Endocytosis of Siglec-8 and Siglec-F on bone marrow-derived eosinophils from a ROSA26-Siglec-8-KI EPX-Cre mouse

- Jeremy O'Sullivan

Materials:

- Bone marrow-derived eosinophils (BMDEs) at day 10-14 of *in vitro* differentiation
- Flow buffer (autoMACS Running Buffer; Miltenyi Biotec, cat# 130-091-221)
- Unconjugated anti-Siglec-8 (2C4)
- Unconjugated anti-Siglec-F (in house hybridomas)
- FITC-labeled anti-mouse IgG1 secondary Ab (BioLegend, cat# 406606)
- PE-labeled anti-rat IgG secondary Ab (BD Pharmingen, cat# 550767)
- Fluoropure-grade DAPI (as viability stain; ThermoFisher Scientific, cat# D21490)
- 5-ml polystyrene round-bottom flow tubes (Falcon, ref. no. 352052)
- RPMI-1640 medium (Gibco, cat# 72400-047)
- BD LSR II flow cytometer
- FlowJo analysis software

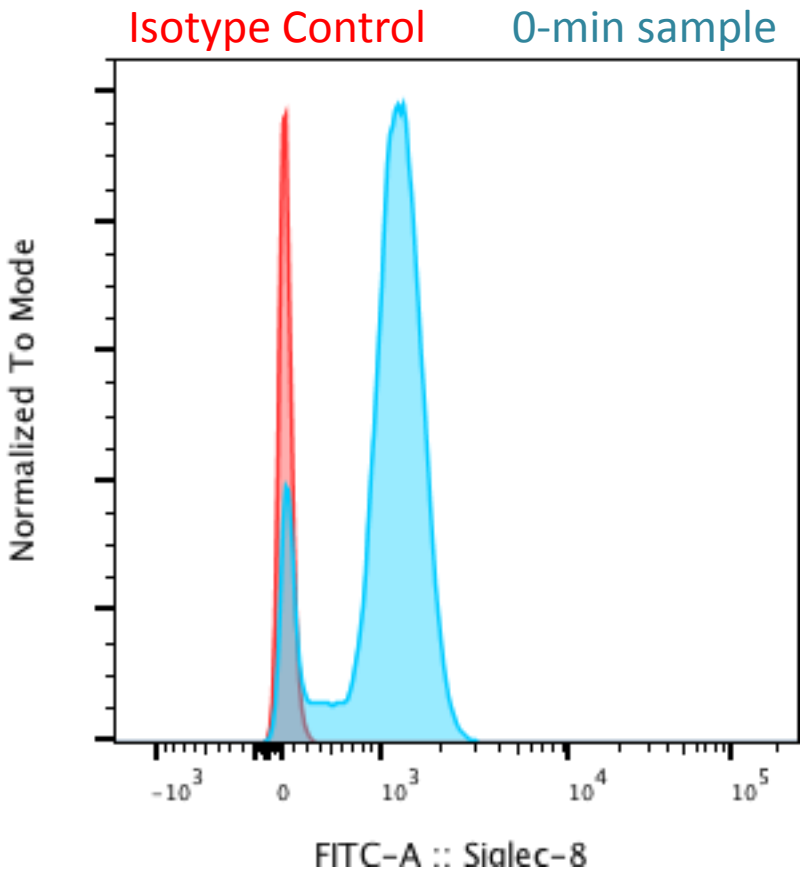
Experimental Procedure:

1. Transfer 2×10^5 to 1×10^6 total cells to each of the flow tubes for staining. Separate tubes will be needed for:
    a. An unstained sample
    b. A sample stained only for Siglec-8 (single stain control for compensation)
    c. A sample stained only for Siglec-F (single stain control for compensation)
    d. A sample stained only with DAPI (single stain control for compensation)
    e. An isotype control sample with isotype controls for both anti-siglec antibodies to identify non-specific and background staining
    f. One sample for each time point of endocytosis, including one at 0 minutes (to indicate initial surface levels of Siglec-8 and Siglec-F)
2. Wash with 1-2 ml of flow buffer and spin down the cells at 300 x *g* (1400 RPM) for 5 min at 4°C.
3. Decant the supernatant and vortex to resuspend the cells.

4. **Unconjugated primary antibody labeling:** Incubate the samples in 100 µl of flow buffer with the appropriate antibody/antibodies for 20 min at 4°C. To do this, make stock 2X dilutions of each of the unconjugated antibodies (5 µg/ml of the anti-Siglec-8 mAb [2C4] and 10 µg/ml of the anti-Siglec-F mAb, the same concentrations for the isotype controls) in flow buffer with 50 µl for each applicable sample, plus about 10% for pipetting error. Add 50 µl of each dilution to the relevant sample tubes and bring to 100 µl with flow buffer, if necessary.
5. Following the 20-min incubation, wash with 1-2 ml of flow buffer and spin down the cells at 300 x *g* (1400 RPM) for 5 min at 4°C.
6. Decant the supernatant, resuspend the cells, and add 100 µl of cold RPMI-1640.
7. **Endocytosis incubation step:** Keep the controls and 0-min sample on ice and incubate the experimental samples for the appropriate duration in the incubator at 37°C.
8. Wash all of the samples with 1-2 ml of flow buffer and spin down the cells at 300 x *g* (1400 RPM) for 5 min at 4°C. While the samples are spinning, make up dilutions of the detection antibodies. Make 2X dilutions of the FITC-conjugated anti-mouse IgG1 (1:100, for a final dilution of 1:200; to detect the anti-Siglec-8 or its isotype control) and the PE-conjugated anti-rat IgG (1:100, for a final dilution of 1:200; to detect the anti-Siglec-F or its isotype control) with 50 µl for each applicable sample, plus about 10% for pipetting error.
9. **Fluorophore-conjugated secondary antibody labeling:** Decant the supernatant, resuspend the cells, and add 50 µl of each dilution to the relevant sample tubes and bring to 100 µl with flow buffer, if necessary. Incubate for 20 min at 4°C.
10. Wash all of the samples with 1-2 ml of flow buffer and spin down the cells at 300 x *g* (1400 RPM) for 5 min at 4°C.
11. Decant the supernatant, resuspend the cells, and add 200 µl of DAPI-containing flow buffer (pre-diluted, at 1.8 µM).
12. Analyze the samples by flow cytometry.

Endocytosis of Siglec-8

…on mouse Siglec-8+ bone marrow-derived eosinophils over 4 hours:



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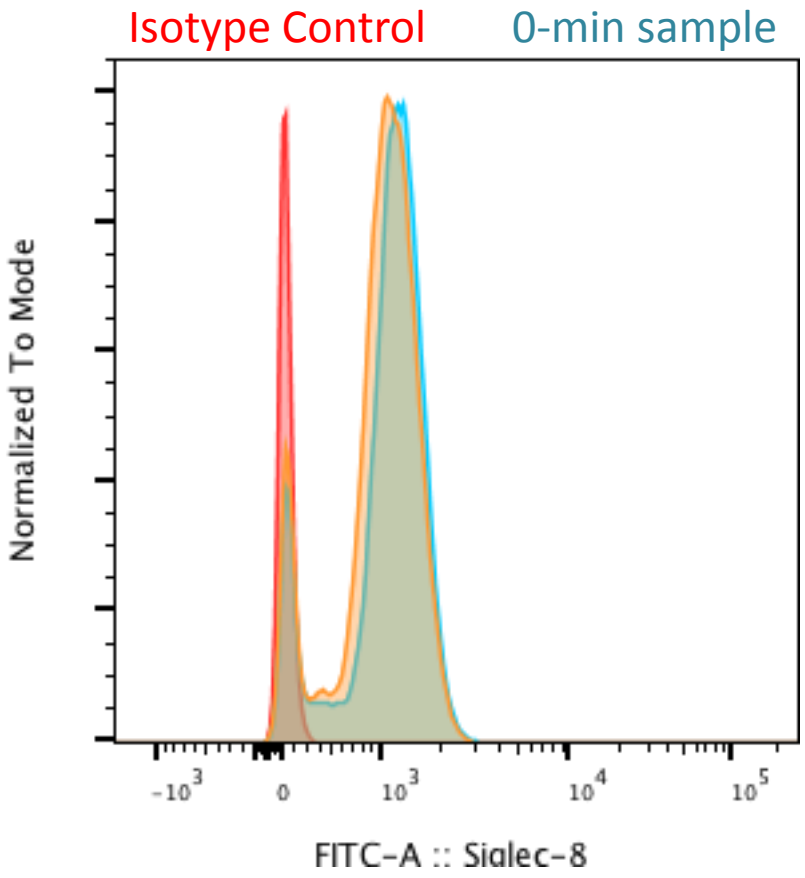

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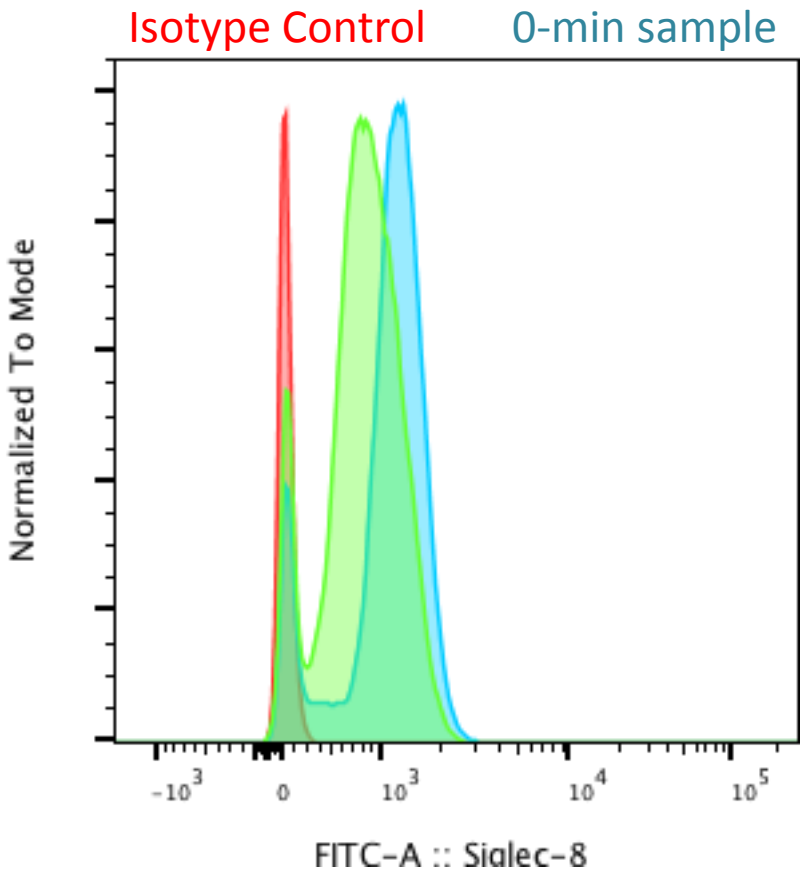

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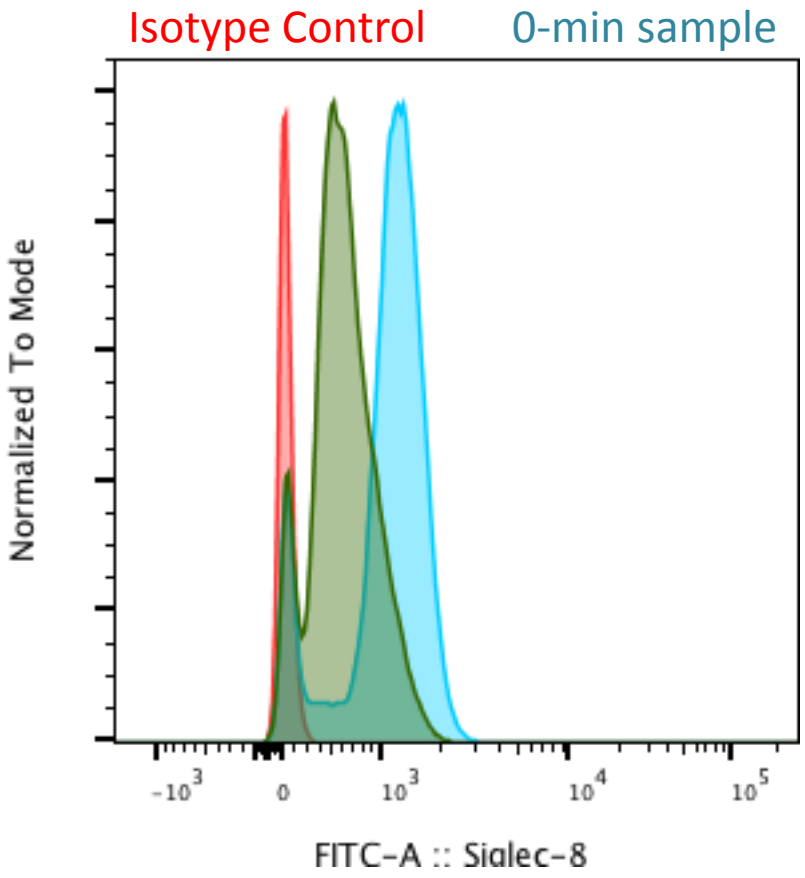

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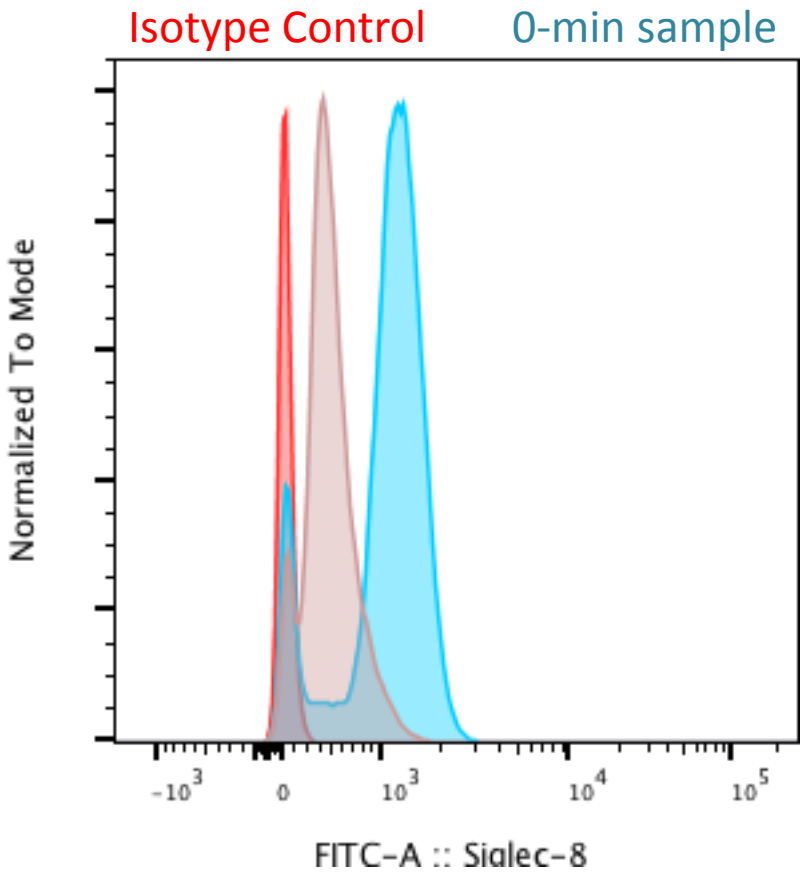

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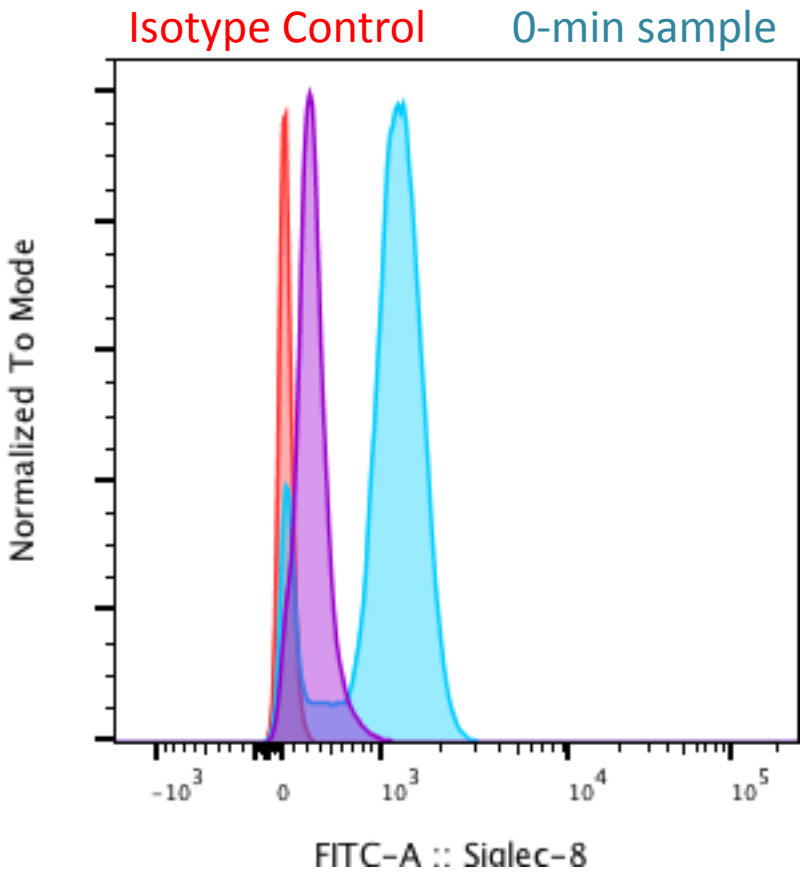

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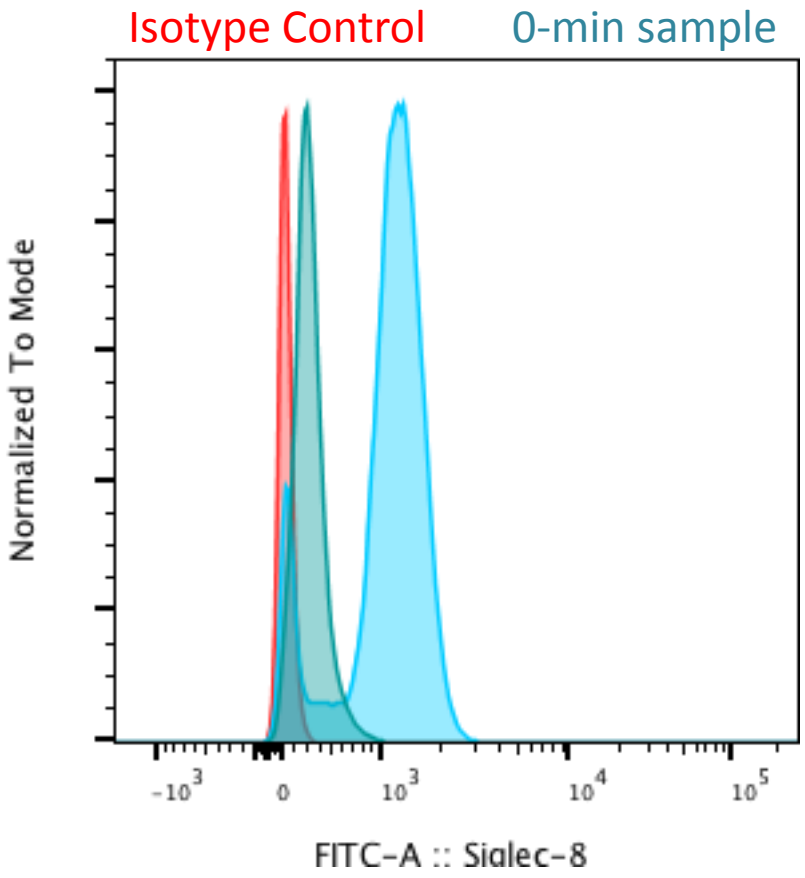

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