Core J Procedure Protocol
Compactin, Kdo2 Lipid A Treatment
in Dye-Free Growth Medium

Setup

1. See Figure 1 for an overview of the treatment time course.
2. See Table 1 for an overview of the four treatment groups.
3. Maintain sterile technique throughout the treatment procedure until harvesting.
4. Plate 2 x 10^6 cells per 60 mm plate in 5 ml of Dye-Free Growth Medium (PS0000002400) as recommended in the LIPID MAPS Thawing and Passage Procedure (PP0000000101). Make triplicate plates for each condition at each time point. Assign a barcode to each plate and enter into LIMS.
5. Incubate 24 hours at 37˚C.

Reagent Preparation

1. Mevalonate 20 mM – prepare from 0.2 M mevalonate (PS0000002200)
2. Compactin 10 mM – thaw from -80˚C
3. Kdo2 Lipid A working solution – prepare from Kdo2 Lipid A stock solution (PS0000001401)
4. Spray the reagent tubes with 70% ethanol and let air dry before using.

Treatment

1. Remove 12 dishes from the incubator and label 24A through 24L.
2. Before treating the cells, remove 0.5 mL of medium from each plate for the TNFα assay, place in labeled Eppendorf tubes, and place at 4˚C until further processing.
3. Treat each dish with the appropriate reagents:

Group 1: -C-K	Dishes A, B, C	11.25 µL mevalonate 22.5 µL PBS 4.5 µL PBS
Group 2: +C-K	Dishes D, E, F	11.25 µL mevalonate 22.5 µL compactin 4.5 PBS
Group 3: -C+K	Dishes G, H, I	11.25 µL mevalonate 22.5 µL PBS 4.5 µL Kdo2 Lipid A
Group 4: +C+K	Dishes J, K, L	11.25 µL mevalonate 22.5 µL compactin 4.5 µL Kdo2 Lipid A

4. Note the time and return the dishes to the incubator.
5. Repeat this treatment procedure for every time point, working backwards from 24 hours to 12, 8, 4, 2, 1, and 0.5.
6. For 0 hours, do not treat with anything, not even mevalonate. Label three dishes A, B, and C, take the medium for TNFα, and proceed with harvesting.

Harvest

1. At the appropriate time, remove the dishes from the incubator and place on ice.
2. Before harvesting the cells, remove 0.5 mL of medium from each plate for the TNFα assay, place in labeled Eppendorf tubes, and place at 4˚C until processing.
3. Transfer remaining medium for Core G to a 15 mL conical polypropylene tube on ice.
4. Gently wash each plate twice with 3 ml of cold PBS.
5. Add 2 mL of PBS/1mM EDTA to each dish and scrape the cells with a scraper.
6. Transfer the cells to a 15 mL conical polypropylene tube and pipette 20x with a p1000 to suspend the cells.
7. Remove 400 µL of the cell suspension to an eppendorf on ice for DNA assay.
8. Proceed with sterol extraction using the remaining 1.6 mL of cells in PBS.

Medium Preparation for Core G

1. Add 100 µL of Core G Eicosanoid Internal Standard to each medium sample, vortex.
2. Remove stray cells by centrifuging the samples at 2400 rpm (1160 rcf) for 5 min at 4˚C. (eppendorf 5810 R with swinging bucket rotor)
3. Decant medium into a 5 mL polypropylene snap-cap tube. Store at -20˚C.
4. Send frozen samples on dry ice to Core G. Include a sample of 4 mL medium spiked with 100 µL Eicosanoid Internal Standard, as well as all remaining internal standard mix.

Medium Processing for TNFα Assay

1. Centrifuge the TNFα aliquots at top speed in coldroom microfuge for 2 min, collect an aliquot of supernatant (~0.3-0.4 ml) and place in a new labeled Eppendorf tube. Assign a barcode, enter into LIMS and freeze the aliquots at -80˚C.
2. The TNFα aliquots from the Kdo_2-lipid A treated cells must be diluted in medium at least 1:80 before assaying. ElisaTech will dilute the samples, if requested. Do not dilute aliquots from cells that were not treated with Kdo_2-lipid A.
3. Send frozen labeled aliquots to ElisaTech, 12635 E. Montview Blvd., Suite 215, Aurora, CO 80010. For assaying in house, use the Quantikine mouse TNFα/TNFSF1A EIA kit (R&D Systems, Cat. #MTA00).

Cell Processing for DNA Assay

1. Add 20 µL of 50% etOH in H_2O to each sample for DNA analysis. Store at -20˚C.
2. Assign a barcode and enter into LIMS. Follow the DNA assay protocol in Molecular Probe's manual with two exceptions: 1) Use 5 ul of standards instead of 10 ul; and 2) Substitute component B with Molecular Probes' Lysis Buffer (cat C-7027) at 1x concentration.

Figure 1

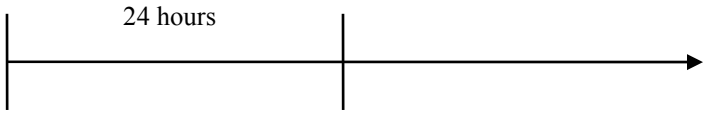

Plate 2 x 10^6 cells
60 mm plate
Dye-Free GMed

Compactin/Kdo2 LipidA
Time course
0-24 hours

Table 1

	Group 1: -C-K	Group 2: +C-K	Group 3: -C+K	Group 4: +C+K
Mevalonate 50 μM	+	+	+	+
Compactin 50 μM	-	+	-	+
Kdo2 LipidA 100 ng/mL	-	-	+	+

Author: Bonne Thompson
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