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## Determination of regulatory T cell subsets in murine thymus, pancreatic draining lymph node and spleen using flow cytometry --Manuscript Draft--

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Corresponding Author:	Kailash Singh Uppsala University Uppsala, N/A SWEDEN
Corresponding Author's Institution:	Uppsala University
Corresponding Author E-Mail:	kailash.singh@mcb.uu.se
Order of Authors:	Zhengkang Luo Lina Thorvaldson Martin Blixt Kailash Singh
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**TITLE:**

Determination of Regulatory T Cell Subsets in Murine Thymus, Pancreatic Draining Lymph Node and Spleen Using Flow Cytometry

**AUTHORS AND AFFILIATIONS:**

Zhengkang Luo<sup>1</sup>, Lina Thorvaldson<sup>1</sup>, Martin Blixt<sup>1</sup>, Kailash Singh<sup>1</sup>

<sup>1</sup>Department of Medical Cell Biology, Uppsala University, Uppsala, Sweden

**Corresponding Authors:**

Kailash Singh

Email: Kailash.Singh@mcb.uu.se

Zhengkang Luo

Email: Zhengkang.Luo@mcb.uu.se

**KEYWORDS:**

Single cell preparation, regulatory T cell, flow cytometry, Foxp3, Helios, Neuropilin 1

**SUMMARY:**

Herein, we present a protocol to prepare single cells from murine thymus, pancreatic draining lymph node and spleen to further study these cells using flow cytometry. In addition, this protocol was used for determining the subsets of regulatory T cells using flow cytometry.

**ABSTRACT:**

Our immune system consists of a number and variety of immune cells including regulatory T cells (Treg) cells. Treg cells can be divided into two subsets, thymic derived Treg (tTreg) cells and peripherally induced Treg (pTreg) cells. They are present in different organs of our body and can be distinguished by specific markers, such as Helios and Neuropilin 1. It has been reported that tTreg cells are functionally more suppressive than pTreg cells. Therefore, it is important to determine the proportion of both tTreg and pTreg cells when investigating heterogeneous cell populations. Herein, we collected thymic glands, pancreatic draining lymph nodes and spleens from normoglycemic non-obese diabetic mice to distinguish tTreg cells from pTreg cells using flow cytometry. We manually prepared single cell suspensions from these organs. Fluorochrome conjugated surface CD4, CD8, CD25, and Neuropilin 1 antibodies were used to stain the cells. They were kept in the fridge overnight. On the next day, the cells were stained with fluorochrome conjugated intracellular Foxp3 and Helios antibodies. These markers were used to characterize the two subsets of Treg cells. This protocol demonstrates a simple but practical way to prepare single cells from murine thymus, pancreatic draining lymph node and spleen and use them for subsequent flow cytometric analysis.

**INTRODUCTION:**

Regulatory T (Treg) cells are critical for the homeostasis of immune system. Treg cells are defined by the expression of surface antigens CD4 and CD25, and the transcription factor forkhead box P3 (Foxp3)<sup>1-3</sup>. Sakaguchi *et al.* first showed that CD25 is constitutively expressed

on T suppressor cells in mice<sup>4</sup>, which provided a pioneering observation for the identification of human Treg cells. Treg cells play a central role in immune tolerance by exerting a suppressive ability via a variety of mechanisms, including cytotoxicity through the secretion of granzymes to induce apoptosis, cytokine consumption and inhibition through the expression of cytotoxic T-lymphocyte antigen 4<sup>5</sup>. Additionally, they can secrete inhibitory cytokines such as transforming growth factor beta (TGF- $\beta$ ), interleukin-10 (IL-10) and IL-35<sup>5</sup>. Treg cells can naturally be derived from the thymus, in which case they are designated thymic derived Treg (tTreg) cells, or they can be induced in the peripheral organs in that case are called peripherally induced Treg (pTreg) cells.

Helios, a member of the Ikaros transcription factor family, was reported to be a marker for distinguishing between tTreg cells and pTreg cells<sup>6</sup>. Later, two other groups reported that Neuropilin 1 (Nrp1), a semaphorin III receptor, could serve as a surface marker for tTreg cells under certain conditions<sup>7,8</sup>. Nevertheless, Singh *et al.* have shown that Helios is a better marker in this context in naïve mice<sup>9</sup>.

The subsets of Treg cells play a pivotal role in the maintenance of the homeostasis of the immune system and in the protection against autoimmunity and infection. Therefore, it is important to determine the numbers and proportions of these populations in both lymphoid and non-lymphoid tissues. To achieve this, one has to prepare single cell suspensions from these organs. A number of protocols have been described or used in previous studies. Mainly, automatic dissociators have been used in previously published reports<sup>10,11</sup>. Using automatic dissociators is convenient and time saving, but it is an expensive procedure. Therefore, we have used a manual method to prepare single cell suspensions from murine thymic glands, pancreatic draining lymph nodes (PDLNs) and spleens from normoglycemic non-obese diabetic (NOD) mice, and isolated single cells from these organs. This method has been used in our previous studies and we found that the manual method for preparing single cell suspension is as efficient as automatic dissociator method<sup>9,12-14</sup>. Furthermore, we used flow cytometry to determine the proportions of CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells and the subsets of Treg cells tTreg and pTreg cells. The tTreg and pTreg cells were distinguished using Helios and Nrp1 as markers for tTreg cell. Thus, our results indicate that the manual method for preparing the single cells does work efficiently and the prepared single cell suspensions can be further used for studies using flow cytometry.

## **PROTOCOL:**

The local animal ethics committee at Uppsala University approved the animal experiments.

### **1. Harvesting Organs from Animals**

1.1. Sacrifice the mice by cervical dislocation.

1.2. Place thymic glands and spleens in 20 mL scintillation vials or 15 mL conical tubes filled with 5 mL of Hanks' balanced salt solution (HBSS). Place PDLNs in 1.5 mL micro tubes filled with 1 mL of RPMI 1640. Use the whole thymus and spleen, and all the PDLNs.

Note: Organs should be kept on ice throughout the procedure and the investigator should try to be fast while preparing single cell suspensions, especially when handling thymic tissue since thymic immune cells can be quickly degraded at high temperatures.

## **2. Single Cell Isolation from Thymus and Spleen**

2.1. Thoroughly squeeze the thymus and spleen with a pair of tweezers to release the immune cells. Discard the remaining thymic and splenic capsule.

2.2. Transfer the cell suspension to a 15 mL conical tube and centrifuge at 433 x g for 5 min at 4 °C to form a pellet. Discard the supernatant.

2.3. To lyse the red blood cells, resuspend the cell suspension in 5 mL of 0.2 M  $\text{NH}_4\text{Cl}$  and incubate at room temperature for 10 min. Invert the tubes upside down gently every 2 min. Add 5 mL of HBSS at the end of the incubation to stop the lysis.

2.4. Centrifuge the tubes at 433 x g for 5 min at 4 °C. Discard the supernatant.

2.5. Resuspend the cell pellet in approximately 5 mL of HBSS. Fill the tubes with HBSS.

2.6. Centrifuge the tubes at 433 x g for 5 min at 4 °C. Discard the supernatant.

2.7. Resuspend the cell pellet in approximately 5 mL of HBSS. Fill the tubes with HBSS.

2.8. Centrifuge the tubes at 433 x g for 5 min at 4 °C. Discard the supernatant.

2.9. Resuspend the cell pellet in in 5 mL of HBSS for thymus and 10 mL for spleen.

2.10. Transfer 1 mL of the thymic cell suspension and 500  $\mu\text{L}$  of the splenic cell suspension to 5 mL round bottom tubes with cell strainer caps. Apply the cell suspension to the cell strainer caps.

Note: A 35  $\mu\text{m}$  nylon mesh is incorporated into the cap. Approximately 5-10 million cells were collected in each tube.

## **3. Single Cell Isolation from PDLN**

3.1. Place a sterile 250  $\mu\text{m}$  metal mesh over a 15 mL conical tube in a rack. Rinse the mesh with 1 mL of RPMI.

3.2. Transfer the lymph nodes to the metal mesh and grind them through the mesh using a pair of tweezers. Apply 1 mL of RPMI on the metal mesh to flush the cells into the tube. Repeat 3 more times and remove the mesh.



133  
134 3.3. Centrifuge the tubes at 433 x g for 5 min at 4 °C. Discard the supernatant.

135  
136 3.4. Resuspend the cell pellet in approximately 5 mL of RPMI. Fill the tubes with RPMI.

137  
138 3.5. Centrifuge the tubes at 433 x g for 5 min at 4 °C. Discard the supernatant.

139  
140 3.6. Resuspend the cell pellet in approximately 5 mL of RPMI. Fill the tubes with RPMI.

141  
142 3.7. Centrifuge the tubes at 433 x g for 5 min at 4 °C. Discard the supernatant.

143  
144 3.8. Resuspend the cell pellet in 2 mL of RPMI. Transfer 2 mL of the cell suspension to 5 mL  
145 round bottom tubes with cell strainer caps. Apply the cell suspension to the cell strainer caps.

#### 146 147 **4. Flow Cytometry**

148  
149 4.1. Centrifuge the cell suspensions from thymus, PDLN and spleen at 433 x g for 5 min at 4  
150 °C. Discard the supernatant.

151  
152 4.2. Stain the cells with the following surface antibodies in 100 µL of flow cytometry staining  
153 buffer (FACS buffer): 0.75 µg/100 µL FITC-conjugated CD4 antibody, 0.60 µg/100 µL APC-H7-  
154 conjugated CD8 antibody, 0.30 µg/100 µL PE-conjugated CD25 antibody, 5 µL (1: 20) APC-  
155 conjugated Nrpl antibody. Incubate the tubes on ice for 40 min.

156  
157 4.3. Add 200 µL of FACS buffer to each tube. Centrifuge the tubes at 433 x g for 5 min at 4 °C.  
158 Discard the supernatant. Repeat one more time.

159  
160 4.4. Fix and permeabilize the cells by resuspending the cell pellet in 500 µL of  
161 Permeabilization Fixation Buffer (PFB).

162  
163 Note: PFB is prepared by mixing 1 part of Fixation/Permeabilization Concentrate with 3 parts of  
164 Fixation/Permeabilization Diluent.

165  
166 4.5. Keep the tubes in the fridge at 4 °C overnight.

167  
168 Note: A 1 h incubation also works.

169  
170 4.6. Centrifuge the tubes at 433 x g for 5 min at 4 °C. Discard the supernatant.

171  
172 4.7. Resuspend the cell pellet in 500 µL of Permeabilization Washing Buffer (PWB).  
173 Centrifuge the tubes at 433 x g for 5 min at 4 °C. Discard the supernatant.

174  
175 Note: PWB is prepared by diluting Permeabilization Buffer (10x) to 1:10 in distilled water.

176

4.8. Stain the cells with the following intracellular antibodies in 100  $\mu$ L of PWB: 0.30  $\mu$ g/100  $\mu$ L PE-Cy7-conjugated Foxp3 antibody, 4  $\mu$ L (1: 25) PacificBlue-conjugated Helios antibody. Incubate the tubes on ice for 1 h.

4.9. Add 500  $\mu$ L of PWB to each tube. Centrifuge the tubes at 433 x g for 5 min at 4  $^{\circ}$ C. Discard the supernatant.

4.10. Resuspend the cell pellet in 500  $\mu$ L of PWB. Centrifuge the tubes at 433 x g for 5 min at 4  $^{\circ}$ C. Discard the supernatant.

4.11. Resuspend the cell pellet in 300  $\mu$ L of FACS buffer.

4.12. Analyze the cells on a flow cytometer.

4.13. Gate the single cells based on Side Scatter-Area, Height and Weight, and Forward Scatter-Area, Height and Weight. Run one million events for analysis.

4.14. Export the FCS files of experiments and analyze them using a flow cytometry analyzer software.

#### REPRESENTATIVE RESULTS:

To investigate the expression of Nr1p and Helios on tTreg and pTreg cells, we prepared single cells from thymic glands, PDLNs and spleens of normoglycemic NOD mice and stained them with the Treg cell markers CD4, CD25, Foxp3, Helios and Nr1p for flow cytometric analysis. The results were analyzed as shown in the representative gating strategies (**Figure 1**). We found that the proportion of Helios<sup>+</sup> cells among CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells was higher than that of Nr1p<sup>+</sup> cells in all three organs (**Figure 2A**). More than 80% of the Treg cells in the thymus expressed Helios, which was higher than in the PDLN and spleen (**Figure 2A**). The proportion of Nr1p<sup>+</sup> cells among Helios<sup>+</sup> Treg cells was higher in the PDLN than those in the thymus and spleen (**Figure 2B**). The majority of Nr1p<sup>+</sup> Treg cells also expressed Helios, and the proportions of Helios<sup>+</sup> cells among Nr1p<sup>+</sup>Treg cells in the thymus and spleen were higher than that in the PDLN (**Figure 2C**). Together, these results indicate that Helios is a better marker to detect tTreg cells than Nr1p.

#### FIGURE AND TABLE LEGENDS:

**Figure 1. Representative gating strategies for flow cytometry.** The live single cells were gated based on FSC and SSC. Then, cells were further gated for CD4 and CD8 expression. CD4<sup>+</sup>CD8<sup>-</sup> cells were further gated for the expression of CD25. Next, CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup> cells were gated for Foxp3 expression. CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells were gated for Nr1p or Helios expression.

**Figure 2: The expression of Nr1p and Helios in Treg cells in the thymus, PDLN and spleen.** Four 8-week-old non-diabetic NOD mice were sacrificed. Single cells were prepared from the thymic glands, PDLNs and spleens. The cells were then stained with fluorochrome conjugated antibodies for flow cytometric analysis. (**A**) The percentages of Nr1p<sup>+</sup> and Helios<sup>+</sup> cells among

CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells. (B) The percentages of Nrp1<sup>+</sup> cells among Helios<sup>+</sup> Treg cells. (C) The percentages of Helios<sup>+</sup> cells among Nrp1<sup>+</sup> Treg cells. Results are expressed as means ± SEM. \*\*\* $p < 0.001$ . One-way ANOVA followed by Tukey's multiple comparisons (A, B and C) and unpaired t-tests (A) were performed.

## DISCUSSION:

In this study, we isolated single cells from thymic glands, PDLNs and spleens of NOD mice, and investigated the expression of Helios and Nrp1 in CD4<sup>+</sup>CD8<sup>+</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> Treg cells using flow cytometry. In the present study, NOD mice were used, which is a murine model of type 1 diabetes. In a previous study, we have used the wild type mouse strains CD-1 and C57BL/6 mice to investigate whether Helios or Nrp1 is a better marker for distinguishing tTreg cells from pTreg cells. In that study, we used a similar protocol to determine the subsets of Treg cells in naïve mice<sup>9</sup>. We found that Helios is a better marker in this context. Therefore, herein we used normoglycemic NOD mice to confirm our earlier finding in this model and found that the results were consistent with our previous results<sup>10</sup>. This protocol provides an efficient, yet economical way to prepare single cells and subsequently use them for flow cytometric analysis. There are several published protocols in which single cells are prepared using an automatic dissociator<sup>11,12</sup>. Instead of using a dissociator, by squeezing the thymic glands and spleens manually and discarding the remaining capsule, we can avoid the presence of fibrous tissue in the cell suspension and make sure the cell suspension is mainly composed of immune cells and red blood cells. We then used NH<sub>4</sub>Cl to lyse the red blood cells. The duration of lysis can be extended for a few more minutes if the organs are larger than the normal size. However, the lysis duration should not be extended too much, otherwise immune cells will also be lysed. It is important to let the cell suspension go through a cap with cell strainer (or other equivalent cell filters), to prevent clog problems during flow cytometry.

Working in the dark is recommended when working with fluorochrome conjugated antibodies to avoid longer exposure to light. We incubate the cells on ice for extracellular and intracellular staining. The incubation can also be done at room temperature to allow a faster binding of antibodies, but there is a risk of unspecific binding.

Some intracellular markers require large numbers of cells to give good signals in flow cytometry. Given the small size of PDLNs, grinding them on a metal mesh requires more care to allow a maximal release of immune cells. Collecting more PDLNs is also a way to increase the cell number. Another limitation of this protocol is that the cells are fixed and permeabilized for flow cytometric analysis, thus they cannot be used for subsequent functional studies. Magnetic sorting is an option to allow functional studies if only surface markers are of interest.

This protocol has been applied in other mouse models of autoimmunity and cancer<sup>13-15</sup>. Single cell preparation is a basic but useful technique to determine the characteristics of immune cells in different immune organs. Using this method for single cell preparation might cause necrosis, which may result in decreasing the total number of cells. The spectral overlap of traditional flow cytometry limits the number of markers that can be detected at the same time. Therefore, several panels and large numbers of cells are required to acquire an overall immune profile. In

the future, this can be overcome by the development of mass cytometry.

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#### DISCLOSURES:

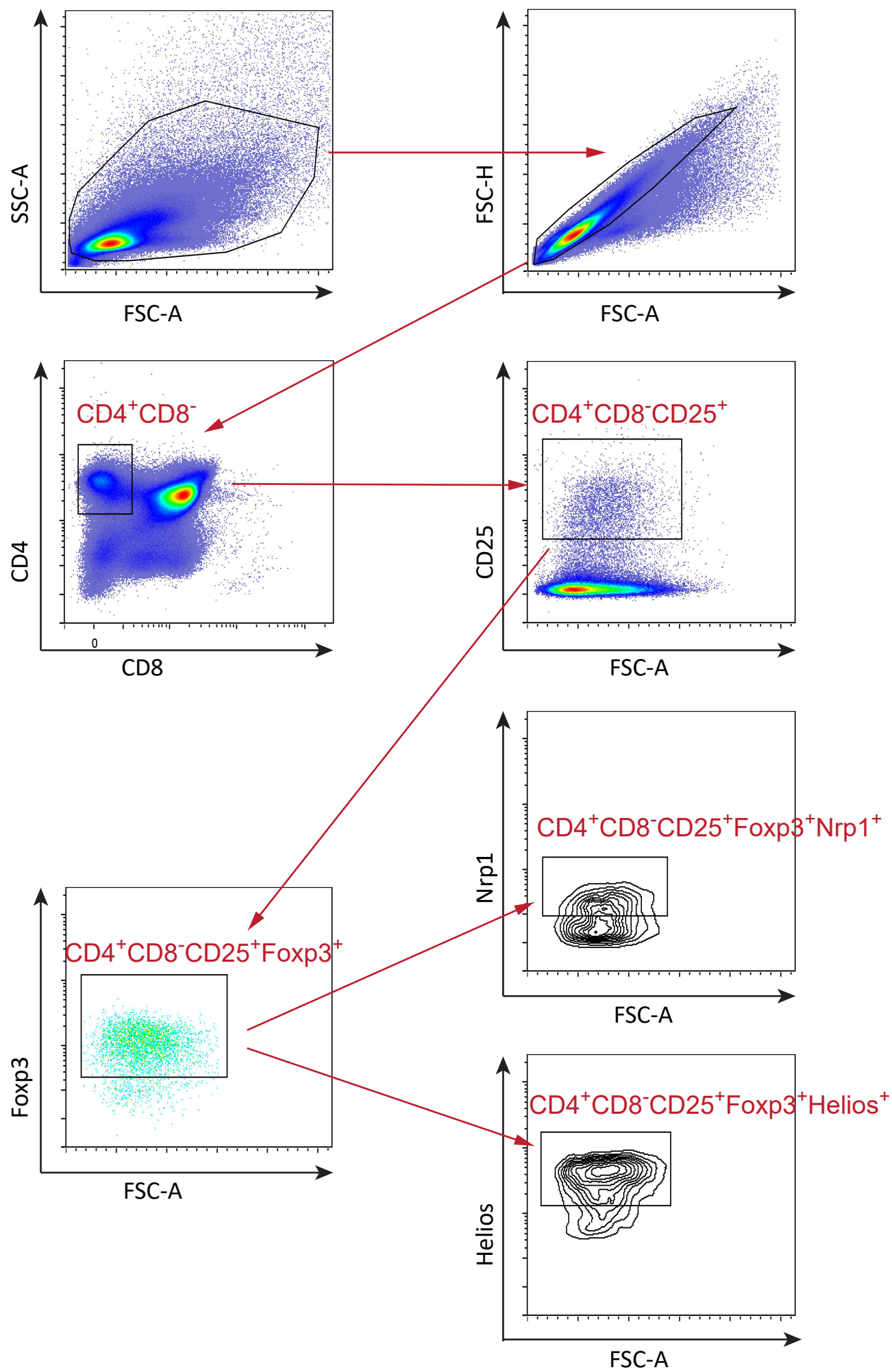
The authors have nothing to disclose.

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314

## Gating strategies for flow cytometry



Bar graph showing the percentage of CD4<sup>+</sup>CD8<sup>-</sup>CD25<sup>+</sup>Foxp3<sup>+</sup> cells in Thymus, PDLN, and Spleen for Nrp1 (black bars) and Helios (grey bars) groups. The y-axis represents the percentage of cells, ranging from 0 to 100. The x-axis lists the tissues: Thymus, PDLN, and Spleen. Significance markers (\*\*\*) indicate statistical differences between the groups in each tissue and between the Helios and Nrp1 groups in Thymus and Spleen.

Tissue	Nrp1 (%)	Helios (%)
Thymus	~18	~82
PDLN	~45	~60
Spleen	~22	~68

Organ	% of Nrp1 <sup>+</sup> cells among Helios <sup>+</sup> Treg cells
Thymus	~14
PDLN	~57
Spleen	~21

Organ	% of Helios <sup>+</sup> cells among Nrp1 <sup>+</sup> Treg cells
Thymus	~91
PDLN	~76
Spleen	~88

Name of Material/ Equipment	Company
NOD mice	Statens veterinärmedicinska anstalt
HBSS	Sigma-Aldrich
RPMI-1640	EMD Millipore
NH <sub>4</sub> Cl	ThermoFisher
eBioscience Foxp3 / Transcription Factor Staining Buffer Set	ThermoFisher
eBioscience Flow Cytometry Staining Buffer	ThermoFisher
CD4 Monoclonal Antibody (RM4-5), FITC, eBioscience	ThermoFisher
CD25 Monoclonal Antibody (PC61.5), PE, eBioscience	ThermoFisher
BD Pharmingen APC-H7 Rat anti-Mouse CD8a	BD Biosciences
Mouse Neuropilin-1 APC-conjugated Antibody	R&D Systems
FOXP3 Monoclonal Antibody (FJK-16s), PE-Cyanine7, eBioscience	ThermoFisher
Pacific Blue anti-mouse/human Helios Antibody	BioLegend
Falcon 5 mL Round Bottom Polystyrene Test Tube, with Cell Strainer Snap Cap	CORNING
Tube 15ml, 120x17mm, PP	Sarstedt
Micro tube 1.5ml	Sarstedt
disposable scintillation vials	WHEATON
Flow cytometry	BD
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### CORRESPONDING AUTHOR

Name:	Zhengkang Luo and Kailash Singh	
Department:	Medical Cell Biology	
Institution:	Uppsala University	
Title:	Determination of regulatory T cell subsets in murine pancreatic draining lymph node and spleen using flow cytometry	
Signature:	Rish. Zhengkang Luo	Date: 2018-08-27

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Authors: done

2. Line 36: Helios antibodies were? Please complete the sentence.

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3. Step 1.1, 1.2: Please write each step in imperative tense.

Authors: We have re-written.

4. 1.2: How much sample is used?

Authors: the information has been provided in the latest version of this paper.

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