

Supplementary Information

Differential location of NKT and MAIT cells within lymphoid tissue

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List of Contents:

Supplementary Methods

Supplementary Figure S1: *Validation of the identification of NKT cells in situ with CD1d- α -GalCer tetramer.*

Supplementary Figure S2: *Positive identification of NKT cells in situ with CD1d- α -GalCer tetramer and their location within C57BL/6 mouse spleens.*

Supplementary Figure S3: *No evidence of an increase in medullary holes in thymuses of BALB/c, V α 19Tg or C57BL/6 MR1^{-/-} mice.*

Supplementary Figure S4: *NKT cells within small intestine.*

Supplementary Figure S5: *NKT cells within lungs.*

Supplementary Figure S6: *NKT cells within Kidneys.*

Supplementary Figure S7: *NKT cells within Heart.*

Supplementary Fig S8: *The expansion and redistribution of NKT cells following in vivo stimulation with α -GalCer.*

Supplementary Figure S9: *CD1d/GD3 tetramer staining of BALB/c liver sections.*

Supplementary Figure S10: *Multiparameter in situ analysis of NKT cells and Antigen Presenting Cells.*

Supplementary Figure S11: *Positive identification of MAIT cells within WT BALB/c and CAST mice spleen with MR1-5-OP-RU tetramer in situ.*

Supplementary Figure S12: *CD1d and MR1 tetramer co-stain for NKT cells MAIT cells within WT C57BL/6 and BALB/c mice spleen in situ.*

Supplementary Figure S13: *No MAIT cells identified within C57BL/6, BALB/c and CAST mice thymus with MR1-5-OP-RU tetramer.*

Supplementary Figure S14: *The expansion of MAIT cells following intranasal administration of 5-OP-RU.*

Supplementary Methods

Count cells within thymus

// Marco to count cells within set distance bands from the ROI. The ROI is drawn by hand based on K5 and DAPI stain to show the CMJ. A distance map is used to determine distance from the ROI. The results output uses the pre-set positions of cells and determine the area integrated of each point. Therefore, dividing the area integrated by 255 gives the number of cells in that area.

// Author: Darryl Johnson, University of Melbourne, with assistance from Ellie Cho, University of Melbourne.

```
// A dialog opens to let you select the source directory, directory is assigned to variable 'inputdir'
inputdir = getDirectory("Select the input directory");
```

```
// A dialog opens to let you select the destination directory, directory is assigned to variable 'outputdir'
outputdir = getDirectory("Select the output directory");
```

```
// Create a list of files from that directory. it comes as array[6] if there are 6 files
filelist = getFileList(inputdir);
```

```
// Sets 16bit depth for the later MAP
run("Options...", "iterations=1 count=1 edm=16-bit do=Nothing");
```

```
setBatchMode(true);
```

```
// Set a loop
```

```
for (i=0; i<filelist.length; i++){
inputfilename = inputdir + filelist[i];
```

```
// Open tif files in the list.
```

```
if(endsWith(inputfilename, ".tif")){
```

```
    open(inputfilename);
```

```
    fileName=getTitle();
```

```
    Name = File.nameWithoutExtension;
```

```
    inputCellPointsName = inputdir + Name+"_cellROI.roi";
```

```
    inputROIname = inputdir + Name+"_ROI.roi";
```

```
        run("Duplicate...", "duplicate channels=1");
```

```
        fileNameDuplicate=getTitle();
```

```
        selectWindow(fileNameDuplicate);
```

```
// Makes mask of cell points
```

```

// Opens the ROI manager and required cell points ROI.
run("ROI Manager...");
roiManager("reset");
roiManager("Open", inputCellPointsName);
roiManager("Select", 0);

run("Create Mask");
rename("Mask of cells");
run("Set Measurements...", "area integrated redirect=[Mask of cells] decimal=3");

// Select the duplicated window
selectWindow(fileNameDuplicate);
run("Select None");

// Makes the distance map inside the ROI (edge to centre)
// Opens the ROI manager and required medulla ROI.
roiManager("reset");
roiManager("Open", inputROIname);
roiManager("Select", 0);

run("Create Mask");
run("Distance Map");

// Count cells within 25micron to 150micron outer bands from centre of the ROI to the edge. The thresholds
are set to the pixel width of the image (in this case 1pixel = 0.41666micros).
// 25micron
setThreshold(1, 60);
run("Create Selection");
run("Measure");
// 50micron
setThreshold(1, 120);
run("Create Selection");
run("Measure");
// 75micron
setThreshold(1, 180);
run("Create Selection");
run("Measure");
// 100micron
setThreshold(1, 241);
run("Create Selection");
run("Measure");
// 125micron
setThreshold(1, 301);
run("Create Selection");
run("Measure");
// 150micron
setThreshold(1, 361);
run("Create Selection");
run("Measure");

// count cells within inner center of medulla. Pixels set as per pixel width above.
// 25-1000 inner center
setThreshold(61, 3613);
run("Create Selection");
run("Measure");
// 50-1000 inner center
setThreshold(121, 3613);

```

```

        run("Create Selection");
        run("Measure");
    // 75-1000 inner center
        setThreshold(181, 3613);
        run("Create Selection");
        run("Measure");
    // 100-1000 inner center
        setThreshold(242, 3613);
        run("Create Selection");
        run("Measure");
    // 125-1000 inner center
        setThreshold(302, 3613);
        run("Create Selection");
        run("Measure");
    // 150-1000 inner center
        setThreshold(362, 3613);
        run("Create Selection");
        run("Measure");
    saveAs("Results", outputdir+Name +"-outter and inner medulla count");
    wait(100);

// Makes the distance map outside the ROI (edge of ROI to the end of the window)
    selectWindow("Mask");
    run("Invert LUT");
    run("Distance Map");

// Count cells within cortex
    setThreshold(1, 3613);
    run("Create Selection");
    run("Measure");

    saveAs("Results", outputdir+Name +"-whole count");
    wait(100);
    close("Results");

// clean up
    run("Close All");
    run("Collect Garbage");

        }
}

setBatchMode("exit & display");
print("Done ;");

```

Thymus hole measurement

//This marco opens an image and applies a pre-drawn ROI onto the image. The data outside the image is removed and the 'holes' between the keratin5 stain is measured.

//Author: Darryl Johnson, University of Melbourne, with assistance from Ellie Cho and Robyn Esterbauer, University of Melbourne.

//Run from Plugins > Macros

//A dialog opens to let you select the source directory, directory is assigned to variable 'inputdir'

```
Dialog.create("Select input folder");
Dialog.show();
inputdir = getDirectory("Select the input directory");
```

//A dialog opens to let you select the destination directory, directory is assigned to variable 'outputdir'

```
Dialog.create("Select output folder");
Dialog.show();
outputdir = getDirectory("Select the output directory");
```

//Optimise which channel to be analysed, analyse particle and threshold settings specific to your application and result of interest

//Dialog: Get values from the user

```
Dialog.create("Set the parameters");
Dialog.addString("K5 channel : ", "C1");
Dialog.addString("DAPI channel : ", "C3");
Dialog.addNumber("K5 Setting lower threshold : ", 0);
Dialog.addNumber("K5 Setting upper threshold : ", 15);
Dialog.addNumber("Minimal Hole size : ", 5000);
Dialog.show();
K5Channel = Dialog.getString();
DAPIChannel = Dialog.getString();
LowThresholdK5Setting = Dialog.getNumber();
HighThresholdK5Setting = Dialog.getNumber();
minHole = Dialog.getNumber();
AnalyzeStromalHoleSettings="size="+minHole+"-Infinity circularity=0.04-1.00 include add slice";
```

//create a list of files from that directory and stat a loop to measure for all files.

```
filelist = getFileList(inputdir);

for (i=0; i<filelist.length; i++){
    inputfilename = inputdir + filelist[i];
    //open tif files in the list and splits channels.
    if(endsWith(inputfilename, ".tif")){
        open(inputfilename);
        fileName=getTitle();
        Name = File.nameWithoutExtension;
        inputROIname = inputdir + Name+"_ROI.roi";
        run("Split Channels");
```

//make result table

```
title1 = "Measurement";
title2 = "["+title1+"];";
if (isOpen(title1)) {selectWindow(title1); run("Close"); }
run("Table...", "name="+title2+" width=1200 height=600");
print(title2, "\\Headings:Image Name \t Area");
```

```
K5_stain = K5Channel + "-" + fileName;
selectWindow(K5_stain);
```

```

run("Duplicate...", "title=proof");run("Grays"); run("RGB Color");
wait(100);

DAPI_stain = DAPIChannel + "-" + fileName;
selectWindow(DAPI_stain);
run("Duplicate...", "title=DAPI_stain");run("Grays"); run("RGB Color");
wait(100);

//Selects the channel window and processes for later area measurement of the holes.
//Threshold settings are set by low and high Threshold K5 Settings above.
selectWindow(K5_stain);
run("Threshold...");
setThreshold(LowThresholdK5Setting, HighThresholdK5Setting);
run("Convert to Mask");
run("Invert LUT");
run("Analyze Particles...", "size=10-Infinity show=Masks");

//Renames the mask that was made.
rename("mask");
selectWindow("mask");

//Fills gaps in the edges of the holes
run("Options...", "iterations=6 count=1 black do=Open");

//Opens the ROI manager and required medulla ROI.
roiManager("reset");
roiManager("Open", inputROIname);

//make proof image for Area selection on the K5 and DAPI stains
setForegroundColor(0,255,0);run("Line Width...", "line=5");
selectWindow("proof");roiManager("select",0);run("Draw", "slice");
selectWindow("DAPI_stain");roiManager("select",0);run("Draw", "slice");
saveAs("jpg", outputdir+Name +"-DAPI.jpg"); run("Close");
wait(100);

//Adds area of the ROI into the first line of the table
selectWindow("mask");roiManager("Select", 0);
run("Clear Results"); List.setMeasurements; area = List.getValue("Area"); print(title2, Name
+ "\t" + area );
setBackground(0, 0, 0);

//Run MeasureHoles function
MeasureHoles();

//Saves the results table
selectWindow("Measurement");
saveAs("Text", outputdir+Name +"-2.csv");
selectWindow("Measurement");
run("Close");

//Make proof image for ROI detected
selectWindow("proof");
ROI = roiManager("count"); setForegroundColor(255,0,0);run("Line Width...", "line=5");
for(r=0;r<ROI;r++){
    roiManager("select",r);run("Draw", "slice");
    wait(100);
}

```

```

        saveAs("jpg", outputdir+Name +"-proof.jpg"); run("Close");
    }

run("Close All");
run("Collect Garbage");
}

setBatchMode("exit & display");
print("Done");
/*-----*//
//A function that measures the area of the base ROI, clears everything outside the ROI, inverts LUT, sets
threshold and measures area of particles.
//Analyse particle settings are set by "AnalyzeStromaHoleSettings" veribles above.
function MeasureHoles(){
    run("Clear Outside");
    roiManager("Deselect");
    roiManager("reset");
    run("Analyze Particles...", AnalyzeStromalHoleSettings);

//and you can get your measurement to be printed out on this general table. One table is made per image
ROI = roiManager("count");
if(ROI>0) {
    for(r=0;r<ROI;r++){
        roiManager("select", r); run("Clear Results"); List.setMeasurements; area =
        List.getValue("Area");
        print(title2, Name + "\t" + area );
    }
    //to save the hole ROIs
        roiManager("deselect");
        roiManager("Save", outputdir + Name+ "-RoiSet.zip");
    }
    else { print(title2, Name + "\t" + "NA" );
    }
}
}

```

Supplementary Fig S1: *Validation of the identification of NKT cells in situ with CD1d- α -GalCer tetramer.*

Sections from BALB/c WT or CD1d^{-/-} mice spleens were stained with CD1d tetramers loaded with α -GalCer or left unloaded. **a**, Representative images showing CD1d tetramer (green), B220 (blue) and TCR β (red) staining, scale bar 25 μ m. **b** Representative image showing non-NKT (CD1d-tetramer⁺, but CD3⁻) staining; merged or single channel CD3 and CD1d tetramer staining. White arrows show the location of CD3⁻ non-specific tetramer stain. **c** Representative voxel density plots depicting correlation between CD1d tetramer and TCR β staining. **d** Pearson correlation coefficients between the indicated groups and **e** area co-stained with CD3 and CD1d- α -GalCer of individual fields of view. Data n=8 combined of 3 independent experiments. Mean \pm SEM, Kruskal-Wallis with Dunn's post-hoc test.

Supplementary Fig S2: *Positive identification of NKT cells in situ with CD1d- α -GalCer tetramer and their location within C57BL/6 mouse spleens.*

Spleen sections from C57BL/6 WT or CD1d^{-/-} mice stained with CD1d tetramers loaded with α -GalCer or GD3 and anti-B220, CD3 and CD4 antibodies. **a** Representative images showing CD1d tetramer (green), B220 (blue) and CD3 (red) staining within splenic Red Pulp (RP), B cell zone (BCZ) and T cell zone (TCZ). **b** Count of CD1d/ α -GalCer⁺CD3⁺ NKT cells per image and **c** proportion of NKT cells within each region of the total number of NKT cells per image, mean \pm SEM. **d** Zoomed regions from C57BL/6 WT and CD1d^{-/-} stained with α -GalCer or GD3 loaded CD1d tetramer showing non-NKT (CD1d-tetramer⁺, but CD3⁻) staining; merged or single channel CD3 and CD1d tetramer staining. White arrows show the location of CD3⁻ non-specific tetramer stain. Data n = 3 to 5 mice of one experiment. Mean SEM, one-way ANOVA with Tukey post-hoc test.

Supplementary Fig S3: *No evidence of an increase in medullary holes in thymuses of BALB/c, V α 19Tg or C57BL/6 MR1^{-/-} mice.*

Sections of **(a)** BALB/c WT, BALB/c Ja18^{-/-} and BALB/c CD1d^{-/-} and **(a)** Va19Tg and C57BL/6 MR1^{-/-} thymuses stained with CD31 (Red), cytokeratin 5 (K5, Gray) and DAPI (not shown). **a** and **b** Representative images showing medulla (yellow line) and medullary holes (green lines). Medulla determined from the K5 and DAPI stains and medullary holes determined with the use of an ImageJ script and defined as areas >5000 μ m² lacking K5 stain and within the medulla. Scale bar 100 μ m of each main image and 25 μ m of zoom region. **c** Mean number of holes and **d** mean hole area per mouse. Symbols represent means of 3 images per mouse thymic section, n = 4 mice. One experiment for each Va19Tg and MR1^{-/-} mice and 2 independent experiments for NOD mice. Mean \pm SEM.

Supplementary Fig S4: *NKT cells within small intestine.*

BALB/c WT small intestine sections stained with CD3, DAPI and either **(a)** CD1d/GD3 or **(b and c)** CD1d/ α -GalCer. Representative image showing CD1d tetramer (green), CD3 (red), DAPI (blue) and autofluorescence (Auto, gray), scale bar 100 μ m, with corresponding zoomed regions of interest, scale

bar 25 μm . White arrows non-NKT (tetramer⁺, but CD3⁻) green arrows NKT cells (tetramer⁺, but CD3⁺). Images representative of 4 mice, 2 independent experiments.

Supplementary Fig S5: *NKT cells within lungs.*

Lung sections from BALB/c WT mice stained with CD3, DAPI and either (a) CD1d/GD3 or (b) CD1d/ α -GalCer. Representative image showing CD1d tetramer (green), CD3 (red), DAPI (blue) and autofluorescence (Auto, gray), scale bar 100 μm , with corresponding zoomed regions of interest, scale bar 25 μm . White arrows non-NKT (tetramer⁺, but CD3⁻) green arrows NKT cells (tetramer⁺, but CD3⁺). Images representative of 4 mice, 2 independent experiments.

Supplementary Fig S6: *NKT cells within Kidneys.*

7 μm sections from BALB/c WT kidneys stained with CD3, DAPI and either (a) CD1d/GD3 or (b) CD1d/ α -GalCer. Representative image showing CD1d tetramer (green), CD3 (red), DAPI (blue) and autofluorescence (Auto, gray), scale bar 100 μm , with corresponding zoomed regions of interest, scale bar 25 μm . White arrows non-NKT (tetramer⁺, but CD3⁻) green arrows NKT cells (tetramer⁺, but CD3⁺). Images representative of 4 mice, 2 independent experiments.

Supplementary Fig S7: *NKT cells within Heart.*

Sections from BALB/c WT heart stained with CD3, DAPI and either (a) CD1d/GD3 or (b) CD1d/ α -GalCer. Representative image showing CD1d tetramer (green), CD3 (red), DAPI (blue) and autofluorescence (Auto, gray), scale bar 100 μm , with corresponding zoomed regions of interest, scale bar 25 μm . White arrows non-NKT (tetramer⁺, but CD3⁻) green arrows NKT cells (tetramer⁺, but CD3⁺). Images representative of 4 mice, 2 independent experiments.

Supplementary Fig S8: *The expansion and redistribution of NKT cells following in vivo stimulation with α -GalCer.*

As in Fig 5c, BALB/c WT mice were injected intraperitoneally with 2 μg of α -GalCer or left unstimulated. On days 3 and 5 post injection the spleens were harvested, samples were snap frozen, cut into 7 μm sections and stained with B220 and CD3 antibodies and CD1d- α -GalCer tetramer for histological analysis. Representative image showing CD1d- α -GalCer tetramer (green), B220 (blue) and CD3 (red) staining within the splenic T cells zone (TCZ), B cells zone (BCZ) and RP (RP), scale bar 100 μm . Corresponding zoomed regions of interest showing CD3 and B200 staining with tetramer (middle) or without tetramer (right), scale bar 25 μm . Images representative of n = 7-8 mice of 2 independent experiments.

Supplementary Fig S9: *CD1d/GD3 tetramer staining of BALB/c liver sections.*

BALB/c WT mice were treated with 2 μg α -GalCer i.p. or left unstimulated. On days 3 and 5 post the livers were harvested. Most of the liver of each mouse were prepared for FACS analysis and stained with anti-mouse B220 and CD3 antibodies and CD1d- α -GalCer tetramer. One lobe of each liver was snap frozen cut into 7 μm sections and stained with CD1d- α -GalCer tetramer, anti-CD3 antibody and counterstained

with DAPI. **a** Representative FACS plots and **(b)** mean number of CD3⁺CD1d/ α -GalCer⁺ cells for each time point. Representative image showing CD3 (red), DAPI (blue) and autofluorescence (gray) and either **(c)** α -GalCer or **(d)** GD3 loaded CD1d tetramer (green), scale bar 100 μ m and zoom regions 25 μ m. Symbols represent each mouse of n = 7-8 mice per group combined from 2 independent experiments, Kruskal-Wallis with Dunn's post-hoc test.

Supplementary Fig S10: *Multiparameter in situ analysis of NKT cells and Antigen Presenting Cells.*

Spleen sections from BALB/c WT mice were cut and stained with anti-mouse B220, CD3, CD4, CD11b, CD11c, MHC-II antibodies, CD1d- α -GalCer tetramer followed by DAPI counterstain. Following spectral imaging, linear unmixing and deconvolution was performed followed by conventional compensation. **a** Montage of the resulting separated channels; top row Autofluorescence (Auto, gray), DAPI (blue) and CD11c (cyan); middle row CD1d- α -GalCer (green) CD3 (red) and B220 (blue); bottom row CD4 (purple), CD11b (orange) and MHC-II (yellow). Single cell masks were made based on the DAPI stain and staining data of the segmented cells exported for histo-cytometry analysis. **b** Representative image of the DAPI channel and **c** centre points resulting from cell segmentation based on the DAPI stain. **d** Corresponding images to Fig 4D showing the original stains; CD11c (cyan), CD1d- α -GalCer (green), CD3 (red), B220 (blue), CD4 (purple), CD11b (orange) and MHC-II (yellow). Scale bar of image in A, B, C 400 μ m, D I and II 150 μ m and III to IX 30 μ m. One mouse representative of 3 independent mice/experiments.

Supplementary Fig S11: *Positive identification of MAIT cells within WT BALB/c and CAST mice spleen with MR1-5-OP-RU tetramer in situ.*

Spleen sections from BALB/c WT (**a** to **c**) and CAST WT, CAST.MR1^{-/-} (**d** to **e**) mice stained with MR1 tetramers loaded with 5-OP-RU or Ac-6-FP and anti-B220 and CD3 antibodies. **a** and **d**, Representative images showing MR1 tetramer (green), B220 (blue) and CD3 (red) staining within splenic Red Pulp (RP), B cell zone (BCZ) and T cell zone (TCZ) of the splenic white pulp, scale bar 100 μ m with corresponding zoomed regions of interest as shown, scale bar 25 μ m. **b** and **e** Count of MR1/5-OP-RU⁺CD3⁺ MAIT cells per image and **c** and **f** proportion of MAIT cells within each region of the total number of NKT cells per image, mean \pm SEM. Data n = 10 of 2 independent experiments, Wilcoxon test for data from BALB/c mice and Kruskal-Wallis with Dunn's post-hoc test for data from CAST mice.

Supplementary Fig S12: *CD1d and MR1 tetramer co-stain for NKT cells MAIT cells within WT C57BL/6 and BALB/c mice spleen in situ.*

Sections of spleens from C57BL/6 (**a**) and BALB/c (**b**) mice stained with CD1d- α -GalCer and MR1-5-OP-RU tetramers and anti-B220 and CD3 antibodies. **a** and **b**, Representative images showing CD1d tetramer (gray), MR1 tetramer (green), B220 (blue) and CD3 (red) staining within splenic Red Pulp (RP), B cell zone (BCZ) and T cell zone (TCZ) of the splenic white pulp, scale bar 100 μ m with corresponding zoomed regions of interest as shown, scale bar 25 μ m. Images representative of n = 4 mice of 2 independent experiments.

Supplementary Fig S13: *No MAIT cells identified within C57BL/6, BALB/c and CAST mice thymus with MR1-5-OP-RU tetramer.*

Thymus sections from C57BL/6 WT and C57BL/6.MR1^{-/-} (a), BALB/c WT (b) and CAST WT and CAST.MR1^{-/-} (c) mice stained with MR1 tetramers loaded with 5-OP-RU or Ac-6-FP and anti-CD3 antibody and DAPI or anti-K5, CD205 and CD3 antibodies. Representative images showing (a) MR1 tetramer (green), CD3 (red) and DAPI (blue) staining, (b) MR1 tetramer (green), CD3 (red) and K5 (blue), and (c) MR1 tetramer (green), CD3 (red), K5 (blue) and CD205 (gray). Cortex (C) and Medulla (M), green arrows MR1-tetramer⁺,CD3⁺, white arrows MR1-tetramer⁺,CD3⁻. Scale bar 100 μm with corresponding zoomed regions of interest as shown, scale bar 25 μm. Images representative of n = 4 of 2 independent experiments.

Supplementary Fig S14: *The expansion of MAIT cells following intranasal administration of 5-OP-RU.*

C57BL/6.CAST WT mice were given 5-OP-RU intranasally or left unstimulated. On day 5 post antigen administration the mLN were harvested and snap frozen. 7μm sections were cut and stained with B220 and CD3 antibodies and either 5-OP-RU or Ac-6-FP loaded MR1 tetramer. Representative images of (a) unstimulated and (b) stimulated mLN showing MR1 tetramer (green), B220 (blue) and CD3 (red) staining within the splenic paracortex (P), follicle (F), intrafollicular space (IF) and medulla (M), scale bar 100μm, with corresponding zoomed regions of interest, scale bar 25μm. c Total count of MAIT cells and d proportion of MAIT cells within each region at each time point. Mean ± SEM. Data n = 3 mice of one experiment, two-way ANOVA with Sidak post-hoc test.