Video Article

In Vitro Differentiation of Mouse Granulocyte-macrophage-colony-stimulating Factor (GM-CSF)-producing T Helper (T_HGM) Cells

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Abstract

The granulocyte-macrophage-colony-stimulating factor (GM-CSF)-producing T helper (T_HGM) cell is a newly identified T helper cell subset that predominantly secretes GM-CSF without producing interferon (IFN)γ or interleukin (IL)-17 and is found to play an essential role in the autoimmune neuroinflammation. A method of isolation of naive CD4+ T cells from a single-cell suspension of splenocytes and T_HGM cell generation from naive CD4+ T cells would be a useful technique in the study of T cell-mediated immunity and autoimmune diseases. Here we describe a method that differentiates mouse naive CD4+ T cells into T_HGM cells promoted by IL-7. The outcome of the differentiation was assessed by the analysis of the cytokines expression using different techniques, including intracellular cytokine staining combined with flow cytometry, a quantitative real-time polymerase chain reaction (PCR), and enzyme-linked immunosorbent assays (ELISA). Using the T_HGM differentiation protocol as described here, about 55% of the cells expressed GM-CSF with a minimal expression of IFNα or IL-17. The predominant expression of GM-CSF by T_HGM cells was further confirmed by the analysis of the expression of GM-CSF, IFNα, and IL-17 at both mRNA and protein levels. Thus, this method can be used to differentiate naive CD4+ T cells to T_HGM cells *in vitro*, which will be useful in the study of T_HGM cell biology.

Video Link

The video component of this article can be found at https://www.jove.com/video/58087/

Introduction

CD4+ T helper (T_H) cells are essential components of the immune system, having crucial roles in the host defense against microbial pathogens, in cancer surveillance, and in autoimmunity[1,2,3]. Upon T cell receptor (TCR) activation, naive CD4+ T cells can be differentiated into T_H1, T_H2, T_H 17, or regulatory T (Treg) cells under the influence of different cytokine milieus[2,4,5]. Recently, a new subset of T_H cells, which predominantly produces GM-CSF, was identified and named T_HGM[6]. The differentiation of T_HGM cells is driven by IL-7 through the activation of a signal transducer and an activator of transcription 5 (STAT5). These cells express a large amount of GM-CSF while having a low expression of other T_H-cell signature cytokines such as IFNγ and IL-17[6]. GM-CSF was found to play a critical role in the development of CD4+ T cell-mediated neuroinflammation[7,8]. Compared to IFNγ- or IL-17-expressing autoreactive T cells, GM-CSF-expressing T cells transferred to wild-type (WT) mice caused an earlier disease onset and higher disease severity. In addition, *Csf2*-/- T cells failed to induce experimental autoimmune encephalomyelitis (EAE) after being adoptively transferred to WT recipients, whereas T cells lacking IFNγ or IL-17A retained the ability to mediate EAE[7]. Moreover, a blockade of GM-CSF using neutralizing antibodies ameliorated EAE disease severity[8]. Furthermore, a deficiency of STAT5 in T cells in mice resulted in a diminished T_HGM generation and, hence, in a resistance of the mice to EAE development[6]. These findings underscore the importance of GM-CSF-expressing T_H cells in autoimmune neuroinflammatory disease. Thus, establishing a method to differentiate GM-CSF-expressing T_H cells from naive CD4+ T cells would be important in the study of the pathogenesis of autoimmune neuroinflammation and T cell-mediated immune responses. However, a protocol that efficiently generates T_HGM cells from murine naive CD4+ has not been established.

Here we present a method that differentiates murine T_HGM cells from naive CD4+ T cells. This protocol describes the whole procedure, including the extraction of the spleen from the mouse, the preparation of a single-cell suspension, the CD4 positive selection, the fluorescence-activated cell sorting (FACS), and the T_H cell differentiation and analysis. The differentiated T helper cells are analyzed by intracellular cytokine staining combined with flow cytometry to determine the cytokine expression at the single-cell level, by a quantitative real-time PCR to determine the cytokine expression at mRNA level, and by ELISA to assess the cytokine expression at protein level. This method can be applied to studies of T_HGM cell biology under various conditions, such as EAE, where GM-CSF plays an important role in pathogenesis.

Protocol

All mice used in this protocol were on the C57BL/6 genetic background and housed under specific pathogen-free conditions at the National University of Singapore. All experiments were performed using protocols approved by the Institutional Animal Care and Use Committee of the National University of Singapore.

1. Reagent and Material Preparation

1. Prepare 500 mL of phosphate-buffered saline (PBS) containing 2% fetal bovine serum (FBS) and 1 mM ethylenediaminetetraacetic acid (EDTA).
   NOTE: Keep the buffer on ice throughout the whole procedure for optimal results.
2. Prepare 50 mL of complete RPMI media containing RPMI 1640, 10% FBS, and 1% penicillin-streptomycin. Use complete RPMI media containing 50 µM β-mercaptoethanol for the T_HGM cell differentiation. Add the β-mercaptoethanol in a fume hood.
3. Prepare 7.5 mL of an anti-CD3e antibody mixture by diluting anti-CD3e concentrated stock to 3 µg/mL in PBS. Coat 48-well plates by adding 150 µL of the antibody mixture to each well and incubate the plate at 37 °C for at least 1 h, or at 4 °C overnight.
4. Sterilize a pair of scissors and forceps and keep them in 70% ethanol between dissections.

2. Preparation of Murine Splenocytes

1. Euthanize the mouse (of 6–8 weeks old) using an institutionally-approved CO_2 asphyxiation or cervical dislocation. Spray the mouse with 70% ethanol and mount it on a polystyrene block on its back. Transfer the mouse to a biological safety cabinet to proceed with the following steps.
2. Hold the skin using a pair of forceps and cut the skin below the rib cage on the left side for about 4 cm, using a pair of scissors. Open the peritoneal sac to expose the spleen and use sterile forceps to extract the spleen.
3. Place a 70 µm cell strainer on a 50 mL tube and pre-wet it with 2 mL of buffer. Place the spleens on the cell strainer (2–3 spleens for one cell strainer) and macerate them using the end of a 5 mL syringe plunger.
4. Rinse the strainer and tube several times with 1–2 mL of cell isolation buffer until all cells are flushed into the tube. Discard the cell strainer and centrifuge the cells at 350 x *g* for 5 min at 4 °C. Discard the supernatant.
5. Resuspend the cell pellet with 5 mL of cold (4 °C) ammonium-chloride-potassium (ACK) lysing buffer (150 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.2–7.4) and gently mix them for 2 min. Add 10 mL of complete RPMI media to neutralize the ACK buffer and immediately centrifuge the cells at 350 x *g* for 5 min at 4 °C. Discard the supernatant after the centrifugation.

3. Isolation of CD4+ T Cells Using Magnetic CD4 Microbeads and a Cell Separation Column

1. Resuspend the cell pellet in 5 mL of cell isolation buffer. Filter the cell suspension using a pre-separation filter (30 µm) into a new 15-mL tube to remove any debris.
2. Take 10 µL of the cell suspension and make a 10x dilution by adding 90 µL of buffer. Take 10 µL of the diluted cell suspension and mix it with 10 µL of trypan blue for cell counting. Count the cells in a hemocytometer to determine the yield of viable cells.
3. Centrifuge the cells at 350 x *g* for 5 min at 4 °C and discard the supernatant.
4. Resuspend the cells in 90 µL of buffer per 10^7 cells. Add 10 µL of magnetic CD4 microbeads per 10^7 cells. Incubate the cells for 15 min at 4 °C. For optimal results, mix the cell suspension gently every 5 min during incubation.
5. While the cells are incubating, place a separation column on the magnetic stand. Pre-wet the column with 2 mL of buffer.
6. At the end of the cell/bead incubation, wash the cells with 5 mL of buffer and centrifuge them at 350 x *g* for 5 min at 4 °C. Discard the supernatant.
7. Resuspend the cells in 1 mL of buffer, load the sample into the cell separation column, and let it flow through. Use a 15-mL centrifuge tube to collect the CD4- cell fraction. Add 1 mL of buffer to wash the reservoir before it is dry. Repeat the washing step 2x.
8. Remove the cell separation column from the magnetic stand and place it on a new 15 mL centrifuge tube. Add 2 mL of cell isolation buffer to the cell separation column and apply the plunger firmly to force the cells out of the column.
9. Centrifuge the fraction at 350 x *g* for 5 min at 4 °C to obtain CD4+ cells. Discard the supernatant.

4. Purification of the Naive CD4+ T cells ($CD4^+CD25^-CD44^{lo}CD62L^{hi}$) by Fluorescence-activated Cell Sorting and T_HGM differentiation

1. Resuspend the obtained CD4+ cell pellet in 500 µL of buffer. Mix the cells with a fluorescent-conjugated antibodies mixture containing CD4-PerCp (2.8 µg/mL), CD44-APC (2.4 µg/mL), CD25-PE (2 µg/mL), and CD62L-FITC (10 µg/mL) (**Table 1**). Incubate the cells on ice for 20–30 min, while protecting them from light.
   NOTE: The concentrations of the antibodies were optimized previously in the lab. The number of antibodies listed is for cells from 1–2 mice.
2. After the incubation, wash the cells with 5 mL of buffer and centrifuge the cells at 350 x *g* for 5 min at 4 °C.
3. Discard the supernatant and resuspend the cells in 500 µL of buffer. Filter the cells again using a nylon mesh.
4. Transfer the cell suspension to a FACS tube for cell sorting. Precoat the collection FACS tubes with 500 µL of buffer.
5. Obtain a $CD4^+CD25^-CD44^{lo}CD62L^{hi}$ population (of naive $CD4^+$ T cells) by FACS sorting. Gate on $CD4^+CD25^-$ first, and then select $CD44^{lo}CD62L^{hi}$ cells from this population.
6. Collect the naive $CD4^+$ T cell fractions into a new 15-mL centrifuge tube. Centrifuge the cells at 350 x *g* for 5 min at 4 °C and discard the supernatant.
7. Resuspend the naive CD4+ T cells at a concentration of 10^6 cells/mL in complete RPMI media containing 50 µM β-mercaptoethanol.

8. Aspirate the anti-CD3e antibodies used for precoating in the 48-well plate. Seed 0.25 million (250 µL) cells in each well with IL-7 (2 ng/mL), anti-CD28 (1 µg/mL), and anti-IFNγ (10 µg/mL) (**Table 1**).
   NOTE: The concentrations of cytokine and antibodies were optimized previously in the lab for a T_HGM cell differentiation.
9. Incubate the cells at 37 °C with 5% CO_2 for 3 days. Do not change the medium during the incubation.

5. Analysis of the Mouse T_HGM Cells Generated *In Vitro*

1. Using a microscope, check the cell differentiation 3 d after the differentiation. Harvest the cells and centrifuge them at 350 x *g* for 5 min at 4 °C. Wash the cells 2x with complete RPMI media.
   NOTE: Differentiated cells are larger in size than naive CD4+ T cells.
2. Resuspend the cells in 1 mL of complete RPMI media. Take 10 µL of cell suspension and mix it well with 10 µL of trypan blue to determine the cell number with a hemocytometer. Divide differentiated cells into three potions for a restimulation and analysis.
   NOTE: Intracellular cytokine staining and ELISA assays require ≥0.5 million cells, and qPCR analysis requires ≥ 1 million cells.
3. **For intracellular cytokine staining, restimulate the cells with phorbol 12-myristate 13-acetate (PMA) (100 ng/mL) and ionomycin (1 µg/mL) in the presence of a protein transport inhibitor (1 µL/mL) for 4–6 h.**
   1. Harvest the cells and stain them with anti-CD4 antibody (PerCP-conjugated, 0.2 mg/mL, 1:100 dilution; or FITC-conjugated, 0.5 mg/mL, 1:100 dilution) for 30 min.
   2. Fix the cells with 200 µL of fixation buffer for 20–60 min. After the fixation, wash the cells 2x with 1 mL of permeabilization buffer.
   3. Perform intracellular staining with antibodies against GM-CSF (PE-conjugated, 0.2 mg/mL, 1:100 dilution), IL-17A (FITC-conjugated, 0.5 mg/mL, 1:100 dilution; APC-conjugated, 0.2 mg/mL, 1:100 dilution), IL-4 (APC-conjugated, 0.2 mg/mL, 1:100 dilution), and IFNγ (APC-conjugated, 0.2 mg/mL, 1:100 dilution; PE-conjugated, 0.2 mg/mL, 1:100 dilution) for 30 min in the dark.
4. For a qPCR analysis of the cytokine gene expression by differentiated cells, activate the cells with plate-bound anti-CD3e (3 µg/mL) (precoating the plate as described in step 1.3). At 3 h after the stimulation, harvest the cells to isolate total RNA using a phenol RNA extraction reagent to prepare cDNA for the qPCR. The primers and program used for the qPCR are listed in **Tables 2** and **3**, respectively.
5. For the analysis of the cytokine protein secretion by differentiated cells, restimulate the cells with plate-bound anti-CD3e (3 µg/mL) for 24 h (precoating the plate as described in step 1.3). Harvest the cell culture supernatant for IL-17 and GM-CSF ELISA to determine their concentrations.

Representative Results

Naive CD4+ T cells isolated from two 8-week-old male C57BL/6 mice were divided into three portions. One portion of the cells was differentiated into T_HGM cells following the protocol described. Another portion was cultured under a T_HGM condition in the presence of anti-IL-4 antibody (10 µg/mL) to test the influence of an IL-4 blockade in the differentiation of T_HGM. The last portion was cultured under a T_H17 differentiation condition (3 µg/mL anti-CD3e, 1 µg/mL anti-CD28, 10 ng/mL TGFβ, 30 ng/mL IL-6, 10 µg/mL anti-IFNγ, and 10 µg/mL anti-IL-4). After 3 days of differentiation, the cells were harvested and restimulated to analyze the cytokine expression by intracellular cytokine staining, qPCR, and ELISA. Results from the intracellular cytokine staining and FACS analysis demonstrated that about 55% of the cells cultured under the T_HGM differentiation condition were GM-CSF-expressing cells (**Figure 1A**), whereas only about 2% of the cells differentiated under the T_H17 condition expressed GM-CSF. In addition, compared to the T_H17 differentiation condition which generated 8.17% IL-17-producing cells, the T_HGM differentiation condition only resulted in about 1% of IL-17-expressing cells. Under the two differentiation conditions tested, only a small fraction of the cells expressed IFNγ (<1%). Furthermore, few IL-4-expressing T cells (<1%) were seen in T_HGM or T_H17 culture (**Figure 1B**). These results showed that using the described T_HGM cell differentiation condition, we have successfully generated T helper cells that predominantly express GM-CSF.

The successful differentiation of T_HGM cells was further confirmed by results from qPCRs and ELISA assays to examine the expression of GM-CSF and IL-17 at both RNA and protein levels in the differentiated cells. The generated T_HGM cells had a significantly higher expression of *Csf2* but a much lower expression of *Il17* compared to the T_H17 cells (**Figure 2A**). As shown in **Figure 2B**, GM-CSF protein was detected in culture supernatants from both T_HGM and T_H17 cells. Nevertheless, the GM-CSF concentration in the T_HGM culture supernatant was about threefold of that in T_H17 cells. In addition, proteins of IL-17 (**Figure 2B**), IFNγ, and IL-4 were undetectable in the T_HGM cell culture supernatant. Since RORγt has been identified as the master transcription factor of T_H17 cells, while STAT3 and STAT5 have been shown to have a great importance in the development of T_H17 and T_HGM cells, respectively, we examined their mRNA expression in the T_HGM and T_H17 cells. It was found that the T_HGM cells had a significantly lower expression of *Rorc* and a higher *Stat5* expression than the T_H17 cells (**Figure 3**). Interestingly, the T_HGM cells had a slightly lower (but not significant) expression of the *Stat3* gene compared to the T_H17 cells. These results indicated that although the T_HGM and the T_H17 differentiation are governed by different transcriptional factors, they may share some common features.

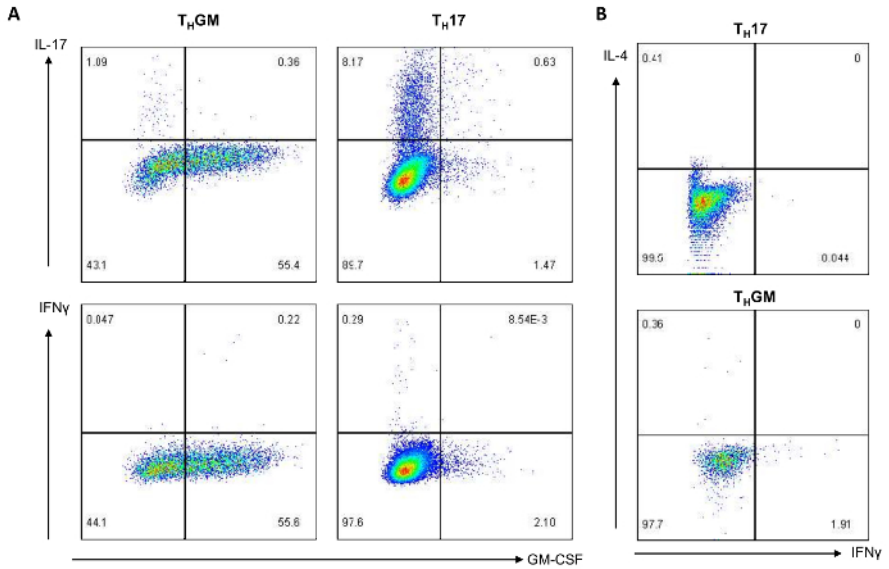

Figure 1: T_HGM cells predominantly express GM-CSF. T_HGM and T_H17 cells were harvested on day 3 after the differentiation. (**A**) For 5 h, the cells were restimulated with PMA and ionomycin in the presence of a protein transport inhibitor. The expression of GM-CSF, IL-17, and IFNγ was analyzed by intracellular cytokine staining followed by flow cytometry. (**B**) The IL-4 and IFNγ expression by T_H17 or T_HGM cells was analyzed. Please click here to view a larger version of this figure.

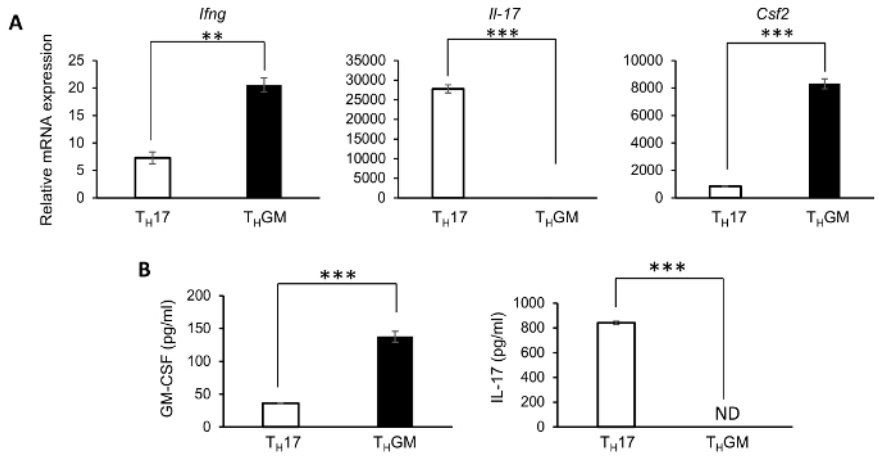

Figure 2: Expression of IL-17, IFNγ, and GM-CSF in T_H17 and T_HGM cells. T_HGM and T_H17 cells were harvested and restimulated with plate-bound anti-CD3e, 3 days after the differentiation. (**A**) Cells were harvested to isolate RNA for cDNA preparation, 3 h after the stimulation. The expressions of *Ifng*, *Il-17*, and *Csf2* were determined by a quantitative real-time PCR (qPCR). (**B**) The culture supernatant was harvested at 24 h after the stimulation, to determine the concentration of GM-CSF in T_HGM cells, or IL-17 in T_H17 cells, by ELISA. (** p <0.01, *** p <0.001.) Please click here to view a larger version of this figure.

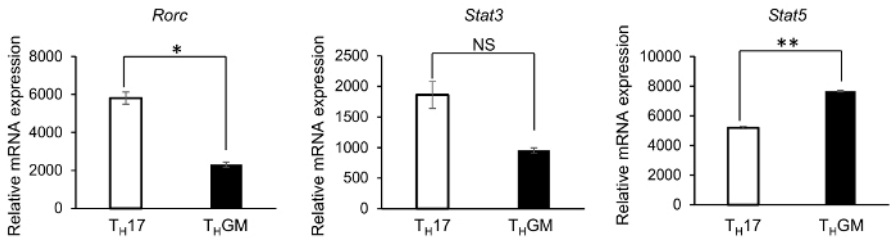

Figure 3: *Rorc, Stat3,* and *Stat5* gene expression in T_H17 and T_HGM cells. Differentiated T_HGM and T_H17 cells were harvested and restimulated with plate-bound anti-CD3e for 3 h. Total RNA was isolated to prepare cDNA. The *Rorc*, *Stat3*, and *Stat5* expressions were determined by qPCR. (* p <0.05, ** p <0.01; NS = not significant.) Please click here to view a larger version of this figure.

Name	Stock concentration	working concentration	Dilution	volume in 500 µL staining buffer
CD4-PerCp	0.2 mg/mL	2.8 µg/mL	1:71	7 µL
CD44-APC	0.2 mg/mL	2.4 µg/mL	1:83	6 µL
CD25-PE	0.2 mg/mL	2 µg/mL	1:100	5 µL
CD62L-FITC	0.5 mg/mL	10 µg/mL	1:50	10 µL
GM-CSF-PE	0.2 mg/mL	2 µg/mL	1:100	
IL-17A-FITC	0.5 mg/mL	5 µg/mL	1:100	
IL-17A-APC	0.2 mg/mL	2 µg/mL	1:100	
IFNγ-APC	0.2 mg/mL	2 µg/mL	1:100	
IFNγ-PE	0.2 mg/mL	2 µg/mL	1:100	
IL-4-APC	0.2 mg/mL	2 µg/mL	1:100	
CD4-FITC	0.5 mg/mL	5 µg/mL	1:100	
IL-7	20 µg/mL	2 ng/mL	1:10000	
Anti-CD28	0.5 mg/mL	1 µg/mL	1:500	
Anti-IFNγ	1 mg/mL	10 µg/mL	1:100	

Table 1: Used cytokines and antibodies.

Primer	Sequence
Ifng Forward	5'-TCAAGTGGCATAGATGTGGAAGAA-3'
Ifng Reverse	5'-TGGCTCTGCAGGATTTTCATG-3'
Il-17 Forward	5'-CTCCAGAAGGCCCTCAGACTAC-3'
Il-17 Reverse	5'-AGCTTTCCCTCCGCATTGACACAG-3'
Csf2 Forward	5'-TTTACTTTTCCTGGGCATTG-3'
Csf2 Reverse	5'-TAGCTGGCTGTCATGTTCAA-3'
Rorc Forward	5'-TTTGGAACTGGCTTTCCATC-3'
Rorc Reverse	5'-AAGATCTGCAGCTTTTCCACA-3'
Stat3 Forward	5'-TGGCCCTTTGGAATGAAGGGTACA-3'
Stat3 Reverse	5'-CACTGATGTCCTTTTCCACCCAAGT-3'
Stat5 Forward	5'-TGCCCGGCTGGAACTACACCTT-3'
Stat5 Reverse	5'-ATGCCCCCGATTTCCGAGTCAC-3'

Table 2: Primers for qPCR.

step	Temp. (°C)	time	repeat	
1	95	2 min		
2	95	3 s		
3	60	30 s	step 2 and 3, 39 times	read plate
4	65–95	5 s	step 4, 30 times, +0.5°C each repeat	read plate

Table 3: PCR program to assess gene expression.

Discussion

Here we described a protocol of an *in vitro* T_HGM differentiation from mouse naive CD4+ cells, followed by an analysis of the differentiated cells to validate the method. Of note, both spleen and lymph nodes can be used for naive CD4+ T cell purification and T_HGM differentiation. The cytokine expression determined by intracellular cytokine staining combined with flow cytometry showed that about 55% of the cells were induced to become GM-CSF-expressing cells under the T_HGM condition (**Figure 1A**). Compared to cells under the T_H17 differentiation condition, the cells that differentiated under the T_HGM condition contained a background level of an IL-17-expressing population; however, the *il17* gene expression is undetectable by qPCR (**Figure 2A**).

Interestingly, despite the addition of an IFNγ neutralizing antibody, we detected a low level of *Ifng* mRNA expression in both the T_H17 and the T_HGM cells, and less than 1% of the T_HGM cells were IFNγ-expressing cells. (**Figures 1** - **2**). This could be due to the insufficient amount of IFNγ-neutralizing antibody used in the T_HGM condition, or to a possible contamination of innate immune cells (it is almost impossible to obtain 100% pure naive CD4+ T cells) that provided a trace amount of IL-12 for the possible T_H1 cell generation. It is also possible that the T_HGM condition we used was not able to completely shut off the transcription of *Ifng*. We will address this issue in future. Nevertheless, IFNγ protein was not detected in the culture supernatants of T_HGM or T_H17 by ELISA.

In the protocol presented here, we only used an IFNγ-blocking antibody together with IL-7 for the T_HGM differentiation. After 3 days of differentiation under this condition, more than 50% of the cells expressed GM-CSF, but not IL-17, IL-4, or IFNγ (**Figure 1**). In addition, the expression of *Rorc*, the gene that encodes RORγt, which is critical for the Th17 differentiation[9], was significantly lower in the T_HGM cells compared to that in the T_H17 cells (**Figure 3**). This protocol is, therefore, a more efficient method for the generation of GM-CSF-expressing T cells than another reported previously[10]. Our results also demonstrated that, in addition to the purity of naive T cells and IL-7 signaling, the prevention of the IFNγ-expressing T cell differentiation is a key for T_HGM generation.

The predominant expression of the GM-CSF cells that were generated using the described protocol demonstrated the successful differentiation of T_HGM. We would like to point out that the quality of cytokines and antibodies from different companies or even of different batches from the same company may not be the same. Therefore, we recommend that the number of cytokines and antibodies used in T helper cell differentiation should be tested in order to find out the optimal condition.

In summary, T_HGM cells that were generated using this protocol express a minimal level of IL-17, IL-4, or IFNγ. We are confident that this protocol works well in generating GM-CSF-expressing T_HGM cells *in vitro*. This method can be used to further study the biology of T_HGM cells in various conditions such as autoimmune neuroinflammation, including experimental autoimmune encephalomyelitis (EAE) in mice and human multiple sclerosis, where GM-CSF plays an important role in the pathogenesis[11].

Disclosures

The authors have nothing to disclose.

Acknowledgements

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