library(pkgbuild)

devtools::install\_github(repo = "satijalab/seurat", ref = "develop")

1

install.packages('circlize')

library(ggplot2)

install.packages("ggrepel")

a

if (!requireNamespace("BiocManager", quietly = TRUE))

install.packages("BiocManager")

BiocManager::install(version = "3.14")

BiocManager::install(c('BiocGenerics', 'DelayedArray', 'DelayedMatrixStats',

'limma', 'S4Vectors', 'SingleCellExperiment',

'SummarizedExperiment', 'batchelor', 'Matrix.utils'))

install.packages("devtools")

devtools::install\_github('cole-trapnell-lab/leidenbase')

BiocManager::install('SummarizedExperiment')

devtools::install\_github('cole-trapnell-lab/monocle3')

devtools::install\_github('https://github.com/cole-trapnell-lab/monocle3',ref='develop')

install.packages("sf")

library(dplyr)

library(Matrix)

library(sctransform)

library(cowplot)

install.packages("grr")

library(grr)

library(RCurl)

library(monocle3)

library(stringr)

library(umap)

library(RCurl)

library(scales)

library(tidyverse)

library(ggplot2)

library(SeuratWrappers)

library(Seurat)

library(SingleCellExperiment)

library(ggbeeswarm)

library(ggthemes)

BiocManager::install("geom\_text\_repel")

a

library(geom\_text\_repel)

#BiocManager::install("clusterExperiment")

library(clusterExperiment)

library(gam)

library(monocle3)

a

library(ggplot2)

library(dplyr)

n

rm(synC4DMMLeft)

for (file in c("synC1DMMleft", "synC1DMMright", "synHFD3DMMLeft", "synHFD3DMMRight", "synC4DMMLeft", "synC4DMMRight", "synC5DMMLeft", "synC5DMMRight", "synC6DMMLeft", "synC6DMMRight", "synH4DMMLeft", "synH4DMMRight", "synH5DMMLeft", "synH5DMMRight", "synH6DMMLeft", "synH6DMMRight")){

seurat\_data <- Read10X(data.dir = paste0("Data/", file))

seurat\_obj <- CreateSeuratObject(counts = seurat\_data, min.cells = 3, min.features = 200, project = file)

assign(file, seurat\_obj)

}

synC1DMMleft@meta.data$stim <- "CL"

synC1DMMleft@meta.data$stim2 <- "DMM"

synC1DMMright@meta.data$stim <- "CR"

synC1DMMright@meta.data$stim2 <- "DMM"

synHFD3DMMLeft@meta.data$stim <- "HL"

synHFD3DMMLeft@meta.data$stim2 <- "DMM"

synHFD3DMMRight@meta.data$stim <- "HR"

synHFD3DMMRight@meta.data$stim2 <- "DMM"

synC5DMMLeft@meta.data$stim <- "CL"

synC5DMMLeft@meta.data$stim2 <- "DMM"

synC4DMMLeft@meta.data$stim <- "CL"

synC4DMMLeft@meta.data$stim2 <- "DMM"

synC4DMMRight@meta.data$stim <- "CR"

synC4DMMRight@meta.data$stim2 <- "DMM"

synC5DMMLeft@meta.data$stim <- "CL"

synC5DMMLeft@meta.data$stim2 <- "DMM"

synC5DMMRight@meta.data$stim <- "CR"

synC5DMMRight@meta.data$stim2 <- "DMM"

synC6DMMLeft@meta.data$stim <- "CL"

synC6DMMLeft@meta.data$stim2 <- "DMM"

synC6DMMRight@meta.data$stim <- "CR"

synC6DMMRight@meta.data$stim2 <- "DMM"

synH4DMMLeft@meta.data$stim <- "HL"

synH4DMMLeft@meta.data$stim2 <- "DMM"

synH4DMMRight@meta.data$stim <- "HR"

synH4DMMRight@meta.data$stim2 <- "DMM"

synH5DMMLeft@meta.data$stim <- "HL"

synH5DMMLeft@meta.data$stim2 <- "DMM"

synH5DMMRight@meta.data$stim <- "HR"

synH5DMMRight@meta.data$stim2 <- "DMM"

synH6DMMLeft@meta.data$stim <- "HL"

synH6DMMLeft@meta.data$stim2 <- "DMM"

synH6DMMRight@meta.data$stim <- "HR"

synH6DMMRight@meta.data$stim2 <- "DMM"

merged\_seurat <- merge(x = synC4DMMLeft, y = c(synC4DMMRight, synC1DMMleft, synC1DMMright, synHFD3DMMLeft, synHFD3DMMRight, synC5DMMLeft, synC5DMMRight, synC6DMMLeft, synC6DMMRight, synH4DMMLeft, synH4DMMRight, synH5DMMLeft, synH5DMMRight, synH6DMMLeft, synH6DMMRight), add.cell.ids = c("synC4DMMLeft", "synC4DMMRight", "synC1DMMleft", "synC1DMMright", "synHFD3DMMLeft", "synHFD3DMMRight", "synC5DMMLeft", "synC5DMMRight", "synC6DMMLeft", "synC6DMMRight", "synH4DMMLeft", "synH4DMMRight", "synH5DMMLeft", "synH5DMMRight", "synH6DMMLeft", "synH6DMMRight"))

#Added metadata for QC analyses

merged\_seurat$log10GenesPerUMI <- log10(merged\_seurat$nFeature\_RNA) / log10(merged\_seurat$nCount\_RNA)

merged\_seurat$mitoRatio <- PercentageFeatureSet(merged\_seurat, pattern = "^mt-")

merged\_seurat$mitoRatio <- merged\_seurat@meta.data$mitoRatio / 100

merged\_seurat$riboRatio <- PercentageFeatureSet(merged\_seurat, pattern = "^Rp[ls]")

merged\_seurat$riboRatio <- merged\_seurat@meta.data$riboRatio / 100

metadata <- merged\_seurat@meta.data

metadata$cells <- rownames(metadata)

metadata <- metadata %>%

dplyr::rename(seq\_folder = orig.ident,

nUMI = nCount\_RNA,

nGene = nFeature\_RNA)

metadata$sample <- NA

metadata$sample[which(str\_detect(metadata$cells, "^synC1DMMleft"))] <- "synC1DMMleft"

metadata$sample[which(str\_detect(metadata$cells, "^synC1DMMright"))] <- "synC1DMMright"

metadata$sample[which(str\_detect(metadata$cells, "^synHFD3DMMLeft"))] <- "synHFD3DMMLeft"

metadata$sample[which(str\_detect(metadata$cells, "^synHFD3DMMRight"))] <- "synHFD3DMMRight"

metadata$sample[which(str\_detect(metadata$cells, "^synC4DMMLeft"))] <- "synC4DMMLeft"

metadata$sample[which(str\_detect(metadata$cells, "^synC5DMMLeft"))] <- "synC5DMMLeft"

metadata$sample[which(str\_detect(metadata$cells, "^synC6DMMLeft"))] <- "synC6DMMLeft"

metadata$sample[which(str\_detect(metadata$cells, "^synC4DMMRight"))] <- "synC4DMMRight"

metadata$sample[which(str\_detect(metadata$cells, "^synC5DMMRight"))] <- "synC5DMMRight"

metadata$sample[which(str\_detect(metadata$cells, "^synC6DMMRight"))] <- "synC6DMMRight"

metadata$sample[which(str\_detect(metadata$cells, "^synH4DMMLeft"))] <- "synH4DMMLeft"

metadata$sample[which(str\_detect(metadata$cells, "^synH5DMMLeft"))] <- "synH5DMMLeft"

metadata$sample[which(str\_detect(metadata$cells, "^synH6DMMLeft"))] <- "synH6DMMLeft"

metadata$sample[which(str\_detect(metadata$cells, "^synH4DMMRight"))] <- "synH4DMMRight"

metadata$sample[which(str\_detect(metadata$cells, "^synH5DMMRight"))] <- "synH5DMMRight"

metadata$sample[which(str\_detect(metadata$cells, "^synH6DMMRight"))] <- "synH6DMMRight"

merged\_seurat@meta.data <- metadata

save(merged\_seurat, file="Data/merged\_filtered\_seurat.RData")

#Quality Metrics###############

#NCells

metadata %>%

ggplot(aes(x=sample, fill=sample)) +

geom\_bar() +

theme\_classic() +

theme(axis.text.x = element\_text(angle = 45, vjust = 1, hjust=1)) +

theme(plot.title = element\_text(hjust=0.5, face="bold")) +

ggtitle("NCells")

#nUMIs

metadata %>%

ggplot(aes(color=sample, x=nUMI, fill= sample)) +

geom\_density(alpha = 0.2) +

scale\_x\_log10() +

theme\_classic() +

ylab("Cell density") +

geom\_vline(xintercept = 1000)

#nGenes

metadata %>%

ggplot(aes(color=sample, x=nGene, fill= sample)) +

geom\_density(alpha = 0.2) +

theme\_classic() +

scale\_x\_log10() +

geom\_vline(xintercept = 500)

metadata %>%

ggplot(aes(color=sample, x=nUMI, fill= sample)) +

geom\_density(alpha = 0.2) +

theme\_classic() +

scale\_x\_log10() +

geom\_vline(xintercept = 500)

#nGenes Boxplot

metadata %>%

ggplot(aes(x=sample, y=log10(nGene), fill=sample)) +

geom\_boxplot() +

theme\_classic() +

theme(axis.text.x = element\_text(angle = 45, vjust = 1, hjust=1)) +

theme(plot.title = element\_text(hjust=0.5, face="bold")) +

ggtitle("NCells vs NGenes")

#Correlation of genes v. UMI

metadata %>%

ggplot(aes(x=nUMI, y=nGene, color=mitoRatio)) +

geom\_point() +

scale\_colour\_gradient(low = "gray90", high = "black") +

stat\_smooth(method=lm) +

scale\_x\_log10() +

scale\_y\_log10() +

theme\_classic() +

geom\_vline(xintercept = 750) +

geom\_hline(yintercept = 500) +

facet\_wrap(~sample)

#mito counts ratio

metadata %>%

ggplot(aes(color=sample, x=mitoRatio, fill=sample)) +

geom\_density(alpha = 0.1) +

scale\_x\_log10() +

theme\_classic() +

geom\_vline(xintercept = 0.2)

#ribo counts ratio

metadata %>%

ggplot(aes(color=sample, x=riboRatio, fill=sample)) +

geom\_density(alpha = 0.2) +

scale\_x\_log10() +

theme\_classic() +

geom\_vline(xintercept = 0.3)

#novelty gene

metadata %>%

ggplot(aes(x=log10GenesPerUMI, color = sample, fill=sample)) +

geom\_density(alpha = 0.2) +

theme\_classic() +

geom\_vline(xintercept = 0.90)

#Filtering Cells ##############################################################

filtered\_seurat <- subset(x = merged\_seurat,

subset= (nUMI >= 500) &

(nGene >= 550) &

(log10GenesPerUMI > 0.80) & (log10GenesPerUMI < 0.90) &

(mitoRatio < 0.1) & (riboRatio <0.5))

#Keep genes expressed in 10 or more cells

counts <- GetAssayData(object = filtered\_seurat, slot = "counts")

nonzero <- counts > 0

keep\_genes <- Matrix::rowSums(nonzero) >= 0

filtered\_counts <- counts[keep\_genes, ]

filtered\_seurat <- CreateSeuratObject(filtered\_counts, meta.data = filtered\_seurat@meta.data)

save(filtered\_seurat, file="Data/seurat\_filtered.RData")

#cell cycle analysis and Supplementary Figure 4

s.genes <- c("Pcna", "Usp1", "Casp8ap2", "Brip1", "Prim1", "Ung", "Atad2", "Gmnn", "Ubr7", "Rpa2", "Mcm2", "Clspn", "Pola1", "Fen1", "Dscc1", "Uhrf1", "Chaf1b", "Dtl", "Blm", "Pold3", "Rrm2", "Rad51ap1", "Tipin", "Ccne2", "Pcna", "Wdr76", "Msh2", "Nasp", "Rad51", "Hells", "Mcm6", "Mcm4", "Mcm5", "Rfc2", "Slbp", "Tyms", "Cdc45", "E2f8", "Cenpu", "Rrm1", "Cdca7", "Cdc6", "Exo1", "Gins2")

g2m.genes <- c("Ube2c","Lbr","Ctcf","Cdc20","Cbx5","Kif11","Anp32e","Birc5","Cdk1","Anln", "Aurka", "Aurkb", "Bub1", "Ccnb2", "Cdc25c", "Cdca2", "Cdca3", "Cdca8", "Cenpa", "Cenpe", "Cenpf", "Ckap2", "Ckap2l", "Ckap5", "Cks1b", "Cks2", "Dlgap5", "Ect2", "Gas2l3", "Gtse1", "Hjurp", "Hmgb2", "Hmmr", "Kif20b", "Kif23", "Kif2c", "Mki67", "Ncapd2", "Ndc80", "Nek2", "Nuf2", "Nusap1", "Psrc1", "Rangap1", "Smc4", "Tacc3", "Tmpo", "Top2a", "Tpx2", "Ttk", "Tubb4b")

Seurat\_CellCycle <- NormalizeData(filtered\_seurat)

Seurat\_CellCycle <- CellCycleScoring(Seurat\_CellCycle,

g2m.features = g2m.genes,

s.features = s.genes, set.ident = TRUE)

#View(Seurat\_CellCycle@meta.data)

Seurat\_CellCycle <- FindVariableFeatures(Seurat\_CellCycle, verbose = F)

Seurat\_CellCycle <- ScaleData(Seurat\_CellCycle)

Seurat\_CellCycle <- RunPCA(Seurat\_CellCycle)

DimPlot(Seurat\_CellCycle, reduction = "pca", group.by = "Phase", split.by = "Phase")

#SCTransform #######################################################################

split\_seurat <- SplitObject(filtered\_seurat, split.by = "sample")

split\_seurat <- split\_seurat[c("synC1DMMleft", "synC1DMMright", "synHFD3DMMLeft", "synHFD3DMMRight", "synC4DMMLeft", "synC4DMMRight", "synC5DMMLeft", "synC5DMMRight", "synC6DMMLeft", "synC6DMMRight", "synH4DMMLeft", "synH4DMMRight", "synH5DMMLeft", "synH5DMMRight", "synH6DMMLeft", "synH6DMMRight")]

options(future.globals.maxSize= 993718400)

#scTransform data without regressing out mitochondrial genes

for (i in 1:length(split\_seurat)) {

split\_seurat[[i]] <- NormalizeData(split\_seurat[[i]], verbose = FALSE)

split\_seurat[[i]] <- CellCycleScoring(split\_seurat[[i]], g2m.features = g2m.genes, s.features = s.genes, set.ident = TRUE, min.cells=1)

split\_seurat[[i]] <- SCTransform(split\_seurat[[i]], method = "glmGamPoi", vars.to.regress = c("S.Score", "G2M.Score"),verbose = F)

}

#Integration of DataSets ###########################################################

integ\_features <- SelectIntegrationFeatures(object.list = split\_seurat, nfeatures = 3000)

split\_seurat <- PrepSCTIntegration(object.list = split\_seurat, anchor.features = integ\_features)

split\_seurat<- lapply(X = split\_seurat, FUN = RunPCA, vars.to.regress = c("S.Score", "G2M.Score"), features = integ\_features)

integ\_anchors2 <- FindIntegrationAnchors(object.list = split\_seurat, normalization.method = "SCT", anchor.features = integ\_features, dims = 1:30, reduction = "rpca", reference = c(1, 2), k.anchor = 20)

Integrated\_Seurat <- IntegrateData(anchorset = integ\_anchors2, normalization.method = "SCT", dims = 1:30)

Integrated\_Seurat <-ScaleData(Integrated\_Seurat, vars.to.regress = c("S.Score", "G2M.Score"))

#Run PCA on integrated dataset

Integrated\_Seurat<- FindVariableFeatures(Integrated\_Seurat, verbose = F)

Integrated\_Seurat <- RunPCA(object = Integrated\_Seurat)

PCAPlot(Integrated\_Seurat, split.by = "sample", verbose = F)

DimHeatmap(Integrated\_Seurat, dims = 1:9, cells = 500, balanced = T)

ElbowPlot(object = Integrated\_Seurat, ndims = 100)

#Run UMAP on integrated dataset

Integrated\_Seurat <- RunUMAP(Integrated\_Seurat, dims = 1:40)

DimPlot(Integrated\_Seurat)

DimPlot(Integrated\_Seurat, split.by = "sample")

#Clustering333###############################

Integrated\_Seurat <- FindNeighbors(object = Integrated\_Seurat, dims = 1:40)

Integrated\_Seurat <- FindClusters(object = Integrated\_Seurat, resolution = c(0.2,0.3, 0.4, 0.6, 0.8, 1.0, 1.4))

Integrated\_Seurat <- FindClusters(object = Integrated\_Seurat, resolution = c(1.8))

#Resolution 0.2

Idents(object = Integrated\_Seurat) <- "integrated\_snn\_res.0.3"

Integrated\_Seurat <- RunUMAP(Integrated\_Seurat, reduction = "pca", dims = 1:40)

DimPlot(Integrated\_Seurat, reduction = "umap", label = T)

DimPlot(Integrated\_Seurat, reduction = "umap", label = F, cols = c("firebrick", "goldenrod", "darkolivegreen3", "deepskyblue3", "darkslategray4", "forestgreen", "orchid", "darkorchid4", "navy"))

#Resolution 0.4

Idents(object = Integrated\_Seurat) <- "integrated\_snn\_res.0.3"

Integrated\_Seurat <- RunUMAP(Integrated\_Seurat, reduction = "pca", dims = 1:40)

DimPlot(Integrated\_Seurat, reduction = "umap", label = T, label.size = 6)

DimPlot(Integrated\_Seurat, label = T, split.by = "sample") + NoLegend()

DefaultAssay(Integrated\_Seurat) <- "integrated"

DefaultAssay(Integrated\_Seurat) <- "RNA"

Idents(Integrated\_Seurat) <- "groups"

Integrated\_Seurat <- RunUMAP(Integrated\_Seurat, reduction = "pca", dims = 1:40)

DimPlot(Integrated\_Seurat, reduction = "umap", label = T, label.size = 6)

DefaultAssay(Integrated\_Seurat) <- "integrated"

DefaultAssay(Integrated\_Seurat) <- "RNA"

my\_cols <- c('#7f0000','#b30000','#662506','#fc8d59','#fdbb84', '#fdd49e',

'#28CECA','#dd3497','#fe9929','#fc8d59','#faf4cf',

'#ef6548','#d7301f','#d7301f','#fc8d59','#fdd49e',

'#a50f15', '#54278f','#238b45', '#74c476', '#a1d99b','#c7e9c0', '#e5f5e0', '#ef6548', '#fa9fb5', '#ae017e', '#49006a')

my\_cols2 <- my\_cols[order(as.integer(names(my\_cols)))]

new.cluster.ids <- c("Mono\_Ly6c\_Low", "Mono\_Ly6c\_High", "Macro\_MhcII","Macro\_Lyve1", "Mono\_Prolif", "Neutrophils", "Mono\_Ly6c\_High", "Mo/DC","Bcells","T/NK/ILcells", "MastCells","Mono\_Interm","Mono\_Ly6c\_High", "T/NK/ILcells", "Fibro", "Mono\_Ly6c\_High", "Macro\_Ccr2", "Bcells", "Mono\_Ly6c\_High", "T/NK/ILcells", "Bcells", "Mono\_Ly6c\_Low", "Mono\_Prolif")

names(new.cluster.ids) <- levels(Integrated\_Seurat)

Integrated\_Seurat <- RenameIdents(Integrated\_Seurat, new.cluster.ids)

table(Integrated\_Seurat@meta.data$integrated\_snn\_res.0.3,Integrated\_Seurat@meta.data$orig.ident)

table(Integrated\_Seurat@meta.data$active.ident,Integrated\_Seurat@meta.data$orig.ident)

head(Integrated\_Seurat@meta.data)

Integrated\_Seurat@meta.data$groups <- paste(Integrated\_Seurat@meta.data$stim, "\_", Integrated\_Seurat@meta.data$integrated\_snn\_res.0.3)

Integrated\_Seurat@meta.data$age <- paste(Integrated\_Seurat@meta.data$stim2, "\_", Integrated\_Seurat@meta.data$integrated\_snn\_res.0.2)

head(Integrated\_Seurat@meta.data)

Integrated\_Seurat$stim <- factor(x=Integrated\_Seurat$stim, levels = c('CR', 'CL', 'HR', 'HL'))

markers0sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 0, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

allmarkers2 <- FindAllMarkers(Integrated\_Seurat, min.pct = 0.25, only.pos = T)

markers1sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 1, grouping.var = "orig.ident", verbose = FALSE , only.pos = T)

cluster1s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 1, min.pct = 0.25, only.pos = T)

cluster0s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 0, min.pct = 0.25, only.pos = T)

markers2sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 2, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster2s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 2, min.pct = 0.25, only.pos = T)

markers3sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 3, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster3s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 3, min.pct = 0.25, only.pos = T)

markers8sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 8, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster8s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 8, min.pct = 0.25, only.pos = T)

markers4sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 4, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster4s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 4, min.pct = 0.25, only.pos = T)

markers5sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 5, grouping.var = "orig.ident", verbose = FALSE , only.pos = T)

cluster5s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 5, min.pct = 0.25, only.pos = T)

markers6sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 6, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster6s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 6, min.pct = 0.25, only.pos = T)

markers7sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 7, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster7s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 7, min.pct = 0.25, only.pos = T)

markers8sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 8, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster8s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 8, min.pct = 0.25, only.pos = T)

markers9s <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 9, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster9s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 9, min.pct = 0.25, only.pos = T)

markers10sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 10 , grouping.var = "orig.ident", min.cells.group= 1 , verbose = FALSE, only.pos = T)

cluster10s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 10, min.pct = 0.25, only.pos = T)

markers11sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 11, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster11.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 11, min.pct = 0.1)

markers12sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 12, grouping.var = "orig.ident", min.cells.group= 1 , verbose = FALSE, only.pos = T)

cluster12.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 12, min.pct = 0.25, only.pos = T)

markers13sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 13, grouping.var = "orig.ident", min.cells.group= 1, verbose = FALSE, only.pos = T)

cluster13s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 13, min.pct = 0.25, only.pos = T)

markers14sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 14, grouping.var = "orig.ident", min.cells.group= 1 , verbose = FALSE, only.pos = T)

cluster14s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 14, min.pct = 0.25, only.pos = T)

markers15sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 15, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster15s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 15, min.pct = 0.25, only.pos = T)

markers16sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 16, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster16s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 16, min.pct = 0.25, min.cell.group = 0, only.pos = T)

cluster17s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 17, min.pct = 0.25, min.cell.group = 0, only.pos = T)

cluster18s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 18, min.pct = 0.25, min.cell.group = 0, only.pos = T)

markers17sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 17, grouping.var = "orig.ident", verbose = FALSE, min.cell.group = 0, only.pos = T)

markers18sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 18, grouping.var = "orig.ident", verbose = FALSE, min.cell.group = 0, only.pos = T)

head(markers18)

cluster18s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 18, min.pct = 0.25, only.pos = T)

markers19sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 19, grouping.var = "orig.ident", min.cells.group= 1, verbose = FALSE, min.cell.group = 0, only.pos = T)

cluster19s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 19, min.pct = 0.25,only.pos = T)

markers20sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 20, grouping.var = "orig.ident", min.cells.group= 1, verbose = FALSE,min.cell.group = 0, only.pos = T)

cluster20s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 20, min.pct = 0.25, only.pos = T)

markers21sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 21, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster21s.markersM <- FindMarkers(Integrated\_Seurat, ident.1 = 21, min.pct = 0.25, only.pos = T)

cluster22s.markersM <- FindMarkers(Integrated\_Seurat, ident.1 = 22, min.pct = 0.25, only.pos = T)

markers22sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 22, grouping.var = "orig.ident", min.cells.feature = 1, verbose = FALSE,min.cell.group = 0, only.pos = T)

cluster22s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 22, min.pct = 0.25, only.pos = T)

cluster23s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 23, min.pct = 0.25, only.pos = T)

markers23sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 23, grouping.var = "orig.ident", min.cells.group= 1, verbose = FALSE,min.cell.group = 0, only.pos = T)

cluster24s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 24, min.pct = 0.25, only.pos = T)

markers24sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 24, grouping.var = "orig.ident", verbose = FALSE, min.cell.group = 0, only.pos = T)

markers25sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 25, grouping.var = "orig.ident", min.cells.group= 1, verbose = FALSE, only.pos = T)

cluster24s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 24, min.pct = 0.25, only.pos = T)

cluster21s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 21, min.pct = 0.25, only.pos = T)

markers26sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 26, grouping.var = "orig.ident", verbose = FALSE, only.pos = T)

cluster27s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 27, min.pct = 0.25, only.pos = T)

markers27s <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 27, grouping.var = "orig.ident", min.cells.group= 1, verbose = FALSE, only.pos = T)

markers28sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 28, grouping.var = "orig.ident", verbose = FALSE, min.cells.group= 1, only.pos = T)

cluster28s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 28, min.pct = 0.25, only.pos = T)

markers29sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 29, grouping.var = "orig.ident", verbose = FALSE, min.cells.group= 1, only.pos = T)

cluster29s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 29, min.pct = 0.25, only.pos = T)

markers30sp <- FindConservedMarkers(Integrated\_Seurat, ident.1 = 30, grouping.var = "orig.ident", verbose = FALSE, min.cells.group= 1, only.pos = T)

cluster30s.markersMp <- FindMarkers(Integrated\_Seurat, ident.1 = 30, min.pct = 0.25, only.pos = T)

3

Macro3 <- subset(Integrated\_Seurat, idents = c( " "Macro\_MhcII", "Macro\_Lyve1", "Mono\_Prolif", "Macro\_Ccr2","))

Macro3 <- SplitObject(Macro3, split.by = "sample")

Macro3 <- lapply(X = Macro3, FUN = function(x) {

x <- NormalizeData(x)

x <- FindVariableFeatures(x, selection.method = "vst", nfeatures = 1000)

})

Macro3 <- lapply(X = Macro3, FUN = function(x) {

x <- NormalizeData(x)

x <- FindVariableFeatures(x, selection.method = "vst", nfeatures = 2000)

})

features <- SelectIntegrationFeatures(object.list = Macro3)

anchors <- FindIntegrationAnchors(object.list = Macro3, anchor.features = features, k.anchor=50)

Macro3 <- IntegrateData(anchorset = anchors, k.weight = 11)

DefaultAssay(Macro) <- "RNA"

DefaultAssay(Macro) <- "integrated"

Macro3 <- FindVariableFeatures(Macro3, selection.method = "vst", nfeatures = 2000)

Macro3 <- ScaleData(Macro3,vars.to.regress = c("S.Score", "G2M.Score"), verbose = FALSE)

Macro3 <- RunPCA(Macro3, npcs = 20, verbose = FALSE)

Macro3 <- RunUMAP(Macro3, reduction = "pca", dims = 1:15)

Macro3 <- FindNeighbors(Macro3, reduction = "pca", dims = 1:15)

Macro3 <- FindClusters(Macro3, resolution = 0.2)

DimPlot(Macro, reduction = "umap", label = TRUE)

new.cluster.ids <- c("Ccr2+", "Lyve1+/MHCII+","Ccr2+/MHCII+" ,"Retnlg+", "Lyve1+", "Prolif", "Prolif", "Prolif" )

names(new.cluster.ids) <- levels(Macro)

my\_levels <- c("Ccr2+", "Lyve1+/MHCII+","Ccr2+/MHCII+" ,"Retnlg+", "Lyve1+", "Prolif", "Prolif", "Prolif")

Idents(Macro) <- factor(Idents(Macro), levels= my\_levels)

Idents(object = Macro3) <- "integrated\_snn\_res.0.2"

DimPlot(Macro3, reduction = "umap", label = TRUE)

Mcluster1.markersM <- FindMarkers(Macro3, ident.1 = 1, min.pct = 0.25)

head(Mcluster1.markersM, n = 5)

Mmarkers1 <- FindConservedMarkers(Macro3, ident.1 = 1, grouping.var = "orig.ident", verbose = FALSE)

Mmarkers0 <- FindConservedMarkers(Macro3, ident.1 = 0, grouping.var = "orig.ident", verbose = FALSE)

head(Treg)

Mcluster0.markersM <- FindMarkers(Macro3, ident.1 = 0, min.pct = 0.25)

head(Mcluster0.markersM, n = 5)

Mmarkers2 <- FindConservedMarkers(Macro3, ident.1 = 2, grouping.var = "orig.ident", verbose = FALSE)

head(Mmarkers2)

Mcluster2.markersM <- FindMarkers(Macro3, ident.1 = 2, min.pct = 0.25)

head(Mcluster2.markersM, n = 5)

Mmarkers3 <- FindConservedMarkers(Macro3, ident.1 = 3, grouping.var = "orig.ident", verbose = FALSE)

head(Mmarkers3)

Mcluster3.markersM <- FindMarkers(Macro3, ident.1 = 3, min.pct = 0.25)

head(Mcluster3.markersM, n = 5)

Mmarkers4 <- FindConservedMarkers(Macro3, ident.1 = 4, grouping.var = "orig.ident", verbose = FALSE)

head(Mmarkers4)

Mcluster4.markersM <- FindMarkers(Macro3, ident.1 = 4, min.pct = 0.25)

head( Mcluster4.markersM, n = 5)

Mmarkers5 <- FindConservedMarkers(Macro3, ident.1 = 5, grouping.var = "orig.ident", min.cell.group = 0, verbose = TRUE)

head(Mmarkers5)

Mcluster5.markersM <- FindMarkers(Macro3, ident.1 = 5, min.pct = 0.25)

head(Mcluster5.markersM, n = 5)

Mmarkers6 <- FindConservedMarkers(Macro3, ident.1 = 6, grouping.var = "orig.ident",min.cell.group = 0, verbose = FALSE)

head(Mmarkers6)

Mcluster6.markersM <- FindMarkers(Macro3, ident.1 = 6, min.pct = 0.25)

head(Mcluster6.markersM, n = 5)

Mmarkers7 <- FindConservedMarkers(Macro3, ident.1 = 7, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

head(Mmarkers7)

Mcluster7.markersM <- FindMarkers(Macro3, ident.1 = 7, grouping.var = "orig.ident", min.cell.group = 0, min.pct = 0.25)

head(Mcluster7.markersM, n = 5)

Mmarkers8 <- FindConservedMarkers(Macro3, ident.1 = 8, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

head(Mmarkers8)

Mcluster8.markersM <- FindMarkers(Macro3, ident.1 = 8, min.pct = 0.25)

head(Mcluster8.markersM, n = 5)

Mmarkers9 <- FindConservedMarkers(Macro3, ident.1 = 9, grouping.var = "orig.ident", min.cells.group = 0, verbose = FALSE)

head(Mmarkers9)

Mcluster9.markersM <- FindMarkers(Macro3, ident.1 = 9, min.pct = 0.25)

head(Mcluster9.markersM, n = 5)

Mmarkers10 <- FindConservedMarkers(Macro3, ident.1 = 10 , grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

head(Mmarkers10)

Mcluster10.markersM <- FindMarkers(Macro3, ident.1 = 10, min.pct = 0.25)

head(Mcluster10.markersM, n = 5)

Mmarkers11 <- FindConservedMarkers(Macro3, ident.1 = 11, grouping.var = "orig.ident", verbose = FALSE)

head(Mmarkers11)

Mcluster11.markersM <- FindMarkers(Macro3, ident.1 = 11, min.pct = 0.25)

head(Mcluster11.markersM, n = 5)

Mmarkers12 <- FindConservedMarkers(Macro3, ident.1 = 12, grouping.var = "orig.ident", verbose = FALSE)

head(markers12)

Mcluster12.markersM <- FindMarkers(Macro3, ident.1 = 12, min.pct = 0.25)

Tcells <- subset(tcell\_combined1)

Tcells <- FindVariableFeatures(object = Tcells)

Tcells <- ScaleData(Tcells, verbose = FALSE)

Tcells <-RunPCA(object=Tcells, npcs = 30, verbose = FALSE)

# t-SNE and Clustering

Tcells <- RunUMAP(Tcells, reduction = "pca", dims = 1:30)

Tcells <- FindNeighbors(Tcells, reduction = "pca", dims = 1:30)

Tcells <- FindClusters(Tcells, resolution = 0.6)

DimPlot(Tcells1, reduction = "umap", label = TRUE)

Tcells1 <- subset(tcell\_combined1, idents = c("0", "1", "2", "3", "4", "5","6", "7", "9", "12", "14"))

DimPlot(Tcells1, reduction = "umap")

DefaultAssay(Tcells1) <- "RNA"

DefaultAssay(Tcells) <- "integrated"

DefaultAssay(tcell\_combined1) <- "RNA"

Idents(Tcells1) <- "groupst"

Idents(Tcells) <- "aget"

Idents(Tcells1) <- "integrated\_snn\_res.0.5"

Idents(Tcells) <- "orig.ident"

table(tcell\_combined1@meta.data$seurat\_clusters,tcell\_combined1@meta.data$orig.ident )

Tcells1@meta.data$groupst <- paste(Tcells1@meta.data$stim, "\_", Tcells1@meta.data$integrated\_snn\_res.0.5)

tcell\_combined1@meta.data$aget <- paste(tcell\_coTcellsmbined1@meta.data$stim2, "\_", tcell\_combined1@meta.data$integrated\_snn\_res.0.3)

head(Tcells1@meta.data)

Th17 <- subset(Tcells1, idents = c("2"))

Idents(Th17) <- "groupst"

Th17.markers <- FindAllMarkers(Th17, only.pos = TRUE, min.pct = 0.25, min.cells.group = 0, logfc.threshold = 0.25)

Th17.markers %>%

group\_by(cluster) %>%

top\_n(n = 30, wt = avg\_log2FC) -> top30

Tcells1$stim <- factor(x=Tcells1$stim, levels = c('CR', 'CL', 'HR', 'HL'))

Th17 <- ScaleData(Th17, features = rownames(Th17), assay="RNA", verbose = FALSE)

DoHeatmap(Th17, features=top30$gene, assay = "RNA")

levels(Tcells1)

levels(Th17) <- c("CR \_ 2","CL \_ 2", "HR \_ 2", "HL \_ 2")

levels(Treg) <- c("CR \_ 14","CL \_ 14", "HR \_ 14", "HL \_ 14")

Treg <- subset(Tcells1, idents = c("14"))

Idents(Treg) <- "groupst"

Treg.markers <- FindAllMarkers(Treg, only.pos = TRUE, min.pct = 0.25, min.cells.group = 0, logfc.threshold = 0.25)

Treg.markers %>%

group\_by(cluster) %>%

top\_n(n = 50, wt = avg\_log2FC) -> top50

Tregstim <- factor(x=Treg$stim, levels = c('CR', 'CL', 'HR', 'HL'))

Treg <- ScaleData(Treg, features = rownames(Treg), assay="RNA", verbose = FALSE)

DoHeatmap(Treg, features=top50$gene, assay = "RNA")

write.csv(Treg.markers, file="Treg.markers.csv")

write.csv(Th17.markers, file="Th17.markers.csv")

Tcellsallmarkers <- FindAllMarkers(Tcells, min.pct = 0.25, only.pos = T)

Tcluster1.markersM <- FindMarkers(tcell\_combined1, ident.1 = 1, min.pct = 0.25)

Tmarkers1 <- FindConservedMarkers(tcell\_combined1, ident.1 = 1, grouping.var = "orig.ident", sverbose = FALSE)

Tmarkers0 <- FindConservedMarkers(tcell\_combined1, ident.1 = 0, grouping.var = "orig.ident", verbose = FALSE)

Tcluster0.markersM <- FindMarkers(tcell\_combined1, ident.1 = 0, min.pct = 0.25)

Tmarkers2 <- FindConservedMarkers(tcell\_combined1, ident.1 = 2, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

Tcluster2.markersM <- FindMarkers(tcell\_combined1, ident.1 = 2, min.pct = 0.25)

Tmarkers3 <- FindConservedMarkers(tcell\_combined1, ident.1 = 3, grouping.var = "orig.ident",min.cell.group = 0, verbose = FALSE)

Tcluster3.markersM <- FindMarkers(tcell\_combined1, ident.1 = 3, min.pct = 0.25)

Tmarkers4 <- FindConservedMarkers(tcell\_combined1, ident.1 = 4, grouping.var = "orig.ident",min.cell.group = 0, verbose = FALSE)

Tcluster4.markersM <- FindMarkers(tcell\_combined1, ident.1 = 4, min.pct = 0.25)

Tmarkers5 <- FindConservedMarkers(tcell\_combined1, ident.1 = 5, grouping.var = "orig.ident", min.cell.group = 0, verbose = TRUE)

Tcluster5.markersM <- FindMarkers(tcell\_combined1, ident.1 = 5, min.pct = 0.25)

Tmarkers6 <- FindConservedMarkers(tcell\_combined1, ident.1 = 6, grouping.var = "orig.ident",min.cell.group = 0, verbose = FALSE)

Tcluster6.markersM <- FindMarkers(tcell\_combined1, ident.1 = 6, min.pct = 0.25)

Tmarkers7 <- FindConservedMarkers(tcell\_combined1, ident.1 = 7, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

cluster7.markersM <- FindMarkers(tcell\_combined1, ident.1 = 7, grouping.var = "orig.ident", min.cell.group = 0, min.pct = 0.25)

Tmarkers9 <- FindConservedMarkers(tcell\_combined1, ident.1 = 9, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

Tcluster9.markersM <- FindMarkers(tcell\_combined1, ident.1 = 9, min.pct = 0.25)

Tmarkers8 <- FindConservedMarkers(tcell\_combined1, ident.1 = 8 , grouping.var = "orig.ident", min.cell.group = 0, verbose = TRUE)

Tcluster8.markersM <- FindMarkers(tcell\_combined1, ident.1 = 8, min.pct = 0.25)

Tmarkers10 <- FindConservedMarkers(tcell\_combined1, ident.1 = 10, grouping.var = "orig.ident",min.cell.group = 0, verbose = FALSE)

Tcluster10.markersM <- FindMarkers(tcell\_combined1, ident.1 = 10, min.pct = 0.25)

Tmarkers11 <- FindConservedMarkers(tcell\_combined1, ident.1 = 11, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

Tcluster11.markersM <- FindMarkers(tcell\_combined1, ident.1 = 11, grouping.var = "orig.ident", min.cell.group = 0, min.pct = 0.25)

Tmarkers12 <- FindConservedMarkers(tcell\_combined1, ident.1 = 12, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

Tcluster12.markersM <- FindMarkers(tcell\_combined1, ident.1 = 12, min.pct = 0.25)

Tcluster13.markersM <- FindMarkers(tcell\_combined1, ident.1 = 13, min.pct = 0.25)

Tmarkers13 <- FindConservedMarkers(tcell\_combined1, ident.1 = 13, grouping.var = "orig.ident", min.cell.group = 0, verbose = TRUE)

Tcluster14.markersM <- FindMarkers(tcell\_combined1, ident.1 = 14, min.pct = 0.25)

Tmarkers14 <- FindConservedMarkers(tcell\_combined1, ident.1 = 14, grouping.var = "orig.ident",min.cell.group = 0, verbose = FALSE)

Tcluster15.markersM <- FindMarkers(tcell\_combined1, ident.1 = 15, min.pct = 0.25)

head(Mcluster6.markersM, n = 5)

Tmarkers15 <- FindConservedMarkers(tcell\_combined1, ident.1 = 15, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

head(Mmarkers7)

Tcluster16.markersM <- FindMarkers(tcell\_combined1, ident.1 = 16, grouping.var = "orig.ident", min.cell.group = 0, min.pct = 0.25)

head(Mcluster7.markersM, n = 5)

Tmarkers16 <- FindConservedMarkers(tcell\_combined1, ident.1 = 16, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

head(Tmarkers8)

Tcluster17.markersM <- FindMarkers(tcell\_combined1, ident.1 = 17, min.pct = 0.25)

Tmarkers17 <- FindConservedMarkers(tcell\_combined1, ident.1 = 17, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

TMacrolefthfdcontrdmmmarkers6 <- FindMarkers(Tcells1, ident.1 = "HL \_ 6", ident.2= "CL \_ 6", min.pct = 0.25)

TMacrolefthfdcontrdmmmarkers6$gene <- rownames(TMacrolefthfdcontrdmmmarkers6)

ggplot(TMacrolefthfdcontrdmmmarkers6, aes(avg\_log2FC, -log10(p\_val))) + geom\_point(size = 1, alpha = 0.5) + theme\_bw() +

ylab("-log10(adjusted p-value)") + geom\_text\_repel(aes(label = ifelse(p\_val < 0.05, gene,

"")), colour = "red", size = 5)

Idents(Tcells1) <- "groupst"

Idents(Tcells) <- "aget"

Idents(Tcells1) <- "integrated\_snn\_res.0.5"

VlnPlot(Tcells1, features = c("Foxp3", "Anxa5","Tox", "Tgfb1", "Stat3", "Stat5b"), idents = "14", split.by = "stim",

pt.size = 0, c("white", "blue", "grey", "lightblue3", "grey", "grey25", "white", "black", "white","blue", "red", "orange", "white", "white", "black", "white", "black", "grey", "grey25", "blue", "red", "orange", "white"))

VlnPlot(Tcells1, features = c("Pdcd1", "Tigit","Tox", "Entpd1", "Lag3", "Stat5b"), idents = "14", split.by = "stim",

pt.size = 0, c("white", "blue", "grey", "lightblue3", "grey", "grey25", "white", "black", "white","blue", "red", "orange", "white", "white", "black", "white", "black", "grey", "grey25", "blue", "red", "orange", "white"))

levels(Tcells1)

write.csv(Tcellsallmarkers, file="Tallmarkers.csv")

write.csv(Tcluster0.markersM, file="Tallclusters0.csv")

write.csv(Tcluster2.markersM, file="Tallclusters2.csv")

write.csv(Tcluster3.markersM, file="Tallclusters3.csv")

write.csv(Tcluster4.markersM, file="Tallclusters4.csv")

write.csv(Tcluster5.markersM, file="Tallclusters5.csv")

write.csv(Tcluster6.markersM, file="Tallclusters6.csv")

write.csv(Tcluster7.markersM, file="Tallclusters7.csv")

write.csv(Tcluster9.markersM, file="Tallclusters9.csv")

write.csv(Tcellsallmarkers, file="Tcellsallmarkers.csv")

write.csv(Tmarkers0, file="Tmarkers0.csv")

write.csv(Tmarkers1, file="Tmarkers1.csv")

write.csv(Tmarkers3, file="Tmarkers3.csv")

write.csv(Tmarkers4, file="Tmarkers4.csv")

write.csv(Tmarkers8, file="Tmarkers8.csv")

write.csv(Tmarkers7, file="Tmarkers7.csv")

write.csv(Tmarkers6, file="Tmarkers6.csv")

write.csv(Tmarkers5, file="Tmarkers5.csv")

write.csv(Tmarkers10, file="Tmarkers10.csv")

write.csv(Tmarkers11, file="Tmarkers11.csv")

write.csv(Tmarkers12, file="Tmarkers12.csv")

write.csv(Tmarkers14, file="Tmarkers14.csv")

write.csv(Tmarkers15, file="Tmarkers15.csv")

write.csv(Tmarkers2, file="Tmarkers2.csv")

write.csv(Tmarkers9, file="Tmarkers9.csv")

write.csv(Macrorightcontrdmmmarkers0, file="Macrorightcontrdmmmarkers0.csv")

write.csv(Mmarkers0, file="Mmarkers0.csv")

write.csv(Mmarkers1, file="Mmarkers1.csv")

write.csv(Mmarkers3, file="Mmarkers3.csv")

write.csv(Mmarkers4, file="Mmarkers4.csv")

write.csv(Mmarkers12, file="Mmarkers12.csv")

write.csv(Mmarkers10, file="Mmarkers10.csv")

write.csv(Mmarkers11, file="Mmarkers11.csv")

write.csv(Mmarkers9, file="Mmarkers9.csv")

write.csv(Mmarkers8, file="Mmarkers8.csv")

write.csv(Mmarkers7, file="Mmarkers7.csv")

write.csv(Mmarkers6, file="Mmarkers6.csv")

write.csv(tMmarkers5, file="Mmarkers5.csv")

write.csv(TMacrohfddmmmarkers0,file="TMacrohfddmmmarkers0.csv")

write.csv(TMacrohfddmmmarkers1,file="TMacrohfddmmmarkers1.csv")

write.csv(TMacrohfddmmmarkers2,file="TMacrohfddmmmarkers2.csv")

write.csv(TMacrohfddmmmarkers3,file="TMacrohfddmmmarkers3.csv")

write.csv(TMacrohfddmmmarkers4 ,file="TMacrohfddmmmarkers4.csv")

write.csv (TMacrohfddmmmarkers5 ,file="TMacrohfddmmmarkers5.csv")

write.csv (TMacrohfddmmmarkers6 ,file="TMacrohfddmmmarkers6.csv")

write.csv ( TMacrohfddmmmarkers7 ,file="TMacrohfddmmmarkers7.csv")

write.csv ( TMacrohfddmmmarkers8 ,file="TMacrohfddmmmarkers8.csv")

write.csv ( TMacrohfddmmmarkers9 ,file="TMacrohfddmmmarkers9.csv")

write.csv(TMacrohfddmmmarkers10,file="TMacrohfddmmmarkers10.csv")

write.csv(TMacrohfddmmmarkers11,file="TMacrohfddmmmarkers11.csv")

write.csv(TMacrohfddmmmarkers12,file="TMacrohfddmmmarkers12.csv")

write.csv(TMacrohfddmmmarkers13,file="TMacrohfddmmmarkers13.csv")

write.csv(TMacrohfddmmmarkers14 ,file="TMacrohfddmmmarkers14.csv")

write.csv ( TMacrocontrdmmmarkers0 ,file="TMacrocontrdmmmarkers0.csv")

write.csv ( TMacrocontrdmmmarkers1 ,file="TMacrocontrdmmmarkers1.csv")

write.csv ( TMacrocontrdmmmarkers2 ,file="TMacrocontrdmmmarkers2.csv")

write.csv ( TMacrocontrdmmmarkers3 ,file="TMacrocontrdmmmarkers3.csv")

write.csv ( TMacrocontrdmmmarkers4 ,file="TMacrocontrdmmmarkers4.csv")

write.csv ( TMacrocontrdmmmarkers5 ,file="TMacrocontrdmmmarkers5.csv")

write.csv ( TMacrocontrdmmmarkers6 ,file="TMacrocontrdmmmarkers6.csv")

write.csv ( TMacrocontrdmmmarkers7 ,file="TMacrocontrdmmmarkers7.csv")

write.csv ( TMacrocontrdmmmarkers8 ,file="TMacrocontrdmmmarkers8.csv")

write.csv ( TMacrocontrdmmmarkers9 ,file="TMacrocontrdmmmarkers9.csv")

write.csv ( TMacrocontrdmmmarkers10 ,file="TMacrocontrdmmmarkers10.csv")

write.csv ( TMacrocontrdmmmarkers11 ,file="TMacrocontrdmmmarkers11.csv")

write.csv ( TMacrocontrdmmmarkers12 ,file="TMacrocontrdmmmarkers12.csv")

write.csv ( TMacrocontrdmmmarkers13 ,file="TMacrocontrdmmmarkers13.csv")

write.csv ( TMacrocontrdmmmarkers14 ,file="TMacrocontrdmmmarkers14.csv")

write.csv ( TMacrohfdcontrdmmmarkers0 ,file="TMacrohfdcontrdmmmarkers0.csv")

write.csv ( TMacrohfdcontrdmmmarkers1 ,file="TMacrohfdcontrdmmmarkers1.csv")

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write.csv ( TMacrorightcontrhfddmmmarkers6,file="TMacrorightcontrhfddmmmarkers62.csv")

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write.csv (TMacrolefthfdcontrdmmmarkers14 ,file="TMacrolefthfdcontrdmmmarkers14.csv")

Treg <- subset(Tcells1, idents = c("14"))

Idents(object = Treg) <- "groupst"

Treg.markers <- FindAllMarkers(Treg, only.pos = TRUE, min.pct = 0.25, logfc.threshold = 0.25)

Treg.markers %>%

group\_by(cluster) %>%

top\_n(n = 30, wt = avg\_log2FC) -> top30

DoHeatmap(Treg, features = top30$gene) + NoLegend()

Treg$stim <- factor(x=Treg$stim, levels = c('CR', 'CL', 'HR', 'HL'))

Treg <- ScaleData(Treg, features = rownames(Treg), assay="RNA", verbose = FALSE)

DoHeatmap(Treg, features=top30$gene, assay = "RNA")+ scale\_fill\_gradientn(colors = c("#7393B3", "grey", "blue"))

Th17 <- subset(Tcells1, idents = c("2"))

Idents(object = Th17) <- "groupst"

Th17.markers <- FindAllMarkers(Th17, only.pos = TRUE, min.pct = 0.25, logfc.threshold = 0.25)

Th17.markers %>%

group\_by(cluster) %>%

top\_n(n = 30, wt = avg\_log2FC) -> top30

DoHeatmap(Th17, features = top30$gene) + NoLegend()

Th17$stim <- factor(x=Treg$stim, levels = c('CR', 'CL', 'HR', 'HL'))

Th17 <- ScaleData(Th17, features = rownames(Treg), assay="RNA", verbose = FALSE)

DoHeatmap(Th17, features=top30$gene, assay = "RNA")+ scale\_fill\_gradientn(colors = c("#7393B3", "grey", "blue"))

Bcells <- subset(Integrated\_Seurat, idents = c("4", "7", "16"))

Bcells <- SplitObject(Bcells, split.by = "sample")

Bcells <- lapply(X = Bcells, FUN = function(x) {

x <- NormalizeData(x)

x <- FindVariableFeatures(x, selection.method = "vst", nfeatures = 3000)

})

featuresb <- SelectIntegrationFeatures(object.list = Bcells)

anchorsb <- FindIntegrationAnchors(object.list = Bcells, anchor.features = featuresb)

Bcells <- IntegrateData(anchorset = anchorsb, k.weight = 20)

Bcells <- ScaleData(Bcells, verbose = FALSE)

Bcells <-RunPCA(object=Bcells, npcs = 30, verbose = FALSE)

# t-SNE and Clustering

Bcells <- RunUMAP(Bcells, reduction = "pca", dims = 1:30)

Bcells <- FindNeighbors(Bcells, reduction = "pca", dims = 1:30)

Bcells <- FindClusters(Bcells, resolution = 0.2)

DimPlot(Bcells, reduction = "umap", label = TRUE)

DimPlot(Bcells1, reduction = "umap", label = FALSE, repel=TRUE, pt.size = 0.5, label.size = 7, cols =c('#7FFFD4','#46C7C7','#3EA99F', '#AAF0D1', '#5F9EA0', '#3EB489','#3CB371','#4AA02C'))+ NoLegend()

new.cluster.ids <- c("Mature\_Naive\_Bcells","Intermediate\_preBcells", "Immature\_Bcells", "Late\_proBcells" , "Mature\_Bcell", "Early\_proBcells", "Activated\_Bcells")

names(new.cluster.ids) <- levels(Bcells1)

Bcells1 <- RenameIdents(Bcells1, new.cluster.ids)

Bcells1 <- subset(Bcells, idents = c("0", "1", "2", "4", "5", "6", "7"))

VlnPlot(Integrated\_Seurat, features = c("Cxcr2"), idents = "8", split.by = "stim",

pt.size = 0, c('#F5EBAB','#70630F' , '#E1D587' , '#660000'))

table(Bcells@meta.data$seurat\_clusters,Bcells@meta.data$orig.ident )

Bcells@meta.data$groupsb <- paste(Bcells@meta.data$stim, "\_", Bcells@meta.data$integrated\_snn\_res.0.2)

Bcells@meta.data$ageb <- paste(Bcells@meta.data$stim2, "\_", Bcells@meta.data$integrated\_snn\_res.0.2)

head(tcell\_combined1@meta.data)

DefaultAssay(Bcells) <- "RNA"

DefaultAssay(Myeloid) <- "integrated"

Idents(Bcells) <- "groupsb"

Idents(Bcells) <- "ageb"

Idents(Bcells) <- "ageb"

Idents(Bcells) <- "integrated\_snn\_res.0.2"

cC

VlnPlot(Bcells1, features = c("Ccr7", "Cd79a", "Cd24a","Cd38", "Cd19", "Tnfrsf13c", "Irf4", "Itga4", "Cd22", "Ms4a1", "Vpreb1", "Pax5"), pt.size = 0, cols =c('#7FFFD4','#46C7C7','#3EA99F', '#AAF0D1', '#5F9EA0', '#3EB489','#3CB371','#4AA02C'))

BTcluster1.markersM <- FindMarkers(Bcells, ident.1 = 1, min.pct = 0.25)

BTmarkers1 <- FindConservedMarkers(Bcells, ident.1 = 1, grouping.var = "orig.ident", verbose = FALSE)

BTmarkers0 <- FindConservedMarkers(Bcells, ident.1 = 0, grouping.var = "orig.ident", verbose = FALSE)

BTcluster0.markersM <- FindMarkers(Bcells, ident.1 = 0, min.pct = 0.25)

BTmarkers2 <- FindConservedMarkers(Bcells, ident.1 = 2, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

BTcluster2.markersM <- FindMarkers(Bcells, ident.1 = 2, min.pct = 0.25)

BTmarkers3 <- FindConservedMarkers(Bcells, ident.1 = 3, grouping.var = "orig.ident",min.cell.group = 0, verbose = FALSE)

BTcluster3.markersM <- FindMarkers(Bcells, ident.1 = 3, min.pct = 0.25)

BTmarkers4 <- FindConservedMarkers(Bcells, ident.1 = 4, grouping.var = "orig.ident",min.cell.group = 0, verbose = FALSE)

BTcluster4.markersM <- FindMarkers(Bcells, ident.1 = 4, min.pct = 0.25)

BTmarkers5 <- FindConservedMarkers(Bcells, ident.1 = 5, grouping.var = "orig.ident", min.cell.group = 0, verbose = TRUE)

BTcluster5.markersM <- FindMarkers(Bcells, ident.1 = 5, min.pct = 0.25)

BTmarkers6 <- FindConservedMarkers(Bcells, ident.1 = 6, grouping.var = "orig.ident",min.cell.group = 0, verbose = FALSE)

BTcluster6.markersM <- FindMarkers(Bcells, ident.1 = 6, min.pct = 0.25)

BTmarkers7 <- FindConservedMarkers(Bcells, ident.1 = 7, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

BTcluster7.markersM <- FindMarkers(Bcells, ident.1 = 7, grouping.var = "orig.ident", min.cell.group = 0, min.pct = 0.25)

BTmarkers9 <- FindConservedMarkers(Bcells, ident.1 = 9, grouping.var = "orig.ident", min.cell.group = 0, verbose = FALSE)

BTcluster9.markersM <- FindMarkers(Bcells, ident.1 = 9, min.pct = 0.25)

BTmarkers8 <- FindConservedMarkers(Bcells, ident.1 = 8 , grouping.var = "orig.ident", min.cell.group = 0, verbose = TRUE)

BTcluster8.markersM <- FindMarkers(Bcells, ident.1 = 8, min.pct = 0.25)

write.csv(BTcluster0.markersM, file="BTallclusters0.csv")

write.csv(BTcluster1.markersM, file="BTallclusters1.csv")

write.csv(BTcluster2.markersM, file="BTallclusters2.csv")

write.csv(BTcluster3.markersM, file="BTallclusters3.csv")

write.csv(BTcluster4.markersM, file="BTallclusters4.csv")

write.csv(BTcluster5.markersM, file="BTallclusters5.csv")

write.csv(BTcluster6.markersM, file="BTallclusters6.csv")

write.csv(BTcluster7.markersM, file="BTallclusters7.csv")

write.csv(BTcluster8.markersM, file="BTallclusters8.csv")

write.csv(BTMacrolefthfdcontrdmmmarkers2, file="TMacrolefthfdcontrdmmmarkers2.csv")

write.csv(BTMacrolefthfdcontrdmmmarkers14, file="TMacrolefthfdcontrdmmmarkers14.csv")

write.csv(BTMacrocontrdmmmarkers14, file="TMacrocontrdmmmarkers14.csv")

write.csv(BTMacrocontrdmmmarkers2, file="TMacrocontrdmmmarkers2.csv")

write.csv(BTMacrohfddmmmarkers2, file="TMacrohfddmmmarkers2.csv")

write.csv(BTMacrohfddmmmarkers14, file="TMacrohfddmmmarkers14.csv")

write.csv(BTmarkers6, file="BTmarkers6.csv")

write.csv(BTmarkers5, file="BTmarkers5.csv")

write.csv(BTMacrohfddmmmarkers0,file="BTMacrohfddmmmarkers0.csv")

write.csv(BTMacrohfddmmmarkers1,file="BTMacrohfddmmmarkers1.csv")

write.csv(BTMacrohfddmmmarkers2,file="BTMacrohfddmmmarkers2.csv")

write.csv(BTMacrohfddmmmarkers3,file="BTMacrohfddmmmarkers3.csv")

write.csv(BTMacrohfddmmmarkers4 ,file="BTMacrohfddmmmarkers4.csv")

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write.csv ( BTMacrocontrdmmmarkers0 ,file="BTMacrocontrdmmmarkers0.csv")

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write.csv(BTmarkers6, file="BTmarkers6.csv")

write.csv(BTmarkers5, file="BTmarkers5.csv")

DimPlot(Bcells, reduction = "umap", split.by = "orig.ident")

DefaultAssay(Bcells) <- "RNA"

DefaultAssay(Bcells) <- "integrated"

table(Bcells@meta.data$seurat\_clusters,Bcells@meta.data$orig.ident )

Mo\_DC <- subset(Integrated\_Seurat, idents = c("15"))

VlnPlot(Mo\_DC, features = c("Tcf4", "Sirpa", "Il7r","Itgax", "Irf8", "Naaa", "Cd209a", "Ccr7", "Clec10a", "Klrd1", "Ccr2", "Csf1r"), pt.size = 0, cols =c('#7FFFD4','#46C7C7','#3EA99F', '#AAF0D1', '#5F9EA0', '#3EB489','#3CB371','#4AA02C'))

VlnPlot(Integrated\_Seurat, features = c("Klf10", "Stap1", "Zeb2", "Pdcd4", "Gzmb", "Fcer1a", "Cebpa", "Fosb", "Csf1"), idents = "18", split.by = "stim",

pt.size = 0, c('#F5EBAB','#70630F' , '#E1D587' , '#660000'))

VlnPlot(Integrated\_Seurat, features = c("Ctsg", "Mpo", "Hist1h1b", "Camp", "Itga6", "Plek", "Tpm4", "Zyx","Tmsb4x", "Bcl2l11"), idents = "13", split.by = "stim",

pt.size = 0, c('#F5EBAB','#70630F' , '#E1D587' , '#660000'))