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Characterization using the MACSQuant® Analyzer 10

Analysis of NKT cell endogenous ligands

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Introduction

The activation of natural killer T (NKT) cells – a regulatory T cell subset – during the immune response is thought to be reliant on endogenous glycolipid display. Many potential candidates for these NKT cell self-antigens have been proposed, including β-linked glycosylceramides. However, determining their actual identity has been difficult due to the low sensitivity of the chemical methods that could not rule out low-level α-anomer contamination of natural or synthetic β-anomer preparations. Here we describe how the limitations of these analytical methods have been overcome by employing highly specific biological and immunological assays. These assays were ideally suited for flow cytometry due to the ability of flow cytometry to process a large number of events, thereby picking up on rare events and making them easily amenable to statistical analysis. This report describes the use of the MACSQuant® Analyzer 10 flow cytometer and MACSQuantify™ Software from Miltenyi Biotec in these assays.

Immunologically relevant lipids are presented to NKT cells by the CD1 family of MHC Class I-like molecules present on the surface of antigen presenting cells (APCs). We used fluorescently labeled CD1d tetramer constructs and loaded them with pure preparations of control, α- or β-anomer glycolipids. These tetramer constructs were then used in binding studies with the MACSQuant Analyzer 10 flow cytometer to detect which glycolipids were capable of binding to both primary and hybridoma NKT cells. The results showed only α-linked glycosylceramides (α-GlyCer) –CD1d complexes bound NKT cells. Further *in vivo* experimentation was done with α-anomer blocking antibodies to confirm the biologically relevant immunological role of α-GlyCer. Flow cytometry is thus a valuable tool for significant scientific discovery especially in the context of rare events.

Materials and methods

Materials

Lipid loaded CD1d-PE labeled tetramers[1]

- DN32.D3 T cells
- Mouse splenocytes
- Fetal thymic organ cultures (FTOC)
- Adult mouse thymus
- Flow buffer
- Antibodies
  - L363 (iGG2a) – a generous gift from Steve Porcelli
  - 14.4.4 (mouse IgG2)[1]
  - 20H2 (rat IgG1)[2]

Methods

Preparation of single-cell suspensions from spleens, adult mouse thymus and FTOC lobes

1. Collect spleen and thymus from adult C57BL/6 mouse and thymic lobes from fetal mouse.
2. Make single-cell suspension by passing through 70µm cell strainer.
3. Deplete erythrocytes using 0.165 M NH4Cl in water.
   a. Not necessary for FTOC lobes
4. Incubate 10 minutes at room temperature and wash with Flow Buffer.
5. FTOCs were cultured for 18 days in DMEM prior to staining.

CD1d-tetramer staining

1. Perform 15 minute FcR block in the presence of avidin on ice.
2. Transfer cells to a 96-well plate (leaving aliquots for compensation).
3. Wash and resuspend cells in empty or lipid loaded CD1d-PE labeled tetramers and incubate 30 min at RT in the dark.
4. Wash and resuspend in buffer with CD3 and CD45R antibodies (add antibodies to compensation controls also) and incubate 15 minutes on ice in the dark.
5. Wash twice and resuspend cells in Flow Buffer.
6. Use the MACSQuant® Analyzer 10 to perform autolabeling of the dead cells with propidium iodide (PI) and run samples using the MACS® MiniSampler automated sampling input.
7. Acquire data with MACSQuantify™ Software and subject to analysis.

Results

α-Glycosylceramides are the endogenous ligands of NKT cells

Although various preparations of β-glycosylceramides have been shown to be strong activators of NKT cells, the limited analytical methods used could not exclude low-level contamination by α-anomers. To test this, CD1d tetramers were loaded with positive control lipid (PBS-57), or C24:1 β-GluCer or C24:1 α-GalCer glycolipids and then used to stain DN32.D3 NKT cells , followed by flow cytometry using the MACSQuant Analyzer 10 (fig. 1).

CD1d tetramers loaded with C24:1 β –GluCer (top panel) did not stain DN32.D3 cells, while the tetramers loaded with the positive glycolipid control PBS-57 or C24:1 α-GalCer did stain the cells, indicating that only α-glycosylceramides are the endogenous ligands required for NKT cell activation.

This conclusion was further strengthened by similar results from primary $CD3^+$ splenocytes) from C57BL/6 mice (bottom panel). Synthetic C24:1 α-GalCer did in fact result in tetramer binding while C24:1 β-GluCer did not, again indicating that α- and not β-glycosylceramides are the endogenous ligands required for activation.

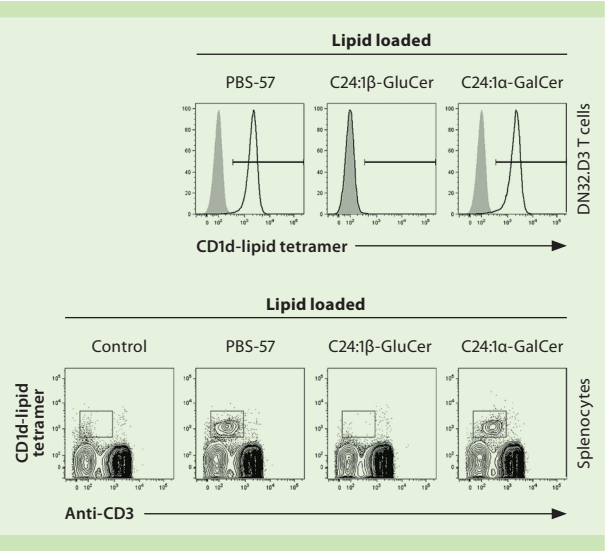

Figure 1: Flow cytometry measurement of binding of labeled Cd1d tetramers to NKT cells. CD1d tetramers loaded with positive control glycolipid PBS-57 C24:1 β-GluCer, or C24:1 α-GalCer were used to stain DN32.D3 NKT hybridoma cells and splenocytes that were examined by flow cytometry.

Blockade of α-GlyCer depletes thymic NKT cells *ex vivo* and *in vivo*

To further test the immunological role of α-GluCer, we utilized the L363 antibody which shows exclusive specificity for α-linked monoglycosylceramides bound to CD1d. FTOCs were treated with L363, and compared to FTOCs treated with a negative control antibody (14.4.4 s) and a positive control anti-CD1 antibody (20H2). Cultures were assayed for NKT cells at day 18 utilizing PE-labeled tetramers that were empty or loaded with positive control lipid (PBS-57). Thymi treated with the anti-α-monoglycosylceramide-CD1d L363 antibody did not contain detectable NKT cells as assayed by flow cytometry on the MACSQuant Analyzer 10 (fig. 2).

This experiment was then performed *in vivo* by injecting either the negative control antibody 14.4.4 or the anti-α-monoglycosylceramide L363 antibody into 8- week-old female C57BL/6 mice for four weeks. The thymic NKT cell numbers were again enumerated via flow cytometry on the MACSQuant Analyzer 10 using PE-labeled PBS-57 loaded tetramers. Thymi in the L363-treated group showed statistically significant lower numbers of NKT cells ($p = 0.0147$).

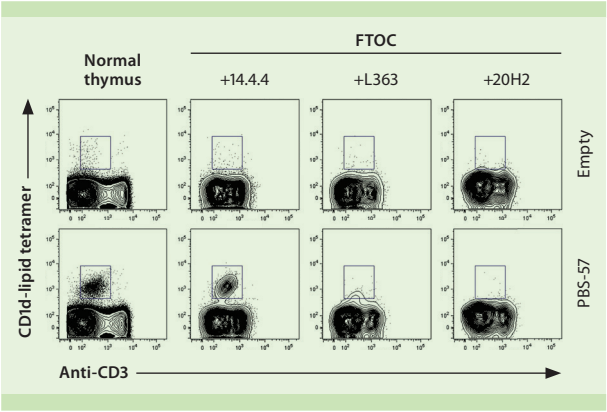

Figure 2: Flow cytometry of adult thymus and cultured thymus cells treated with L363 antibody. Day 14.5 C57BL/6 mouse thymic lobes were cultured for 18 days in the presence of 40–60 µg of antibody. 14.4.4 s was the negative control antibody, and 20H2 was the positive control antibody. Percentages of CD1-PBS-57-positive cells were 0.27%, 8.02%, 1.14%, and 0.27% for adult thymus, 14.4.4 s, L363, and 20H2, respectively. The experiment was repeated three times. Similar results were obtained using four other thymic lobes.

Conclusion

This study demonstrates the utility of flow cytometry for the detection and enumeration of primary and hybridoma NKT cells. Assays performed on the MACSQuant® Analyzer 10 allowed for the identification of α-linked glucosylceramides as the endogenous ligands of NKT cells.

References

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