Imbalance in T follicular helper cells producing IL-17 promotes pro-inflammatory responses in MuSK antibody positive myasthenia gravis

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ABSTRACT

A detailed understanding of the role of Tfh cells in MuSK-antibody positive myasthenia gravis (MuSK-MG) is lacking. We characterized phenotype and function of Tfh cells in MuSK-MG patients and controls. We found similar overall Tfh and follicular regulatory (Tfr) T cell frequencies in MuSK-MG and healthy controls, but MuSK-MG patients exhibited higher frequencies of Tfh17 cells and a higher ratio of Tfh:Tfr cells. These results suggest imbalanced Tfh cell regulation, further supported by increased frequencies of CD4 T cells co-producing IL-21/IL-17 and IL-17/IFN-γ, and increased Tfh-supported IgG production. These results support a role for Tfh cell dysregulation in MuSK-MG immunopathology.

1. Introduction

Myasthenia Gravis (MG) is an autoantibody-mediated autoimmune disorder of the neuromuscular junction characterized by fluctuating muscle fatigue and weakness (Conti-Fine et al., 2006; Vincent and Rothwell, 2004). MG may be classified into subtypes based on the presence of specific autoantibodies directed against proteins in the postsynaptic membrane of the neuromuscular junction, such as anti-acetylcholine receptor antibodies (AChR-MG), anti-muscle specific tyrosine kinase antibodies (MuSK-MG) and anti-lipoprotein receptor-related protein-4 antibodies (Conti-Fine et al., 2006; Meriggioli, 2009). In addition to different autoantibodies, patients with AChR-MG and MuSK-MG have further differences in clinical phenotype, response to treatments, and thymic changes (Guptill and Sanders, 2010; Leite et al., 2005). These differences, along with recently described differences in immune system function (Balandina et al., 2005; Yi et al., 2014; Yi et al., 2018), strongly support the need to further elucidate the immunopathology of these distinct MG subtypes.

Follicular helper T (Tfh) cells are pivotal in the generation of high-affinity memory B cells (Vinuesa et al., 2009). Tfh cells express CXCR5 (Morita et al., 2011), which directs them to the B cell follicle, where they provide vital signals to B cells through CD40L-CD40 interactions and the secretion of IL-21, thereby promoting differentiation and class switching (MacLennan et al., 2003). Although the interaction between Tfh and B cells take place in the germinal center, circulating Tfh cells have been identified by CXCR5 expression and are believed to represent a memory compartment of Tfh lineage cells (Brenna et al., 2020; Chevalier et al., 2011; Morita et al., 2011; Vella et al., 2019). Phenotypic differences exist between circulating Tfh cells and Tfh cells in lymphoid organs, and a subset of circulating Tfh cells have the capacity to support B cell maturation (Brenna et al., 2020; Chevalier et al., 2011; Morita et al., 2011; Sage et al., 2014; Schmitt et al., 2014; Vinuesa and Cook, 2011). Based on the differential expression of CXCR3 and CCR6, circulating Tfh cells can be subdivided into Tfh1 (CXCR3 + CCR6-), Tfh2 (CXCR3-CCR6-), and Tfh17 (CXCR3-CCR6+) cell subsets (Bentebibel et al., 2013; Morita et al., 2011). Of these Tfh subsets, only Tfh2 and Tfh17 cells are efficient in inducing naïve B cells to undergo class switching and secrete IgG (Boswell et al., 2014; Locci et al., 2013;

Morita et al., 2011). Thus, an in-depth evaluation of circulating Tfh cell subsets in autoimmunity permits a greater understanding of the potential for circulating Tfh cells to contribute to B-cell dysregulation.

Abnormal levels of circulating Tfh cells have been demonstrated in several autoimmune diseases, such as systemic lupus erythematosus (SLE) (Simpson et al., 2010), autoimmune thyroid disease (Zhu et al., 2012) and MG (Luo et al., 2013; Saito et al., 2005; Zhang et al., 2016a). In AChR-MG, higher frequencies of circulating Tfh cells were observed compared to healthy controls and this increase positively correlated with serum anti-AChR antibodies (Luo et al., 2013). In addition, decreased Tfr-like cells were demonstrated in AChR-MG patients (Wen et al., 2016; Zhang et al., 2016a). Collectively, the imbalance in Tfh and Tfr frequencies in AChR-MG patients supports a possible mechanism for enhanced memory B cell generation. Considering the different pathogenic mechanisms and response to treatment in AChR-MG and MuSK-MG, it is important to understand the role of Tfh cells in patients with MuSK-MG for supporting B cells and production of anti-MuSK autoantibodies.

We performed detailed phenotypic and functional profiling of circulating Tfh cells and their subsets in 31 MuSK-MG patients. We demonstrate that MuSK-MG patients exhibit an enhanced frequency of Tfh17 cells and an increased Tfh:Tfr cell ratio. MuSK-MG derived CD4 T cells also produced higher frequencies of IFN-γ, IL-17, and IL-21, and supported the production of IgG. Collectively, these data support a role for Tfh cells, particularly the Tfh17 subset, in supporting autoantibody production in MuSK-MG.

2. Materials and methods

2.1. Participants

MuSK-MG patients were recruited during visits to the Duke and the University of North Carolina-Chapel Hill (UNC) MG Clinics. All patients had detectable anti-MuSK antibodies according to commercially available testing (Athena Diagnostics, Worcester, MA and Mayo Laboratories, Rochester, MN), and clinical and electrodiagnostic features consistent with MG. Patients who had received rituximab were excluded from the study. Clinical information including demographics, age of symptom onset, disease duration, pharmacologic treatments, thymectomy status, Myasthenia Gravis Foundation of America (MGFA) severity class, and MG manual muscle testing (MG-MMT) were collected. This study was conducted in accordance with the guidelines of the World Medical Association's Declaration of Helsinki. This study was approved by the Duke University and UNC Institutional Review Boards and informed consent was obtained from each patient and control donor.

2.2. Isolation and storage of peripheral blood mononuclear cells (PBMCs)

Peripheral blood was obtained by venipuncture and collected in acid-citrate-dextrose tubes (BD Vacutainer, Franklin Lake, NJ). PBMCs were separated by Ficoll (GE Healthcare, Uppsala, Sweden) density gradient centrifugation, washed and counted prior to storage. Cells were re-suspended in a 90% FBS (Gemini, West Sacramento, CA) and 10% DMSO (Sigma, Aldrich, St. Louis, MO) solution, and progressively cooled to −80 °C in a CoolCell cell freezing container (BioCision, Larkspur, CA). The next day, the cells were stored in vapor phase liquid nitrogen for future use.

2.3. Antibodies

The following antibodies from BioLegend (San Diego, CA) were used for flow cytometry: APC-Cy7-conjugated anti-CD3 (clone SK7); BV711-conjugated anti-CD4 (clone RPA-T4); APC-Cy7-conjugated anti-CD8 (clone SK1); BB515-conjugated anti-CD25 (clone 2A3); BV650-conjugated anti-CD38 (clone HB-7); BV650-conjugated anti-CXCR3 (clone

G025H7); PE-Cy7-conjugated anti-CCR6 (clone G034E3); PerCP-eFluor 710-conjugated anti-CD39 (clone eBioA1); AlexaFluor 700-conjugated anti-CD45RA (clone HI100); BV421-conjugated anti-PD-1 (clone EH12.2H7); PE-Dazzle-conjugated anti-CCR4 (clone L291H4); BV605-conjugated anti-Ki-67 (clone Ki-67); PE-conjugated anti–FOXP3 (clone 206D); FITC-conjugated anti-IFN-γ (clone B27); PerCP-Cy5.5-conjugated anti IL-17A (clone BL168); PE-conjugated anti-IL-21 (clone 3A3-N2); Live/Dead violet dye; and PE-Dazzle-conjugated anti-IL-4 (clone MP4-25D2). AlexaFluor 647-conjugated anti-CXCR5 (clone RF8B2) was from BD and PE-Cy7-conjugated anti-ICOS (clone C398.4A) was from eBioscience.

2.4. Cellular analysis and flow cytometry

After thawing PBMCs and washing twice with RPMI medium contained 10% FBS (R10) (Gemini), cell number and viability were calculated. A total of 2×10^6 PBMCs were plated in 96-well round-bottom plates in R10. After centrifugation and removal of media, cells were surface stained with LIVE/DEAD violet dye (Life Technologies, Grand Island, NY) for 15 min at room temperature. Cells were washed with PBS, then stained with a surface stain cocktail mix for 30 min at 4 °C. Following cell surface staining, cells were permeabilized with the FOXP3/Transcription Factor and Fixation/Permeabilization buffer according to the manufacturer's recommendations (eBioscience, San Diego, CA) for intranuclear staining or Cytofix/Cytoperm buffer (BD Biosciences, San Jose, CA) for intracellular staining. Afterwards, intranuclear or intracellular cytokine staining was performed. Lastly, cells were fixed with 1% paraformaldehyde (BD Biosciences) and acquired on a LSRII flow cytometer (BD Biosciences).

2.5. Co-culture of Tfh and naïve B cells in vitro

For Tfh and naïve B cell sorting, 2×10^7 PBMC were stained with Live/Dead, CD3, CD4, CD8, CXCR5, CD19, IgD, CD27 at room temperature for 30 min. An Astrios EQ Sorter was used to purify Live $CD3^+CD8\text{-}CD4^+CXCR5^+$ Tfh cells and Live $CD3\text{-}CD19^+IgD^+CD27\text{-}$ naïve B cells. The purity of sorted cell population was confirmed > 95% by flow cytometry. Isolated Tfh cells from four MuSK-MG patients and four healthy controls were incubated with allogenic naïve B cells in a 96-well plate, at a ratio of 1:1 in R10 medium with the presence of anti-CD3 and anti-CD28. Naïve B cells only served as a control group. After 7 days of co-culture, the supernatants were preserved for total IgG testing.

2.6. ELISA test for total IgG in supernatant

Human IgG total ELISA Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA) was used to measure total IgG concentration in cell culture supernatant, according to the manufacturer's instruction. Optical density were measured at 450 nm using a 1420 multilabel counter (PerkinElmer life and analytical sciences, Boston, MA).

2.7. Data analysis and statistics

Data analysis was performed using Flowjo software (Tree Star, Ashland, OR). For analysis of multiple cytokine producing T cells graphs and pie charts of the various combinations of intracellular cytokines were produced using "Simplified Presentation of Incredibly Complex Evaluations" (SPICE) software (Roederer et al., 2011). Student's t-tests or Mann-Whitney U test were used to determine statistical significance between two groups. Analysis of variance (ANOVA) were used to compare the differences among three or more groups. Summary data are presented as mean values and the standard error of the mean. The p values were calculated using Prism software (Graph Pad, LaJolla, CA).

Table 1
Clinical characteristics of MuSK-MG patients at the time of blood draw ($N = 31$).

Patient NO.	Age(Yr)	Gender	Onset-age(Yr)	Disease duration(mo)	Race	ThymX	MGFA	MMT	Treatment (daily dose)
1	23	Female	22	13	B		2B	3	Pred 20 mg
2	48	Female	36	150	W		I	1	MMF 2000 mg
3	60	Female	45	186	B		2B	3	Pred 10 mg + MMF2000mg
4	23	Female	10	154	B		2A	5	MMF 1500 mg
5	46	Female	45	14	B		I	3	Pred 20 mg
6	29	Female	20	109	B		0	0	None
7	67	Female	49	218	W		2A	3	MMF 2000 mg
8	56	Female	42	161	W	Yes	2B	2	None
9	65	Female	53	139	W	Yes	1	3	Pred 1.25 mg + MMF 2000 mg
10	63	Female	51	146	W		3B	23	Pred 5 mg + AZA 150 mg
11	64	Female	30	402	W	Yes	2B	9	MMF 500 mg
12	31	Female	26	58	B	Yes	3B	20	Plasma exchange
13	47	Female	34	157	B		2B	3	Pred 7.5 mg + MMF 2000 mg
14	23	Female	21	20	B		2A	2	Pred 30 mg + MMF 2000 mg
15	51	Male	43	94	B		1	1	AZA 75 mg
16	26	Female	17	112	Mixed	Yes	2B	2	Pred 20 mg + MMF 2500 mg
17	58	Female	43	187	B	Yes	2B	5	MMF 2000 mg
18	40	Female	34	78	B		2B	5	MMF 2000 mg
19	31	Female	20	138	W		0	0	None
20	44	Female	29	185	B		2B	6	Pred 10 mg
21	48	Female	32	184	B		2B	7	Pred 15 mg
22	66	Female	48	215	W		2B	4	Pred 1 mg
23	35	Female	33	28	B		3B	26	Mestinon 180 mg + pred 30 mg
24	45	Male	37	104	Unknown		2B	9	None
25	28	Female	4	288	W		2A	7	Pred 7.5 mg + MMF 3000 mg
26	48	Female	9	476	W		3B	31	Plasma exchange
27	56	Female	36	235	B		2A	21	None
28	58	Female	44	173	B		3B	27	Pred 20 mg
29	53	Male	41	146	B	Yes	1	3	Pred 5 mg
30	28	Female	27	16	W		2B	17	MMF 2000 mg
31	25	Female	5	239	B	Yes	3A	4	Pred 3.8 mg

Abbreviations: AZA = azathioprine; B = black; d = day; F = female; mg = milligrams; M = male; MGFA = Myasthenia Gravis Foundation of America; MM = minimal manifestations; MMF = mycophenolate mofetil; MMT = myasthenia gravis manual muscle testing score at time of blood draw; Mo = months; Pred = predinison; ThymX = thymectomy; W = white; Yr = years.

3. Results

3.1. Patient characteristics

Thirty-One MuSK-MG patients (3 male/28 female; mean age: 44; range: 23–67 years old) were included in this study from Duke and UNC MG clinics (Table 1). The duration from onset of symptoms to blood sample collected was more than one year in all MuSK-MG patients. Thymectomy had been performed in 9 patients: one had thymic hyperplasia, but none had a thymoma. Nearly all patients were on immunosuppressant treatment, either monotherapy with prednisone, azathioprine or mycophenolate mofetil, or combination therapy. The control group consisted of twenty-two healthy individuals (11 male/11 female; mean age: 46.2; range: 24–66 years) matched for age as closely as possible, who weighed more than 110 pounds and were not receiving treatment for any chronic disease.

3.2. Overall circulating Tfh cell frequencies are not increased in MuSK-MG

Tfh cells play a critical role in several autoimmune diseases, such as MS, SLE, autoimmune thyroid desease and Sjögren's syndrome (Le Coz et al., 2013; Li et al., 2012; Tzartos et al., 2011; Zhu et al., 2012), also in AChR-MG (Luo et al., 2013; Saito et al., 2005; Zhang et al., 2016a). However, the role of Tfh cells in MuSK-MG patients remain unknown to date. To explore this question, PBMCs were phenotyped by flow cytometry to quantitate overall Tfh cell frequencies and activation status. Tfh cells were identified by the expression of CXCR5 on CD4 T cells (Supplementary Fig. S1). Comparison of Tfh frequencies between healthy controls and MuSK-MG patients, and between MGFA classifications among MuSK-MG patients revealed no significant differences (Fig. 1A). Furthermore, we were unable to detect differences in the frequency of activated and proliferating Tfh cells (Pilkinton et al., 2017)

between healthy and MuSK-MG patients (Fig. 1B-E). Thus, the overall frequency of total circulating Tfh cells, activated Tfh cells, and proliferating Tfh cells did not differ between MuSK-MG and healthy controls.

3.3. Tfh17 subset is increased in MuSk-MG

Next, we examined the proportion of Tfh1, Tfh2, and Tfh17 cell subsets contained within the total Tfh population. Using CXCR3 and CCR6, Tfh cells can be divided into three Tfh subsets: Tfh1 (CXCR3$^+$CCR6$^-$), Tfh2 (CXCR3$^-$CCR6$^-$), and Tfh17 (CXCR3$^-$CCR6$^+$) (Fig. 2A) (Bentebibel et al., 2013; Morita et al., 2011). The frequency of Tfh1 and Tfh2 subsets were similar between MuSK-MG patients and healthy controls (Fig. 2B and C). Notably, MuSK-MG patients demonstrated a significant increase in the frequency of Tfh17 cells (Fig. 2D) and in the ratio of Tfh17/Tfh1 cells (Fig. 2E). When Tfh1, Tfh2, Tfh17 subsets and the ratio of Tfh17/Tfh1 were stratified by MGFA classification, we observed no differences between disease severity (data not shown). These data show that although the frequency of total circulating Tfh cells are similar between MuSK-MG patients and healthy controls, there is a disparity in Tfh17 cell frequencies, the subset that provides the strongest support to antibody producing B cells.

3.4. Increased ratio of circulating Tfh:Tfr cells in MuSK-MG patients

After demonstrating similar frequencies of total Tfh cells between MuSK-MG patients and healthy controls we investigated the possibility of Tfr cell dysregulation in MuSK-MG. Circulating Tfr cells were defined as CD4$^+$CXCR5$^+$FOXP3+ (Sage et al., 2014) (Fig. 3A). Overall frequencies of Tfr cells or Tfr cells stratified by MGFA classification were not significantly different from healthy controls (Fig. 3B, C); however, the ratio of circulating Tfh:Tfr was significantly increased in MuSK-MG
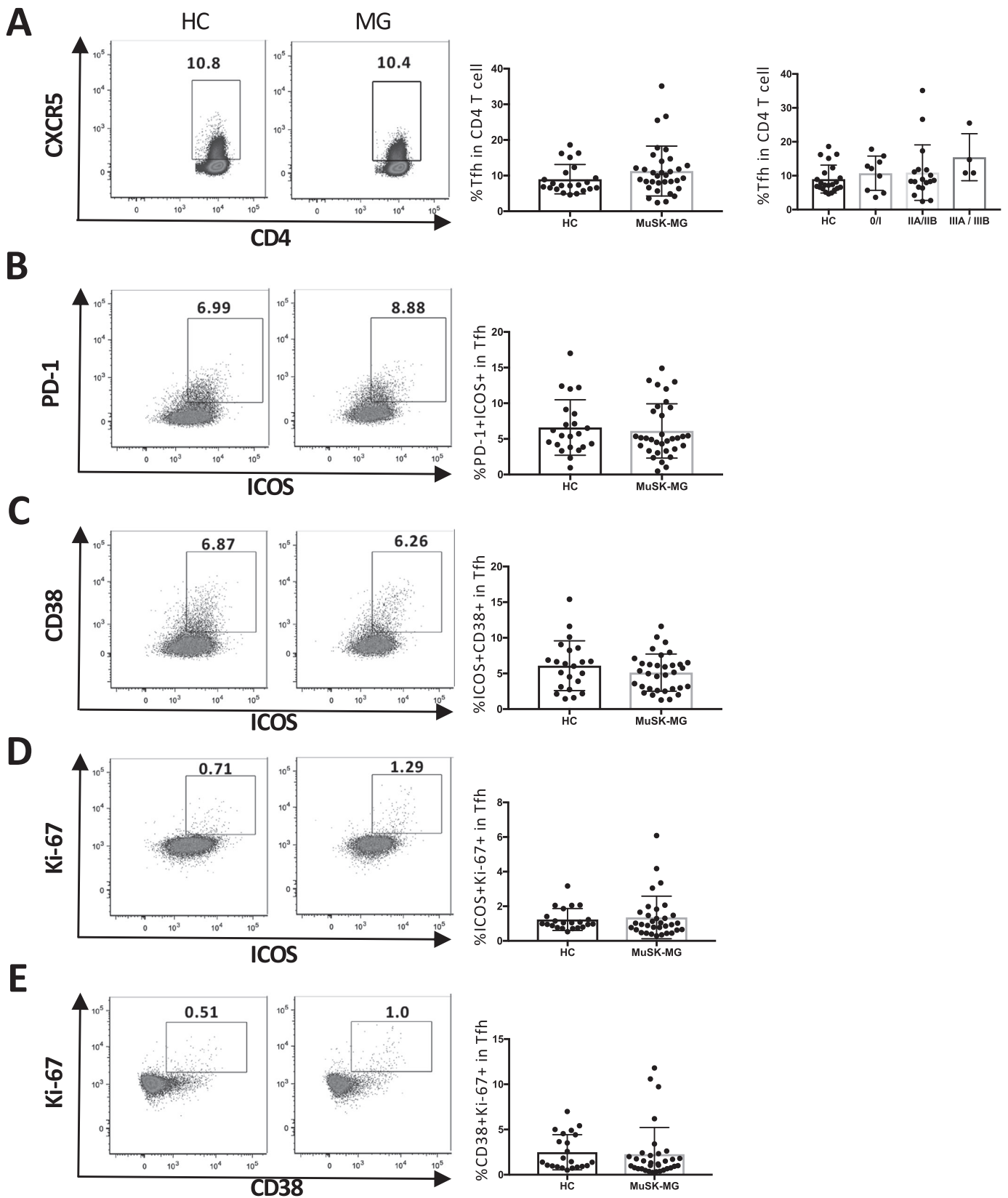
Fig. 1. Comparable frequencies of circulating Tfh cells in healthy controls and MuSK-MG. (1A) Tfh cells were identified by gating CXCR5 on CD4 T cells. Percentages of Tfh cells in CD4 T cells in lymphocytes in MG patients were not different than healthy controls. The overall frequencies of Tfh cells in MuSK-MG patients according to MGFA classification were similar. Frequencies of PD-1$^+$/ICOS$^+$ Tfh cells (1B), CD38$^+$/ICOS$^+$ Tfh cells (1C), Ki-67$^+$/ICOS$^+$ Tfh cells (1D) and Ki-67$^+$/CD38$^+$ Tfh cells (1E) were not different in MuSK-MG patients compared with controls.
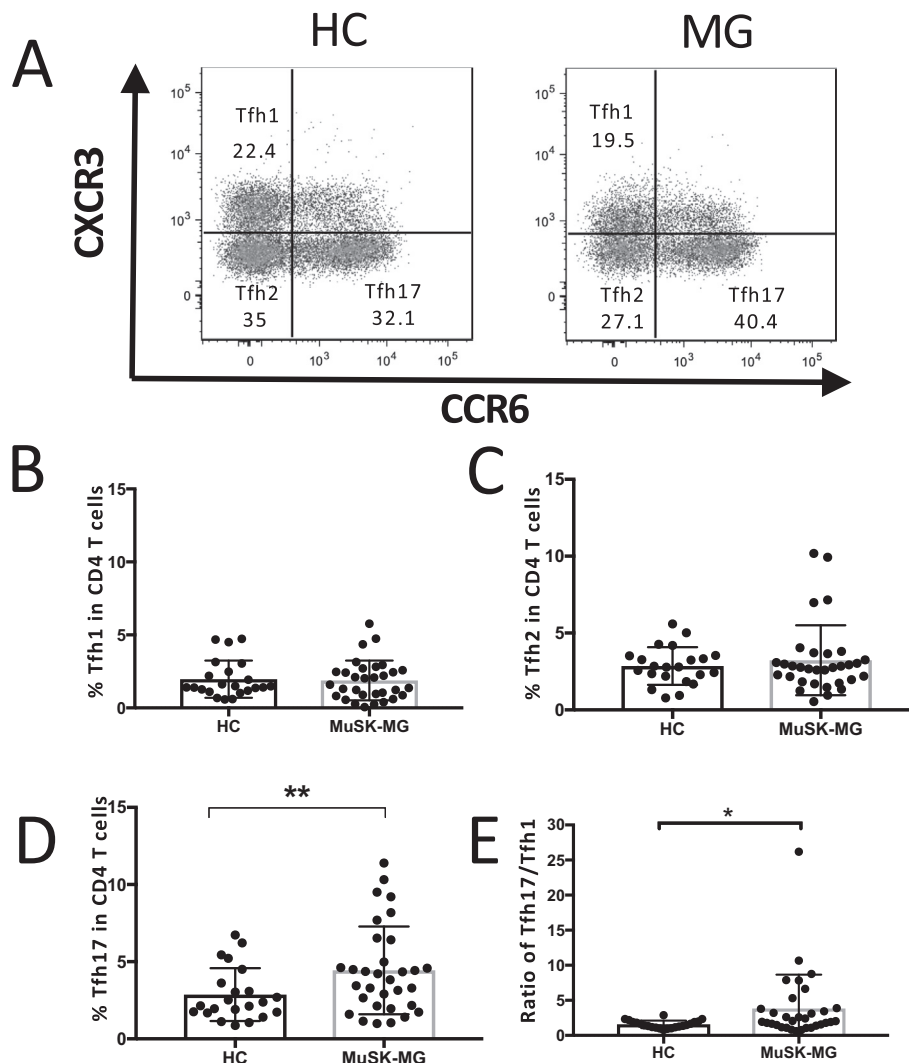
Fig. 2. Tfh subsets in MuSK-MG patients. (2A) Gating strategy of Tfh subsets in CD4 T cells using CXCR3 and CCR6 in MuSK-MG and healthy controls. Tfh1 was defined as CXCR3$^+$CCR6$^-$. Tfh2 was defined as CXCR3$^-$CCR6$^-$. Tfh17 was defined as CXCR3$^-$CCR6$^+$. (2B, 2C) Tfh1 and Tfh2 frequencies were not different between MuSK-MG and healthy controls. (2D) Tfh17 frequencies were significantly increased in MuSK-MG patients. (2E) The ratio of Tfh17/Tfh1 subsets is increased in MuSK-MG patients compared with healthy controls. *, significant difference, $p < .05$; **, significant difference, $p < .01$.

(Fig. 3D), with a trend towards a higher ratio with increasing severity (Supplement Fig. S2A). To analyze the Tfh:Tfr ratio in more detail we calculated the ratios of Tfh1:Tfr, Tfh2:Tfr and Tfh17:Tfr. We found that only the Tfh17:Tfr ratio was significantly increased in MuSK-MG (Fig. 3E). Therefore, Tfh17 subsets primarily contributed to the increased ratio of circulating Tfh:Tfr in MuSK-MG patients.

3.5. Enhanced pro-inflammatory cytokines in MuSK-MG

We also explored whether Tfh cells from MuSK-MG patients and healthy controls exhibited functional differences. Intracellular cytokine staining following PMA and ionomycin stimulation revealed a significant increase in the production of IL-21 by CD4 T cells in MuSK-MG (Fig. 4A). MuSK-MG patients also produced higher frequencies of IFN-γ and IL-17, cytokines associated with Th1 and Th17 profiles, respectively (Fig. 4B, C). We also observed increased frequencies of a subset of CD4 T cells in MuSK-MG patients that co-produced IL-21 and IL-17, and IFN-γ and IL-17 (Fig. 4D, E). However, production of IL-4 by CD4 T in MuSK-MG was not significantly different from HC. Furthermore, when Boolean gating was performed using the combination of IFN-γ, IL-4, IL-17, and IL-21, and stratified according to MGFA classification, MuSK-MG patients with the highest disease severity had a greater proportion of CD4 T cells producing two or more cytokines (Supplementary Fig. S3). Overall, these data shows the enhancement of Th1, Th17, and Tfh associated cytokines in MuSK-MG patients.

3.6. Tfh cells promote IgG production by autologous naïve B cells

To further assess the function of MuSK-MG derived Tfh cells, we investigated whether MuSK-MG derived Tfh cells have a higher capacity to promote B cell help. In this study, Tfh and naïve B cells were sorted and co-cultured for 7 days. After 7 days of culture, supernatant was collected and used in an ELISA to measure IgG production by B cells. Tfh cells and naïve B cells from healthy controls along with a naïve B cell only condition were included as control groups. In the naïve B cell only conditions, no differences were observed between MuSK-MG patients and healthy controls (Fig. 5). However, IgG production increased significantly with the addition of Tfh cells, and IgG production was increased in MuSK-MG compared with healthy controls ($p < 0.01$). These results suggest that Tfh cells derived from MuSK-MG patients have a more robust capacity in promoting B cell antibody production.
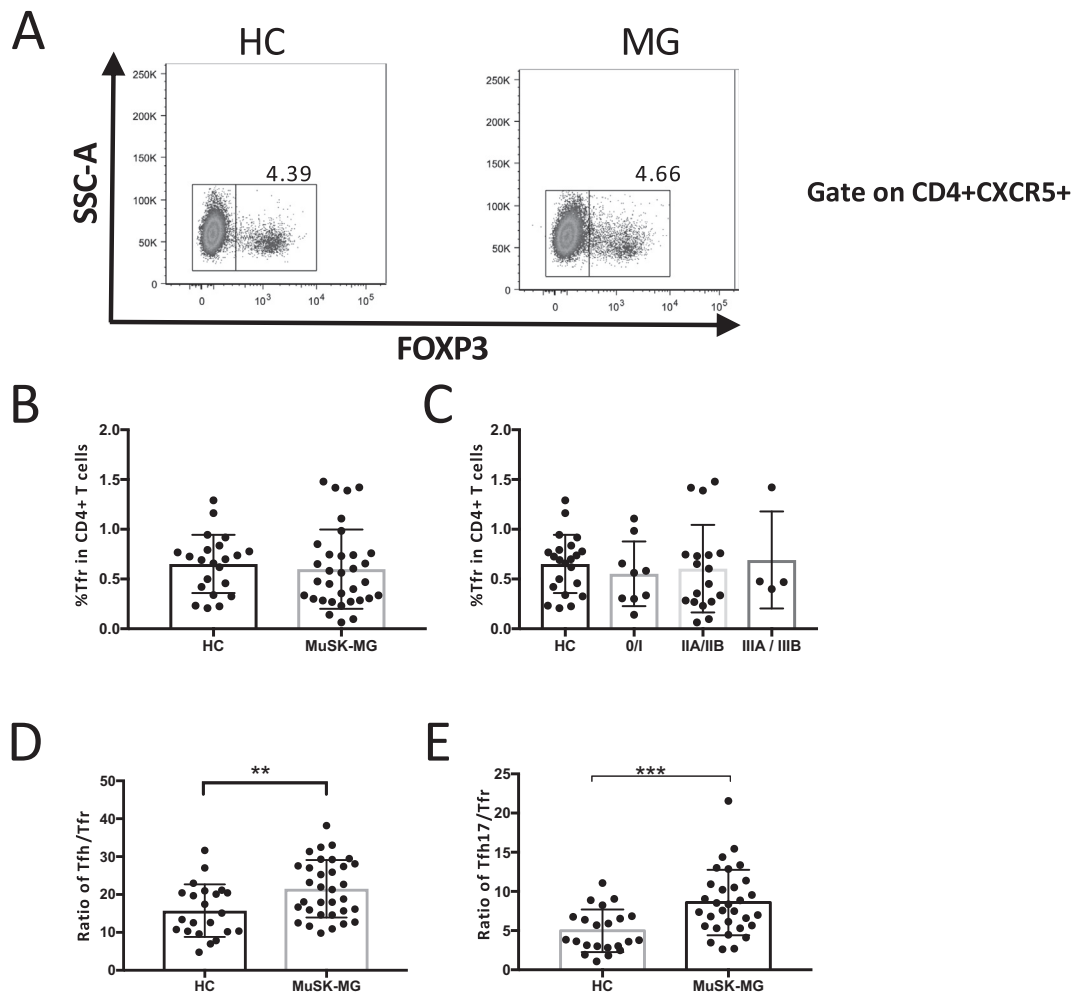
Fig. 3. Comparison of Tfr subsets between MuSK-MG and healthy control groups. (3A) Gating strategy Tfr cells in controls and MuSK-MG patients. (3B) Overall Tfr frequencies and (3C) Tfr frequencies among MuSK-MG patients with different MGFA classifications were not significantly different compared to controls. (3D) The overall Tfh:Tfr ratio is increased in MuSK-MG patients compared to controls. (3E) The Tfh17/Tfr ratio is also increased in MuSK-MG patients. **, significant difference, $p < .01$; ***, significant difference, $p < .001$.

4. Discussion

Previous studies demonstrated that CD4 T cells, particularly Tfh cells, play a critical role in supporting autoantibody production by B cells in autoimmune diseases such as MuSK-MG (Nutt et al., 2015). Tfh cells are a subset of CD4 T cells located in germinal centers (GC) that facilitate B cell hyper-proliferation, somatic hyper-mutation, and class switching (Nutt et al., 2015). In recent years circulating peripheral Tfh cells are regarded as a memory subset of Tfh cells (Brenna et al., 2020; Vella et al., 2019), as they provide rapid and robust responses for naïve and memory B cells producing antibodies (Ma and Deenick, 2017). Up to this point the role of circulating peripheral Tfh cells in the pathogenesis of MuSK-MG remained undefined.

In contrast to AChR-MG patients where previous studies showed that circulating peripheral Tfh populations are expanded (Luo et al., 2013; Zhang et al., 2016a), our study showed that overall Tfh-like population frequencies in MuSK-MG patients were similar to healthy control subjects. Our sub-analysis of activated Tfh subsets identified by the markers ICOS, PD-1, CD38, and Ki-67 (Pilkinton et al., 2017), yielded the same conclusion (Supplement Fig. S4). These observations further support that different immunopathological mechanisms may underly MuSK-MG and AChR-MG (Guptill and Sanders, 2010). GC abnormalities with thymic hyperplasia are commonly found in the thymus of AChR-MG patients (Berrih et al., 1984), with fewer thymic

histopathologic changes observed in MuSK-MG patients; the thymus is currently not felt to play a strong role in disease pathogenesis (Guptill and Sanders, 2010; Marx et al., 2013). Given the prominent GC abnormalities and thymic hyperplasia in AChR-MG and the localization of Tfh cells within areas of thymic hyperplasia leading to AChR-Ab production (Zhang et al., 2016b), it is reasonable that circulating Tfh cells with a memory phenotype of GC Tfh cells are increased in AChR-MG patients (Ma and Deenick, 2017). The absence of thymic changes in the majority of MuSK-MG patients (Lauriola et al., 2005) may explain why the circulating Tfh cell population is not increased.

Although total circulating Tfh cells showed no significant changes in MuSK-MG patients, we demonstrate an imbalance in specific Tfh subsets. Using the surface markers CXCR3 and CCR6 (Bentebibel et al., 2013; Morita et al., 2011), we divided Tfh cells into Tfh1 (CXCR3$^+$CCR6$^-$), Tfh2 (CXCR3$^-$CCR6$^-$) and Tfh17 (CXCR3$^-$CCR6$^+$) subpopulations (Morita et al., 2011). These recognized circulating Tfh subsets differ in their ability to promote antibody production by B cells via IL-21. We found a significant increase in the Tfh17 subset in MuSK-MG patients. This population is characterized by production of the cytokine IL-17, expression of the transcription factor ROR-γt, and is known to provide potent help for B cells producing auto-antibodies, including in autoimmune diseases such as dermatomyositis (Morita et al., 2011). The ratio of Tfh17/Tfh1 has also been shown to correlate with a pro-inflammatory and enhanced humoral immune response
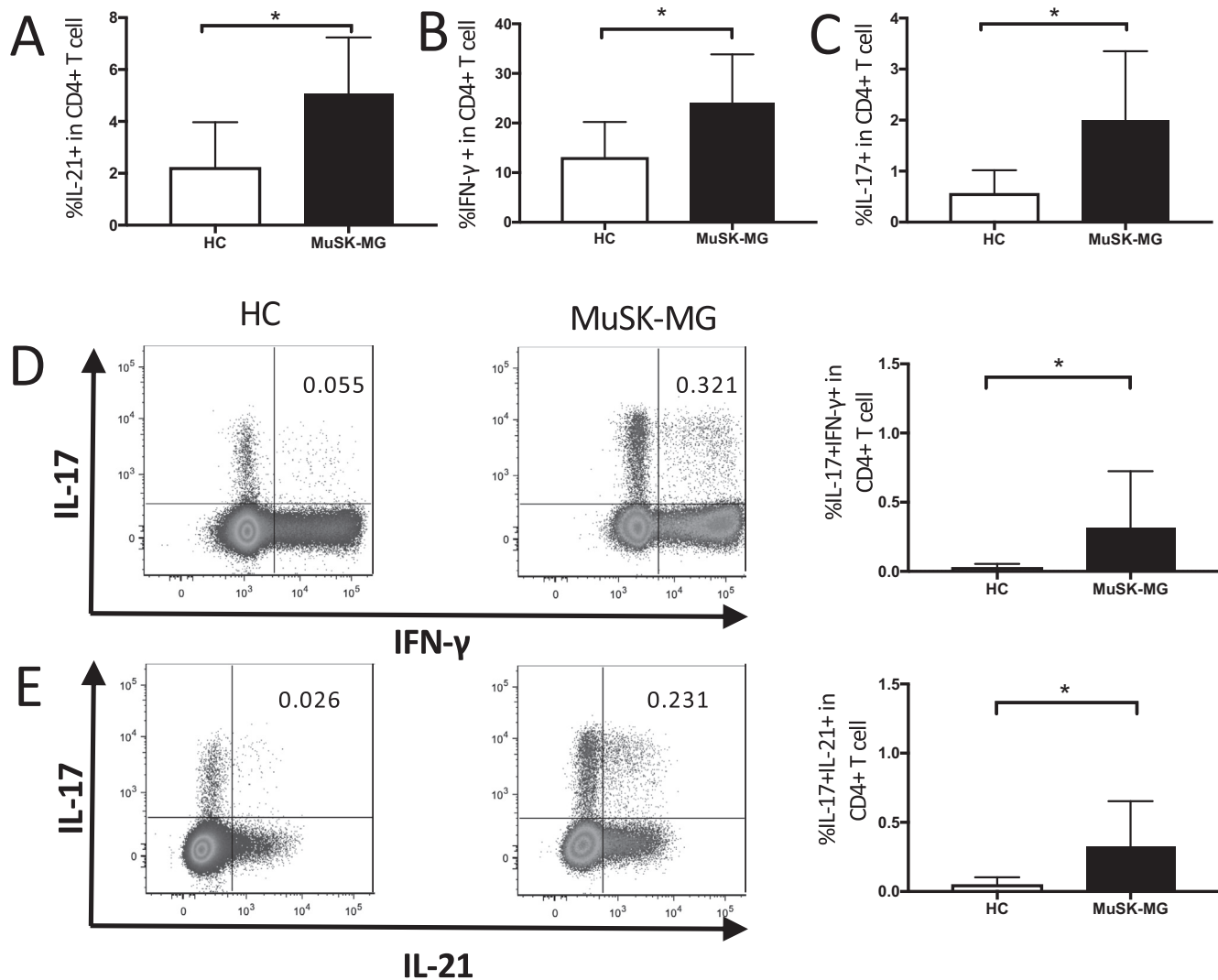
Fig. 4. CD4 T cell function assessed by intracellular cytokine production in MuSK-MG and healthy controls. (4A) IL-21, (4B) IFN-γ and (4C) IL-17 cytokine production is increased in CD4 T cells in MuSK-MG compared with healthy controls. (4D) Demonstration of IL-17 $^+$/IFN-γ $^+$ gating strategy among CD4 T cells (left). IL-17 $^+$/IFN-γ $^+$ CD4 $^+$ pathogenic Th17 cells are increased in MuSK-MG compared with controls (right). (4E) IL-17 $^+$/IL-21 $^+$ gating strategy in CD4 T cells between controls and patients with MuSK-MG (left). IL-17 $^+$/IL-21 $^+$ CD4 T cells are significantly increased in MuSK-MG patients (right). *, significant difference, $p < .05$.

(Locci et al., 2013). In addition, we observed increases in total CD4 T cells in MuSK-MG patients co-producing IL-17 and IL-21 suggesting that these Tfh17 cells are another source for immunopathology in MuSK-MG patients.

Morita et al. previously demonstrated that peripheral CXCR5 $^+$CD4 Tfh cells help naïve B cells produce antibodies via IL-21 (Morita et al., 2011), while others have established that IL-21 $^+$CD4 T cells represent peripheral Tfh cells with helper functions (Schultz et al., 2016). Due to the experimental limitation of sorting cells following intracellular cytokine staining, we sorted total CXCR5+ Tfh cells and stimulated the cells with a polyclonal T cell stimulus to induce a cytokine response, in a co-culture with naïve B cells. Our ex-vivo data demonstrates a higher frequency of IL-21 producing CD4 T cells and we predict that the enhanced IgG production in the co-culture with MuSK-MG cells is associated with the availability of IL-21.

The results from this study expands upon previous reports demonstrating an increase in total IFN-γ, IL-17, and IL-21 (Yi et al., 2014; Yilmaz et al., 2015). Using flow cytometry analysis, we show that MuSK-MG patients are capable of co-producing cytokines that are conventionally designated to a specific subset, and the frequencies of T cells co-producing IFN-γ and IL-17, IL-17 and IL-21 (Fig. 4), and cells

that are triple positive for IFN-γ, IL-17, and IL-21 are increased in MuSK-MG patients compared with controls, particularly in more severely affected patients (Supplement Fig. S3). In addition to Tfh-like cells, pathogenic Th17 cells, defined by co-production of IFN-γ and IL-17 in Th17 cells (Stockinger and Omenetti, 2017), were increased in MuSK-MG (Fig. 4D). This latter finding is in accordance with our research both in AChR-MG and MuSK-MG patients (Li et al., 2019; Yi et al., 2020).

Circulating Tfr cells are considered the counterpart for GC Tfr cells (Brenna et al., 2020; Vella et al., 2019), and to our knowledge this T cell subpopulation has not been studied in MuSK-MG. We found that circulating Tfr cell frequencies were similar in MuSK-MG and healthy control subjects. However, previous studies have demonstrated significant decrease in Tfr levels in AChR-MG patients (Wen et al., 2016), SLE (Xu et al., 2017) and MS (Dhaeze et al., 2015). These discrepancies might be due to the differences in disease pathogenesis among these autoimmune diseases. Despite similar frequencies of Tfr cells, the increased Tfh:Tfr ratio in MuSK-MG suggests that this imbalance favoring Tfh activity is capable of supporting B cell activation.

Limitations of this study include a relatively small sample size of MuSK-MG patients, the use of varied immunosuppressive medications,
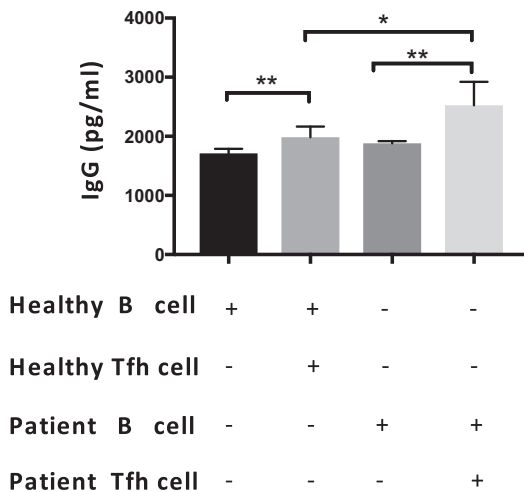
Fig. 5. Tfh cells promote B cell IgG production in vitro. Sorted peripheral CD19$^+$CD127$^-$IgD$^-$ naïve B cells (2x10^5/well) were co-cultured with Tfh cells (2x10^5/well) from MuSK-MG patients ($N = 4$) or healthy controls (N = 4) in the presence of CD3/CD28. Naïve B cells only served as a control condition. Production of IgG Abs in cultured supernatants was detected by ELISA. In the B cell only condition IgG production was not different between MuSK-MG patients or healthy controls. As expected, adding Tfh cells to naïve B cells resulted in increased IgG production in both controls and MuSK-MG patients. However, IgG production was significantly increased in MuSK-MG patients compared with controls. *, significant difference, $p < .05$; **, significant difference, $p < .01$.

and limited number of patients with more severe disease. Ideally, patients would be immunosuppressive naïve with an even distribution of disease severity, but given the rarity and severity of the disease, this is not feasible and the study remains among the largest of its kind in the MuSK-MG patient population.

In summary, these data collectively suggest that CD4 T cell populations including Tfh-like cells with a Tfh17 (CXCR3$^-$CCR6$^+$) phenotype and enhanced IL-21 production, as well as pathogenic Th17 cells, are strongly implicated in MuSK-MG immunopathogenesis. In addition, Tfh abnormalities do not appear related to reduced Tfr frequencies. The imbalance of circulating Tfh-like cells driven by Tfh17 reactivity is likely a strong contributor to B cell activation and antibody production in MuSK-MG patients. Future studies will explore the impact of therapy on Tfh subpopulations. It would be particularly interesting to determine whether rituximab therapy, which often results in dramatic and durable clinical responses in MuSK-MG, indirectly impacts Tfh cell reactivity, including the Tfh17 subset.

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Declarations of Competing Interest

None.

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