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Altered naive CD4$^+$ T cell homeostasis in myasthenia gravis and thymoma patients

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ABSTRACT

In Myasthenia Gravis (MG) thymic pathologies are often present and thymectomy is used as treatment. By flow cytometry we elucidated alterations of naïve CD4$^+$ T cell homeostasis in MG patients and patients with thymoma. MG patients showed increased absolute numbers of CD31$^{-\ central}$naïve CD4$^+$ T cells. Thymoma patients displayed a significantly higher fraction of peripheral blood CD31$^{+\ thymic}$naive T cells. We show an altered naive CD4$^+$ T cell homeostasis in MG patients that might predispose to autoimmunity. Aberrant generation of T cells in thymoma can be detected by an increased frequency of CD31$^{+\ thymic}$naive CD4$^+$ T cells in the periphery.

1. Introduction

Myasthenia gravis is a chronic autoimmune disease characterized by muscle fatigability and weakness. In the majority of patients pathogenic antibodies against the AChR can be detected. For a long time it has been known that myasthenia gravis is associated with pathologies of the thymic gland. Around 10–15% of patient do harbour a thymic tumour that is differentiated according to histology in rather epithelial or lymphocytic subtypes. Furthermore most patients with an early onset form of the disease show a thymic lymphoid hyperplasia, an enlargement of the thymus with histological signs of germinal centers (Gilhus and Verschuuren, 2015). Consequently, thymectomy is used for treatment of MG and it has been shown to provide a significant clinical benefit (Wolfe et al., 2016). This argues for an important role of the thymus not only in the pathogenesis but also in the maintenance of the disease. In patients with thymic hyperplasia, the thymus might be regarded as starting point of T cell activation where consecutive generation of germinal centers and production of pathogenic antibodies takes place (Weiss et al., 2013). In contrast, in MG associated thymoma the abnormal generation of T cells is regarded to be important for disease pathogenesis. As measured by T cell receptor excision circle (TREC) content, CD4$^+$ and CD8$^+$ T cell output into the peripheral blood has been observed to be increased in thymoma patients with MG (Buckley et al., 2001), whereas in thymoma patients without MG, CD4 TREC levels were comparable to healthy controls(Buckley et al., 2001). In contrast, the frequency of CD45RA$^+$ CD62L$^+$ naïve CD4$^+$ T cells has been reported to be decreased in thymoma patients without MG, whereas thymoma patients with MG showed frequencies comparable to healthy controls (Strobel et al., 2002).

Some years ago, it has been described that CD45RA$^+$ CD62L$^+$ naïve CD4$^+$ T cells can be further differentiated into two distinct subpopulations by analysing their surface expression of CD31(Kimmig et al., 2002a; Kohler and Thiel, 2009; Kohler et al., 2005b). CD31$^{+\ thymic}$naive CD4$^+$ T cells are enriched in T cell receptor circles (TRECs), contain recent thymic emigrants (RTEs) display a polyclonal repertoire and might therefore be especially relevant in the generation of primary CD4$^+$ T cell responses. In contrast, CD31$^{-\ central}$naïve CD4$^+$ T cells display a rather low TREC content, are oligoclonal, seem to be generated by homeostatic proliferation of naïve CD4$^+$ T cells in the periphery and might contain autoreactive clones (Kohler and Thiel, 2009). In healthy donors the frequency and absolute count of CD31$^{+\ thymic}$naive CD4$^+$ T cells declines in correlation with age and the associated

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decrease of thymic function and these cells can be regarded as indicators of thymic function. In contrast, the absolute number of $CD31^{-\ central}$naïve $CD4^+$ T cells remains stable over time implying a peripheral regulation independent of thymic activity (Kohler et al., 2005b; Silva et al., 2017).

Using these markers, we demonstrate that naïve $CD4^+$ T cell homeostasis in an unselected cohort of MG patients is comparable to healthy controls. However, after exclusion of patients with potential confounding clinical characteristics, we detected an age-dependent increase in $CD31^{-\ central}$ naïve $CD4^+$ T cells in MG that is not present in healthy donors. We demonstrate here that an aberrant production of naïve $CD4^+$ T cells in thymoma can be detected in the peripheral blood by rather simple flow cytometric detection of $CD31^{+\ thymic}$naive $CD4^+$ T cells.

2. Materials and methods

2.1. Study participants

The study was approved by the local ethical committee at the Charité-University Medicine Berlin. All patients gave written informed consent in accordance with the Declaration of Helsinki in its currently applicable form. Patients with confirmed diagnosis of MG were included in this prospective study independently of disease duration, severity (excluding myasthenic crisis) and current treatment. Diagnosis of MG was confirmed by a neurologist with experience with myasthenia gravis patients. It was based on clinical symptoms, anti-AChR antibody titer, a positive electrophysiological measurement (decrement or single fiber testing) and/or a positive response to treatment with acetylcholinesterase inhibitors. No anti-Musk positive patients were included in the study. Patients were recruited in the Myasthenia Gravis outpatient clinic, the Departments of Neurology and the Department of Thoracic Surgery, Charité—University Medicine Berlin, Germany. Peripheral blood samples were obtained from 76 patients with MG with a median age of 49y. The majority of patients was anti-AChR antibody positive, 9 patients were seronegative (LRP4 was not tested), none was MuSk positive (Table 1).

Healthy controls consisted of 36 (15 male and 14 female) patients from the outpatient clinic of the Department of Orthopedic Surgery, Center for Musculoskeletal Surgery, Charité-University Medicine Berlin, Germany and healthy volunteers with a median age of 51 years and without any evidence of acute or chronic infection (Table 1).

Additionally, patients with thymoma and no co-existing MG were included in the study.

When the absolute number and frequency of $CD31^{+\ thymic}$naïve $CD4^+$ T cells was compared between thymoma patients and controls, both thymoma patients with and thymoma patients without MG were included in the thymoma group.

Table 1
Characteristics of MG patients and healthy controls included in the study.

	MG patients	Controls
Total number	$N = 76$	$N = 36$
Male/female	39.2%/60.8%	60.1%/39.9%[a]
Age (median)	49.0 years	52.4 years[b]
Ocular MG	26.3%	–
Antibody status	88.2% AChR/0% MuSK	–
No IS/CS only/IS	42.1% /15.8%/39.5%	–
EOMG/LOMG/T-MG	52.6% /27.6%/15.8%	–

No IS = no immunosuppression, CS only = corticosteroids only, IS = long term immunosuppression (azathioprine, mycophenolic acid or ciclosporin.
EO-MG = early onset MG, LO-MG late onset MG, T-MG = thymoma associated MG.
[a] Non significant (Fisher's exact test $p = .06$).
[b] Non significant (Mann Whitney U Test).

Thymic tissue was obtained from patients undergoing surgery for myasthenia gravis as an immunomodulatory therapy or if thymoma was suspected. The routine histopathological analysis of thymus tissue was performed by a clinical pathologist independent of the study.

2.2. Sample analysis

Freshly drawn heparinized blood samples were subjected to centrifugation on a Ficoll-Hypaque gradient (PAA laboratories). Cell suspensions were then washed twice in PBS/BSA/2 mM EDTA. Parts of representative hyperplastic or thymoma thymic tissue were removed under the supervision of a pathologist. Afterwards, tissue was mechanically disrupted and filtered. Cell suspensions were subjected to centrifugation on a Ficoll-Hypaque (PAA laboratories) gradient, followed by two wash steps with PBS/BSA/2 mm EDTA.

Resulting PBMCs/thymus mononuclear cells were incubated for 10 min with antibodies specific for CD3, CD4, CD45RA, CD62L and CD31, washed and subsequently analyzed on a LSR2 flow cytometer (Becton-Dickinson, Heidelberg, Germany). For analysis of $CD4^+$ T cell subpopulations we used FlowJo software (Treestar). A representative analysis is shown in Fig. 1. For determination of absolute numbers 50 µl of whole blood was stained in TruCount™ tubes (Becton Dickinson) with anti-CD45, CD3, CD4, CD8, CD19 and CD56 antibodies. After staining and erythrocyte lysis, cells were analyzed on a FACSCalibur (Becton Dickinson). Total $CD4^+$ T cells were identified as CD45, CD3 and CD4 positive cells and absolute $CD4^+$ T cell counts/µl of whole blood were determined by the ratio between analyzed cells and fluorescent TruCount™ beads according to manufacturer's instructions. By determining the frequency of $CD31^{+\ thymic}$ naive and $CD31^{-\ central}$ naive $CD4^+$ T among total $CD4^+$ T cells, their absolute numbers could be calculated. In some cases determination of absolute numbers of $CD4^+$ T cells was not possible for technical reasons and consequently absolute numbers of $CD4^+$ T cell subsets could not be calculated. Therefore there is a discrepancy in the sample size of groups used for determination of statistical differences of relative compared to absolute frequencies of $CD4^+$ T cell subsets.

2.3. Statistical analysis

Statistical analysis was performed with GraphPad prism and SPSS software. The individual statistical test used is indicated in figure legends or in the text. $P < .05$ (2-tailed) was regarded as statistically significant. If testing for normal distribution was negative, we carried out logarithmic transformation of data.

3. Results

3.1. Naive $CD4^+$ T cell homeostasis in MG patients is age dependent

First, we analyzed whether naive $CD4^+$ T cell homeostasis in MG patients is comparable to healthy controls. As previously described for normal blood donors (Kimmig et al., 2002a), we detected an age-dependent decrease in the frequency of $CD31^{+\ thymic}$naïve among naïve $CD4^+$ T cells (Fig. 2A, $p < 0{,}001$ Spearman-Rho Test). This was also evident on the level of absolute numbers of $CD31^{+\ thymic}$naive $CD4^+$ T cells, although this missed the level of statistical significance (Fig. 2B, $p = .063$, Spearman-Rho Test). On the contrary, absolute counts of $CD31^{-\ central}$naïve $CD4^+$ T cells showed no age-dependent decline again in line with previous observations in healthy controls (Fig. 2C) (Kohler et al., 2005b). Other parameters, like absolute counts of total $CD4^+$ or naïve $CD45RA^+CD62L^+CD4^+$ T cells, were not significantly correlated with age (data not shown).

Thus, naive T cell homeostasis in an unselected cohort of MG patients showed features like previously described for healthy controls with an age-dependent decline of $CD31^{+\ thymic}$naive $CD4^+$ T cells while numbers of $CD31^{-\ central}$naïve $CD4^+$ T cells remain constant (Kimmig

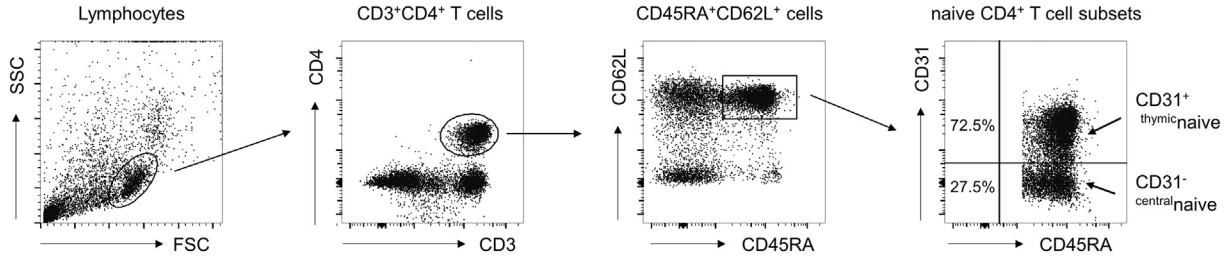

Fig. 1. Flow cytometric identification of naïve $CD4^+$ T cell subsets.
In Fig. 1 an exemplary analysis of $CD31^+$ ^{thymic}naive and $CD31^-$ ^{central}naive $CD4^+$ T cells as described in *Materials and Methods* is depicted. First lymphocytes were identified according to FSC/SSC characteristics. Then $CD4^+CD3^+$ cells were gated and subdivided according to CD62L and CD45RA expression. Among $CD62L^+$ and $CD45RA^+$ naive $CD4^+$ T cells, the frequency of $CD31^+$ ^{thymic}naive and $CD31^-$ ^{central}naive $CD4^+$ T cells was determined.

et al., 2002a; Kohler and Thiel, 2009; Kohler et al., 2005b).

3.2. Increased $CD31^-$ ^{central} naïve $CD4^+$ T cells in MG patients

Since we had included in our representative cohort a heterogeneous population of MG patients, we further excluded patients with any kind of immunosuppressive therapy, corticosteroids or history of thymoma, which might have confounding effects on naive $CD4^+$ T cell homeostasis.

Similar to healthy controls, there was a negative correlation between age and percentage of $CD31^+$ ^{thymic}naive $CD4^+$ T cells in the resulting cohort of MG patients (Fig. 3a, $p < .001$; Spearman-Rho, $n = 26$, median age 35y), but not in absolute numbers (Fig. 3b, $n = 19$, median age 35y). In contrast to healthy controls, we observed an age-dependent increase in numbers of $CD31^-$ ^{central}naïve $CD4^+$ T cells (Fig. 3c, $p < .003$; Spearman-Rho). Changes of absolute numbers of $CD31^-$ ^{central}naïve $CD4^+$ T cells were not associated to disease duration and our findings were not different when patients that had undergone thymectomy were excluded (data not shown).

We conclude that also in the absence of factors potentially modulating thymic activity, the frequency of $CD31^+$ ^{thymic}naive $CD4^+$ T cells is correlated to age in MG patients. However, in contrast to healthy donors, these patients are characterized by an age-correlated increased number of $CD31^-$ ^{central}naïve $CD4^+$ T cells.

3.3. Aberrant naïve $CD4^+$ T cell generation in thymoma patients

Finally, we aimed to evaluate whether the reported aberrant generation of $CD4^+$ T cells in thymoma patients (Buckley, Douek, 2001) can be detected on the cellular level in the peripheral blood by flow cytometric assessment of $CD31^+$ ^{thymic}naive and $CD31^-$ ^{central}naïve $CD4^+$ T cells. To this end, we restricted our statistical analysis to the subgroup of patients in which frequencies and absolute counts of $CD31^+$ ^{thymic}naive and $CD31^-$ ^{central}naïve $CD4^+$ T cells were analyzed on the day of thymectomy.

After adjusting for age the frequency of $CD31^+$ ^{thymic}naive $CD4^+$ T cells differed significantly depending on the histopathological subtype of the removed thymus tissue ($p = .035$, ANCOVA), while this was not the case for percentages of total naive $CD45RA^+$ $CD62L^+$ $CD4^+$ T cells ($p = 0{,}369$, ANCOVA, data not shown). Comparison of thymoma against normal thymus tissue revealed significant differences ($p = .011$ Fig. 4a), whereas results for thymoma versus hyperplastic thymus tissue were not significantly different. Similar findings were obtained for absolute counts of $CD31^+$ ^{thymic}naive $CD4^+$ T cells ($p = .038$ after logarithmic adjusting Fig. 4b). Differences became even more evident after exclusion of two thymoma patients with a WHO A and a A/B subtype, when in fact only Type B thymomas remained (for frequencies $p = .003$ and $p = .008$ for absolute counts, data not shown).

3.4. Impact of thymectomy on naïve $CD4^+$ T cell subsets

We also explored to what extent the therapeutic removal of the thymus in a disease modifying intention in MG does effect peripheral naïve $CD4^+$ T cell homeostasis. Excluding thymoma patients, we first compared patients that had been thymectomized to non-thymectomized MG patients and did not detect a significant difference. Moreover, the interval between thymectomy and the timepoint of our analysis correlates neither with naive $CD31^+$ ^{thymic} or $CD31^-$ ^{central} naïve $CD4^+$ T cells (regression analysis, $p = .428$ for $CD31^+$ ^{thymic}naive).

4. Discussion

This study aims to investigate the naïve $CD4^+$ T cell homeostasis in MG patients by determining the relative frequency among naïve $CD4^+$ T cells and the absolute numbers of $CD31^+$ ^{thymic}naive and $CD31^-$ ^{central} naïve $CD4^+$ T cells. First, we demonstrate that naïve $CD4^+$ T cell homeostasis in an unselected cohort of MG patients is comparable to healthy controls. However, after exclusion of patients with potential confounders such as immunosuppressive treatment, thymectomy or

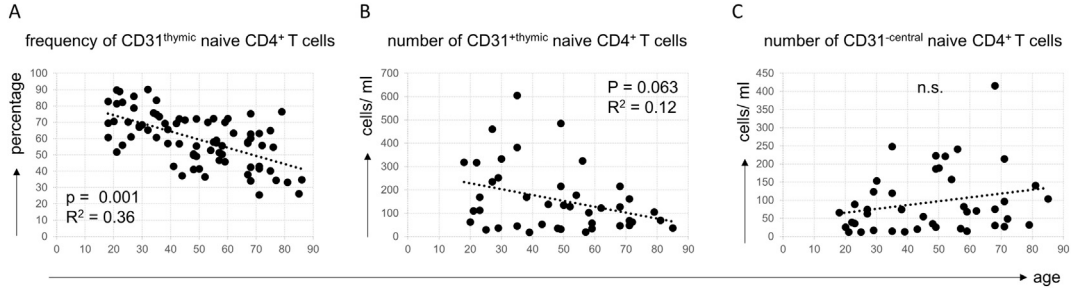

Fig. 2. Naïve $CD4^+$ T cell homeostasis in unselected MG patients.
Shown is an age dependent decline in frequencies (A, left, $n = 76$,median age 49y (range 18-85y)) and absolute numbers (B, middle, $n = 42$, median age 49y (range 18-85y)) of $CD31^+$ ^{thymic}naive $CD4^+$ T cells in MG patients. On the right (C, n = 42) the absolute number of $CD31^-$ ^{central}naïve $CD4^+$ T cells in this group of MG patients is depicted. P and R^2 values refer to results in Spearman-Rho testing.

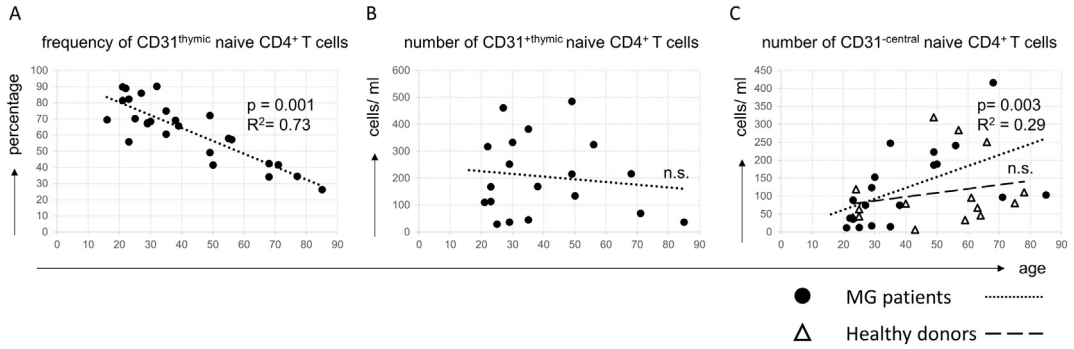

Fig. 3. Naïve CD4$^+$ T cell homeostasis in selected MG patients.
Shown is the age dependent decline in frequencies (A, left, $n = 26$, median age 35 years(range 18-85y)) and absolute numbers (B, middle, $n = 19$, median age 35 years (range 21-85y)) of CD31$^+$ ^{thymic}naive CD4$^+$ T cells in a cohort of MG patients without immunsuppressive therapy (IS), corticosteroids or history of thymoma. On the right(C) the absolute number of CD31$^-$ ^{central}naïve CD4$^+$ T cells in this group of MG patients is depicted (n = 19). Healthy controls consisted of 15 persons with a median age of 57y(range 25-78y). P and R^2 values refer to results in Spearman-Rho testing.

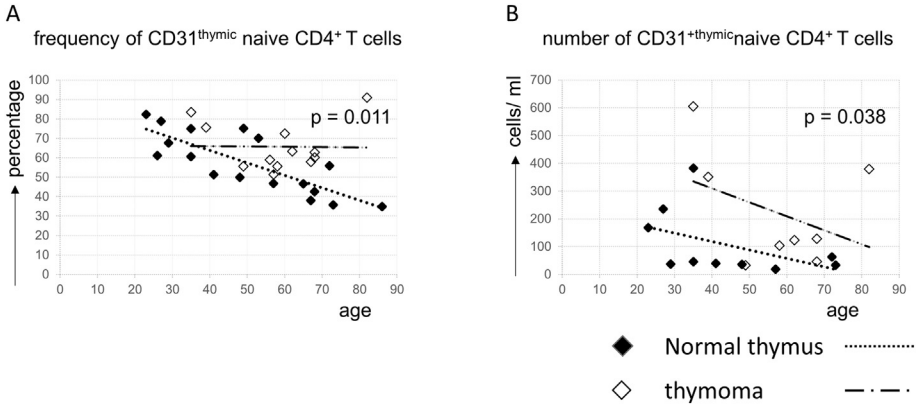

Fig. 4. Alterations of naïve CD4$^+$ T cells in thymoma.
Shown is the age dependent decline in frequencies (A, left, normal thymus group $n = 17$, median age 49y (range 23-86y), n = 4 w/o MG, $n = 5$ with IS, thymoma group $n = 12$, median age 59y (range 35-82y), n = 2 w/o MG, $n = 3$ with IS) and absolute numbers (B, right, normal thymus group $n = 10$, median age 38y (range 23-72y), n = 2 w/o MG, n = 3 with IS, thymoma group $n = 8$, median age 60y (35-82y), n = 2 w/o MG, n = 3 with IS) of CD31$^+$ ^{thymic}naive CD4$^+$ T cells in MG patients with normal thymus (closed squares), as compared to patients with thymoma (open squares). *P* values refer to results in Spearman-Rho testing.

thymoma, we detected an age-dependent increase in CD31$^-$ ^{central}naïve CD4$^+$ T cells in MG patients not present in normal healthy controls. Furthermore, we demonstrate for the first time that an aberrant production of CD4$^+$ T cells in thymoma patients can be revealed by flow cytometric assessment of CD31$^+$ ^{thymic}naive and CD31$^-$ ^{central}naïve CD4$^+$ T cells in peripheral blood.

It has been reported previously that absolute numbers of naïve and memory CD4$^+$ T cells in the peripheral blood are similar between thymectomized and non-thymectomized MG patients, as well as healthy controls. In contrast, CD3$^+$ T cell TREC levels were reduced in MG patients compared to healthy donors, an effect which was independent of previous thymectomy (Sempowski et al., 2001).

Our study partly confirms these findings with respect to counts of naïve CD62L$^+$ CD4$^+$ T cells, where we also did not detect significant differences. In contrast to the above mentioned study (Sempowski, Thomasch, 2001), in our representative MG group the distribution of CD31$^+$ ^{thymic}naive CD4$^+$ T cells is similar compared to healthy controls, indicating similar thymic output of naïve CD4$^+$ T cells. Also CD31$^-$ ^{central}naïve CD4$^+$ T cells were similar.

On the other hand, when patients with potentially confounding clinical characteristics like thymoma or immunosuppressive therapy were excluded, we detected an increased count of peripherally expanded TREC low CD31$^-$ ^{central}naïve T cells in MG patients compared to healthy controls. While this finding might be in line with the previous observations (Sempowski et al., 2001) their results were based on the analysis of total CD3$^+$ T cells and they do not provide a detailed description of their patient population and presence of potential confounders (Sempowski et al., 2001). Our results demonstrate that naïve CD4$^+$ T cell homeostasis in MG patients is altered, although currently we cannot provide a clear mechanistic explanation. However, as in particular the subset of CD31$^-$ ^{central}naïve CD4$^+$ T cells known to be clonally constricted most likely in response to self antigen in the periphery (Kohler and Thiel, 2009; Kohler et al., 2005b) is expanded, this might imply a predisposition to autoimmunity in MG patients. Therefore MG does share some similarity with multiple sclerosis and rheumatoid arthritis, where a dysbalanced thymic output and altered CD4$^+$ T cell homeostasis is equally implicated in disease pathogenesis(Koetz et al., 2000) (Duszczyszyn et al., 2010; Haegert, 2011; Haegert et al., 2011). We can only speculate why this is in our cohort not reflected in lower percentages of CD31$^+$ ^{thymic}naive CD4$^+$ T cells in MG patients. One reason might be an insufficient number of patients included in this analysis that results in lower sensitivity. Furthermore, we tested whether immunosuppressive drugs or clinical subtype (EOMG, LOMG or TMG) might influence the naive CD4$^+$ T cell parameters in our setting. However, we could not detect any statistically relevant difference.

The PCR-based analysis of TRECs in general is laborious, technically challenging and comparison of results obtained in different laboratories is difficult as different normalization standards exist. Moreover the analysis is restricted to analysis of cell populations (Douek et al., 1998; Jamieson et al., 1999; Sodora et al., 2000; Ye and Kirschner, 2002). In contrast, flow cytometric determination of naïve CD4 T cell homeostasis by measurement of frequencies and absolute counts of CD31$^+$ ^{thymic}naive or CD31$^-$ ^{central}naïve CD4$^+$ T cells determination represents a sensitive, robust and rather simple method requiring only minute amounts of blood (Kohler et al., 2005b). This method has reproducibly been demonstrated to correlate with thymic function in health (Duszczyszyn et al., 2006; Junge et al., 2007; Kilpatrick et al., 2008; Kimmig et al., 2002b; Kohler et al., 2005a) and disease (Bofill et al., 2006; Duszczyszyn et al., 2006; Isgro et al., 2005; Thiel et al., 2008) and can thereby be regarded as reliable tool to investigate thymic function (Kohler and Thiel, 2009).

In this study we wanted to confirm the reported increased naïve

$CD4^+$ T cell generation by MG-associated thymoma (Buckley et al., 2001), that has been detected by determination of TREC levels, by our method. Here, in line with the previously reported abnormal thymopoesis in thymoma patients (Buckley et al., 2001), we have detected a significantly increased frequency of $CD31^+$ ^{thymic}naive $CD4^+$ T cells in peripheral blood in correlation with thymic pathology. This difference is even more striking after exclusion of thymoma subtypes A and A/B where histologically by definition only a low content of lymphatic cells is present (Marx et al., 2015). With respect to this one might expect a drastically lower export of lymphocytes to the periphery as compared to other, more lymphatic, subtypes. Our findings not only confirm previous results but also imply that by peripheral blood analysis of $CD31^+$ ^{thymic}naive $CD4^+$ T cells a differentiation into thymomas with rather epithelial or lymphatic subtype could be possible (Marx et al., 2015). Unfortunately we did not have enough numbers of thymoma patients with different subtypes to prove this statistically. As the thymoma group included patients with and without coexisting MG, we can not draw any conclusion whether aberrant naïve $CD4^+$ T cell generation might be specific for MG.

We could not identify differences when comparing MG patients with thymoma and patients with thymic hyperplasia. This can be explained by the fact that, as reflected in our cohort, in which the oldest patient with thymic hyperplasia in our study was only 45 years, patients with thymic hyperplasia are usually of younger age (Tsinzerling et al., 2007) when high frequencies of $CD31^+$ ^{thymic}naive $CD4^+$ T cells are also detected in healthy donors (Kimmig et al., 2002a; Kohler and Thiel, 2009; Kohler et al., 2005b). Therefore, due to the high level of physiological $CD31^+$ ^{thymic}naive $CD4^+$ T cells in this age group, it is more difficult to detect aberrant thymopoesis. In contrast thymectomy in our samples has been performed in adult patients some of them well over the age of 50 years, thus at an age in which thymic function is already low. In this setting the sensitivity for the detection of a further decrease of thymopoesis is rather low and this might explain that in our study we could not show a significant impact of thymectomy on naïve $CD4^+$ T cell subsets.

In conclusion, we have been able to confirm altered naïve $CD4^+$ T cell homeostasis in MG patients in a cohort selected for the absence of potentially modulating factors like immunosuppressive therapy or thymoma. Additionally we have been able for the first time to detect aberrant thymopoesis in thymoma patients in the peripheral blood on the cellular level. Yet, further studies are needed in order to determine, whether this tool can be used as follow-up method for thymoma patients in order to analyse for signs of thymoma recurrence.

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Conflicts of interest

S.K., T.K., T.A., A.T., M.S., M.I., J.C.R. and A.M. report no conflict of interest.

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