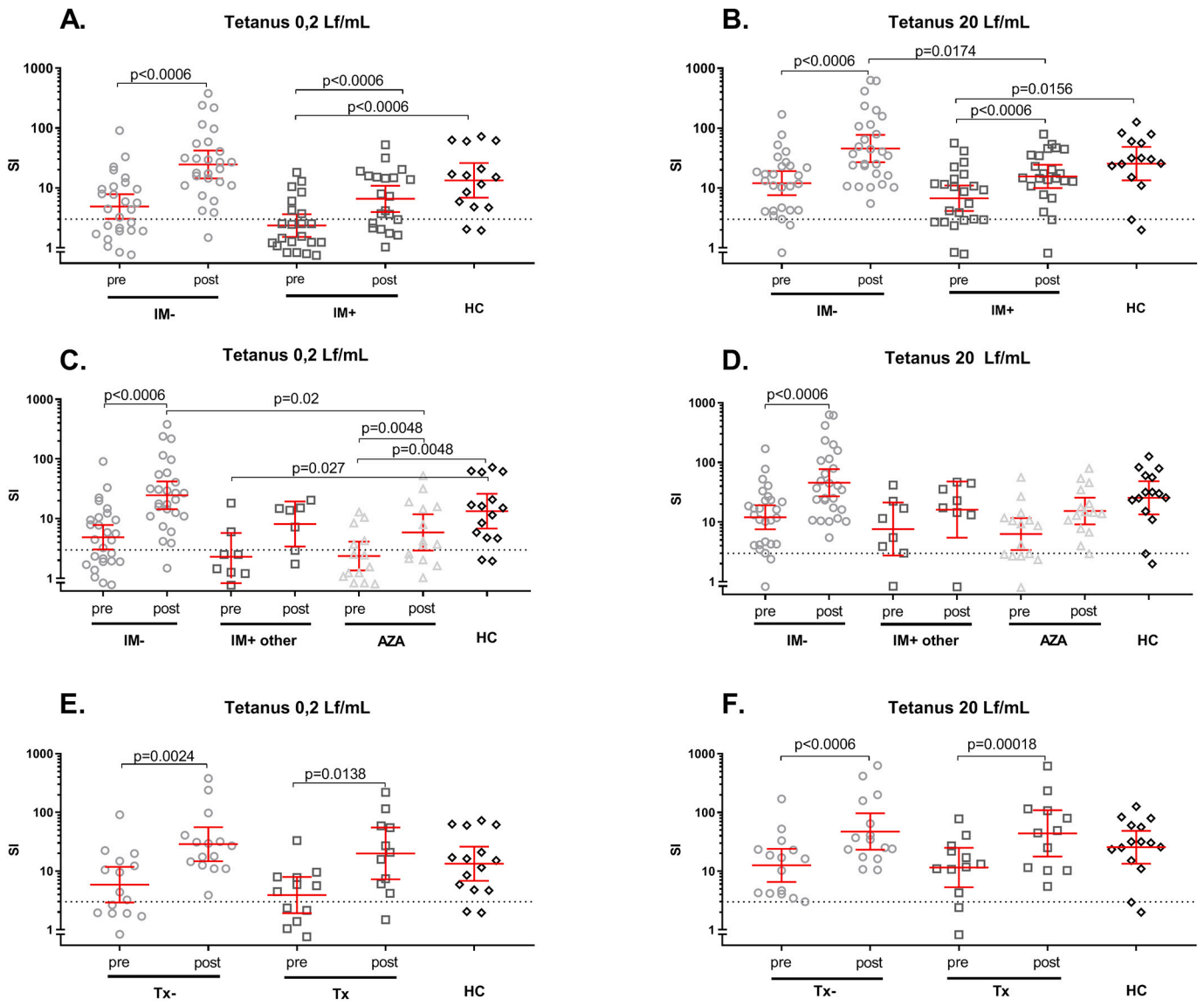

Fig. 1. Proliferative response of peripheral blood mononuclear cells (PBMC) after *in vitro* stimulation with tetanus toxoid. Responses of PBMC taken pre and 4 weeks post tetanus revaccination from myasthenia gravis patients were presented and expressed as stimulation index (SI). Panel A and B: comparison of patients treated with immunosuppressive medication (IM+) and those without (IM-). Panel C and D: comparison of IM- patients and IM+ patients, who are divided in patients receiving IM containing azathioprine (AZA) or not (IM+ other). Panel E and F: comparison of patients without (Tx-) and with (Tx+) thymectomy amongst the IM- group.
Two doses of tetanus toxoid were used for stimulation, *i.e.,* 0.2 (panel A, C and E) and 20 Lf/mL (panel B, D and F). HC: healthy age-matched controls who are not recently revaccinated.

were observed (Figure e-3). The same holds for the CD8+ T-cell subset, except that there was a significant effect ($p = 0{,}0275$) of azathioprine for the CD8+ central memory (CM) stage when comparing the AZA group with the IM- group (Figure e-4). Overall, we found that AZA usage is associated with a reduction of the numbers of memory T cells. The pattern pre TT revaccination was similar to that post revaccination.

3.4. Functional T-cell subsets

Since we found a trend towards reduced numbers of memory T cells in AZA treated patients, we investigated whether this trend was also observed in functional T-cell subsets. Defined on the basis of their discriminative phenotypical characteristics, various functional subpopulations in the memory and effector CD4+ T-cells, *i.e.*, helper T-cells type 1 (Th1), type 2 (Th2), type 17 (Th17), regulatory T-cells (Treg) and follicular T-helper cells (Tfh) were identified (Table e-1) and quantified. Again there was a trend, but not a significant difference, to lower counts across all of these functional CD4+ T-cell subsets in the group of AZA treated patients in comparison with the IM-, IM+ other and HC groups (Fig. 5). Counts pre and post TT revaccination were similar. In comparison with HC, the ratios of Treg versus Th17 and Th1 versus Th2 tended to be higher in AChR MG patients, with exception of the ratio of Th1 versus Th2 in the AZA group. This difference was only significant for the Th1 versus Th2 ratio when comparing the IM- and the IM+ other group and the HC (Figure e-5).

3.5. Absolute numbers of B-cells compared to IgG total anti-TT titers

We previously reported that MG patients demonstrated significant antibody responses after TT revaccination, and in the present study we observed that AZA is associated with lower B-cell numbers. Therefore, we investigated whether there was a correlation between numbers of B-

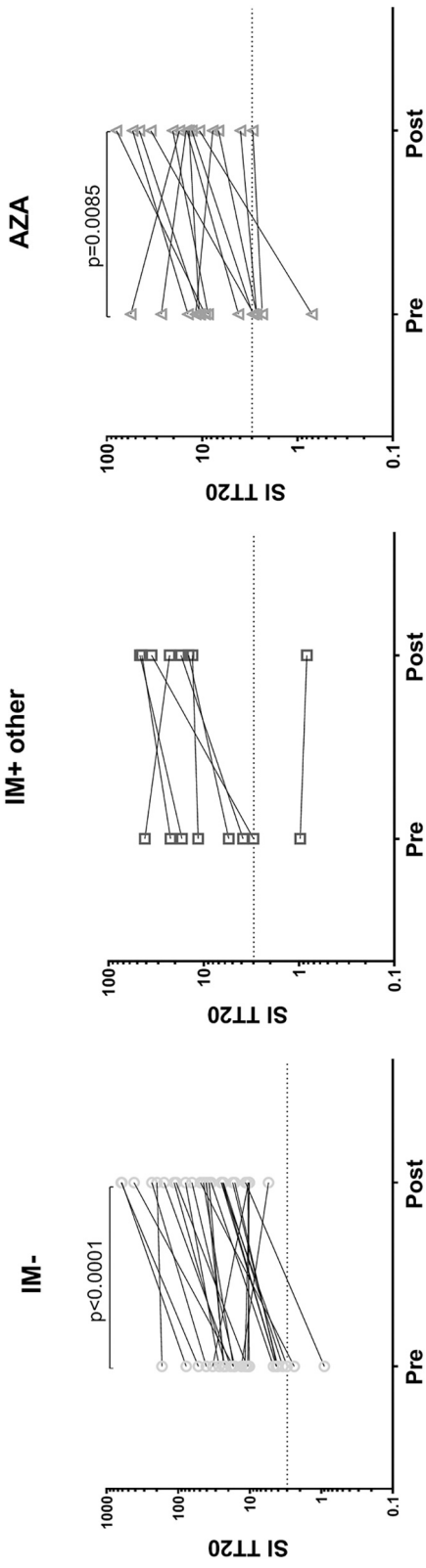

Fig. 2. Proliferative response of peripheral blood mononuclear cells (PBMC) after *in vitro* stimulation with tetanus toxoid. Responses of PBMC taken pre and 4 weeks post tetanus revaccination from myasthenia gravis patients were presented on an individual basis and expressed as stimulation index (SI). IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine.

cells and total IgG anti-TT levels pre and post revaccination. Interestingly, the results indicate that the absolute number of B-cells was not associated with levels of antibodies before or after revaccination. (Fig. 6).

4. Discussion

In the current, observational study, we investigated the proliferative response of T cells to a tetanus revaccination and the circulating numbers of B- and T-cell subsets and NK-cells. In a previous study we described the safety aspects and humoral immune response in this patient group (Strijbos et al., 2017). The study was performed in a cohort of AChR MG patients with stable disease, defined as having an unchanged dose of immunosuppressive medication or no immunosuppressive treatment for at least 3 months. Prior to revaccination the majority of PBMCs of these MG patients showed a specific proliferative response after *in vitro* stimulation with 20 Lf/mL tetanus. Patients without IM showed a higher increase of the proliferation post revaccination than patients with IM. Furthermore, tetanus revaccination had neither influence on the composition of the B-cells and T-cell subsets nor on the number of NK cells. Cell numbers in this AChR MG cohort were comparable to healthy controls, except in AZA treated patients, in whom B- and NK-cell numbers were significantly decreased. AZA is commonly used as treatment in MG and is known to affect the B- and T-cell compartments (Gilhus et al., 2011; Gilhus and Verschuuren, 2015; Jack et al., 2016). Despite lower absolute numbers of circulating B-cells in the AZA group, this was not associated with lower titers of IgG total anti-TT antibodies upon revaccination. To date, thymectomy is an accepted therapeutic intervention in AChR MG without thymoma (Wolfe et al., 2016). Thymectomy status did neither impact the tetanus-specific proliferative response nor the differentiation stages in $CD4^+$ and $CD8^+$ T-cell subsets. Treg/Th17 and Th1/Th2 ratios tended to be higher in patients than in controls. One could hypothesize that timing since thymectomy, in combination with IM, can influence the differentiation stages of the T-cell subsets. However, a limitation of this study is the sample size and that it is not powered to detect differences between these subgroups in our analyses. A study in a larger cohort should be performed to confirm these findings. Staining with a marker of cell proliferation during *in vitro* tetanus stimulation combined with phenotyping of distinct cell populations afterwards might elucidate more precisely the cell types proliferating upon tetanus simulation. However, the experimental design and small amounts of cells available hampered these studies. Fattorossi *et al.* found higher numbers of Treg pre thymectomy in patients with IM and described normalization of Treg numbers 12–16 months after thymectomy in these patients (Fattorossi et al., 2005). The patients without IM in their study had significantly lower Treg than both HC and patients with IM. On the other hand, Sun *et al.* reported higher Treg in thymectomized patients, but they did not differentiate between IM and no IM (Sun et al., 2004). In our study, we did not find an effect of thymectomy on Treg in both the IM+ and IM- groups (data not shown).

As shown in our study, the usage of azathioprine is associated with a strong decrease of B- and NK-cell counts in blood and, to a lesser extent, of T-cell differentiation stages and subpopulations of functional $CD4^+$ Th-cells. IM (prednisolone alone or in combination with azathioprine and other IM) has also been described to have an influence on the functionality of cells (Thiruppathi et al., 2012b; Yilmaz et al., 2015) and this is confirmed by the lower TT induced proliferative response of cells from patients who received IM in our study. We did not analyse the function of Treg. The effect of AZA on B- and NK-cells has also been reported in other autoimmune diseases like lupus and chronic glomerulonephritis, Bechet's disease and inflammatory bowel disease (Hasan et al., 2017; Orandi et al., 2017; Tareyeva et al., 1980). In MG the effect of azathioprine resulting in lower NK-cell numbers has not been described before. Kohler *et al.* reported a lower frequency of CD27− IgD+ naïve B cells and a higher frequency of CD27+ IgD− memory B

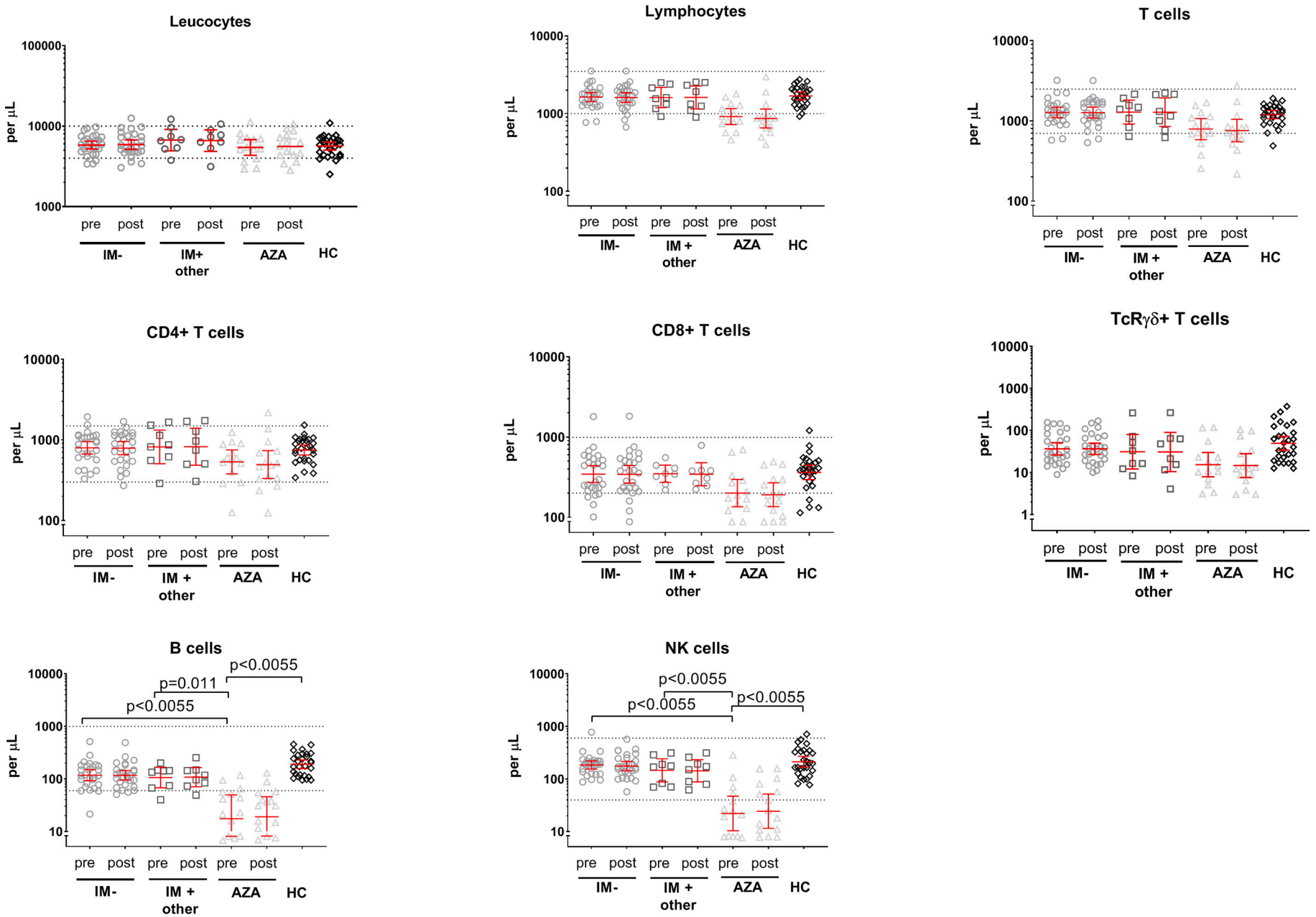

Fig. 3. Numbers of leukocytes, lymphocytes, T cells and CD4+, CD8+ and TCRγδ+ T-cell subsets, B cells and NK cells. Blood of myasthenia gravis patients was taken pre and 4 weeks post tetanus revaccination. IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine. HC: healthy age-matched controls who are not recently revaccinated.

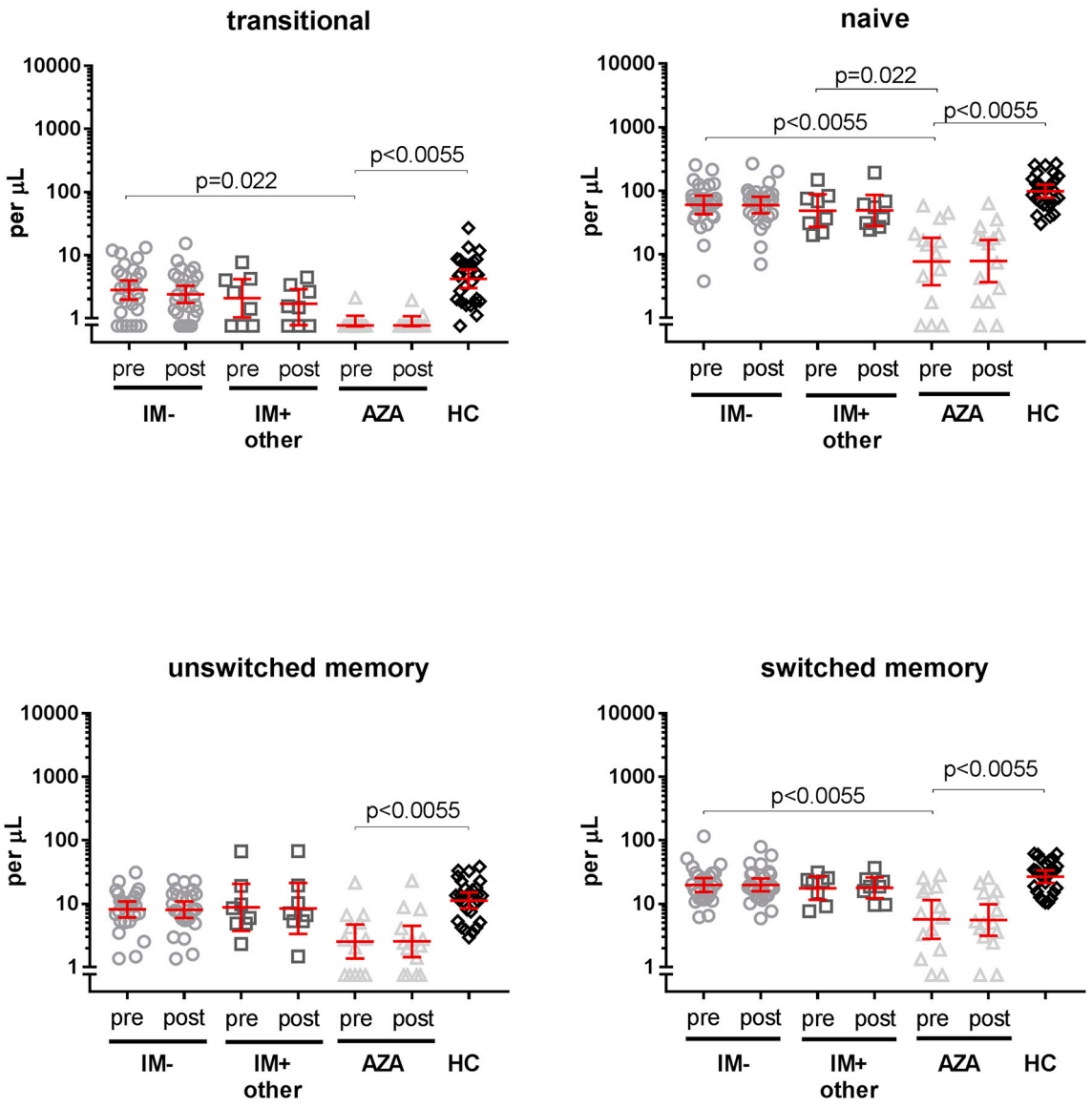

Fig. 4. Numbers of transitional, naive, unswitched memory and switched memory differentiation stages within the B-cell population. Blood of myasthenia gravis patients was taken pre and 4 weeks post tetanus revaccination.
IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine. HC: healthy age-matched controls who are not recently revaccinated.

cells in MG patients with IM, but which IM other than steroids was given is not specified (Kohler et al., 2013). The most known effect of AZA is lymphopenia (Gilhus and Verschuuren, 2015; Jack et al., 2016).

Overall, studies in MG on differences in the B- and T-cell compartments are often performed in heterogeneous patient populations, making it difficult to compare the results of these studies and to point out a specific cause for differences in immune parameters when compared to healthy controls. Patient selection is of great importance to compare these types of studies.

Our previous studies in MG patients on the humoral response to tetanus and influenza showed that (re)vaccination is effective and safe, based on stable AChR antibodies (*i.e.* no significant change of the AChR antibody titer pre- and post-vaccination) and stable clinical outcome measures (Strijbos et al., 2017; Strijbos et al., 2019). Such studies on vaccination in MG are rarely performed, but are of practical relevance for both patients and medical specialists, in order to relieve concerns about worsening or exacerbation of symptoms (Strijbos et al., 2017; Strijbos et al., 2019). At present, there are several studies (Boekel et al., 2022; Farina et al., 2022; Sansone and Bonifati, 2022) reporting that SARS-CoV-2 vaccination is safe in patients with MG. This was also what could be expected based on earlier studies (Strijbos et al., 2017, 2019). However caution should be considered when exposing severely immunosuppressed individuals. To our knowledge, this study is the first to describe the proliferative response to revaccination with a T-cell dependent antigen and the effect of this revaccination on the composition of the T- and B-cell compartments in autoimmune MG.

4.1. Conclusion

In conclusion, revaccination of MG patients with stable disease leads to a clear increase of the *in vitro* tetanus-specific proliferative response of T cells in both MG patients with or without IM, although, the SI's in patients treated with IM are lower. Surprisingly, we could not detect a significant effect of a preceding thymectomy on the proliferative response nor on composition of the T- and B-cell compartments. A significant effect of IM with or without azathioprine on the proliferative response was found. However, a significant decrease of the numbers of B-cell subsets and NK cells was only associated with azathioprine.

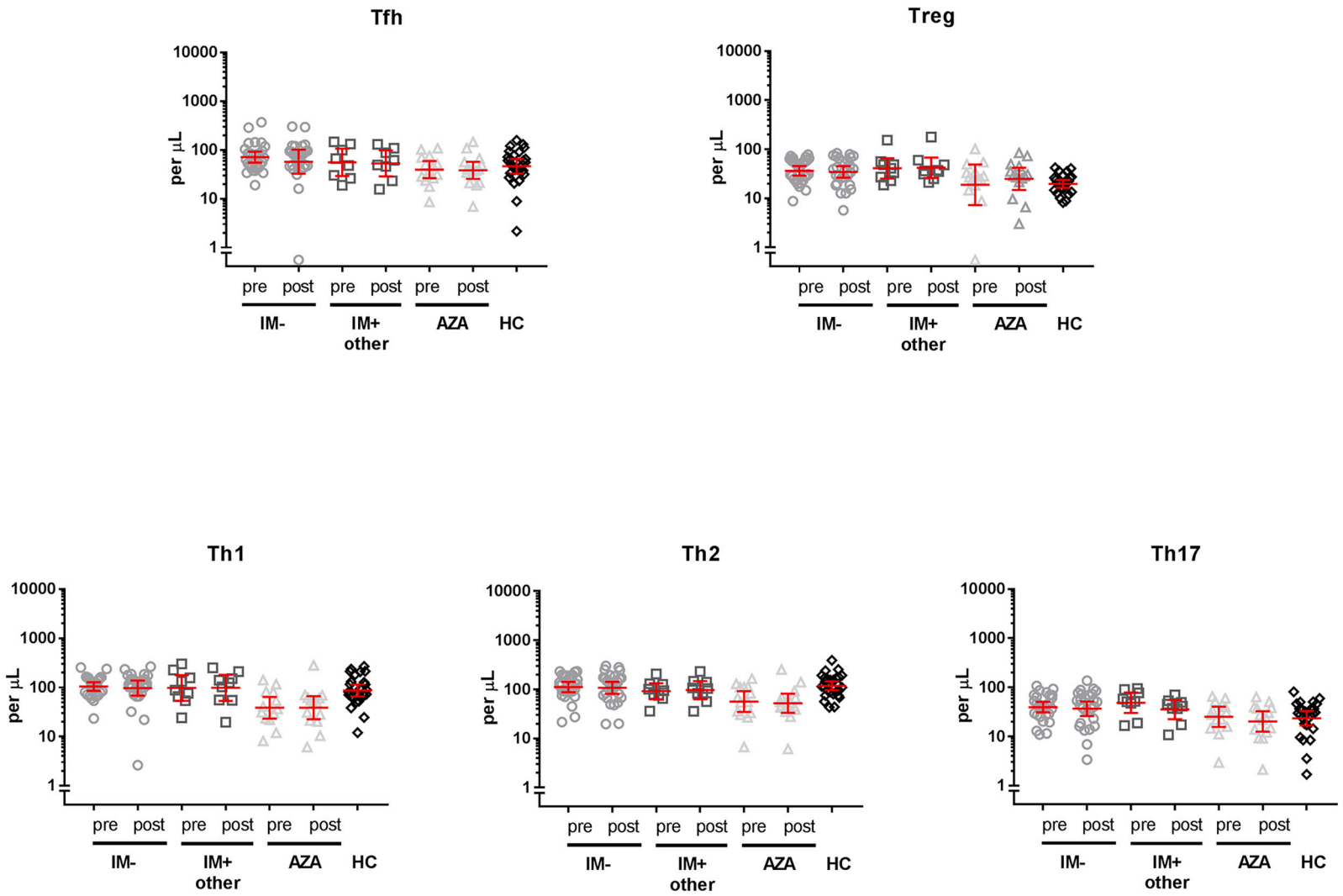

Fig. 5. Number of follicular T helper cells (Tfh), regulatory T cells (Treg), helper T cells type 1 (Th1), helper T cells type 2 (Th2) and helper T cells type 17 (Th17). Blood of myasthenia gravis patients was taken pre and 4 weeks post tetanus revaccination. IM-: patients not receiving immunosuppressive medication; IM+ other: patients treated with medication not containing azathioprine; AZA: patients treated with medication containing azathioprine. HC: healthy age-matched controls who are not recently revaccinated.

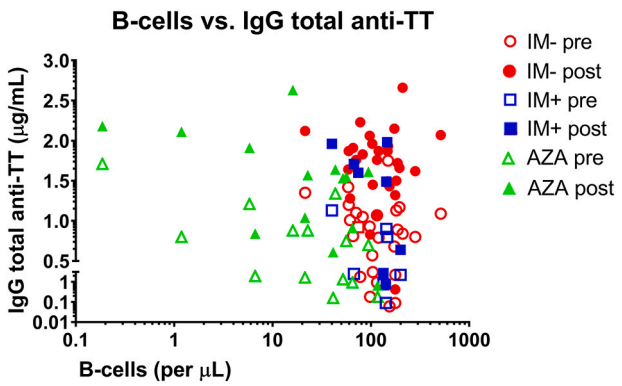

Fig. 6. Relation between numbers of B cells and levels of IgG anti-Tetanus toxoid (TT) antibodies.
Samples were taken from myasthenia gravis patients pre and 4 weeks post tetanus revaccination.
IM- (red circles): patients not receiving immunosuppressive medication; IM+ (blue squares): patients treated with medication not containing azathioprine; AZA (green triangles): patients treated with medication containing azathioprine. Open symbols: pre revaccination; closed symbols: 4 weeks post revaccination.

However, this azathioprine associated decrease in B-cell numbers had no impact on the IgG anti-tetanus response upon revaccination. Together with our previous study on the humoral response to tetanus revaccination in this cohort, our data supports the notion that tetanus revaccination is safe and effective in MG patients with stable disease and does not affect B- and T-cell responsiveness nor absolute numbers of T- and B-cell subsets.

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Disclosures

Ellen Strijbos reports no disclosures.
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MGH and JJGMV are coinventors on patent applications based on MuSK-related research. LUMC, MH, and JJGMV receive license income from these patents. The LUMC receives royalties for a MuSK antibody assay.

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Author appendix

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Monique van Ostaijen-ten Dam, BSc	Department of Paediatrics, Laboratory of Immunology, Leiden University Medical Centre, the Netherlands	Conceptualization, Data curation, formal analysis, investigation, methodology, writing - original draft, writing-review.
Carly Vervat, BSc	Department of Paediatrics, Laboratory of Immunology, Leiden University Medical Centre, the Netherlands	Investigation.
Marco Schilham, MD, PhD	Department of Paediatrics, Laboratory of Immunology, Leiden University Medical Centre, the Netherlands	Conceptualizaation, formal analysis, writing - original draft, writing - review.
Maartje Huijbers, MD, PhD	Department of Neurology and department of human genetics, Leiden University Medical Centre, the Netherlands	Conceptualizaation, formal analysis, writing - original draft, writing - review.t
Maarten van Tol, MD, PhD	Department of Paediatrics, Laboratory of Immunology, Leiden University Medical Centre, the Netherlands	Conceptualizaation, methodology, formal analysis, writing - original draft, writing - review.
Jan Verschuuren, MD, PhD	Department of Neurology, Leiden University Medical Centre, the Netherlands	Conceptualizaation, methodology, formal analysis, writing - original draft, writing - review.

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