**MIFlowCyt Components**

**Detection of autoreactive type II NKT cells: a pilot study of comparison between healthy individuals and patients with vasculitis**

**1. Experiment Overview**

**1.1. Purpose**

The goal of this study was to establish a detection method of autoreactive type II NKT cells in human peripheral blood samples and to compare the cytokine profile of the cells between patients with vasculitis and healthy controls by flow cytometry. Based on our previous results that demonstrated the recognition of P518-532 of sterol carrier protein 2 (SCP2) by the vasculitis-inducible rat type II NKT cell clone, VASC-1, we attempted to detect human type II NKT cells in peripheral blood using hSCP2518-532, the human counterpart of rat SCP2518-532, together with a CD1d tetramer. First, we determined the binding of hSCP2518-532 to CD1d. Next, we detected CD3-positive hSCP2518-532-loaded CD1d (hSCP2518-532/CD1d) tetramer-binding cells in the peripheral blood of healthy donors. The abundance of TGF-β-producing cells rather than TNF-α-producing cells in CD3-positive hSCP2518-532/CD1d tetramer-binding cells suggests the anti-inflammatory property of autoreactive type II NKT cells in healthy individuals. Furthermore, we compared the cytokine profile of autoreactive type II NKT cells between healthy individuals and patients with vasculitis in a pilot study.

**1.2. Keywords**

type II NKT cells

sterol carrier protein 2

CD1d tetramer

cytokine profile

TGF-β

TNF-α

vasculitis

**1.3. Experiment Variables**

The percentage of hSCP2518-532/CD1d tetramer-binding cells in peripheral CD3-positive cells

The percentage of TGF-β-and TNF-α-producing cells in peripheral hSCP2518-532/CD1d tetramer-binding cells

**1.4. Organization**

**1.4.1. Name**

Division of Medical Laboratory Science

Hokkaido University

**1.4.2. Address**

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**1.5. Primary Contact**

**1.5.1. Name**

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**1.6. Date**

April 1 – August 10, 2014

August 1, 2016 – March 31, 2017

**1.7. Conclusions**

The percentage of TGF-β-producing cells in hSCP2518-532/CD1d tetramer-binding type II NKT cells in vasculitic patients was significantly lower than that in healthy controls despite the greater number of these cells.

**1.8. Quality Control Measures**

*Peripheral Blood Samples.* At the earliest opportunity (within 30 min) after blood sampling, PBMCs were isolated using Ficoll-Paque Plus. RPMI 1640 medium supplemented with 10% FBS was used as culture medium. PBMCs were assayed immediately without being frozen for storage. To exclude the artifact caused by dead cells in flow cytometry, we utilized the LIVE/DEAD Fixable Dead Cell Stain Kit (Thermo Fisher Scientific) in some experiments.

**1.9. Other Relevant Experiment Information**

This work was supported by a grant-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan with grant number 26293082 (Akihiro Ishizu).

 **2. Flow Sample/Specimen Details**

The samples (1 × 106 cells/1 ml) were acquired from the tube set into the cytometer. Sample flow rate, 6 µl/sec.

**2.1. Sample/Specimen Material Description**

**2.1.1. Biological Samples**

**2.1.1.1. Biological Sample Description**

Human PBMCs.

**2.1.1.2. Biological Sample Source Description**

Peripheral blood (10 ml) was obtained from eight healthy donors and five patients with vasculitis, such as microscopic polyangiitis and Takayasu arteritis. All patients with vasculitis were actively suffering from the disease. This study was approved for practice by our institutional ethics committee, the Ethical Committee of the Faculty of Health Sciences, Hokkaido University (Permission No. 16-83).

**2.1.1.3. Biological Sample Source Organism Description**

**2.1.1.3.1. Taxonomy**

Human.

**2.1.1.3.2. Age**

Healthy controls: 22-51 years old.

Patients with vasculitis: 50-79 years old.

**2.1.1.3.3. Gender**

Healthy controls: three females and five males.

Patients with vasculitis: three females and two males.

**2.1.1.3.4. Phenotype**

Healthy versus sick.

**2.1.1.3.5. Genotype**

Not examined.

**2.1.1.3.6. Treatment**

All patients with vasculitis were actively suffering from the disease. Peripheral blood was drawn before treatment.

**2.1.1.3.7. Other Relevant Biological Sample Source Organism Information**

None.

**2.1.1.4. Other Relevant Biological Sample Information**

None.

**2.1.2. Environmental Samples**

N/A.

**2.1.3. Other Samples**

N/A.

**2.2. Sample Characteristics**

No specific characteristics.

**2.3. Sample Treatment(s) Description**

For the detection of autoreactive type II NKT cells, PBMCs were assayed immediately without being frozen for storage. For cytokine profiling, PBMCs were adjusted to 2.5 × 106/ml in the culture medium and stimulated with PE-conjugated anti-human CD3 antibody, UCHT-1 (20 μl/1 × 106 cells) (BD Pharmingen) and anti-human CD28 antibody (final concentration, 3.5 μg/ml) (BioLegend) in the presence of GolgiStop (BD Pharmingen) for 16 h at 37°C.

**2.4. Fluorescence Reagent(s) Description**

For the detection of autoreactive type II NKT cells, APC-conjugated human CD1d tetramer (Medical & Biological Laboratory) was employed. One hundred nanograms of hSCP2518-532 and a different peptide hSCP2526-540, TGNMGLAMKLQNLQL (generated by Scrum), as control were independently mixed overnight with 10 μl of the CD1d tetramer at room temperature. On the next day, PBMCs (1 × 106) were stained with 20 µl of PE-conjugated anti-human CD3 antibody, UCHT-1 (BD Pharmingen), for 30 min on ice. After washing with PBS, cells were stained with the APC-conjugated CD1d tetramer without antigen loading or APC-conjugated SCP2/CD1d tetramer for 30 min on ice (total volume, 100 μl). In some experiments, PBMCs were stained with PerCP-conjugated anti-TCR Vα24-Jα18 antibody, 6B11 (Thermo Fisher Scientific) together with the PE-conjugated anti-CD3 antibody and APC-conjugated hSCP2518-532/CD1d tetramer.

For cytokine profiling, PBMCs stimulated by CD3 and CD28 antibodies were then stained with 10 µl of the APC-conjugated hSCP2518-532/CD1d tetramer or the APC-conjugated α-GalCer/CD1d tetramer (Proimmune) and then fixed with a fixation buffer (BioLegend, Cat. No. 420801). After being washed twice with a permeabilization buffer (BioLegend, Cat. No. 421002), cells were stained with 10 µl of FITC-conjugated anti-human TNF-α antibody (Miltenyi) or 5 μl of FITC-conjugated anti-human TGF-β antibody (BioLegend) for 30 min at room temperature. The corresponding IgG1 isotype control (BD Pharmingen) was used for the evaluation of background staining.

**3. Instrument Details**

**3.1. Instrument Manufacturer**

BD Biosciences.

**3.2. Instrument Model**

BD FACS CantoTM II.

**3.3. Instrument Configuration and Settings**

The cytometer has been configured by the manufacturer and has not been altered from the configuration.

**3.3.1. Flow Cell and Fluidics**

The cytometer has factory-installed fluidics and a standard flow cell and has not been altered from the configuration.

**3.3.2. Light Source(s)**

The optics of the BD FACSCanto™ II System consist of an excitation source with up to three lasers: blue (488 nm, air-cooled, 20 mW solid state), red (633 nm, 17 mW HeNe), and violet (405 nm, 30 mW solid state).

**3.3.2.1. Light Source Polarization**

N/A.

**3.3.2.2. Other Relevant Light Source Information**

None.

**3.3.3. Excitation Optics Configuration**

Laser excitation optics illuminate cells in the sample and collection optics direct light scatter and fluorescence signals through spectral filters to the detectors.

**3.3.4. Optical Filters**

**3.3.4.1. Optical Filter Type**

**3.3.4.2. Transmitted Wavelengths**

See diagram.



**3.3.4.3. Optical Filter Installation Date**

Installed by the manufacturer

**3.3.4.4. Optical Filter Manufacturer**

Not known.

**3.3.4.5. Optical Filter Model Number**

Not known.

**3.3.4.6. Other Relevant Optical Filter Information**

None.

**3.3.5. Optical Detectors**

**3.3.5.1. Optical Detector Name**

**3.3.5.2. Optical Detector Type**

**3.3.5.3. Optical Detector Voltage**

**3.3.5.4. Optical Detector Amplification Type**

See Table.

|  |  |  |  |  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- | --- | --- | --- | --- |
| Laser | Detector | Detector type | Filter type | Wavelength | Common parametername | Target channel range(MFI) | Sample voltage (V) | Amplification | Threshold |
| 488 nm | Wide  | Photodiode | BP | 488 | FSC |  | 297 | Linear |  |
| 488 nm |  | PMT | BP | 488 | SSC |  | 395 | Log |  |
| 488 nm | Green | PMT | LP | 530/30 | FITC |  | 571 | Log |  |
| 488 nm | Yellow | PMT | LP | 585/42 | PE |  | 687 | Log |  |
| 488 nm | Far Red | PMT | LP | 670LP | PerCP-Cy5.5 |  | 691 | Log |  |
| 663 nmHeNe | Red | PMT |  | 660/20 | APC |  | 654 | Log |  |

**3.3.5.5. Other Relevant Optical Detector Information**

None.

**3.3.6. Optical Paths**

See diagram.

**3.4. Other Relevant Instrument Details**

None.

**4. Data Analysis Details**

Data was processed by FACSDiva software version 7.0 (BD Biosciences).

**4.1. List-Mode Data File**

FSC.

**4.2. Compensation Details**

**4.2.1. Compensation Description**

Spectral compensation was carried out using single fluorescence-labeled cells in each experiment.

**4.2.2. Other Relevant Compensation Information**

None.

**4.3. Data Transformation Details**

**4.3.1. Purpose of Data Transformation**

N/A.

**4.3.2. Data Transformation Description**

N/A.

**4.3.3. Other Relevant Data Transformation Details**

None.

**4.4. Gating (Data Filtering) Details**

**4.4.1. Gate Description**

For the detection of autoreactive type II NKT cells, PBMCs of healthy donors were stained with PE-conjugated anti-CD3 antibody and APC-conjugated CD1d tetramer loaded with or without SCP2 peptides. At first, cells gated in lymphocytes were selected based on the characteristic FSC/SSC profile. CD3-positive cells within the lymphocyte gate were assayed for the binding to the CD1d tetramer loaded with or without SCP2 peptides.

For cytokine profiling, PBMCs stimulated by CD3 and CD28 antibodies were then stained with APC-conjugated hSCP2518-532/CD1d tetramer followed by fixation. After permeabilization of the plasma membrane, cells were stained with the FITC-conjugated anti-human TNF-α antibody or FITC-conjugated anti-human TGF-β antibody. At first, cells gated in lymphocytes were selected based on the characteristic FSC/SSC profile. Next, CD3-positive cells within the lymphocyte gate were assayed for the binding to the CD1d tetramer loaded with or without SCP2 peptides. Subsequently, SCP2/CD1d tetramer-binding cells were gated for the assessment of the intracellular cytokine staining. For this purpose, the corresponding IgG1 isotype control was used for the evaluation of background staining.

**4.4.2. Gate Statistics**

The percentage of hSCP2518-532/CD1d tetramer-binding cells in peripheral CD3-positive cells and the percentage of TGF-β- and TNF-α-producing cells in peripheral hSCP2518-532/CD1d tetramer-binding cells were compared between healthy donors and vasculitic patients. The Mann-Whitney *U*-test was applied for the comparison of the two groups. *p*<0.05 was considered statistically significant.

**4.4.3. Gate Boundaries**

Lymphocytes were gated based on the characteristic FSC/SSC profile. CD3-negative and CD3-positive cells were separated clearly. For the detection of autoreactive type II NKT cells, the threshold was determined by the background staining of the APC-conjugated CD1d tetramer without peptide loading. For cytokine profiling, the corresponding IgG1 isotype control was used for the evaluation of background staining.

**4.4.4. Other Relevant Gate Information**

None.