JC Applied Immunology SoSe 2022

Diabetes mellitus exacerbates experimental autoimmune myasthenia gravis via modulating both adaptive and innate immunity

by Zhang et al.
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Introduction

- ***Background:***

"Diabetes mellitus (DM) is a common concomitant disease of late-onset myasthenia gravis (MG). However, the impacts of DM on the progression of late-onset MG were unclear."

Introduction – Brief review of selected topics on Diabetes mellitus and Myasthenia gravis relevant to this paper

→ Overall global prevalence of MG: 150-250 per 1x10e6 individuals.

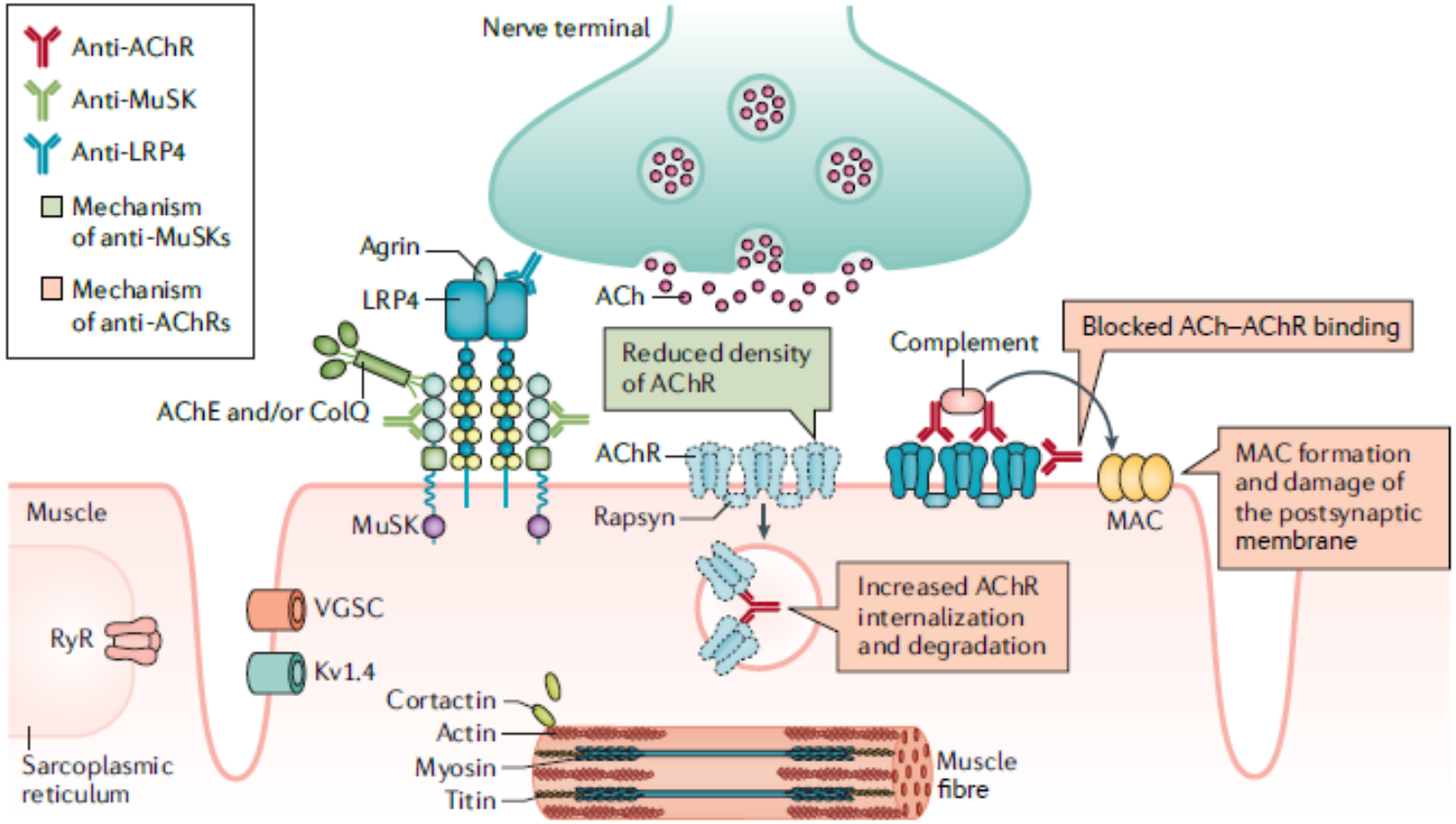
→ Overall global prevalence of DM In 2014, 8.5% of adults aged 18 years and older had diabetes (95% T2DM)

Table 1 | **Classification of MG subgroups**

Subgroup	Autoantibody	Age at onset	Thymus abnormalities
Early-onset MG[a]	AChR	<50 years of age	Hyperplasia common
Late-onset MG	AChR	>50 years of age	Atrophy common
Thymoma MG	AChR	Any	Type AB and B thymoma
MuSK MG	MuSK	Any	Normal
LRP4 MG	LRP4	Any	Normal
Seronegative MG	None detected	Any	Variable
Ocular MG[b]	AChR, MuSK, LRP4 or none	Any	Variable

AChR, acetylcholine receptor; LRP4, lipoprotein-receptor-related protein 4; MG, myasthenia gravis; MuSK, muscle-specific kinase. [a]Juvenile MG is not considered a separate subgroup and is part of early-onset MG. All patients at one time point can belong only to one subgroup. [b]Ocular MG includes the patients with ocular symptoms only and no clinical weakness in other muscles.

https://www.who.int/news-room/fact-sheets/detail/diabetes
Gilhus NE, Tzartos S, Evoli A, Palace J, Burns TM, Verschuuren JJGM. Myasthenia gravis. Nat Rev Dis Primers. 2019 May 2;5(1):30. doi: 10.1038/s41572-019-0079-y. PMID: 31048702.



- **Pathophysiology of MG at the neuromuscular junction**

→ Muscle weakness, typically increasing with repetitive muscle use.

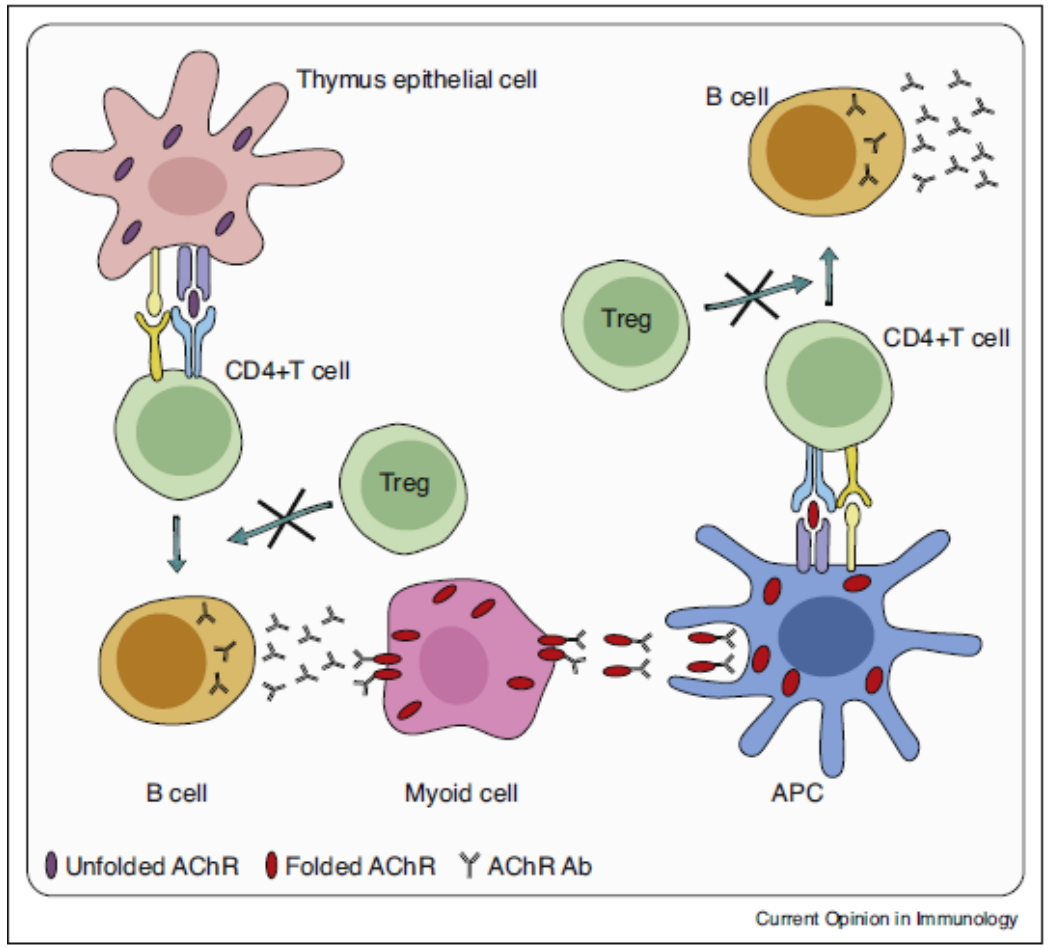
→ Commonly involves ocular muscles -> Diplopia and Ptosis (often asymmetrical)

→ Facial, neck, limb and truncal muscles involved in varying degrees (typically more symmetrical)

→ Bulbar and respiratory involvment can be life threatening!

Gilhus NE, Tzartos S, Evoli A, Palace J, Burns TM, Verschuuren JJGM. Myasthenia gravis. Nat Rev Dis Primers. 2019 May 2;5(1):30. doi: 10.1038/s41572-019-0079-y. PMID: 31048702.

An oversimplified introduction on Auto-Ab generation in Myasthenia gravis



- The exact mechanisms of Auto-Ab generation are not fully elucidated.
- In 10–15% of MG patients a thymoma is present, and up to 50% of thymoma patients develop MG
- **Briefly:** An abnormal expression of epitops mimicking neuromascular junction proteins is thought to occur in the thymus, accompanied by the brakedown of critical checkpoints of selftolerance in the adaptive immune system at large

Romi F, Hong Y, Gilhus NE. Pathophysiology and immunological profile of myasthenia gravis and its subgroups. Curr Opin Immunol. 2017 Dec;49:9-13. doi: 10.1016/j.coi.2017.07.006. Epub 2017 Aug 4. PMID: 28780294.

Introduction: Putative links between diabetes mellitus pathophysiological hallmarks and the progression of myasthenia gravis highlighted by the authors

- Focus on hyperglycemia -> Increased proinflammatory cytokine levels, altered function of leukocytes, e.g. defective immunosuppressive function of Tregs.
- The authors highlight the importance of advanced glycation end products (AGEs)

-> Particularly in modulating the proliferation and maturation of dendritic cells and their capacity to influence T-cells e.g. inducing CD4+ to Th1 and Th17 differentiation.

-> Abnormal Th1, Th17 and Tfh cells have been implicated in the generation of auto-reactive antibodies during MG pathogenesis.

Introduction: Research question

- How does DM influence adaptive and innate immune functions in the course of MG pathogenesis ?
- Main experimental model: EAMG – rodent model of myasthenia gravis

Materials and Methods: Animals and experimental design

Female Lewis rats, 6-8 weeks.

- 2 Experimental conditions: EAMG vs. EAMG +DM
- DM model: STZ, 60 mg/kg/BW single shot i.p. – Vehicle control: Citrate acid buffer

→ Positive control: Hyperglycemia (blood glucose > 200 mg/dl) 3 days post STZ

- EAMG – model: Initiated 4 days after STZ injection

75 µg of R 97-116 peptide emulsified in CFA subcutaneously boosted with 75 µg of R97-116 peptide emulsified in IFA in 200 µl injected at the tail base 30 days later.

→Positive control: Serological evaluation of peptide R97-116-specific Ab production, Body weight monitoring, qualitative assessment of strength and motricity

Materials and methods: Read outs

- **Biological material:**
- **Spleens and lymph nodes**, harvested on day 50 post immunization -> weighed and purified to single cell suspensions.

-> Flow cytometry

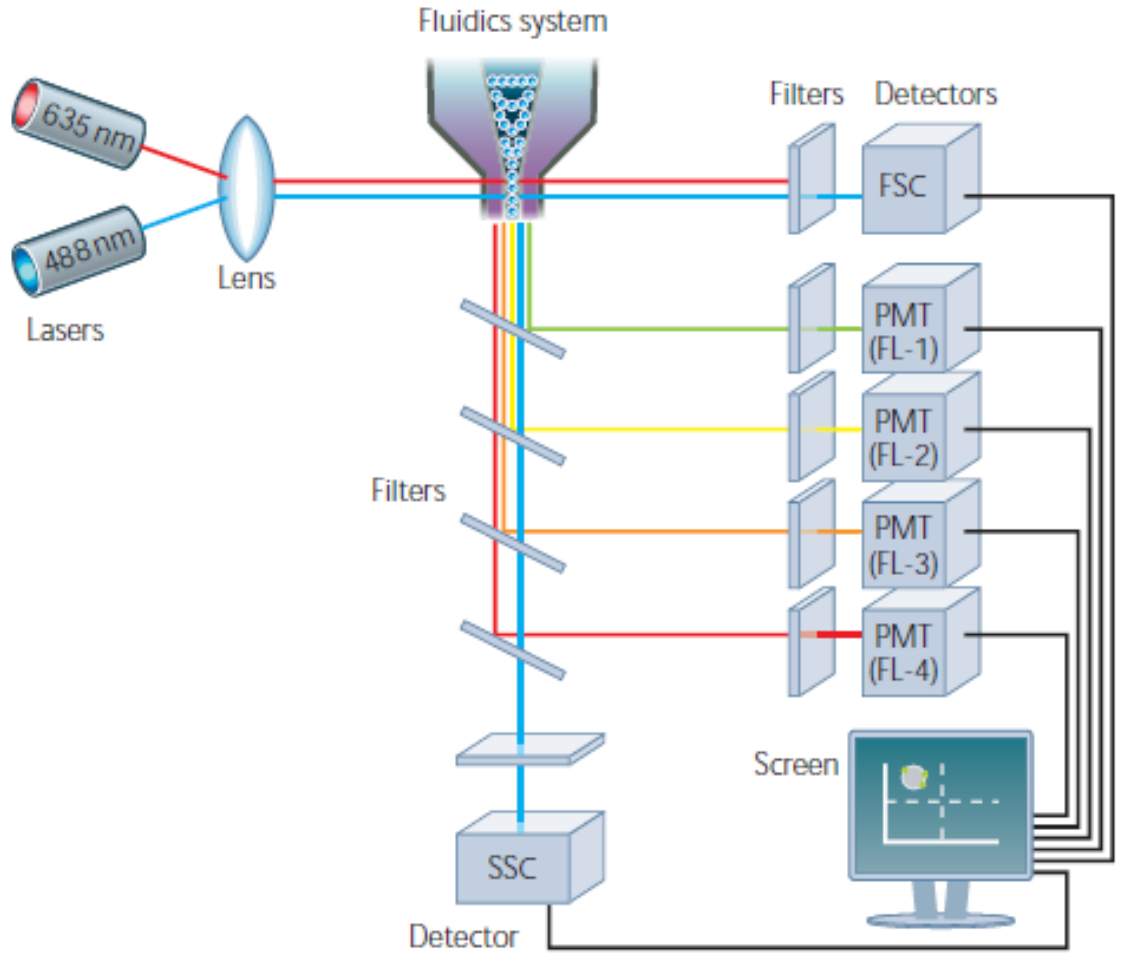
-> Intracellular cytokine and antibody-secreting cells analysis

-> Isolation of T, B, and NK cells and co-culture assays in vitro

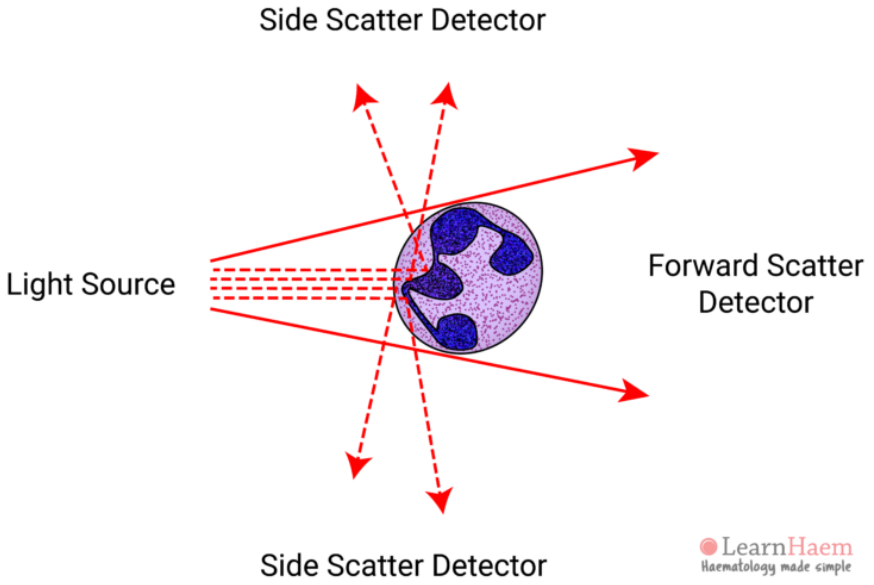
Additional in vitro assays:

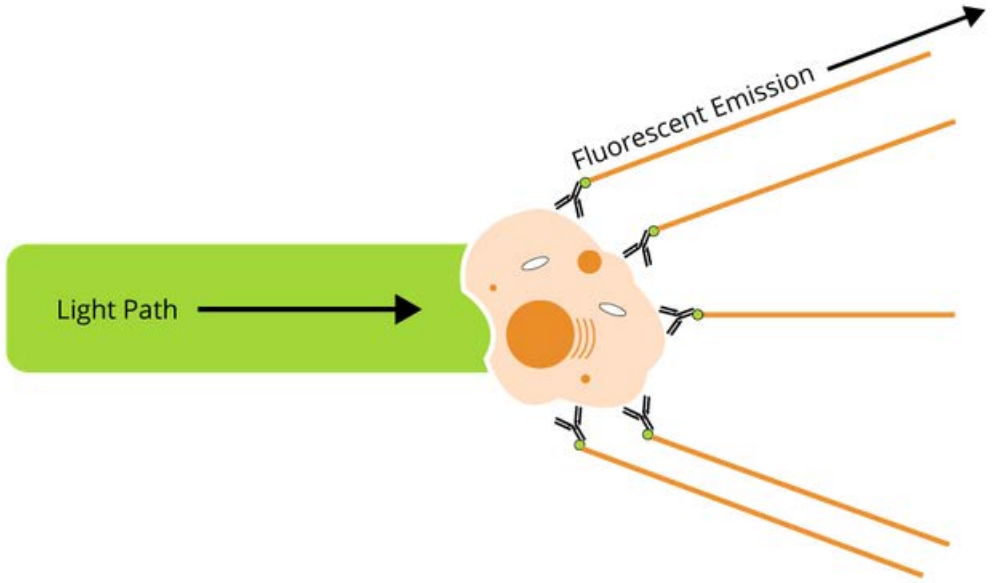
Isolation of T, B, and NK cells and co-culture assays in vitro

Key method: Flow cytometry

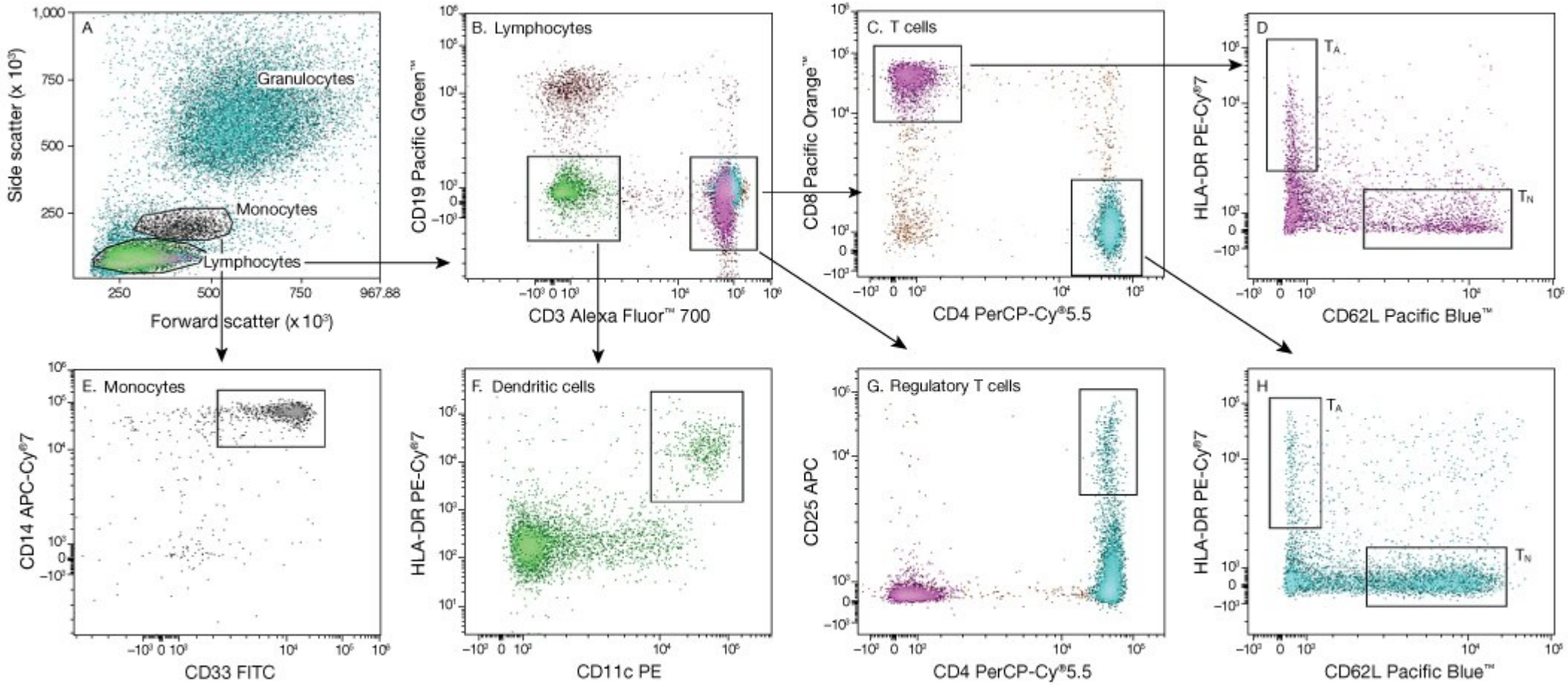


Schematic overview of a typical flow cytometer setup





Pictures taken from: https://www.bosterbio.com/protocol-and-troubleshooting/flow-cytometry-principle
https://www.learnhaem.com/courses/flow-cytometry/lessons/light-scatter/
https://www.researchgate.net/publication/309923040_introduction_to_flow_cytometery



https://www.thermofisher.com/at/en/home/references/newsletters-and-journals/bioprobes-journal-of-cell-biology-applications/bioprobes-71/bioprobes-71-flow-cytometry-panel-design.

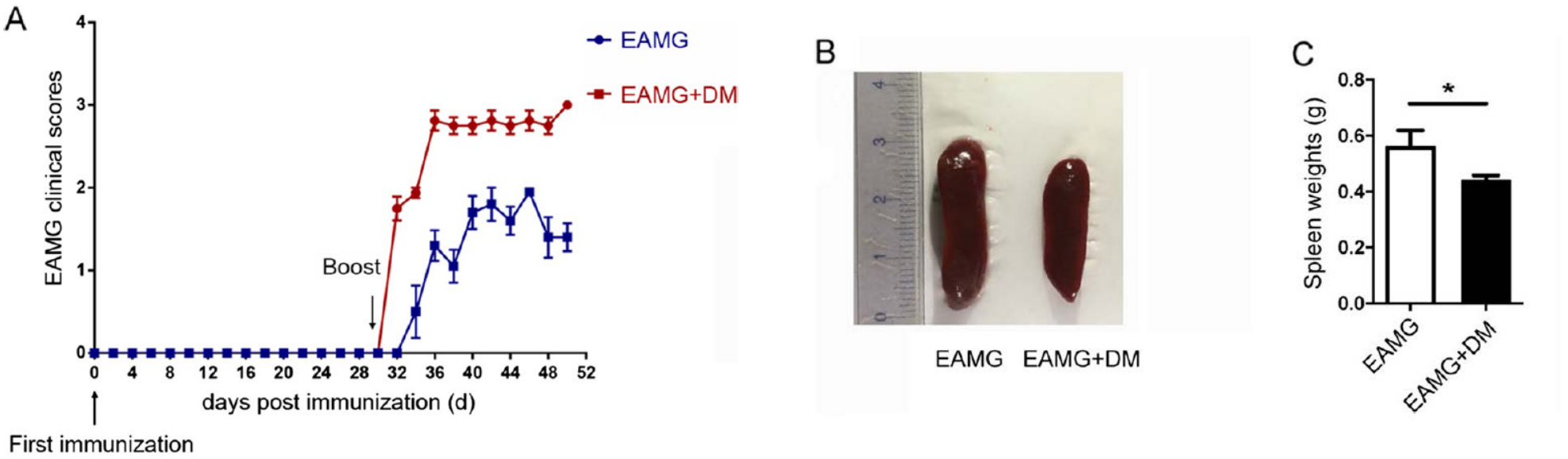
Results

Table 1 BG level of rats in the two groups

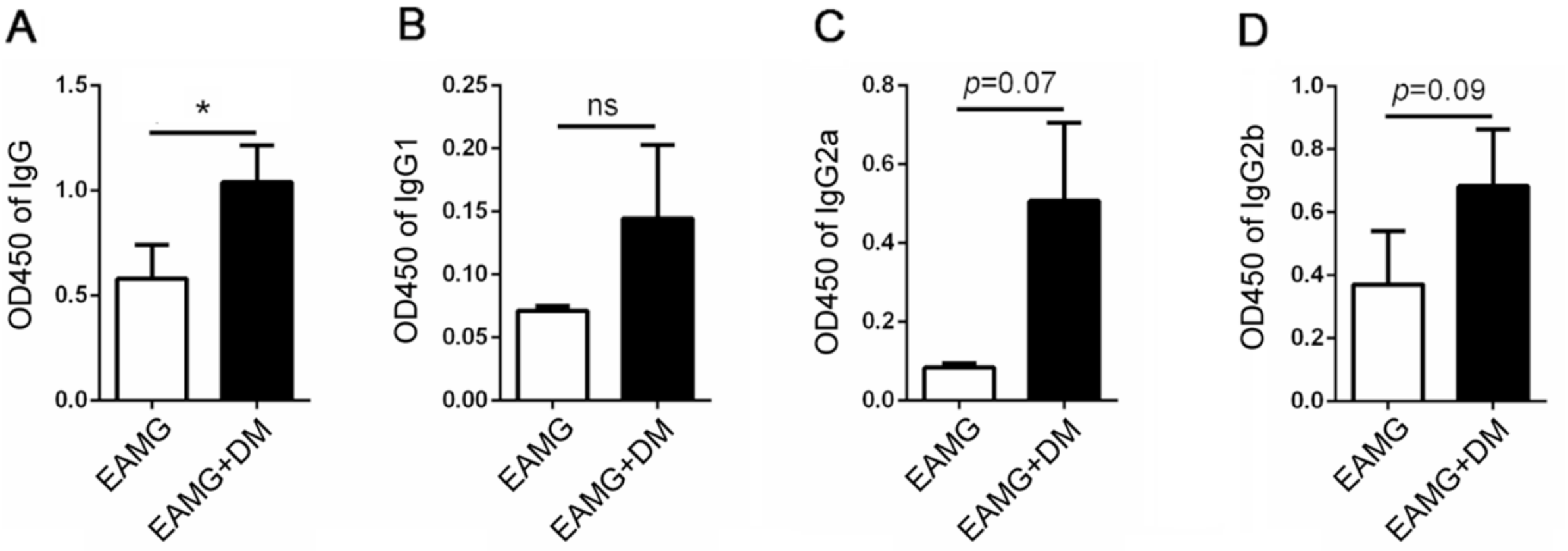
Post STZ injection	Day 3			Day 13			Day 53		
	DM+EAMG	EAMG	*p* value	DM+EAMG	EAMG	*p* value	DM+EAMG	EAMG	*p* value
BG (mg/dl)	449.8 ± 26.2	122.1 ± 4.4	0.001	451.3 ± 21.2	119.8 ± 6.7	0.001	549.0 ± 12.7	105.4 ± 3.9	0.001

Values are mean ± SEM. $n = 8$ in the DM+EAMG group and $n = 7$ in the EAMG group. Unpaired Student's *t* test was used

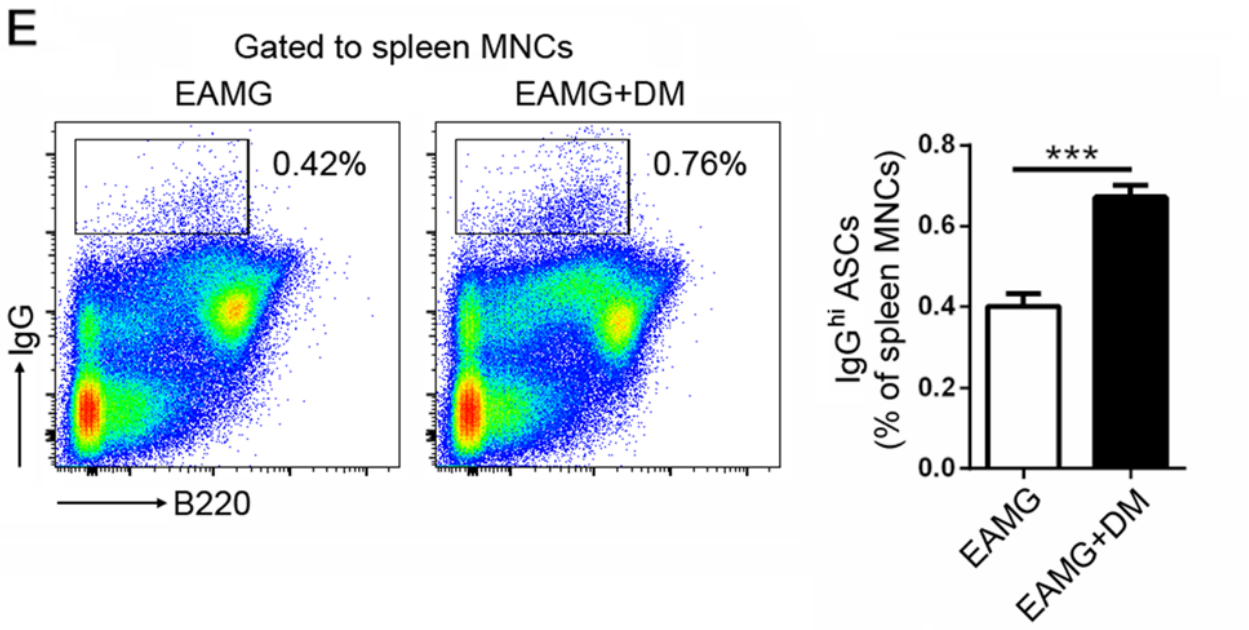
BG blood glucose

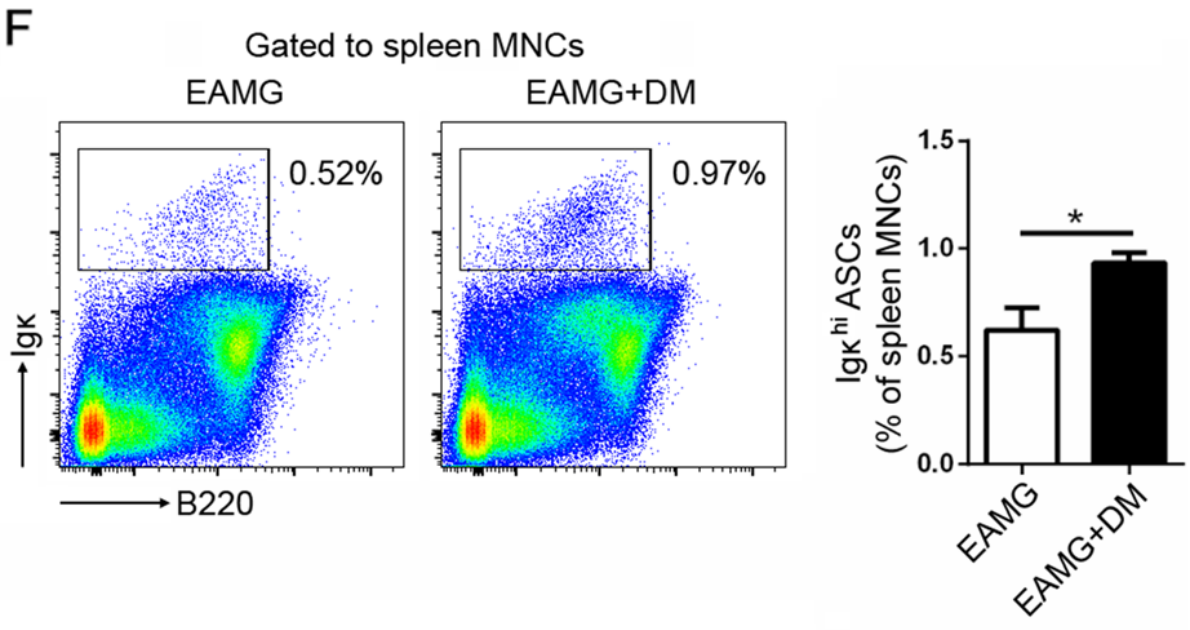


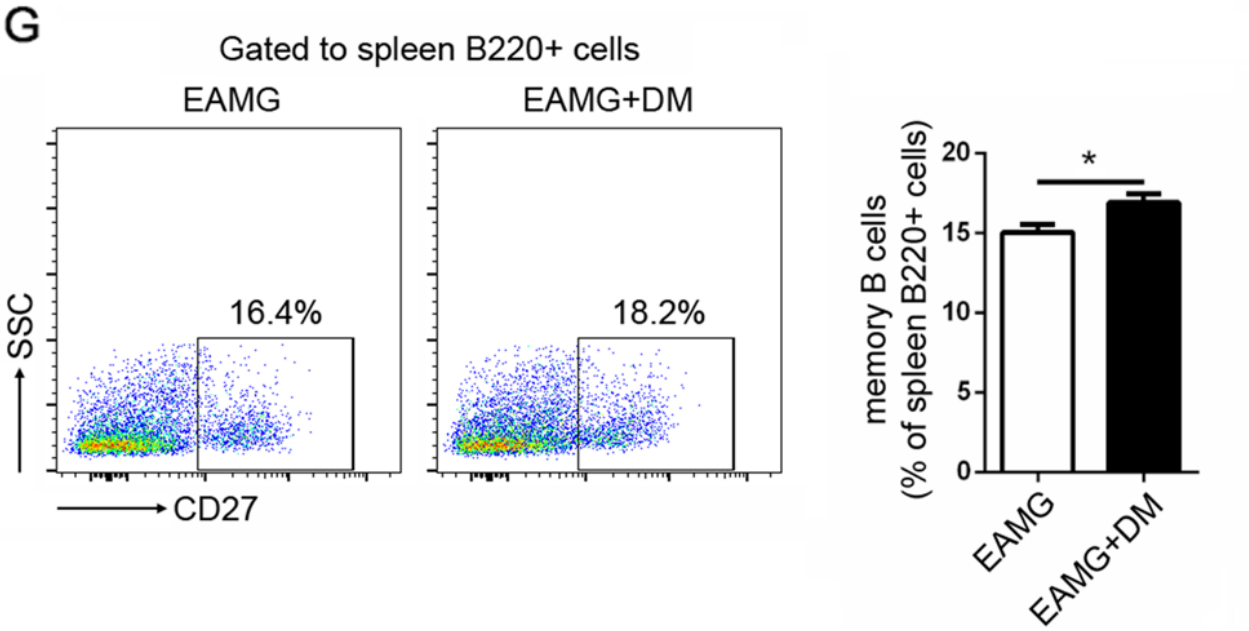
- Diabetes exacerbated the clinical severity and reduced the splenic volume in EAMG rats



- Levels of total anti-R97–116 IgG but not IgG1, IgG2a, and IgG2b subsets were elevated in the sera of EAMG + DM animals as determined by ELISA assays

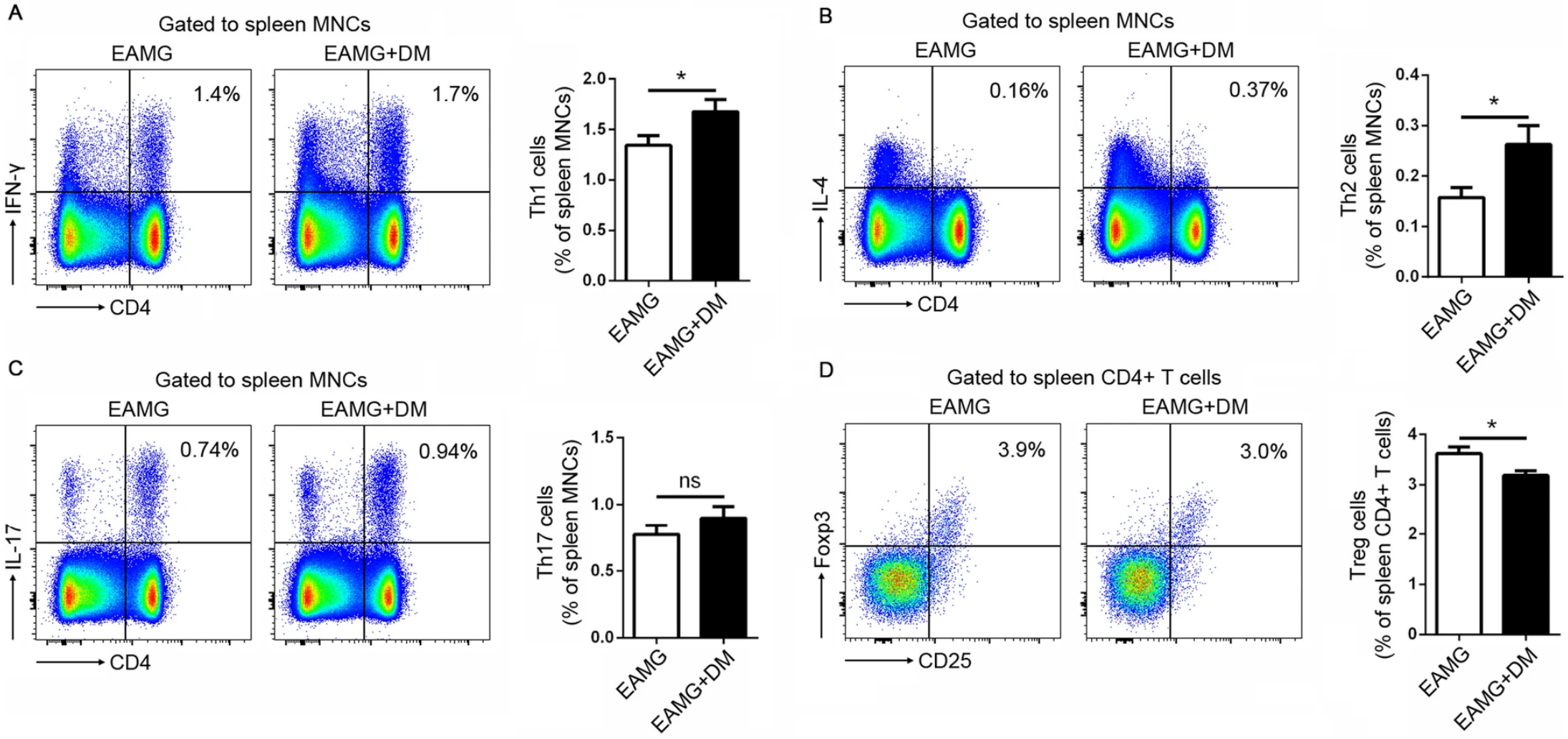






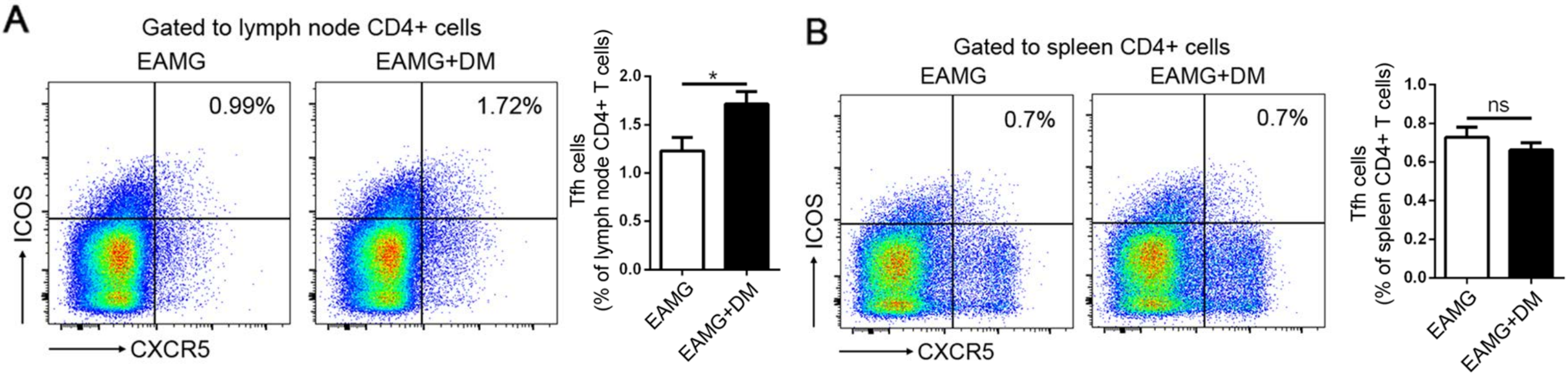
→ Significantly more ASCs (Fig. 2E, F), (B220− IgGhi or B220−Igκhi)in the spleens of rats in the DM+EAMG group

→The percentages of memory B cells (defined as B220+ CD27+) were upregulated in the DM+EAMG group

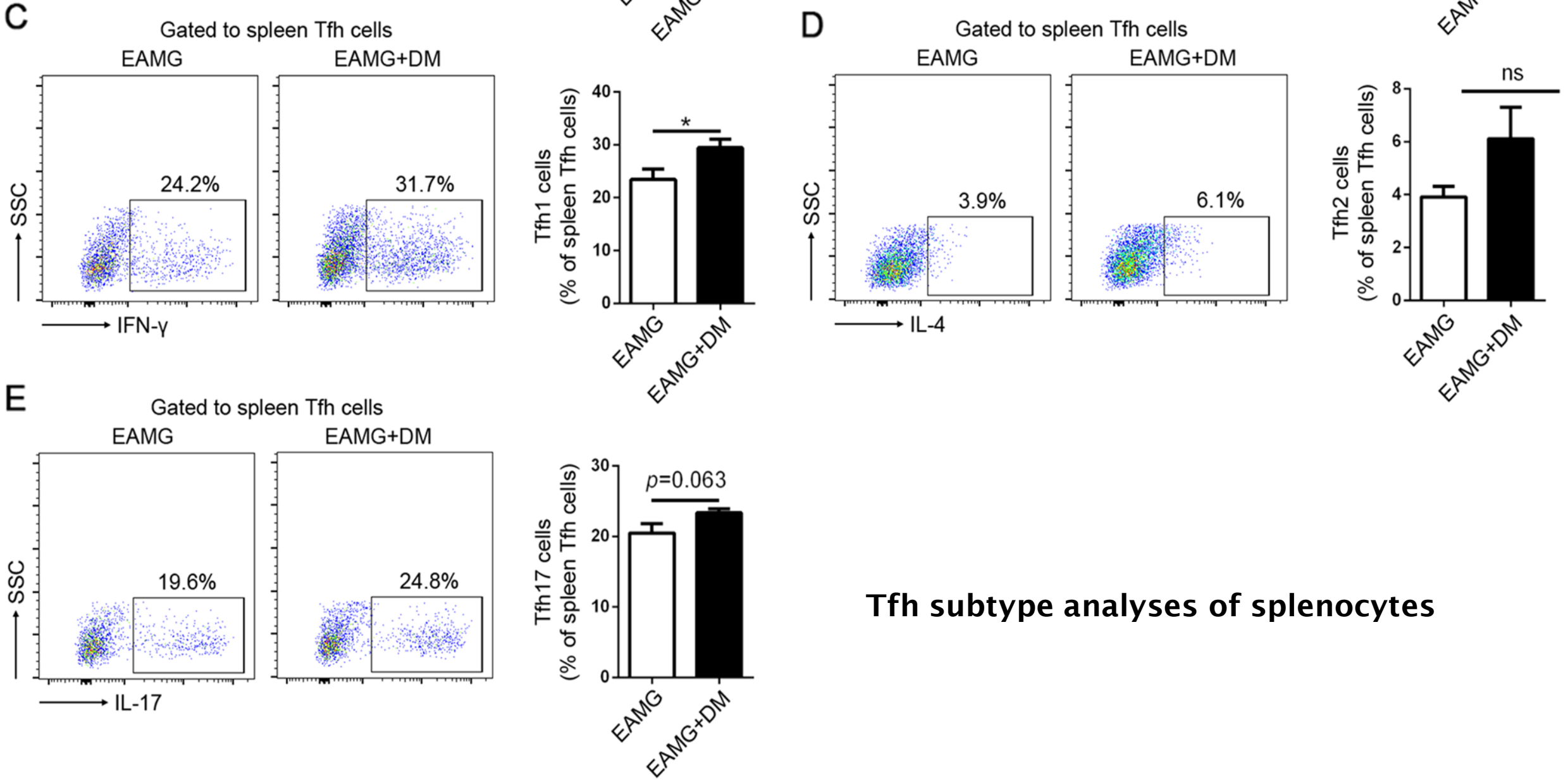


In EAMG + DM upregulated percentages of Th1 cells (CD4+IFN-γ+) downregulated Treg cells (CD4+CD25+Foxp3+)

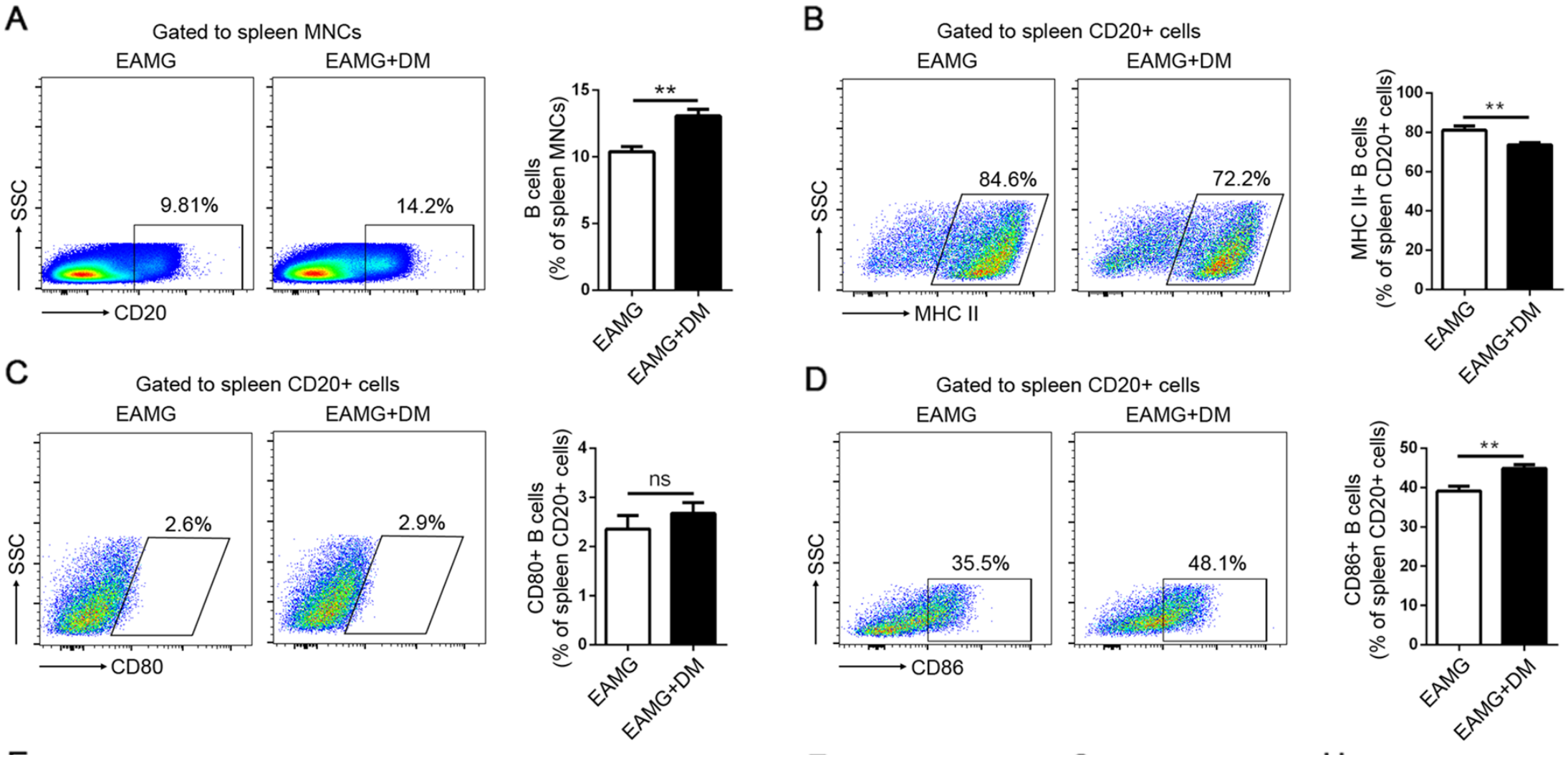
Diabetes increased Tfh cells in lymph nodes and upregulated Tfh1 and Tfh17 cells in the spleen of EAMG rats



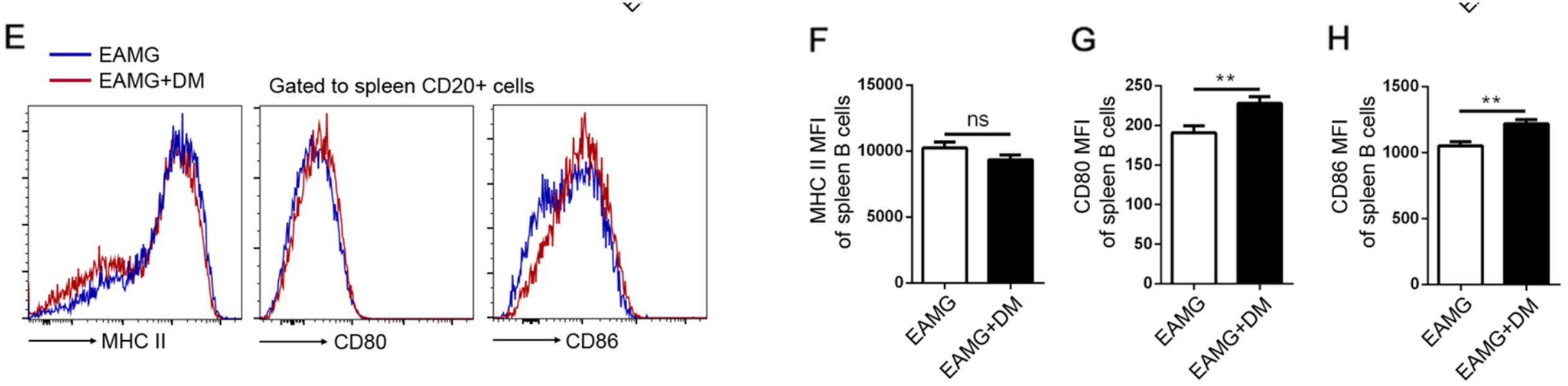
Percentages of Tfh cells in the lymph nodes but not the spleen, were enhanced significantly in the DM+EAMG group



Tfh subtype analyses of splenocytes



- CD20+ B cells were upregulated in the DM+EAMG group compared to the EAMG group, the percentages of MHC II positive splenocytes were decreased but the percentages of CD86 positive splenocytes were increased in the DM+EAMG


E
EAMG
EAMG+DM
Gated to spleen CD20+ cells
MHC II
CD80
CD86
F
MHC II MFI of spleen B cells
15000
10000
5000
0
ns
EAMG
EAMG+DM
G
CD80 MFI of spleen B cells
250
200
150
100
50
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EAMG
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CD86 MFI of spleen B cells
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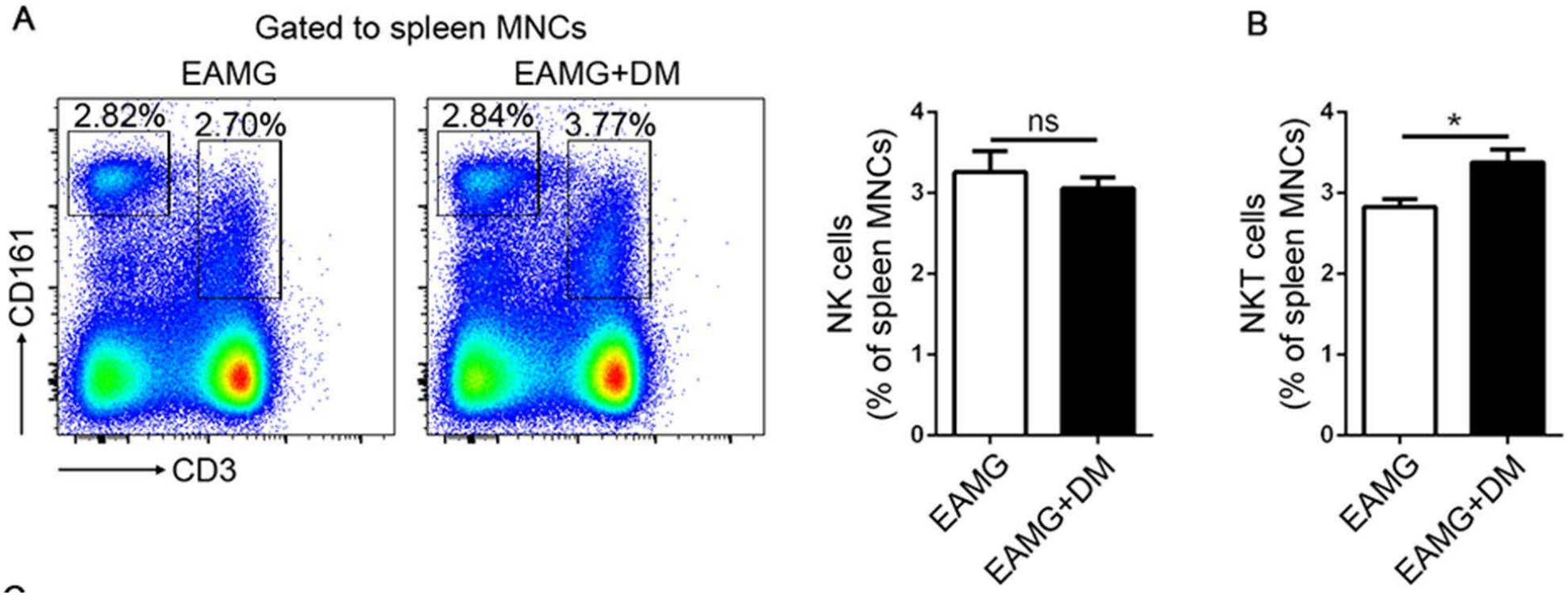
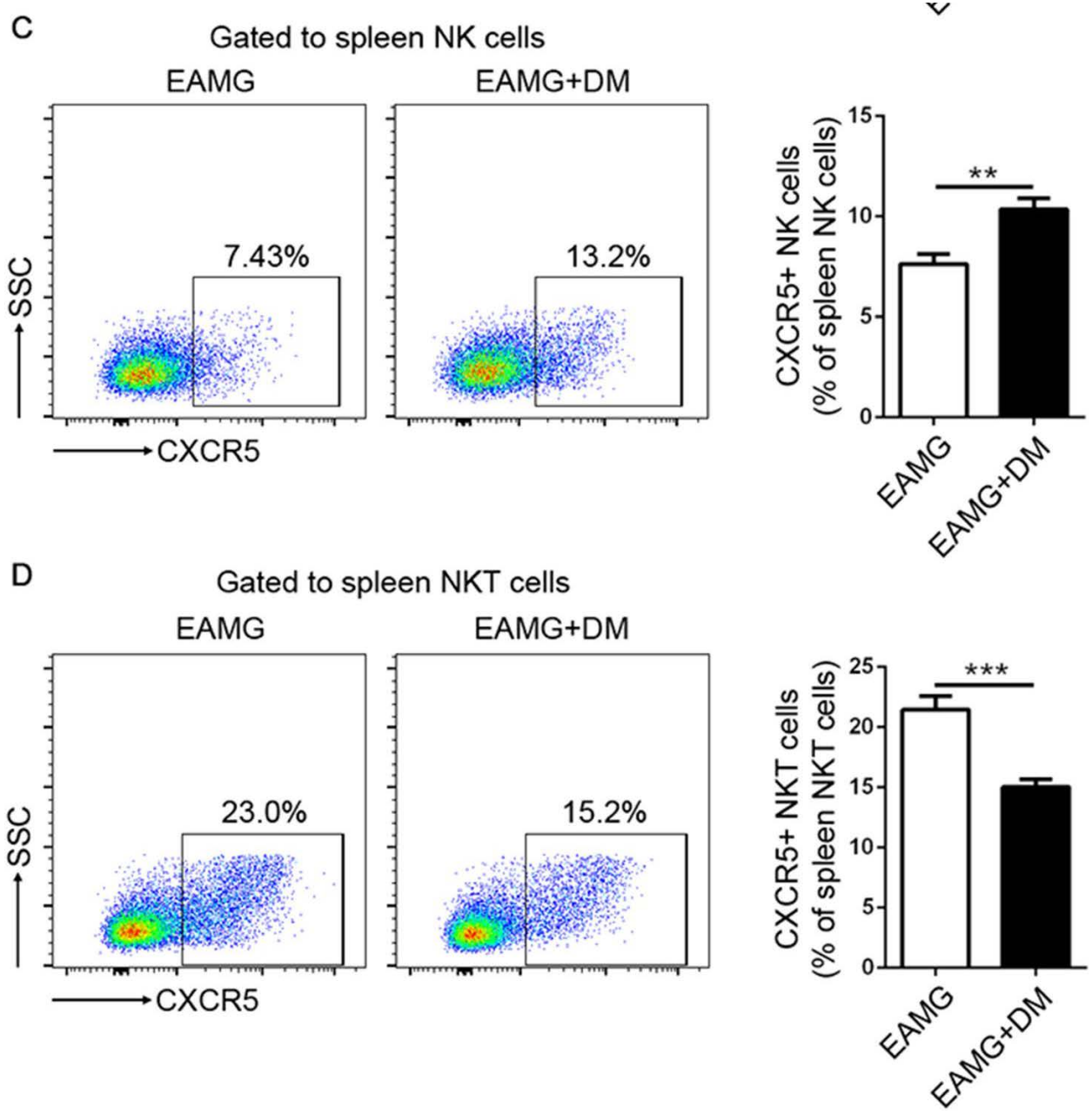
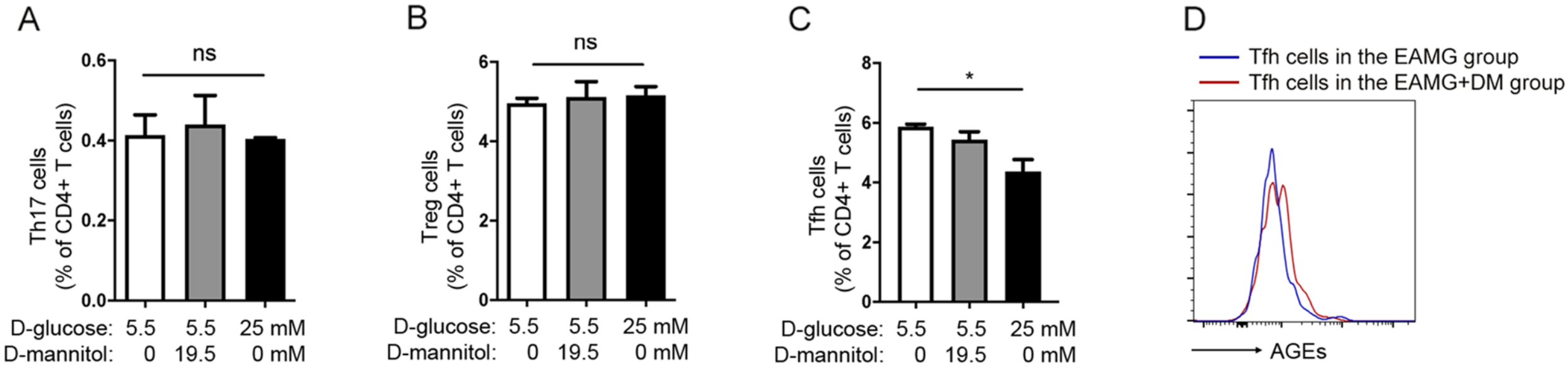
EAMG
EAMG+DM



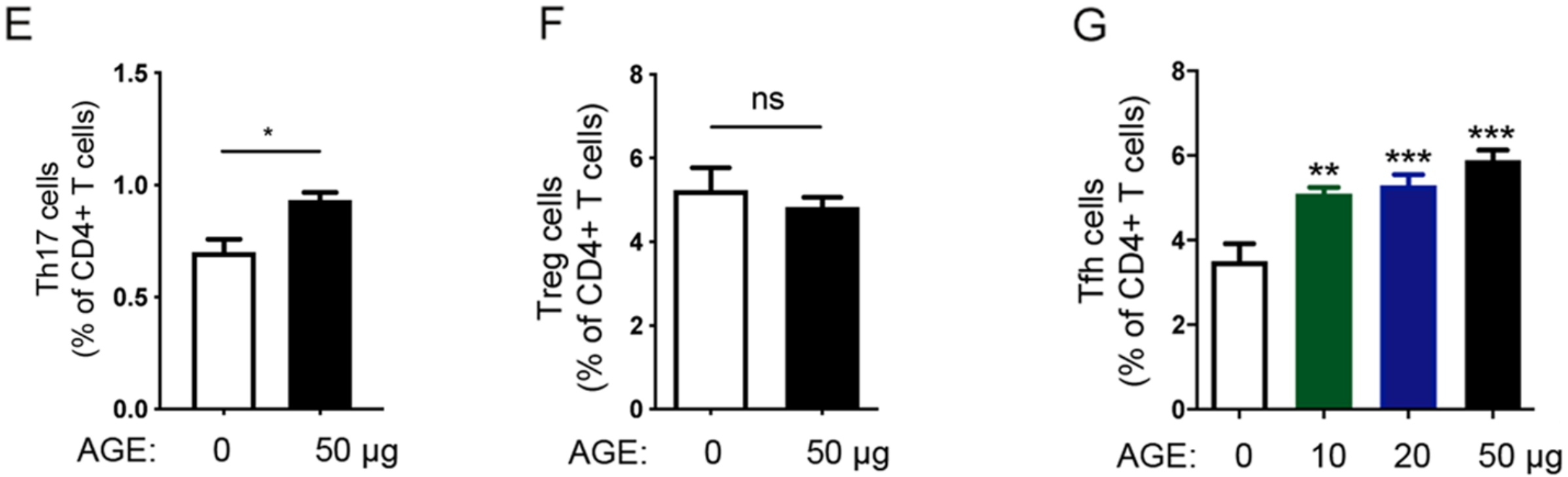
- NK and NKT cells were defined as CD3− CD161hi and CD3+ CD161+
- Significantly less NKT but not NK cells in EAMG + DM mice



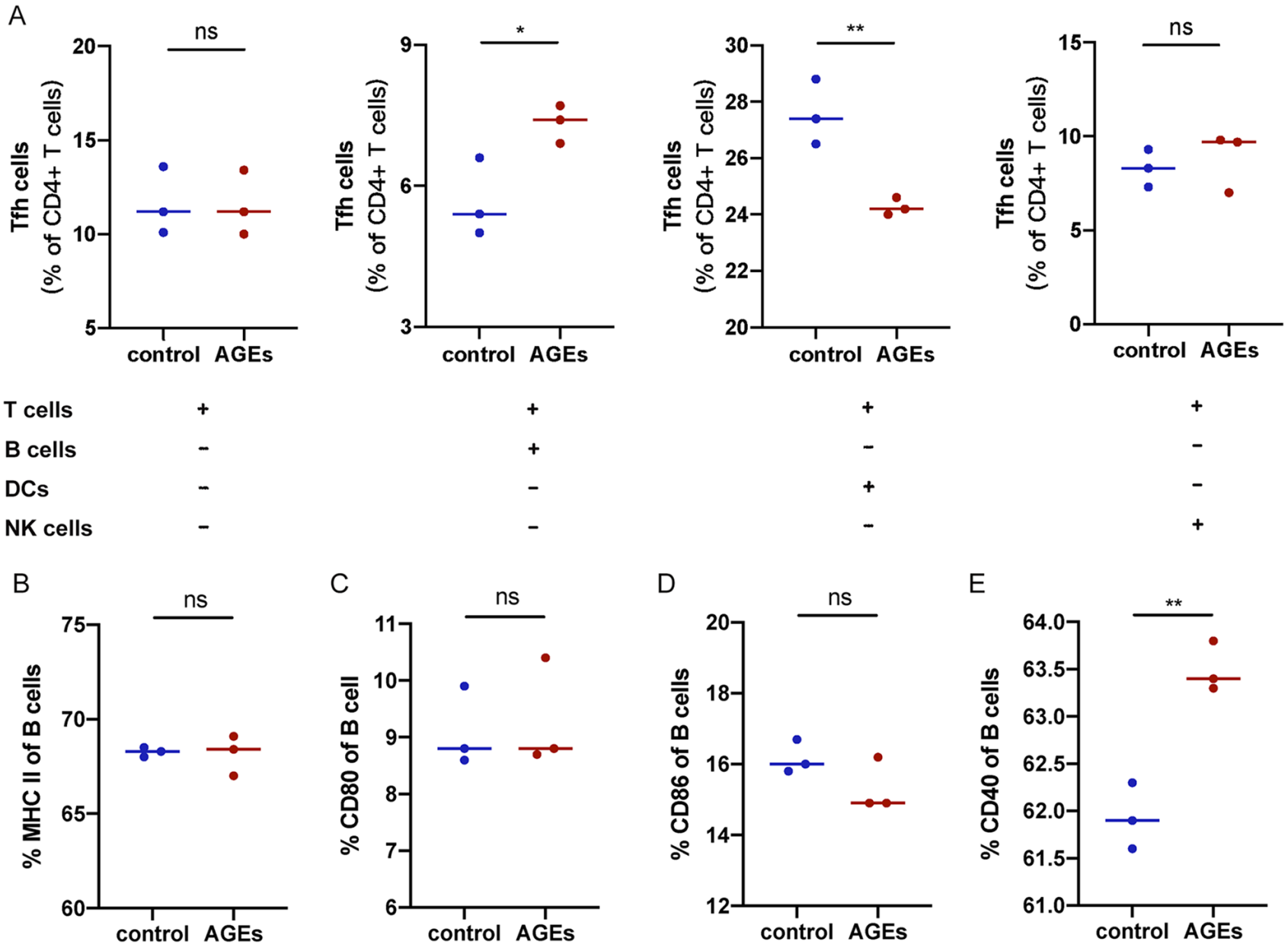
- Increase of CXCR5 expression on NK cells
- Decrease of CXCR5 in EAMG + DM splenocytes



- „However, neither low-level nor high-level concentration of D-glucose boosted the differentiation of CD4+ T cells towards Th17, Treg, or Tfh cells (Fig. 7A–C).”
- „Studies have shown (?) that the high level of AGEs may partially account for the pro-inflammatory conditions of DM”
- “Tfh cells from EAMG+DM rats exhibited increased intracellular AGE accumulation“



- Spleen MNCs were treated with or without AGEs
- In vitro treatment with AGEs induced a shift towards Th17 but not Treg differentiation and increased the percentage of Tfh cells in a dose dependent matter



→ No difference in the percentages of Tfh cells between various concentrations of AGEs (A1)

→ AGEs could specifically promote the differentiation of Tfh cells in the presence of B cells (A2)

→ When co-cultured with DCs the percentage of Tfh is lowered in the precence of AGEs.

→ "A slight but statistically significant increase of CD40 on B cells was observed in the AGEs-treated group.
However, there were no significant differences in the expression of MHC II, CD80, and CD86"

Discussion

- Diabetes exacerbated the clinical symptoms of EAMG rats
- The elevated production of the elevated anti-R97-116 peptide IgG antibody in DM rats may be the key role to aggravate EAMG, as they directly bind to the AchR at the neuromuscular junction
- Diabetes augmented Th1 and Th17 response in EAMG rats, while the percentage of Tregs was reduced in the spleen of EAMG + DM rats.

→ A putative shift to a proinflammatory phenotype (previously characterized in vivo in animal models and DM patients)

- Total Tfh cells and Tfh1 and Tfh17 subsets were upregulated

→ In accordance with increased disease severity, Elevated IgG levels, and ASCs the authors suggest that DM might foster a deletorius upregulation of humoral autoimmunity

- AGEs are a well described culprit in a variety of cardiovascular, metabolic and degenerative diseases associated to DM pathophysiology.
- The levels of AGEs were higher in EAMG + DM rats
- “Furthermore, our ex vivo data showed there were higher percentages of Tfh cells when spleen MNCs were treated with AGEs“
- „However, no differences were observed in Treg, Th17, and Tfh cells when co-cultured with or without d-glucose. These results provided strong evidence that the enhanced immune response in the DM+EAMG group was mediated by AGEs”
- In vitro data indicated that the effect of AGEs on Tfh differentiation depends on B-cells.

JC Discussion of the paper

- **Some suggestions:**
- Consider you are working on a similar research questions

→ Which results do you trust enough to consider them in planing your own project?

→ Does the paper provide you with all the information you need to replicate the results presented?

→ You are offered a collaboration with this laboratory, which tissue samples would from the EAMG + DM model would you like to analyse?

→ Is the narrative compelling? Would you further explore the main pathways of interest suggested, or are there other putative mechanisms of action you would like to learn more about?