

Monocytes can Proliferate in Vacant Tissue Niches prior to Differentiation into Macrophages

12 - cMAF- and Mafb-deficient IM

2022-01-30 23:26:51 +0100

Abstract

Resident tissue macrophages (RTM) are differentiated immune cells populating distinct niches and exhibiting important tissue-supportive functions. RTM maintenance is thought to depend either on monocyte engraftment and differentiation, or on the self-renewal of mature RTM. Here, we discovered that monocytes can re-enter cell cycle and proliferate locally before their differentiation into RTM. We developed a mouse model of inducible lung interstitial macrophage (IM) depletion in which the vacant niche is repopulated by BM-derived monocytes giving rise to fully differentiated IM subsets. By performing time-course single-cell RNA-sequencing analyses of myeloid cells during niche refilling, we found that few Ly6C+ classical monocytes could self-renew locally in a CSF1R-dependent manner. We further showed that the transcription factor MafB restricted such proliferation and was essential to mediate RTM specification and identity in our model. Our data provide evidence that, in the mononuclear phagocyte system, self-renewal is not merely restricted to myeloid progenitor cells and mature macrophages, but is also a tightly regulated capability of mature monocytes developing into RTM *in vivo*.

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1 Description

We would like to know the functions of cMAF and MAFb transcription factors in IM differentiation or IM refilling in empty niche. We analyze the IM with cMAF-KO, MAFb-KO, cMAF/MAF-dKO and control phenotype using scRNASeq (10X Genomics). These mice are Lyz2-Cre induced recombination KO mice, thus gene KO is dependent on the Lyz2 expression.

In this experiment, we had 4 groups of mice:

- Five littermate control mice (Control group);
- Five Lyz2-Cre Maf-flox mice (cMAF-KO group);
- Five Lyz2-Cre Mafb-flox mice (MAFb-KO group);
- Five Lyz2-Cre Maf-flox Mafb-flox mice (dKO group).

Lung monocytes and macrophages were sorted separately and pooled with ratio of 3:7. Pooled cells from each sample were stained with unique anti-MHCI-hashtag (Biolegend Hashtag) before pooled sequencing.

The analysis pipeline was the same as DT treatment time-course analysis.

2 Demultiplexing by hashtag sequences

```
suppressMessages({  
  library(Seurat)  
  library(ggpubr)  
})
```

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2.1 Read data

```
# Load in the UMI matrix (from CR) 1  
  
umis <- Read10X("../counts/scRNASeq/Experiment-7-12-21-ScRNA_NGS21- 2  
U976/outs/filtered_feature_bc_matrix/") 3  
  
# For generating a hashtag count matrix from FASTQ files, please refer to 4  
# https://github.com/Hoohm/CITE-seq-Count. 5  
  
# Load in the HTO count matrix 6  
htos <- Read10X("../counts/HTO/read_count/", gene.column=1) 7
```

```
# change cell names to "-1" mode. 1  
colnames(htos) <- paste0(colnames(htos), "-1") 2  
  
# remove the unmapped as it's not a barcode. It's the last row. 3  
htos <- htos[-nrow(htos), ] 4  
  
# Select cell barcodes detected by both RNA and HTO 5  
# In the example datasets we have already filtered the cells for you, but 6  
# perform this step for clarity. 7  
joint.bcs <- intersect(colnames(umis), colnames(htos)) 8  
  
# Subset RNA and HTO counts by joint cell barcodes 9  
umis <- umis[, joint.bcs] 10  
htos <- as.matrix(htos[, joint.bcs]) 11  
  
# Confirm that the HTO have the correct names 12
```

```
rownames(htos)
```

17

```
## [1] "HT5_Control-CTTGTCTTGAG" "HT6_cMAF_KO-TATGCTGCCACGGTA"  
## [3] "HT7_MAFb_KO-GAGTCTGCCAGTATC" "HT8_dKO-TATAGAACGCCAGGC"
```

1
2
3

Setup Seurat object and add in the HTO data

```
# Setup Seurat object  
hashtag <- CreateSeuratObject(counts = umis)  
  
# Normalize RNA data with log normalization  
hashtag <- NormalizeData(hashtag)  
# Find and scale variable features  
hashtag <- FindVariableFeatures(hashtag, selection.method = 'mean.var.plot'  
'')  
hashtag <- ScaleData(hashtag, features = VariableFeatures(hashtag))
```

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2.2 Adding HTO data as an independent assay

You can read more about working with multi-modal data here

```
# Add HTO data as a new assay independent from RNA  
hashtag[['HTO']] <- CreateAssayObject(counts = htos)  
# Normalize HTO data, here we use centered log-ratio (CLR) transformation  
hashtag <- NormalizeData(hashtag, assay = 'HTO', normalization.method = '  
CLR')
```

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2.3 Demultiplex cells based on HTO enrichment

Here we use the Seurat function HTODemux() to assign single cells back to their sample origins.

```
# If you have a very large dataset we suggest using k_function = "clara".  
# This is a k-medoid clustering function for large applications  
# You can also play with additional parameters (see documentation for  
# HTODemux()) to adjust the threshold for classification  
# Here we are using the default settings  
hashtag <- HTODemux(hashtag, assay = "HTO", positive.quantile = 0.94)
```

1
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2.4 Visualize demultiplexing results

Output from running HTODemux() is saved in the object metadata. We can visualize how many cells are classified as singlets, doublets and negative/ambiguous cells.

```
# Global classification results  
table(hashtag$HTO_classification.global)
```

1
2

```
##  
## Doublet Negative Singlet  
## 2094 2591 6653
```

1
2
3

Visualize enrichment for selected HTOs with ridge plots

```

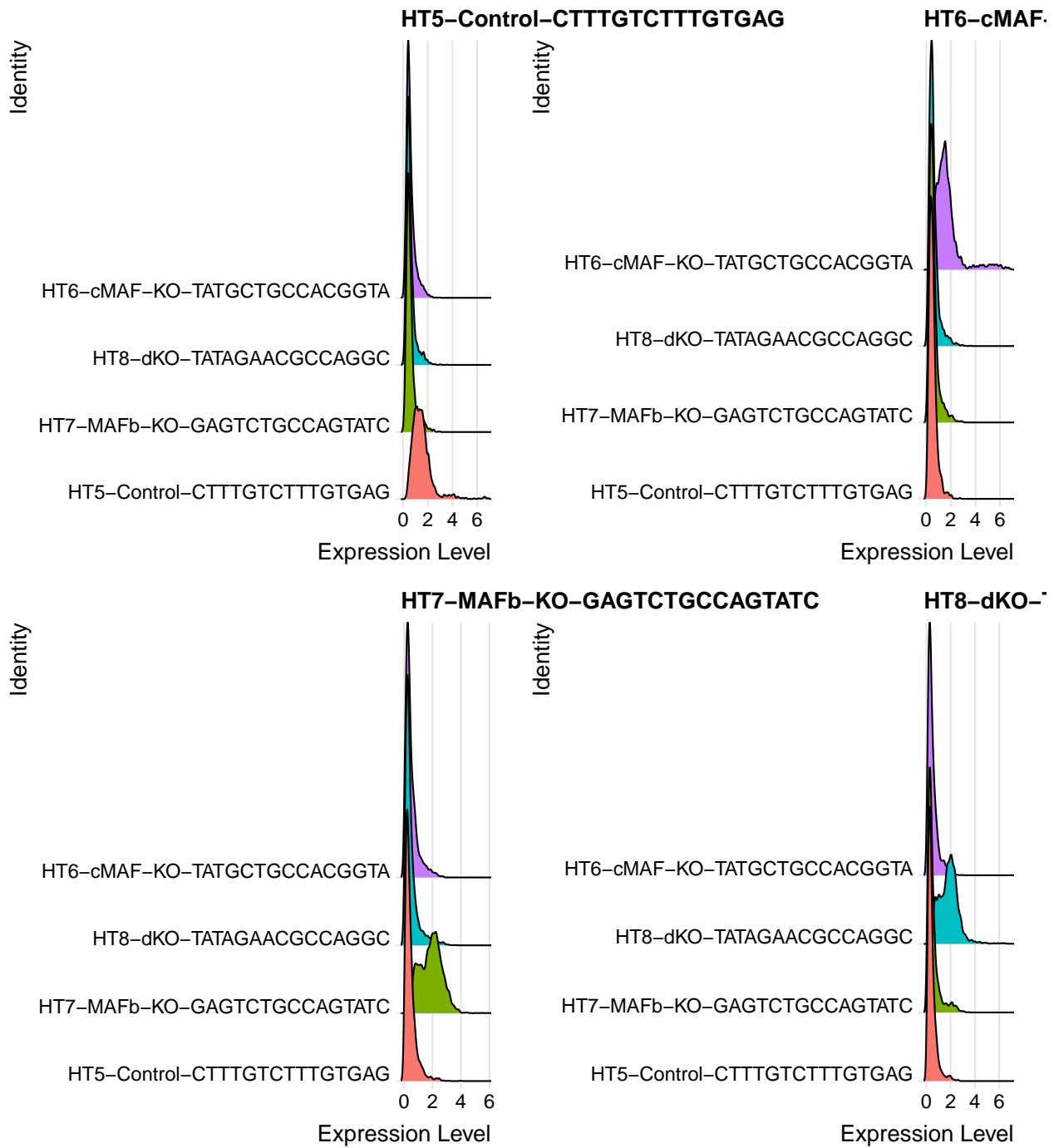
# Group cells based on the max HTO signal
1
2
3

```

```

Idents(hashtag) <- 'HTO_maxID'
RidgePlot(hashtag, assay = 'HTO', features = rownames(hashtag[['HTO']]),
  ncol = 2)

```

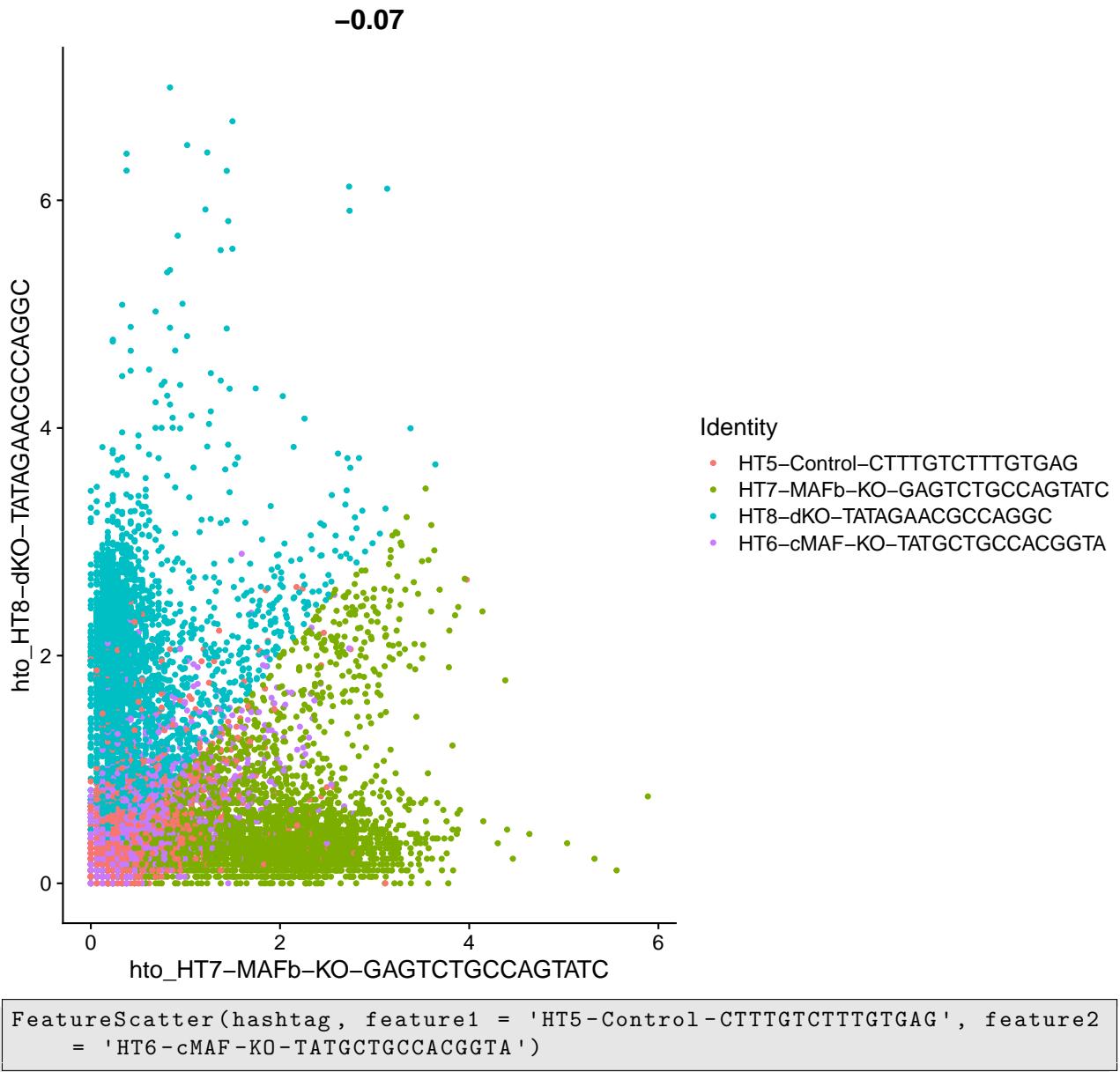


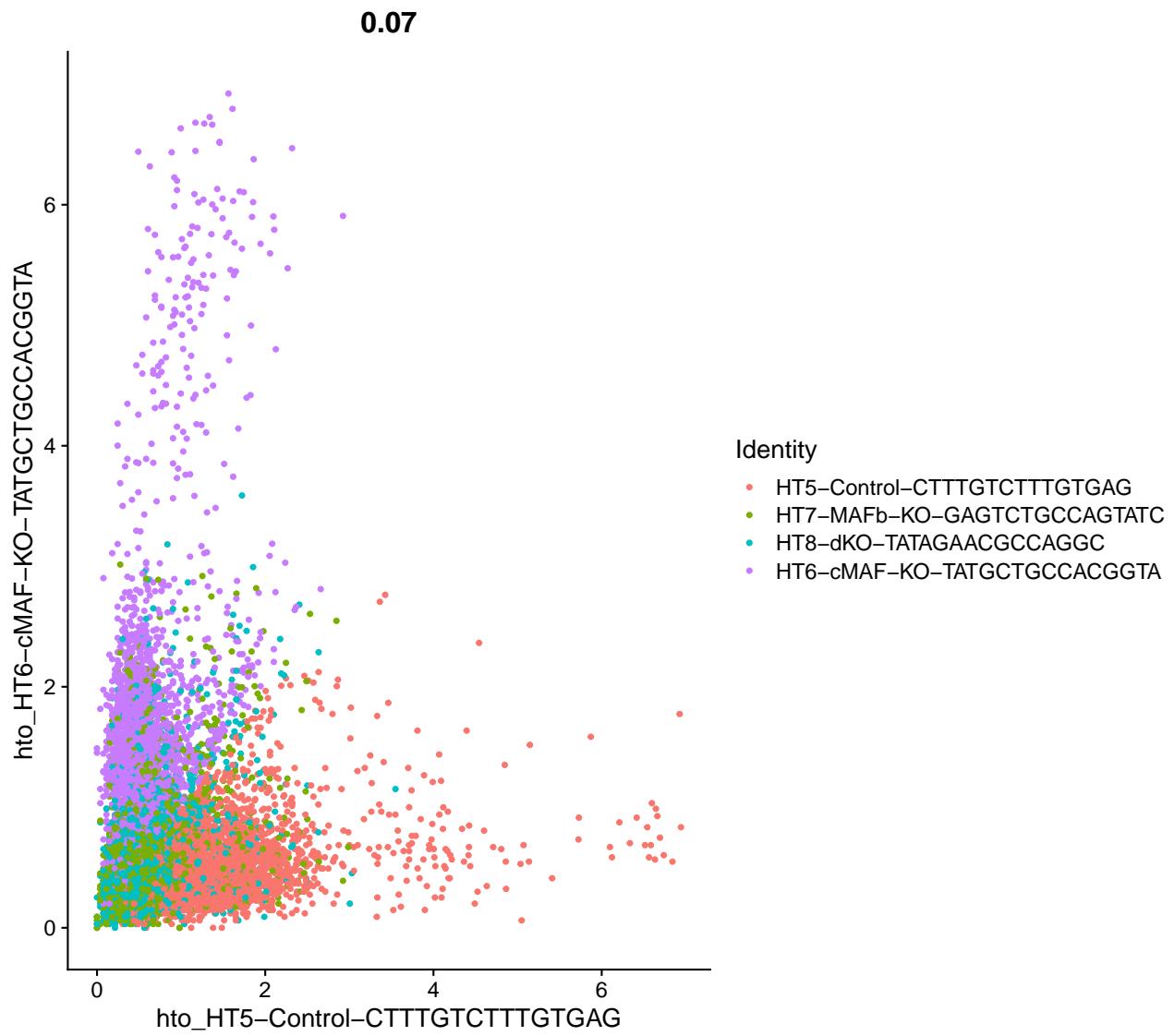
Visualize pairs of HTO signals to confirm mutual exclusivity in singlets

```

FeatureScatter(hashtag, feature1 = 'HT7-MAFb-KO-GAGTCTGCCAGTATC', feature2 = 1
  = 'HT8-dKO-TATAGAACGCCAGGC')

```

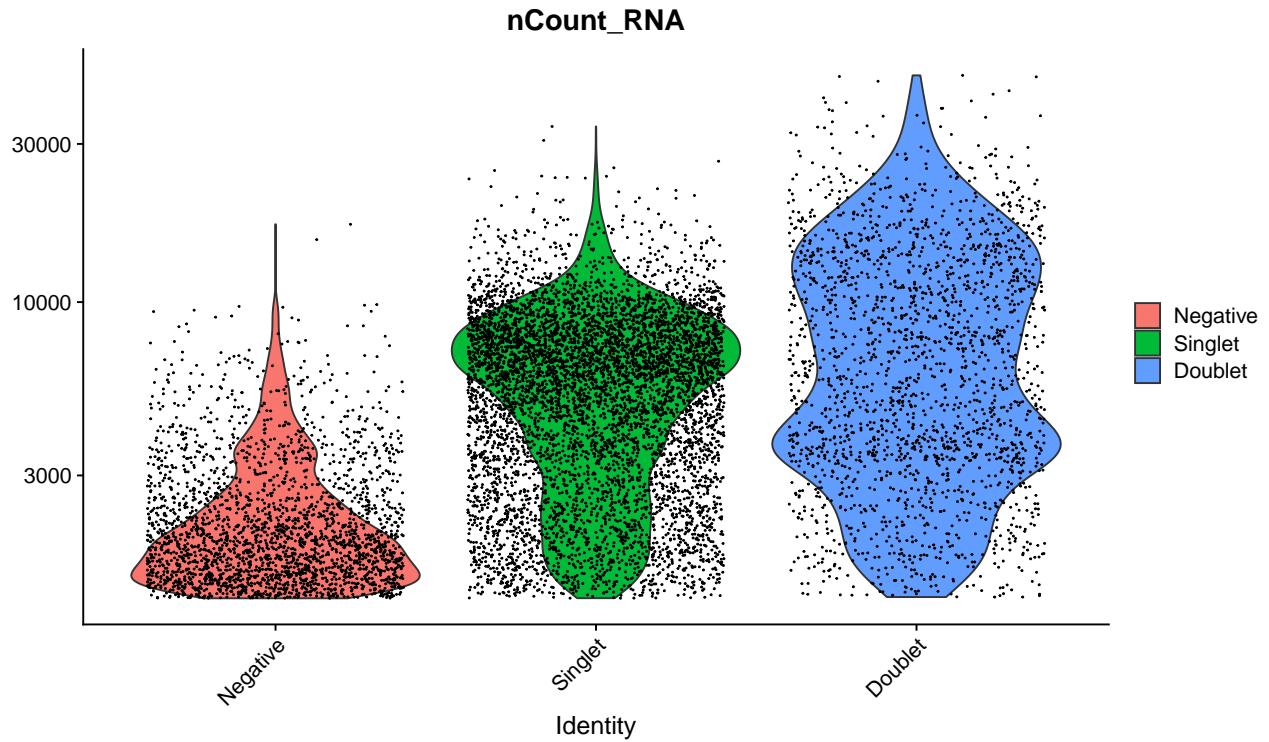




Compare number of UMIs for singlets, doublets and negative cells

```
Idents(hashtag) <- 'HT0_classification.global'
VlnPlot(hashtag, features = 'nCount_RNA', pt.size = 0.1, log = TRUE)
```

1
2

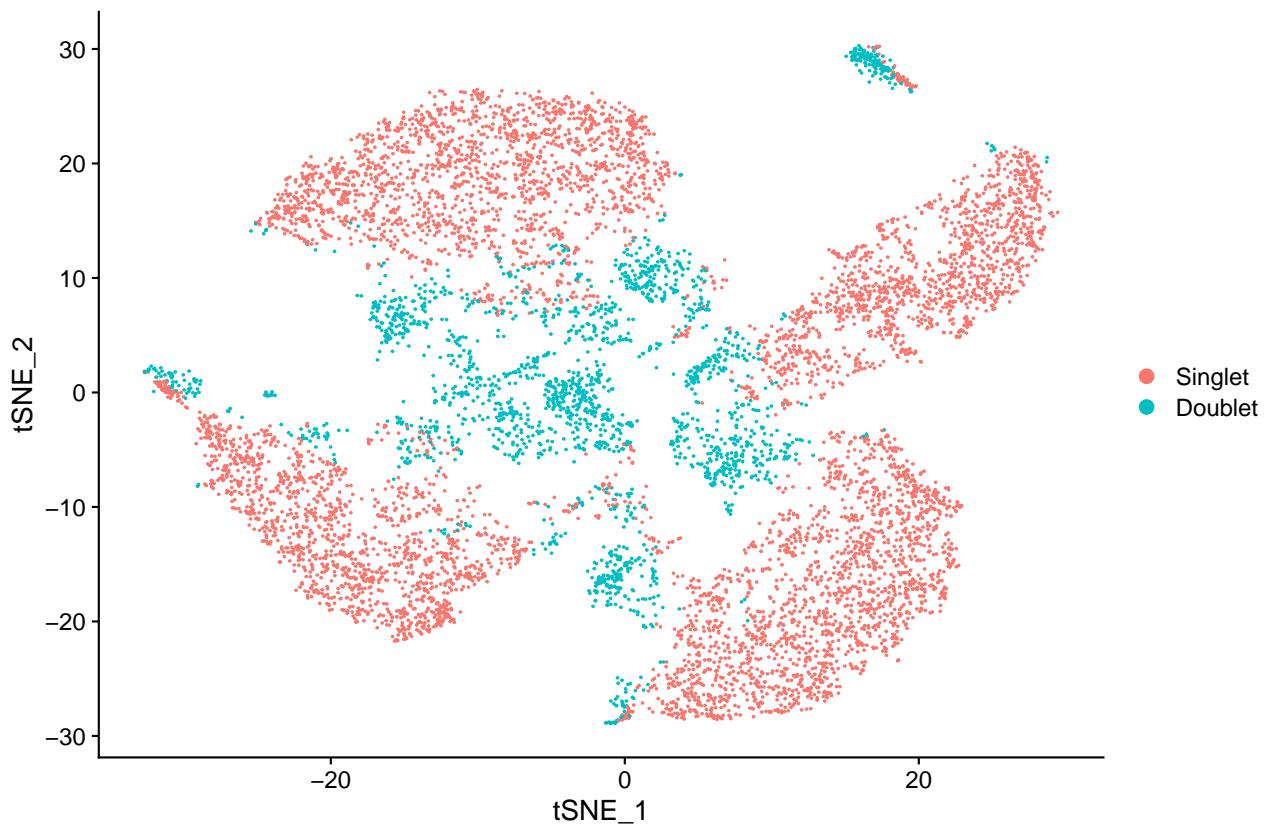


Generate a two dimensional tSNE embedding for HTOs. Here we are grouping cells by singlets and doublets for simplicity.

```

#First, we will remove negative cells from the object
hashtag.subset <- subset(hashtag, idents = 'Negative', invert = TRUE)          1
                                                               2
                                                               3
# Calculate a tSNE embedding of the HTO data
DefaultAssay(hashtag.subset) <- "HTO"                                         4
                                                               5
hashtag.subset <- ScaleData(hashtag.subset, features = rownames(hashtag.      6
  subset), verbose = FALSE)
hashtag.subset <- RunPCA(hashtag.subset, features = rownames(hashtag.        7
  subset), approx = FALSE)
hashtag.subset <- RunTSNE(hashtag.subset, dims = 1:8, perplexity = 100,       8
  check_duplicates = FALSE)
                                                               9
DimPlot(hashtag.subset)                                                       10

```



Create an HTO heatmap, based on Figure 1C in the Cell Hashing paper.

```
#To increase the efficiency of plotting, you can subsample cells using the num.cells argument
HTOHeatmap(hashtag, assay = 'HTO', ncells = 1000)
```



Cluster and visualize cells using the usual scRNA-seq workflow, and examine for the potential presence of batch effects.

```

# Extract the singlets
singlet <- subset(hashtag, idents = 'Singlet')  

# Select the top 1000 most variable features
singlet <- FindVariableFeatures(singlet, selection.method = 'mean.var.plot'  

    ')  

# Scaling RNA data, we only scale the variable features here for  

# efficiency
singlet <- ScaleData(singlet, features = VariableFeatures(singlet))  

# Run PCA
singlet <- RunPCA(singlet, features = VariableFeatures(singlet))

```

```

# We select the top 10 PCs for clustering and tSNE based on PCElbowPlot
singlet <- FindNeighbors(singlet, reduction = 'pca', dims = 1:10)  

singlet <- FindClusters(singlet, resolution = 0.6, verbose = FALSE)  

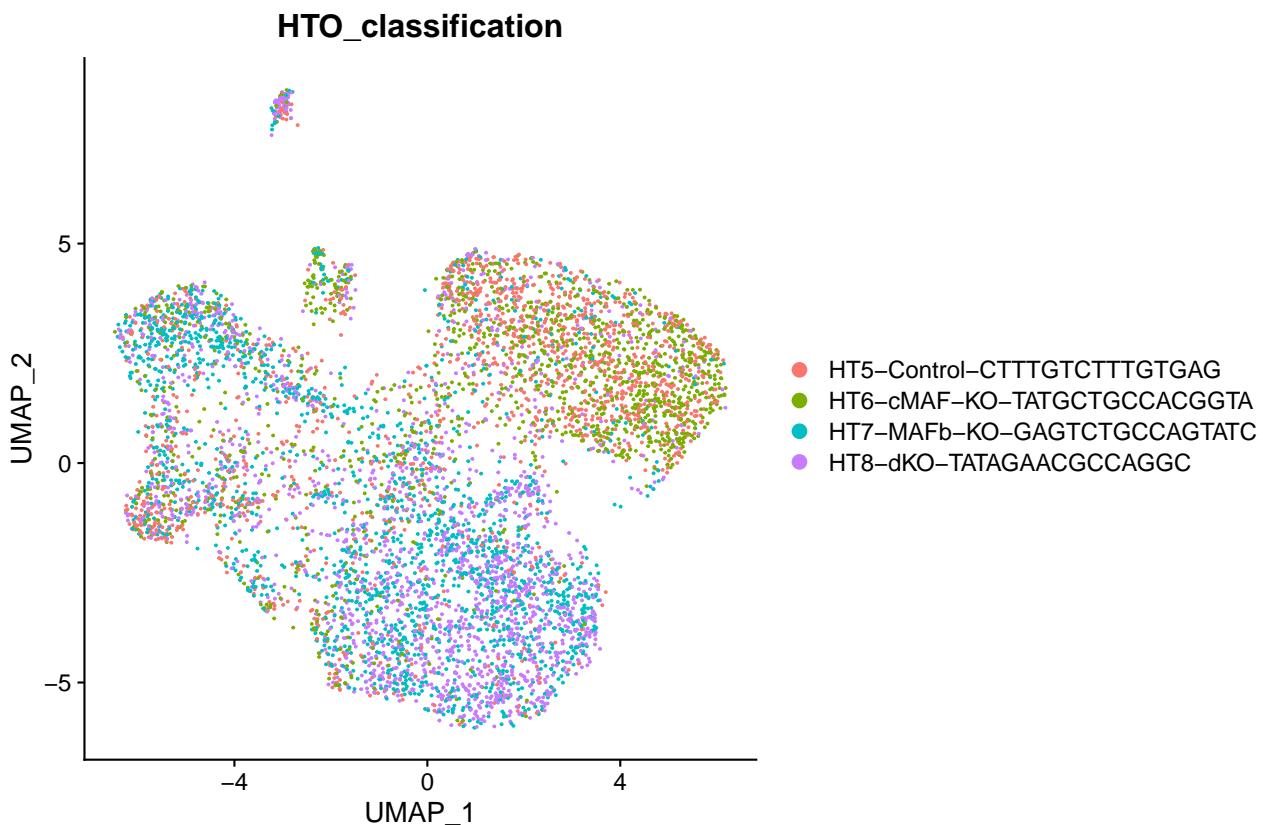
singlet <- RunTSNE(singlet, reduction = 'pca', dims = 1:10)  

singlet <- RunUMAP(singlet, reduction = 'pca', dims = 1:10)  

# Projecting singlet identities on TSNE visualization
DimPlot(singlet, group.by = "HTO_classification")

```



save to seurat object:

```
saveRDS(singlet, file = "./demultiplexed.seuratObject.rds")
```

3 scRNAseq initiation - QC

Given the low apoptotic rate in the sample, we consider cells with >10% mt genes as apoptotic cells and filter them out from the further analyses.

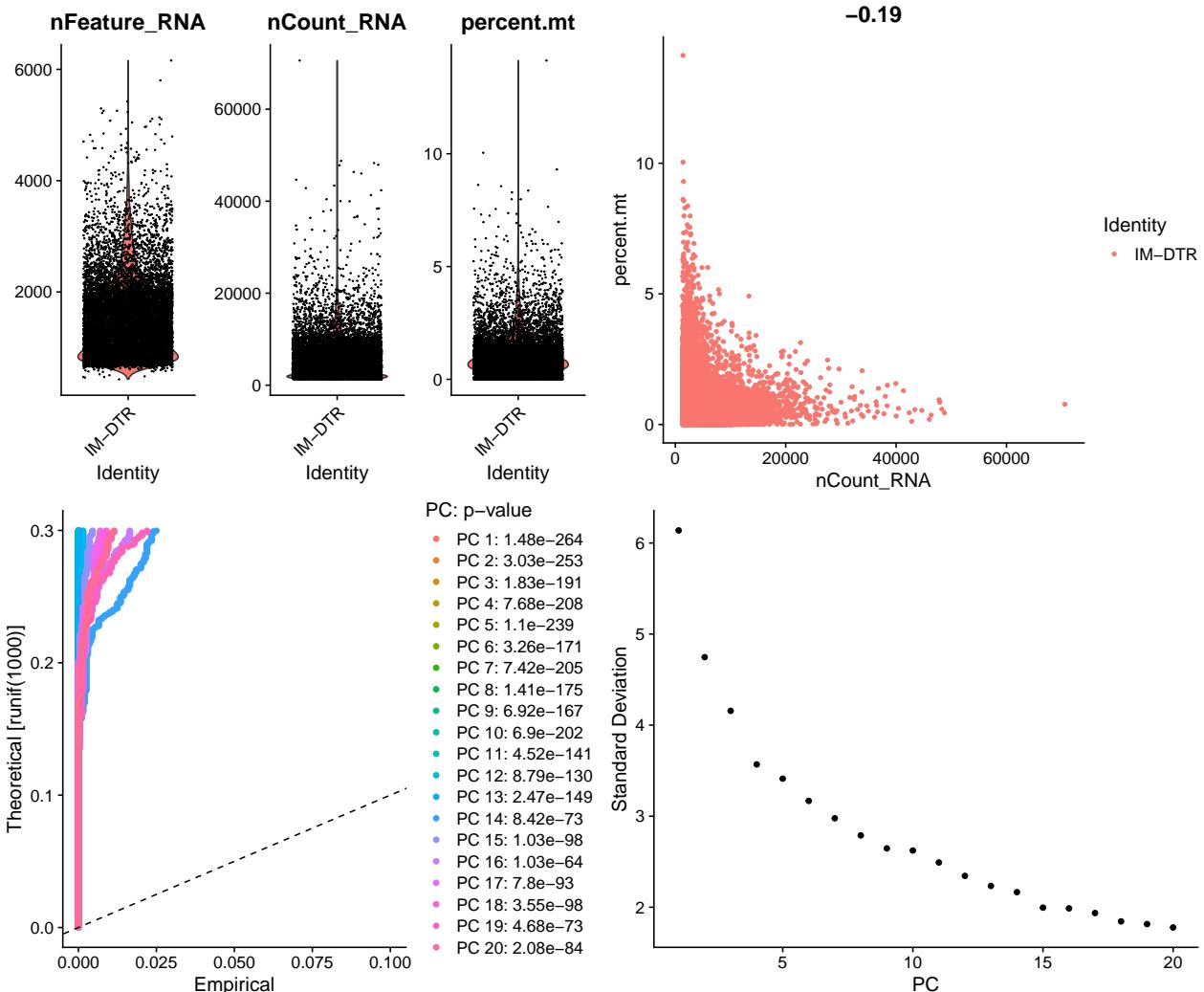
```
# load package and data  
library(Seurat)  
source("../R/seurat.setup.R")  
mt.percentage <- 10  
dimensionality <- 1:20
```

3.1 QC

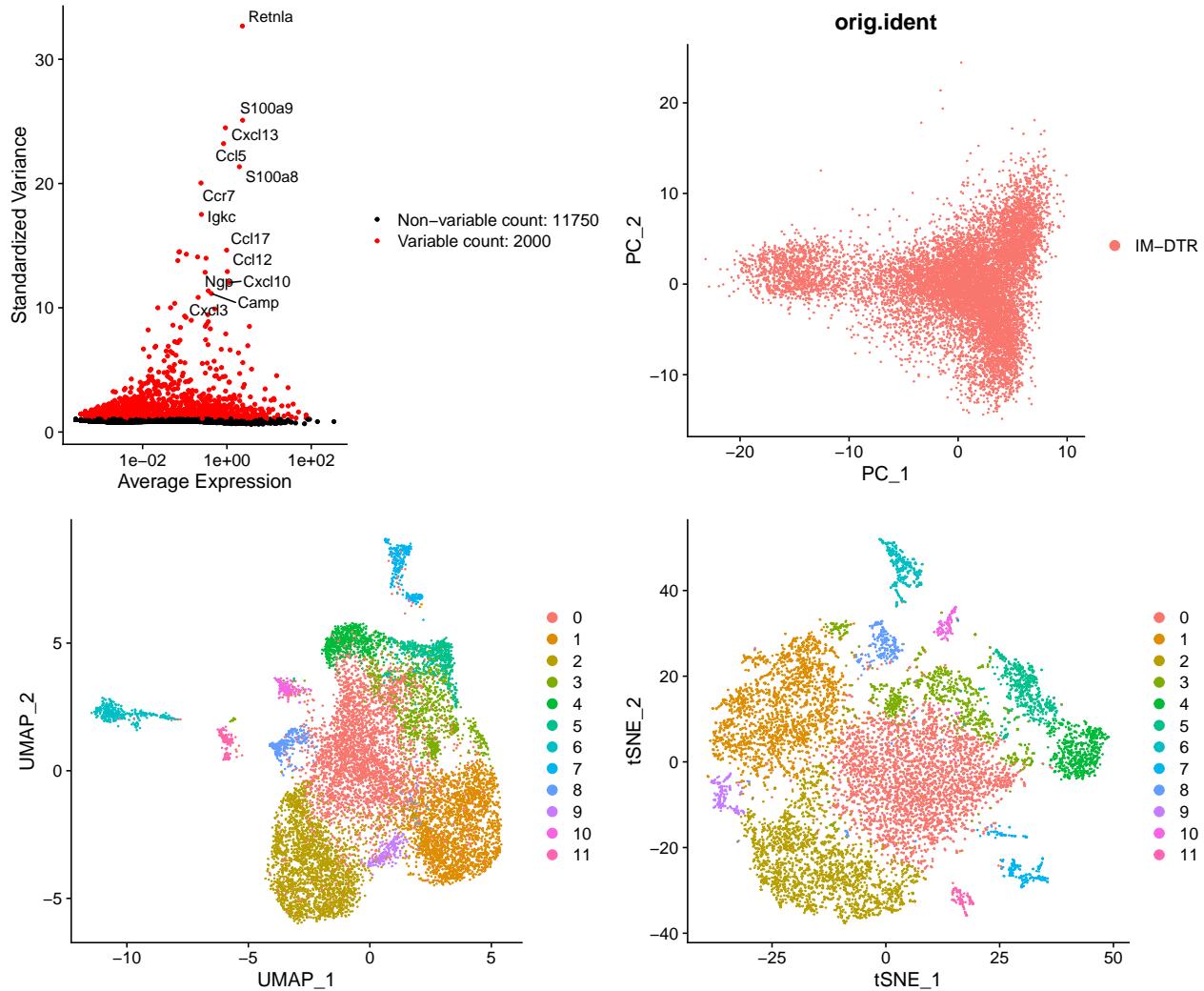
```
IM_Maf <- seurat.setup(path.10x = "/mnt/Data/Single-cell_Analysis/Projects  
/IPL/IM_DTR/IM-DTR_MAF/counts/scRNAseq/Experiment-7-12-21-ScRNA_NGS21-  
U976/outs/filtered_feature_bc_matrix/", project = "IM-DTR",  
dimensionality = dimensionality, mt.percentage = mt.percentage, human =  
FALSE)
```

```
## Modularity Optimizer version 1.3.0 by Ludo Waltman and Nees Jan van Eck  
##  
## Number of nodes: 11452  
## Number of edges: 367228  
##  
## Running Louvain algorithm...  
## Maximum modularity in 10 random starts: 0.8751  
## Number of communities: 12  
## Elapsed time: 1 seconds
```

```
ggarrange(IM_Maf$plots$feature_vln, IM_Maf$plots$RNA_mt.pct.scatter, IM_  
Maf$plots$JackStrawPlot, IM_Maf$plots$ElbowPlot, ncol = 2, nrow = 2)
```



```
ggarrange(IM_Maf$plots$variable_features, IM_Maf$plots$PCA_plot, IM_Maf$plots$UMAP_plot, IM_Maf$plots$TSNE_plot, ncol = 2, nrow = 2) 1
```



4 Annotate cells by hashtags

4.1 Load Chromium/HTO data

```
IM_Maf.seuratObject <- IM_Maf$seuratObject
demultiplexed.seuratObject <- singlet
```

1
2

Assign HTO annotation to cells

```
common <- intersect(colnames(IM_Maf.seuratObject), colnames(demultiplexed.
    seuratObject))

IM_Maf.seuratObject <- subset(IM_Maf.seuratObject, cells = common)
```

1
2
3
4

```
# make intersect between hto and rna cells:
hto <- demultiplexed.seuratObject@meta.data$HTO_classification
names(hto) <- colnames(demultiplexed.seuratObject)

# remove the sequence chars
hto <- sub(hto, pattern = "[C,T,G,A]{0,15}$", replacement = "")
```

1
2
3
4
5
6

```

# assign to seurat object:
IM_Maf.seuratObject$group <- hto[rownames(IM_Maf.seuratObject)]

```

7
8
9

4.2 Make metadata for samples

```

# cell type
IM_Maf.seuratObject$cell.type0 <- "CD45+"

```

1
2

Save individual samples for other use

```

obj <- IM_Maf.seuratObject
for (i in unique(obj$group)) {
  obj.sub <- subset(obj, subset = group == i)
  file.name <- paste("IM_mono", i, "seuratObject.rds", sep = ".")
  saveRDS(object = obj.sub, file = file.path(".", file.name))
}

```

1
2
3
4
5
6

4.3 Data processing and cell clustering

```

IM_Maf.seuratObject <- NormalizeData(IM_Maf.seuratObject)
IM_Maf.seuratObject <- FindVariableFeatures(IM_Maf.seuratObject, selection
  .method = "vst", nfeatures = 2000)
IM_Maf.seuratObject <- ScaleData(IM_Maf.seuratObject, features = rownames(
  IM_Maf.seuratObject))

```

1
2
3

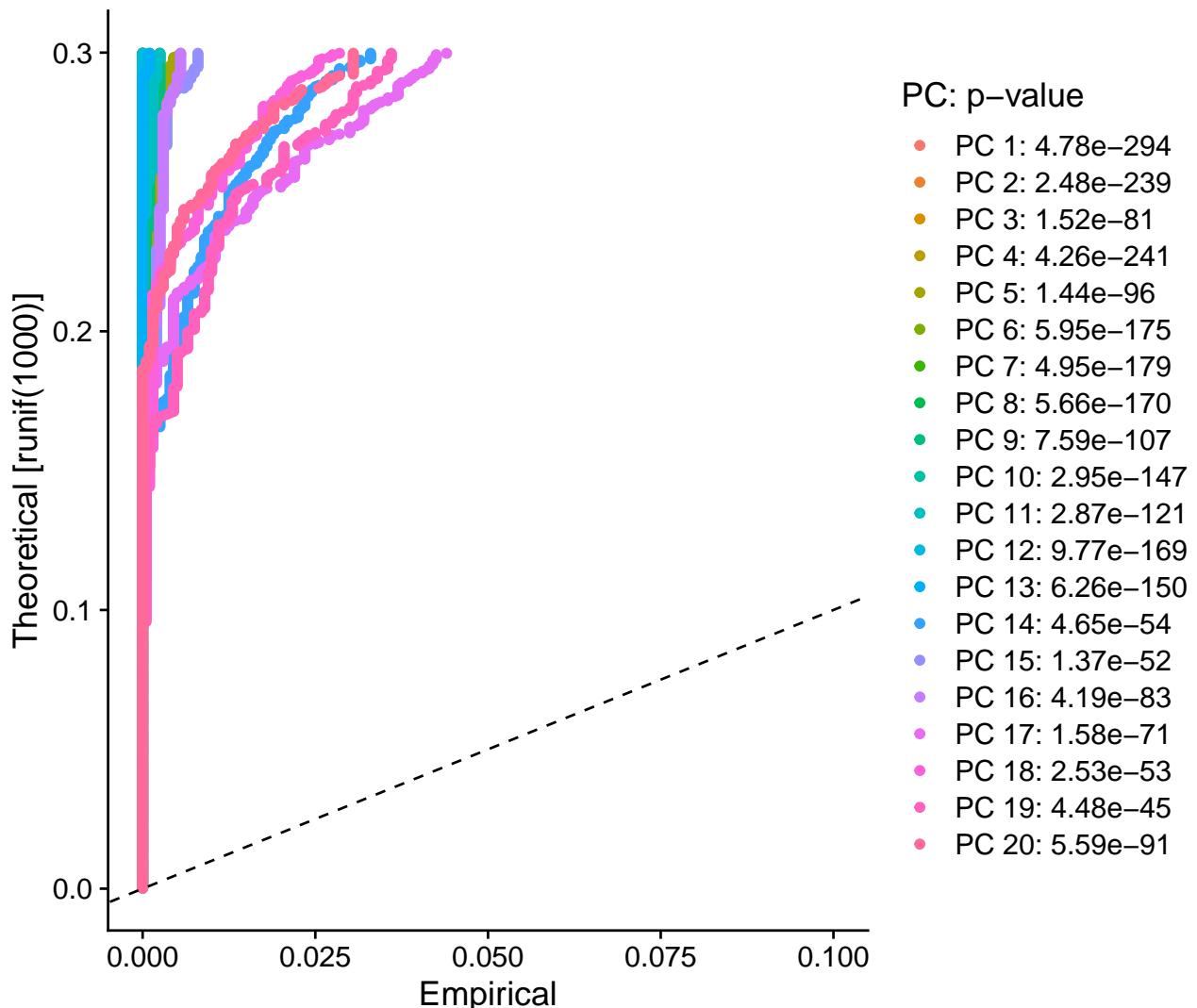
Linear dimension reduction:

```

IM_Maf.seuratObject <- RunPCA(IM_Maf.seuratObject,
  features = VariableFeatures(
    object = IM_Maf.seuratObject
  ))
IM_Maf.seuratObject <- JackStraw(IM_Maf.seuratObject, num.replicate = 100)
IM_Maf.seuratObject <- ScoreJackStraw(IM_Maf.seuratObject, dims = 1:20)
JackStrawPlot(IM_Maf.seuratObject, dims = 1:20)

```

1
2
3
4
5

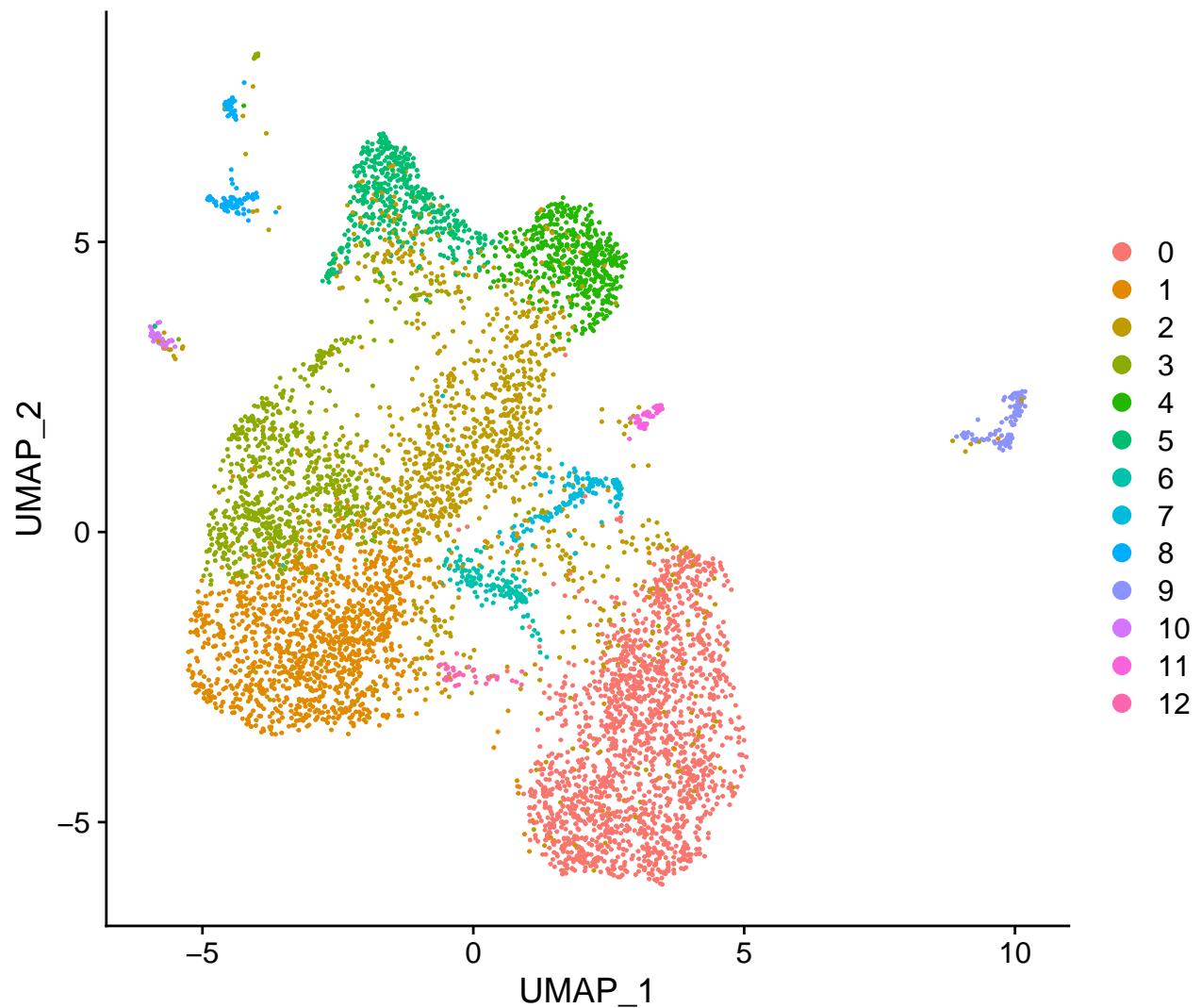


```
IM_Maf.seuratObject <- FindNeighbors(IM_Maf.seuratObject, dims = 1:30) 1
IM_Maf.seuratObject <- FindClusters(IM_Maf.seuratObject, resolution = 0.5) 2
```

```
## Modularity Optimizer version 1.3.0 by Ludo Waltman and Nees Jan van Eck 1
## 2
## Number of nodes: 6652 3
## Number of edges: 247502 4
## 5
## Running Louvain algorithm... 6
## Maximum modularity in 10 random starts: 0.8791 7
## Number of communities: 13 8
## Elapsed time: 0 seconds 9
```

```
IM_Maf.seuratObject <- RunTSNE(IM_Maf.seuratObject, dims = 1:30) 1
IM_Maf.seuratObject <- RunUMAP(IM_Maf.seuratObject, dims = 1:30) 2
```

```
DimPlot(IM_Maf.seuratObject) 1
```



4.4 Celltyping

```

source("../R/seurat2singleR.R")
1
2
results.singleR <- seurat2singleR(IM_Maf.seuratObject, ref = "ImmGenData")
3
4
saveRDS(results.singleR, file = "./IM_Maf.ImmGenData.singleR.Rds")
5
6
saveRDS(IM_Maf.seuratObject, file = "./IM_Maf.seuratObject.rds")

```

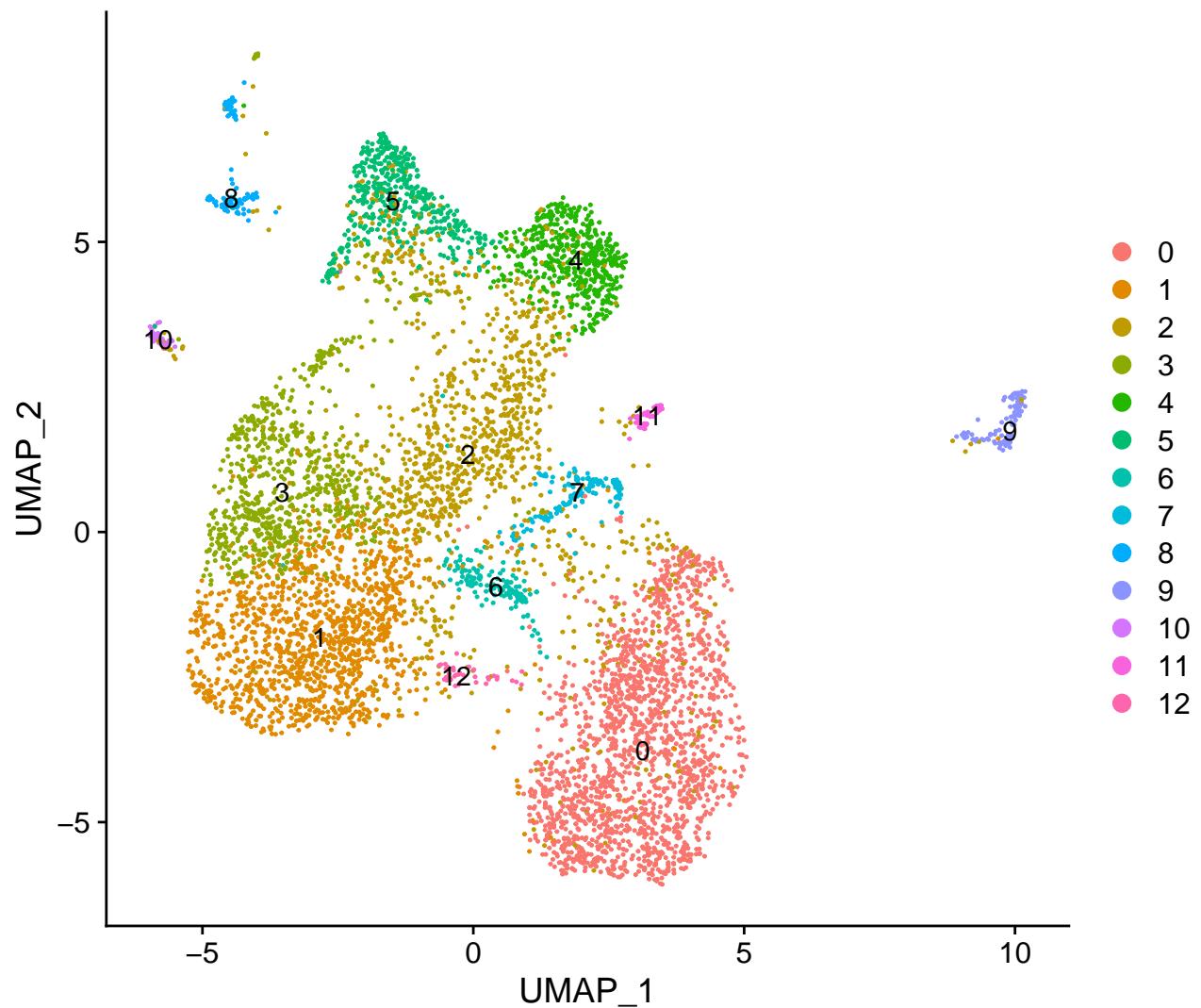
5 Remove contaminated cell types

5.1 Plot with key markers

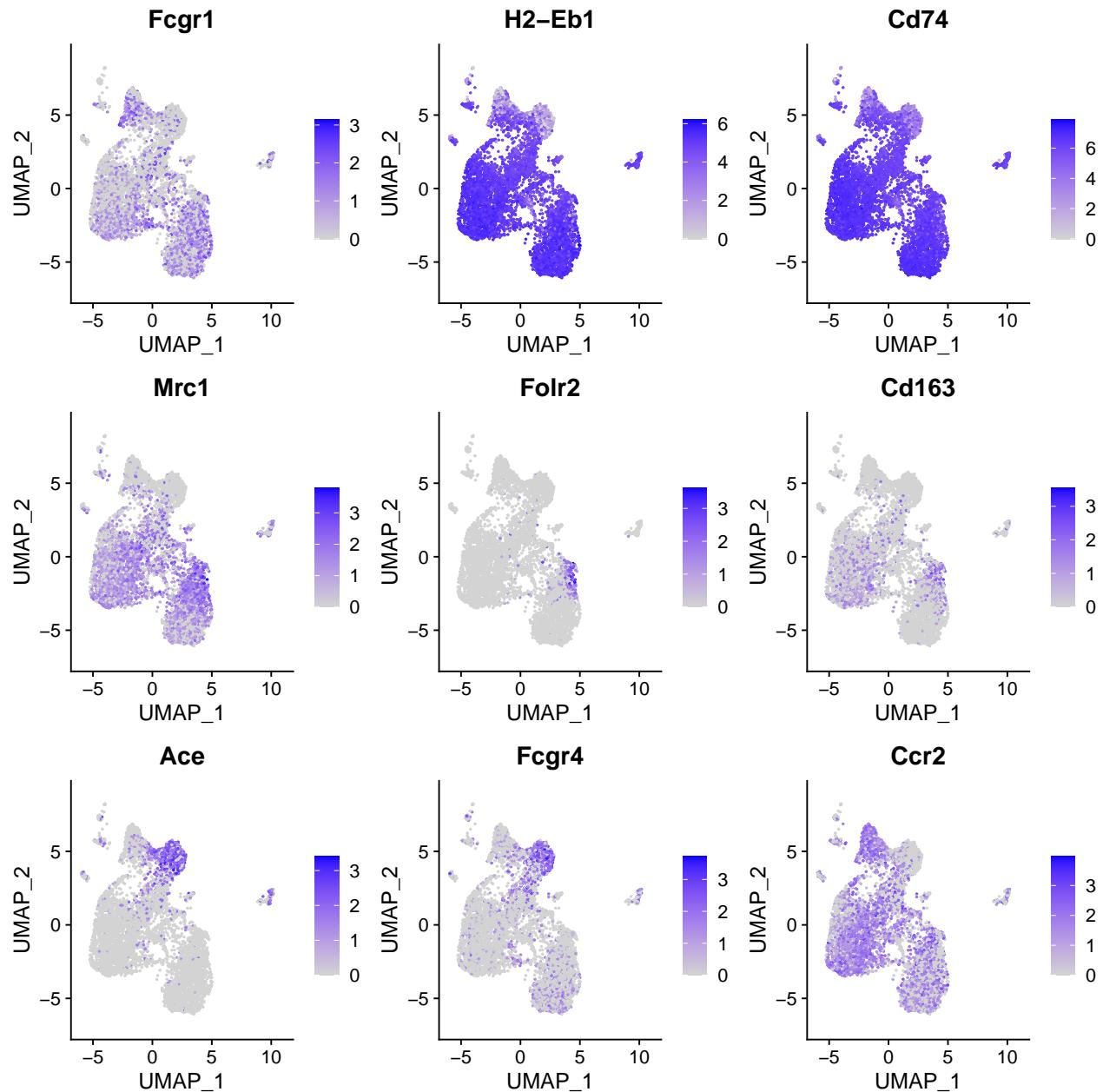
```

library(ggplot2)
1
library(dplyr)
2
results <- IM_Maf.seuratObject
3
DimPlot(results, reduction = "umap", label = TRUE)
4

```



```
DefaultAssay(results) <- "RNA"  
FeaturePlot(results, features = c("Fcgr1", "H2-Eb1", "Cd74", "Mrc1", "  
Folr2", "Cd163", "Ace", "Fcgr4", "Ccr2"), reduction = "umap")
```



5.2 Use results of SingleR celltyping to annotate cells

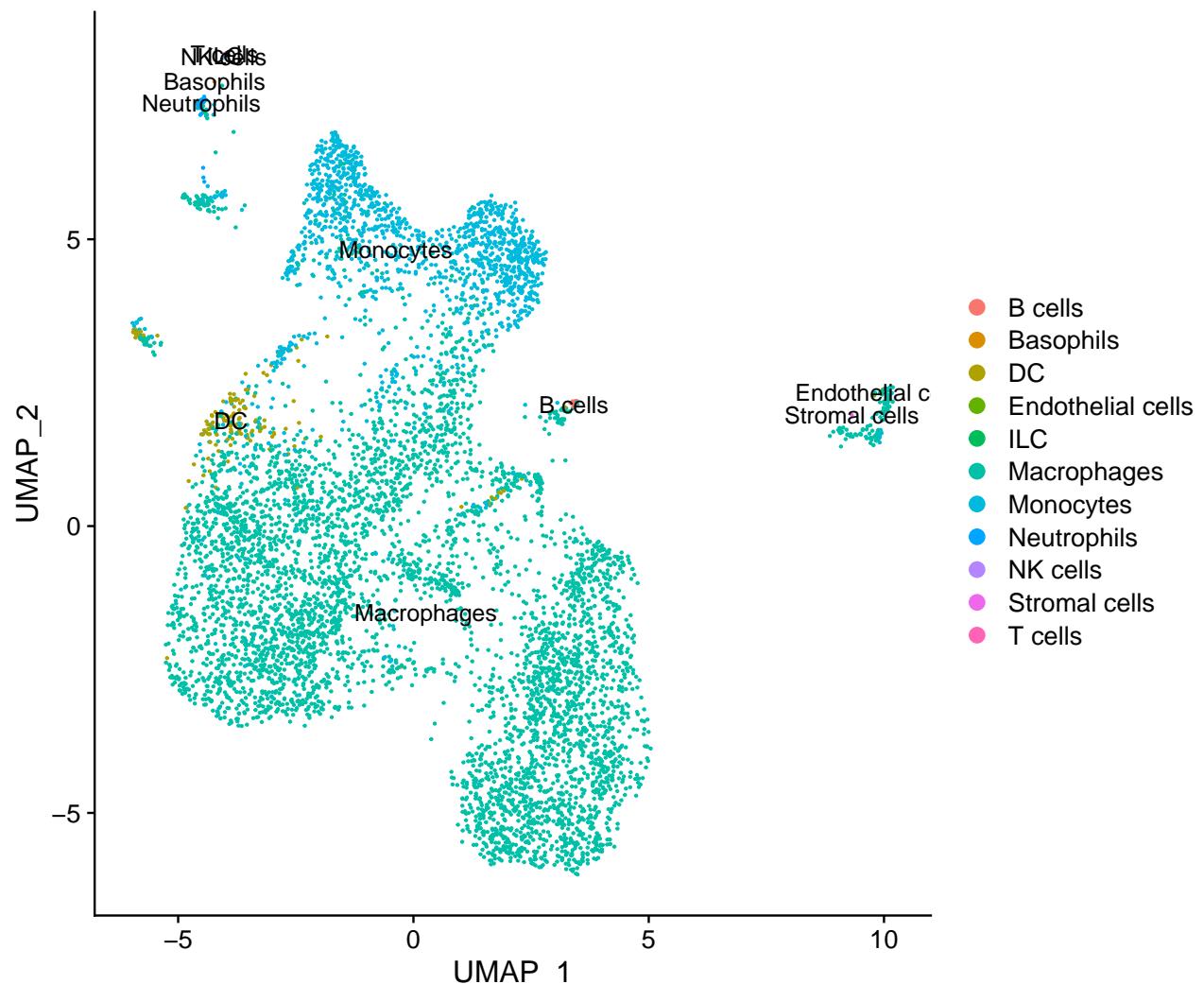
Check if SingleR results contain the same cells:

```
identical(colnames(results), rownames(results.singleR)) 1
## [1] TRUE 1
```

UMAP plot show cell types:

```
results$singleR.celltype <- results.singleR$labels 1
DimPlot(results, group.by = "singleR.celltype", reduction = "umap", label 2
= T) + ggtitle("DatabaseImmuneCellExpressionData - SingleR Identity") 3
```

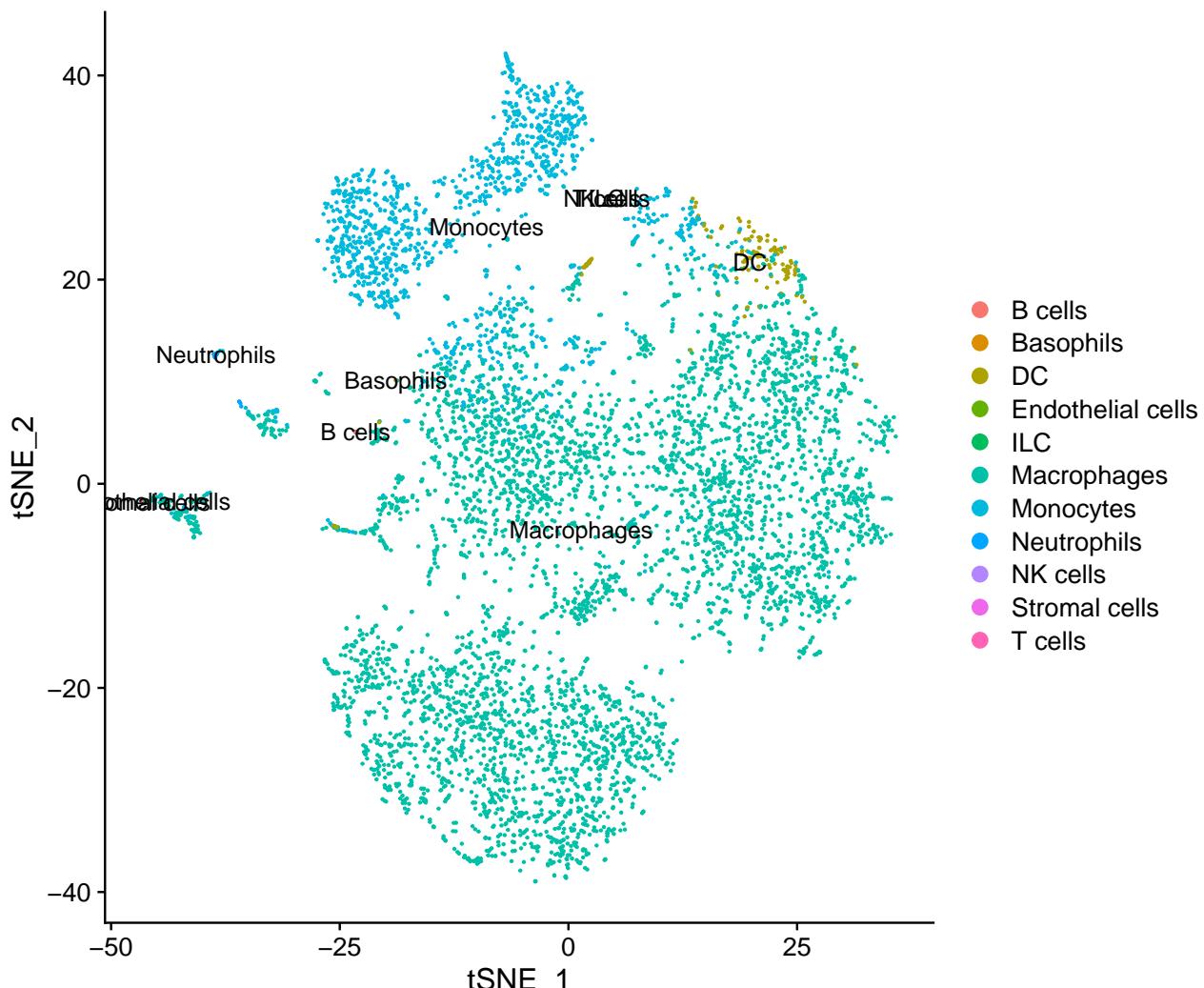
DatabaseImmuneCellExpressionData – SingleR identity



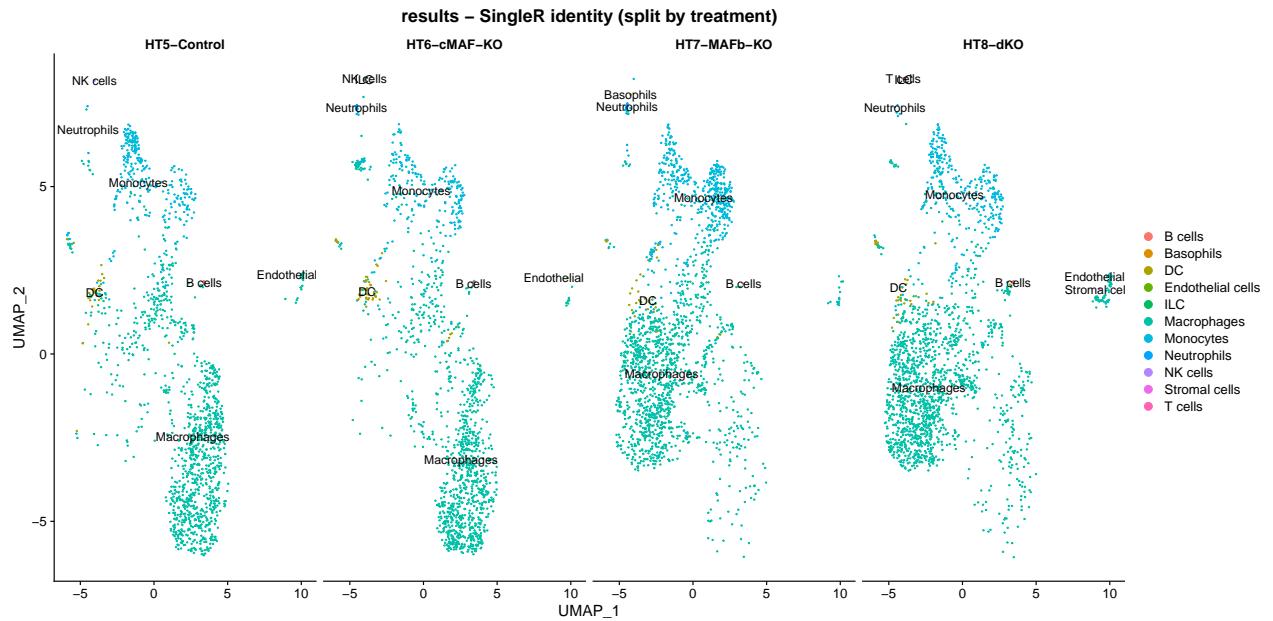
TSNE plot show cell types:

```
DimPlot(results, group.by = "singleR.celltype", reduction = "tsne", label  
= T) + ggtitle("DatabaseImmuneCellExpressionData - SingleR identity") 1
```

DatabaseImmuneCellExpressionData – SingleR identity



```
DimPlot(results, group.by = "singleR.celltype", reduction = "umap", label  
= T, split.by = "group") + ggtitle("results - SingleR identity (split  
by treatment)")
```



5.3 Remove contamination

Here's all the cell types and number:

```
table(results$singleR.celltype)
```

##							1
##	B cells	Basophils		DC	Endothelial cells		2
##	13	1		145		3	3
##	ILC	Macrophages		Monocytes	Neutrophils		4
##	2	5049		1403		30	5
##	NK cells	Stromal cells		T cells		6	
##	4	1		1			7

Remove all the other cell types and keep only monocytes and macrophages.

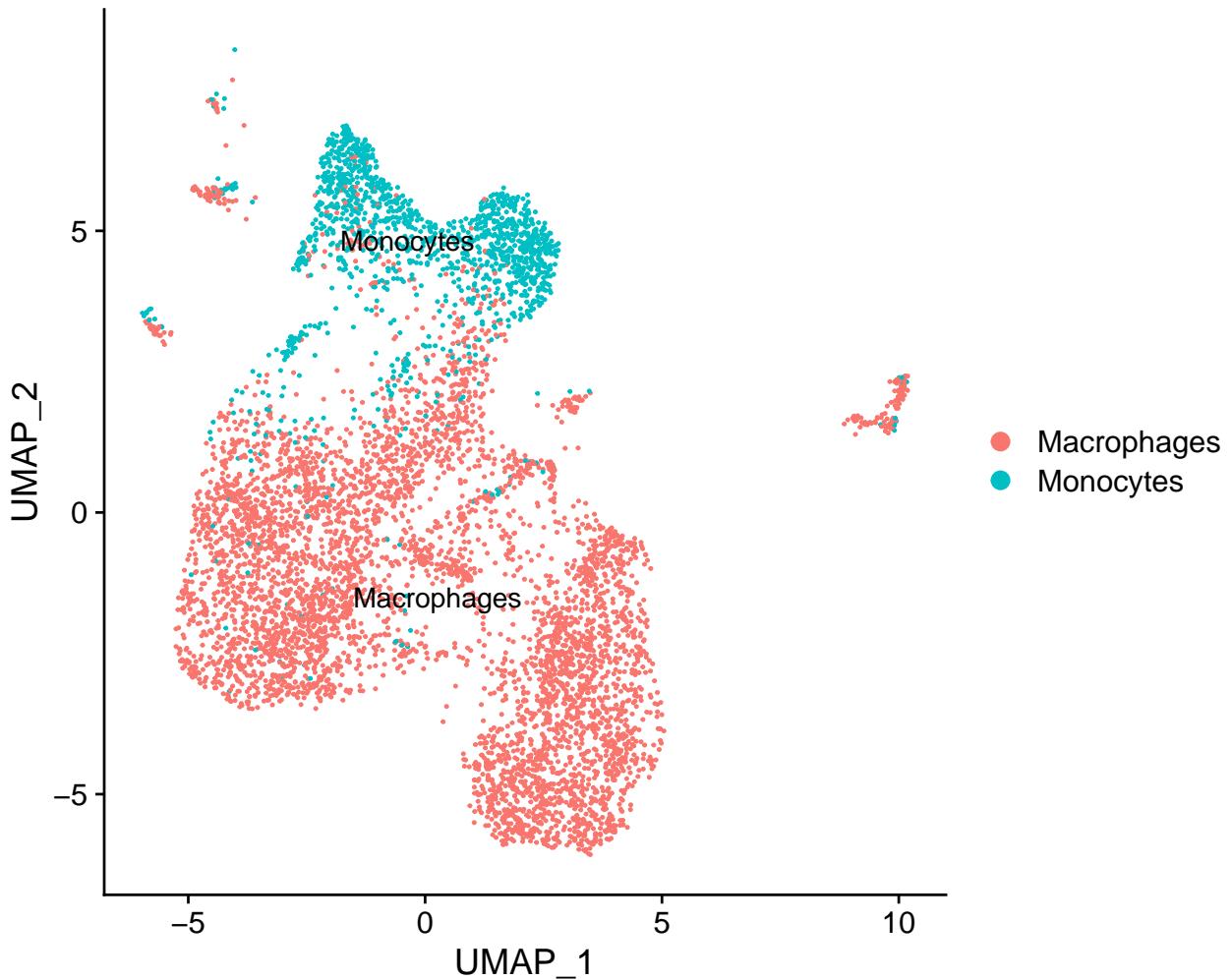
```
results <- subset(results, subset = singleR.celltype %in% c("B_cells", "Basophils", "Endothelial_cells", "Stromal_cells", "Neutrophils", "NK_cells", "DC", "ILC", "T_cells"), invert = TRUE) # DC removed!!!
```

```
table(results$group)
```

##	HT5-Control	HT6-cMAF-KO	HT7-MAFb-KO	HT8-dKO
##	1429	1340	1815	1868

```
DimPlot(results, group_by = "singleR.celltype", reduction = "umap", label = T) + ggtitle("DatabaseImmuneCellExpressionData-SingleR-identity")
```

DatabaselImmuneCellExpressionData – SingleR identity



6 Reanalysis data after contamination removal

Remove old snn:

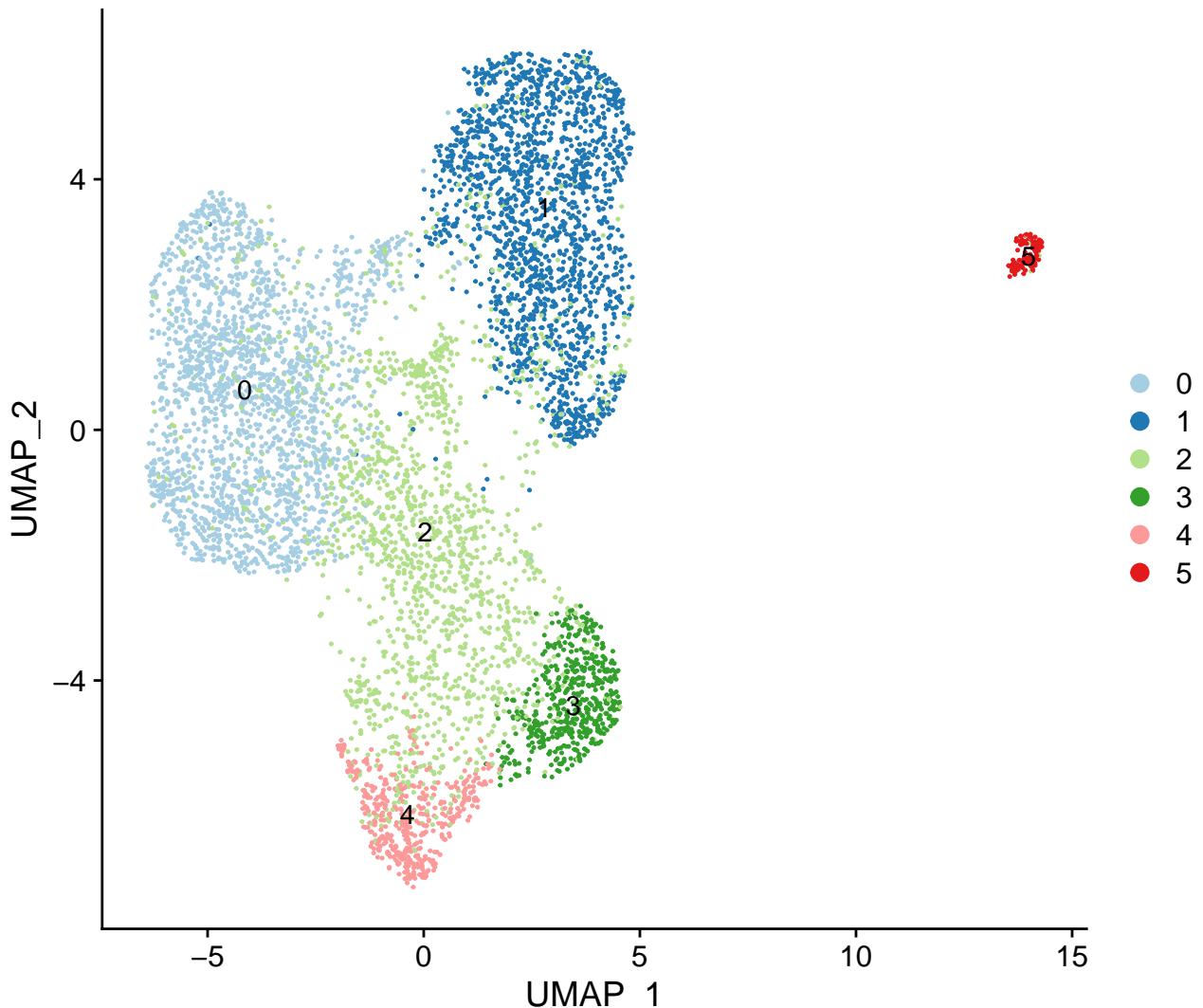
```
meta.names <- names(results@meta.data)
old.snn <- meta.names[startsWith(meta.names, "RNA_snn")]
for (i in old.snn) {
  results[[i]] <- NULL
}
```

```
# DefaultAssay(results) <- "RNA"
results <- NormalizeData(results, verbose=FALSE)
results <- FindVariableFeatures(results, selection.method = "vst",
  nfeatures = 2000, verbose=FALSE)
results <- ScaleData(results, features = rownames(results), verbose=FALSE)
results <- RunPCA(results, features = VariableFeatures(results), verbose=
  FALSE)
results <- RunTSNE(results, dims = 1:10, verbose=FALSE)
results <- RunUMAP(results, dims = 1:10, verbose=FALSE)
```

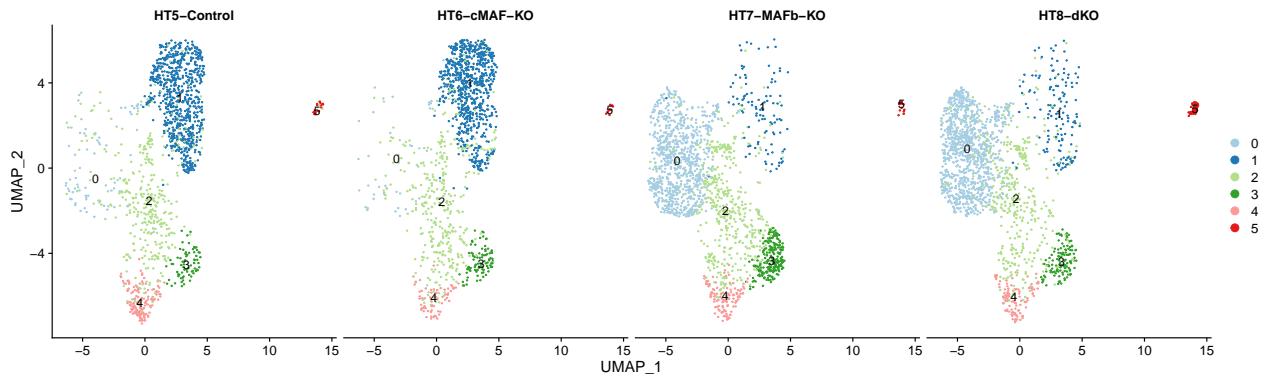
6.1 Cell clustering

```
results <- FindNeighbors(results, dims = 1:10, verbose = FALSE) 1  
results <- FindClusters(results, resolution = 0.12, verbose = FALSE) 2
```

```
DimPlot(results, label = TRUE, cols = "Paired", reduction = "umap") 1
```



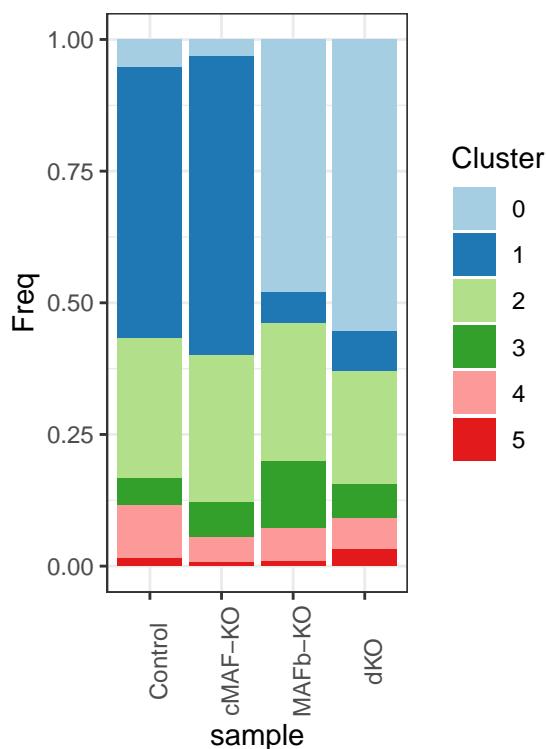
```
DimPlot(results, label = TRUE, split.by = "group", cols = "Paired") 1
```



```

source("../R/SeuratFreqTable.R")
freq.celltype.list <- list(
  `Control` = Seurat2CellFreqTable(subset(results, subset = group == "HT5-
    Control"), slotName = "RNA_snn_res.0.12"),
  `cMAF-KO` = Seurat2CellFreqTable(subset(results, subset = group == "HT6-
    cMAF-KO"), slotName = "RNA_snn_res.0.12"),
  `MAFb-KO` = Seurat2CellFreqTable(subset(results, subset = group == "HT7-
    MAFb-KO"), slotName = "RNA_snn_res.0.12"),
  `dKO` = Seurat2CellFreqTable(subset(results, subset = group == "HT8-dKO"
    ), slotName = "RNA_snn_res.0.12")
)

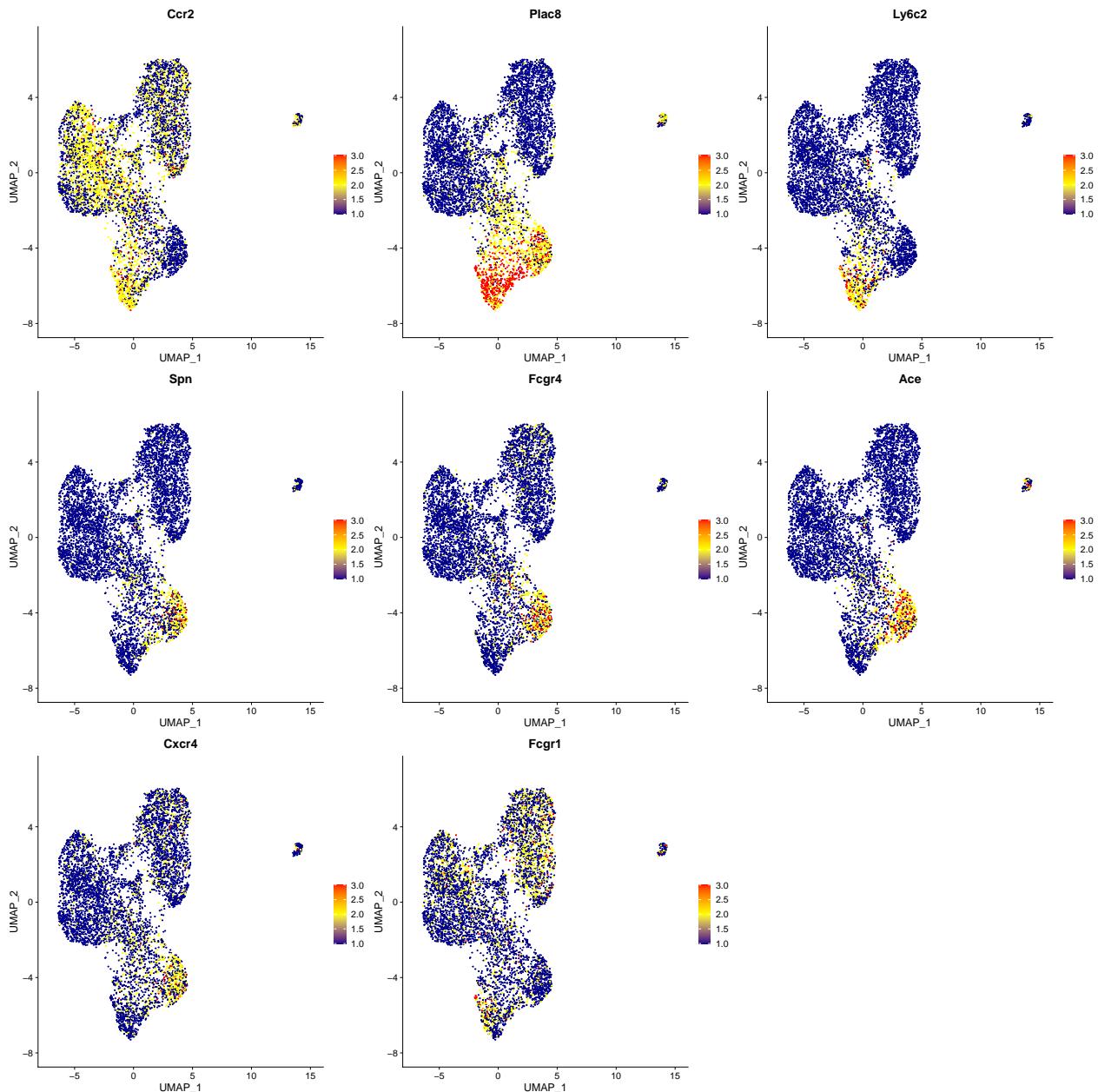
source("../R/barChart.R")
barChart(freq.celltype.list) + labs(fill = "Cluster") + scale_fill_manual(
  values = brewer.pal(6, "Paired")) + theme(axis.text.x = element_text(
  angle = 90))
  
```



6.2 population characterization

6.2.1 Show expression of important monocyte markers

```
FeaturePlot(results, features = c("Ccr2", "Plac8", "Ly6c2", "Spn",
                                 "Fcgr4", "Ace", "Cxcr4",
                                 "Fcgr1"),
            ncol = 3, reduction = "umap", cols = c("darkblue", "yellow", "red"))
```

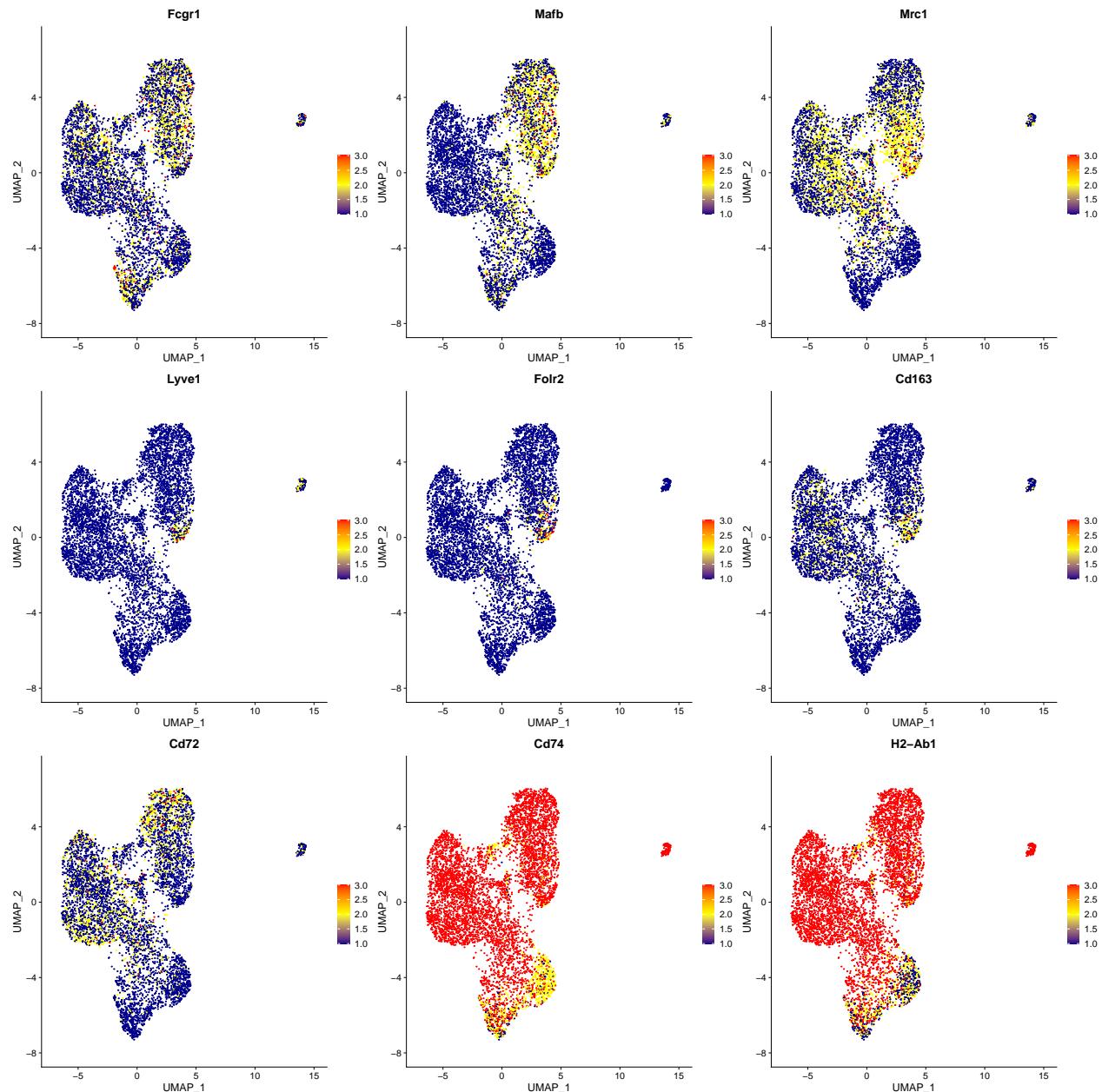


6.2.2 Show expression of important IM markers

```
FeaturePlot(results, features = c(
  "Fcgr1", "Mafb", "Mrc1", "Lyve1", "Folr2",
  "Cd163",
```

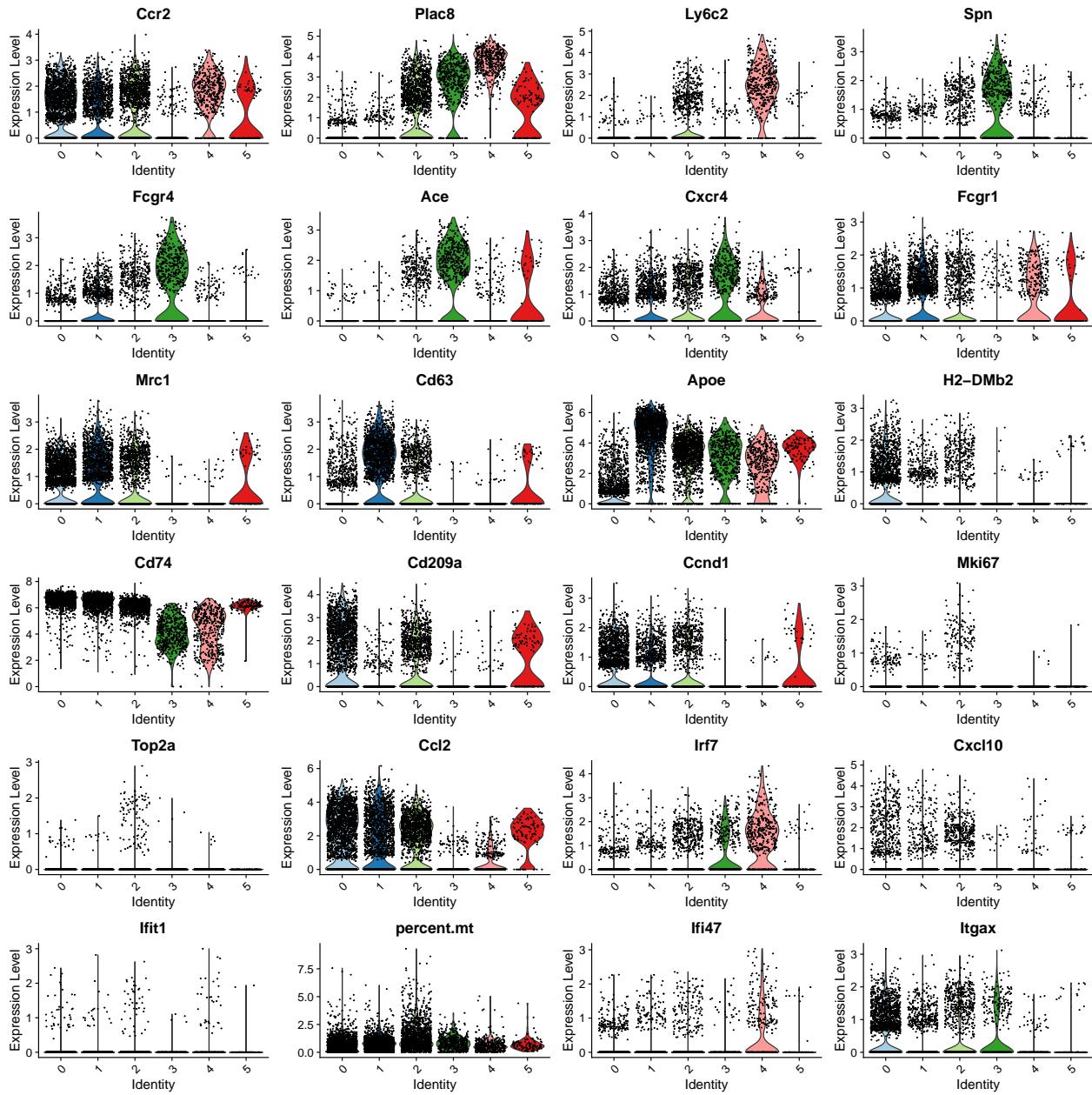
```
"Cd72", "Cd74", "H2-Ab1"),
ncol = 3, cols = c("darkblue", "yellow", "red"))
```

3
4



```
VlnPlot(results, features = c("Ccr2", "Plac8", "Ly6c2", "Spn",
"Fcgr4", "Ace", "Cxcr4",
"Fcgr1", "Mrc1", "Cd63", "Apoe",
"H2-DMb2", "Cd74", "Cd209a",
"Ccnd1", "Mki67", "Top2a", "Ccl2",
"Irf7", "Cxcl10", "Ifit1", "percent.mt",
"IFI47", "ITGAX"),
ncol = 4, cols = brewer.pal(6, "Paired"))
```

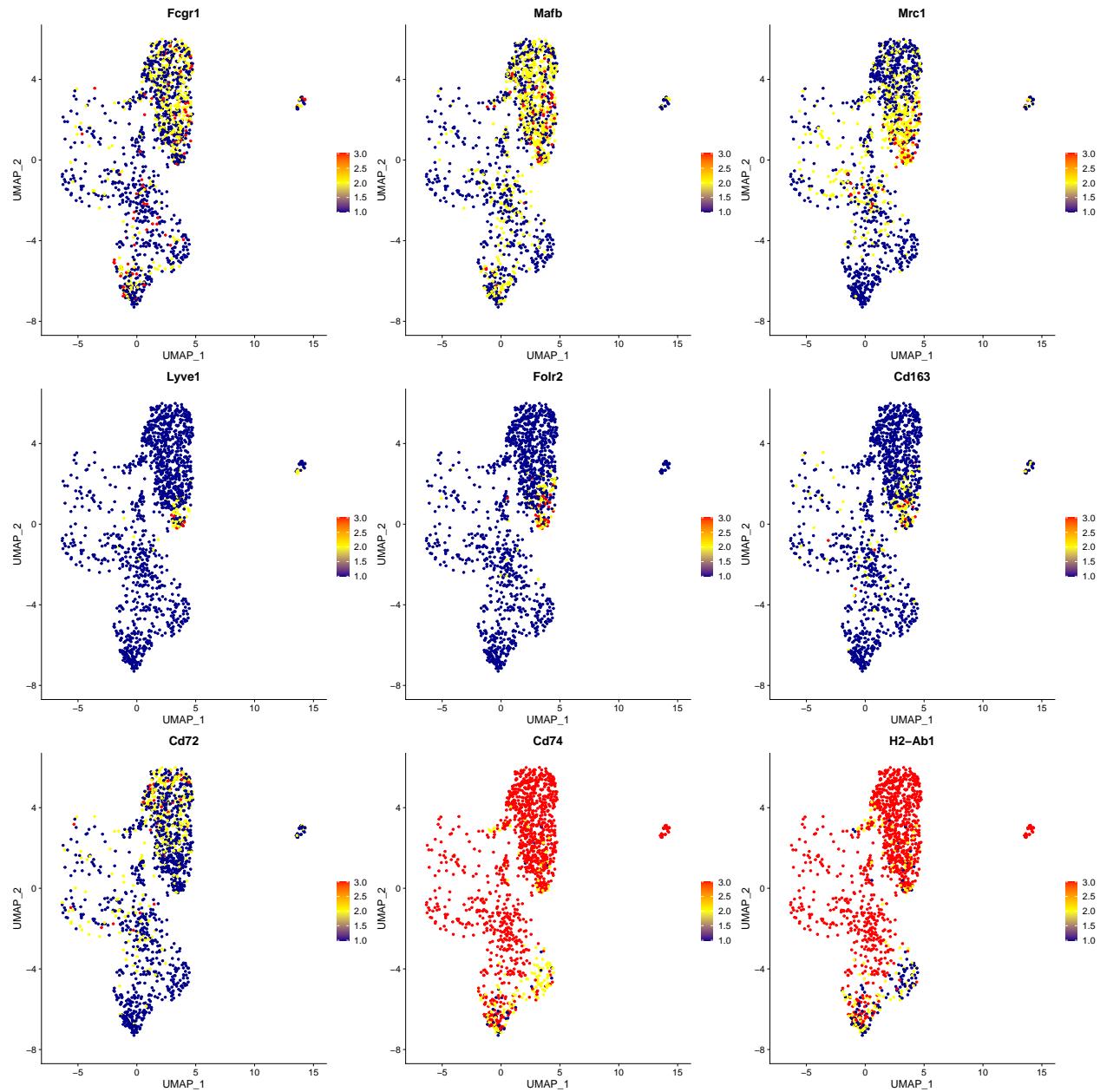
1
2
3
4
5
6
7



All the IMs are in cluster 1 while cluster 0 only presents Mafb- cells. To confirm we try to identify the populations only in control sample.

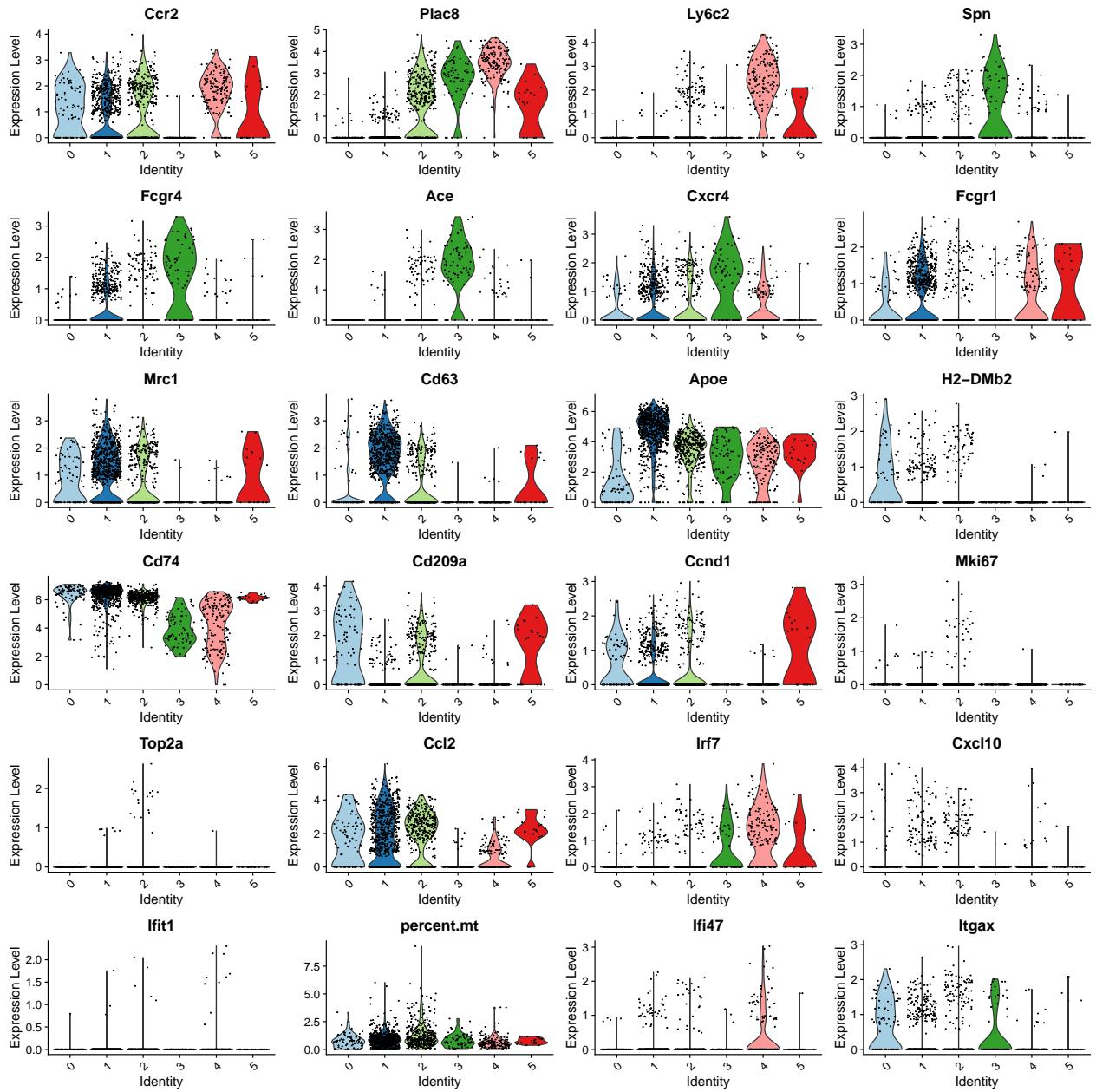
6.2.3 Show expression of important IM markers in Control sample

```
FeaturePlot(
  subset(results, subset = group == "HT5-Control"),
  features = c("Fcgr1", "Mafb", "Mrc1", "Lyve1", "Folr2", "Cd163",
  "",
  "Cd72", "Cd74", "H2-Ab1"),
  ncol = 3, cols = c("darkblue", "yellow", "red"))
  1
  2
  3
  4
  5
```



```
VlnPlot(
  subset(results, subset = group == "HT5-Control"),
  features = c("Ccr2", "Plac8", "Ly6c2", "Spn",
              "Fcgr4", "Ace", "Cxcr4",
              "Fcgr1", "Mrc1", "Cd63", "Apoe",
              "H2-DMb2", "Cd74", "Cd209a",
              "Ccnd1", "Mki67", "Top2a", "Ccl2",
              "Irf7", "Cxcl10", "Ifit1", "percent.mt",
              "Ifi47", "Itgax") ,
  ncol = 4, cols = brewer.pal(6, "Paired"))

```



6.2.4 Subset characterization

cluster 0: Mafb- trapped cluster 1: All IMs (both CD206+ and CD206- IM) cluster 2: Intermediate cluster 3: Patrolling Mono cluster 4: Classical Mono cluster 5: unknown

6.3 Clustering and Annotate CD206+ and CD206- IMs

As Mafb-deficiency introduced a bigger variance which made CD206+/CD206- IMs unable to be clustered separately. We will isolate cluster 1 (contains all IMs) and redo cluster.

```
ims <- subset(results, idents = "1")
```

1

Normalize and find variable genes

```
ims <- NormalizeData(ims, verbose=FALSE)
```

1

```

1 ims <- FindVariableFeatures(ims, selection.method = "vst", nfeatures =
2   2000, verbose=FALSE)
3 ims <- ScaleData(ims, features = rownames(ims), verbose=FALSE)
4 ims <- RunPCA(ims, features = VariableFeatures(ims), verbose=FALSE)
5 ims <- RunTSNE(ims, dims = 1:5, verbose=FALSE)
6 ims <- RunUMAP(ims, dims = 1:5, verbose=FALSE)

```

Cell clustering within cluster 1

```

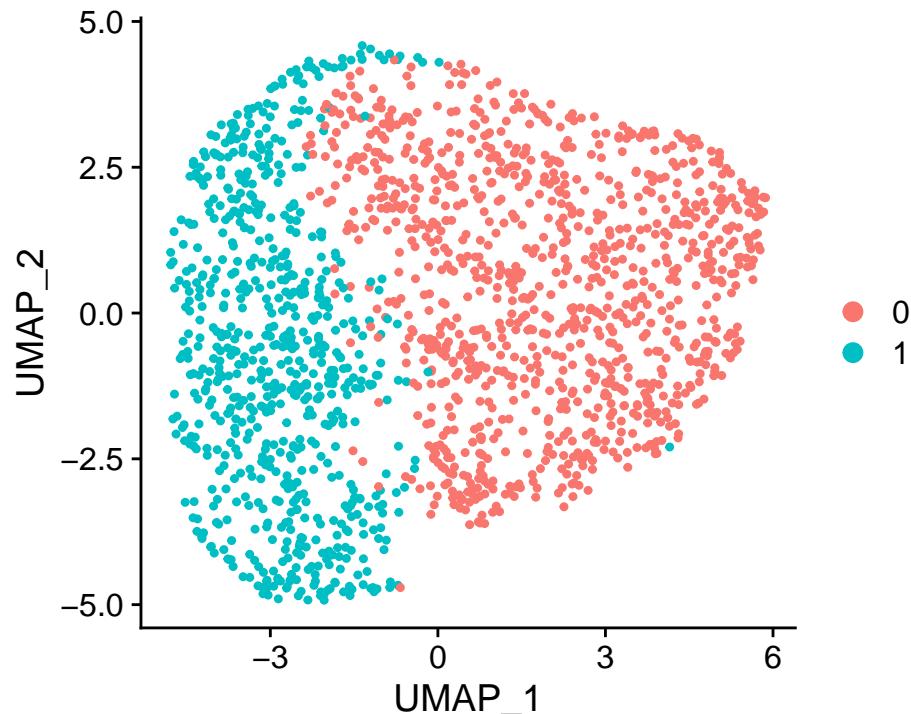
1 ims <- FindNeighbors(ims, dims = 1:5, verbose = FALSE)
2 ims <- FindClusters(ims, resolution = 0.2, verbose = FALSE)

```

Show CD206+ and CD206- IMs:

Clusters:

```
DimPlot(ims)
```

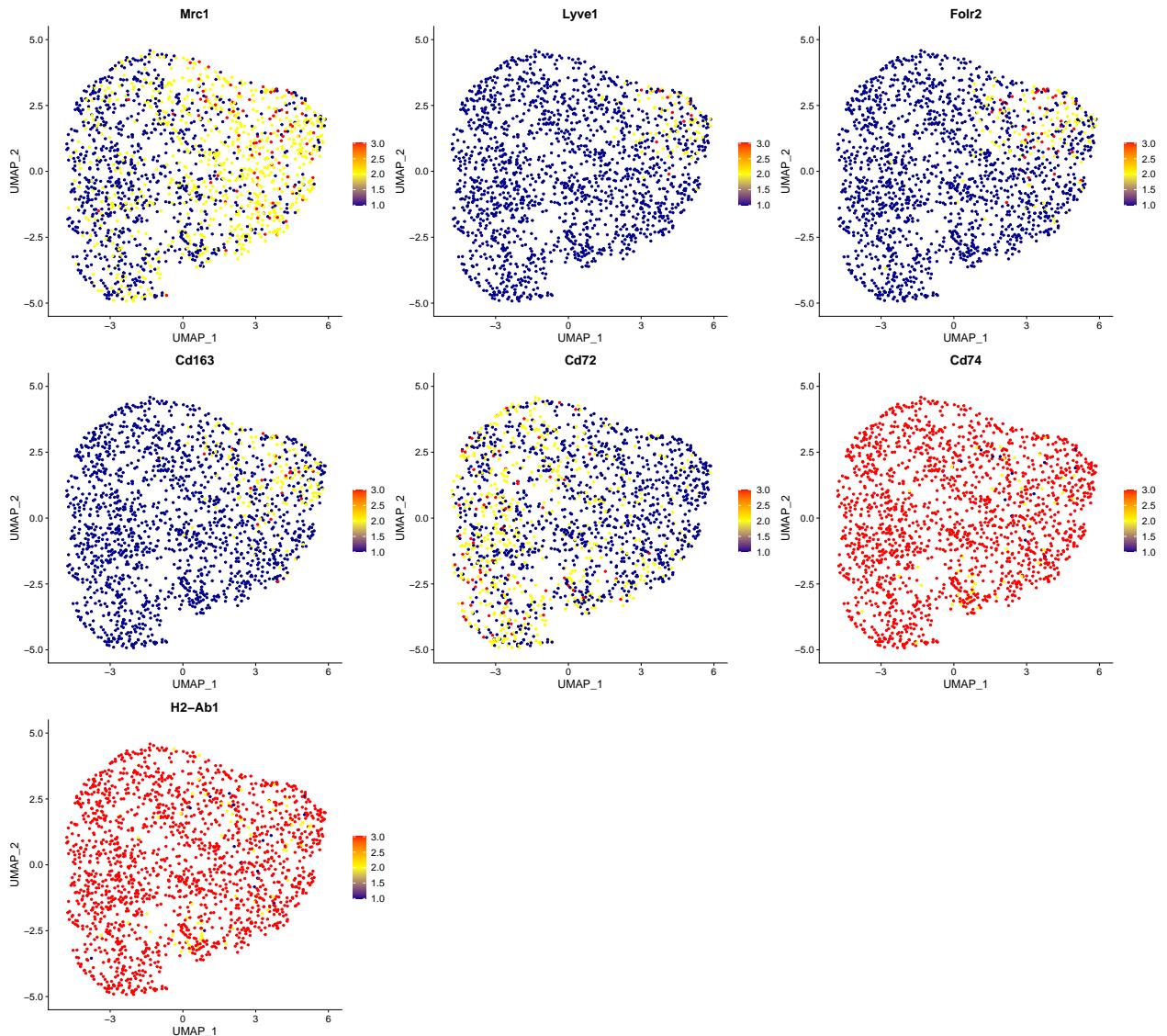


Show marker expression:

```

1 FeaturePlot(ims,
2   features = c("Mrc1", "Lyve1", "Folr2", "Cd163",
3     "Cd72", "Cd74", "H2-Ab1") ,
4   ncol = 3, cols = c("darkblue", "yellow", "red"))

```



Within the cluster1: subcluster 0: CD206+ IMs subcluster 1: CD206- (MHCII hi) IMs

6.4 Annotate with cell types

```
results$cell.type2 <- factor(Idents(results), labels = c("Mafb-deficient",  
"IM", "Intermediate", "Patrolling_Mono", "Classical_Mono", "Unknown"))  
  
results$cell.type2 <- as.character(results$cell.type2)
```

Overide IM cluster with subcluster annotations in new cell.type3 annotation.

```
results$cell.type3 <- results$cell.type2  
  
# override  
results$cell.type3[colnames(ims)] <- as.character(factor(Idents(ims),  
labels = c("CD206+_IMs", "CD206-_IMs")))  
  
# Now make annotation into proper factors:
```

```

results$cell.type2 <- as.factor(results$cell.type2)                                7
results$cell.type3 <- factor(results$cell.type3, levels = c("Classical"           8
    "Mono",
                               "Patrolling"           9
    "Mono",
                               "Intermediate"        10
                               ,
                               "CD206- IMs"          11
                               "CD206+ IMs"          12
                               "Mafb-               13
                               deficient",
                               "Unknown"))            14
                                         15
Idents(results) <- "cell.type3"                                              16
levels(results)                                                               17

```

```

## [1] "Classical Mono"   "Patrolling Mono"  "Intermediate"      "CD206- IMs"  1
## [5] "CD206+ IMs"       "Mafb-deficient" "Unknown"          2

```

6.5 Make plots with annotated cells

Make color palette for plot (3 new colors were Paired number 8 and 9 from RColorBrewer, and light grey for unknown population):

```

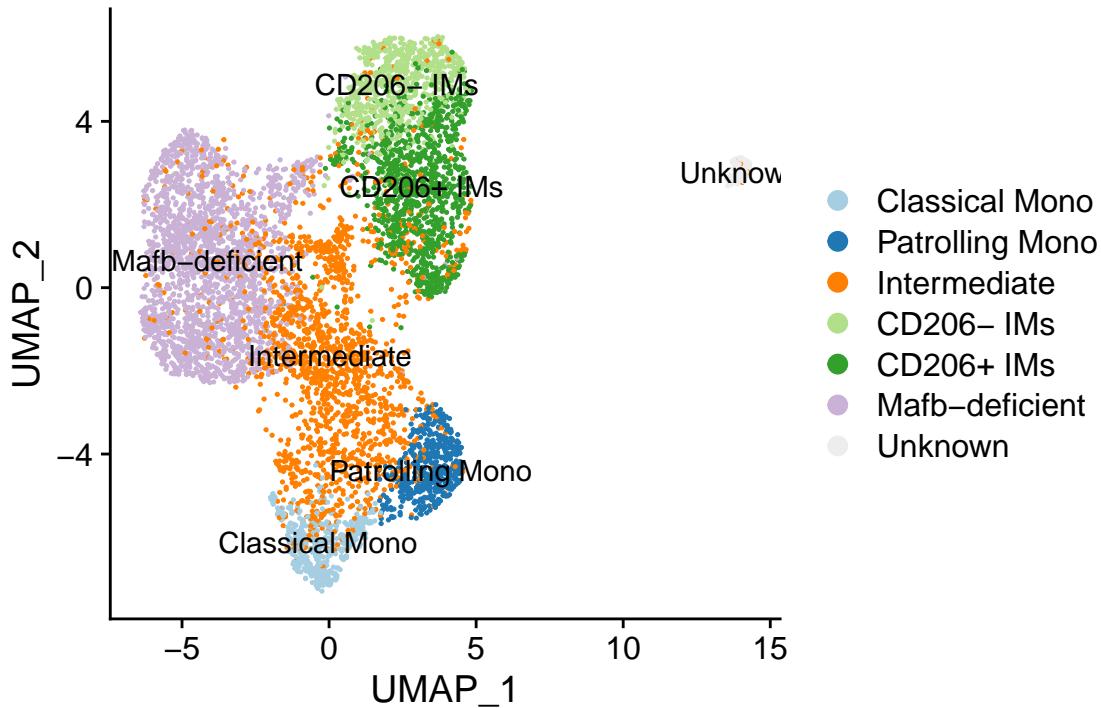
pal3 <- c(
  "#A6CEE3", # cMo
  "#1F78B4", # pMo
  "#FF7F00", # Intermediate
  "#B2DF8A", # MHCII IM
  "#33A02C", # CD206 IM
  "#CAB2D6", # Mafb- neo
  "#eddede" # Unknown
)

```

```

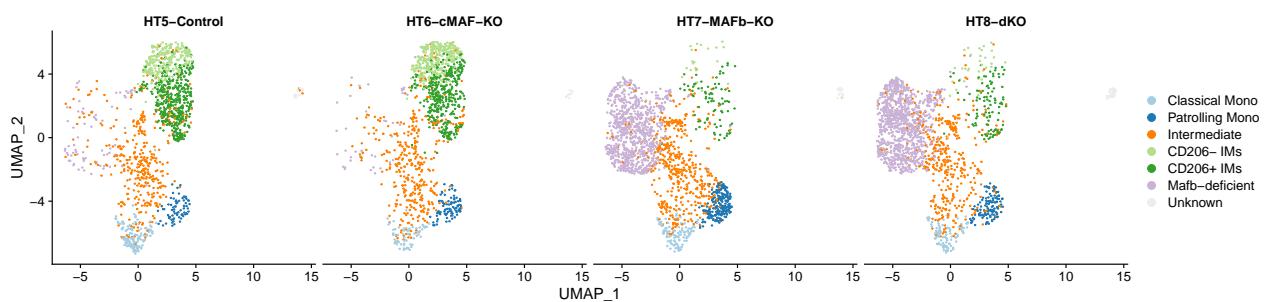
DimPlot(results, label = TRUE, cols = pal3)

```



```
ggsave(filename = ".../Figures/UMAPplot_All_samplesMaf_label.pdf", width = 1  
       6, height = 4)
```

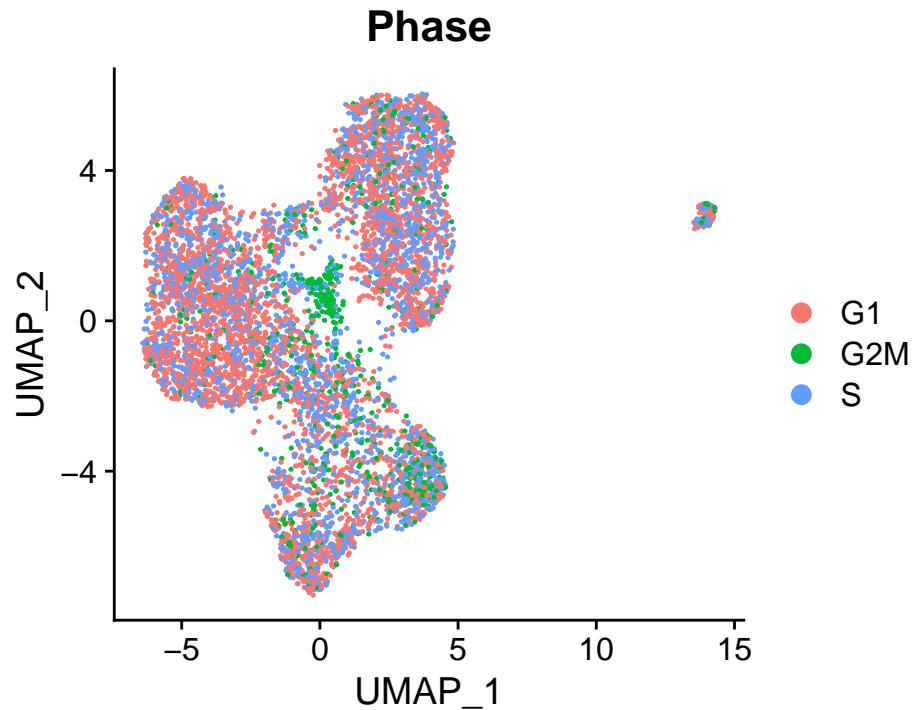
```
DimPlot(results, cols = pal3, split.by = "group")
```



```
ggsave(filename = ".../Figures/UMAPplot_all_separate_samplesMaf.pdf", width = 1  
       16, height = 4)
```

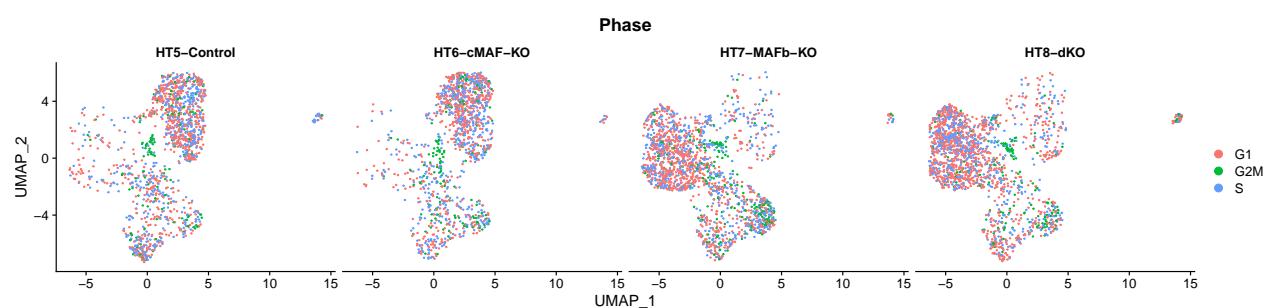
6.6 Cell cycle analysis

```
library(cowplot)  
data("geneinfo_human", package = "nichenetr")  
s.genes <- nichenetr::convert_human_to_mouse_symbols(cc.genes.updated.2019  
           $s.genes)  
g2m.genes <- nichenetr::convert_human_to_mouse_symbols(cc.genes.updated  
           .2019$g2m.genes)  
results <- CellCycleScoring(results, s.features = s.genes, g2m.features =  
           g2m.genes, set.ident = FALSE)  
DimPlot(results, group.by = "Phase")
```



We see a clear cycling core in Intermediate population.

```
DimPlot(results, group.by = "Phase", reduction = "umap", split.by = "group")
```



```
ggsave(filename = "../Figures/UMAPplot_all_separate_samplesMaf_Phase.pdf", width = 16, height = 4)
```

```
saveRDS(results, file = "./All_samples_Maf.seuratObject.Rds")
```

7 Session information

R session:

```
sessionInfo()
```

```
## R version 4.0.3 (2020-10-10)
## Platform: x86_64-pc-linux-gnu (64-bit)
## Running under: Ubuntu 20.04.3 LTS
##
```

```

## Matrix products: default                                5
## BLAS:   /usr/lib/x86_64-linux-gnu/openblas-pthread/libblas.so.3    6
## LAPACK: /usr/lib/x86_64-linux-gnu/openblas-pthread/liblapack.so.3    7
##
## locale:                                                 8
## [1] LC_CTYPE=en_US.UTF-8      LC_NUMERIC=C          10
## [3] LC_TIME=en_GB.UTF-8       LC_COLLATE=en_US.UTF-8    11
## [5] LC_MONETARY=en_GB.UTF-8   LC_MESSAGES=en_US.UTF-8    12
## [7] LC_PAPER=en_GB.UTF-8     LC_NAME=C            13
## [9] LC_ADDRESS=C             LC_TELEPHONE=C        14
## [11] LC_MEASUREMENT=en_GB.UTF-8 LC_IDENTIFICATION=C    15
##
## attached base packages:                               16
## [1] parallel stats4 stats      graphics grDevices utils    18
## datasets
## [8] methods  base
##
## other attached packages:                            19
## [1] cowplot_1.1.1           RColorBrewer_1.1-2   S4Vectors_0.28.1 22
## [4] BiocGenerics_0.36.1     dplyr_1.0.7         ggpubr_0.4.0      23
## [7] ggplot2_3.3.5           SeuratObject_4.0.4  Seurat_4.0.5      24
##
## loaded via a namespace (and not attached):           25
## [1] backports_1.4.0          Hmisc_4.6-0        systemfonts_1.0.3 27
## [4] plyr_1.8.6                igraph_1.2.9       lazyeval_0.2.2      28
## [7] splines_4.0.3             listenv_0.8.0     scattermore_0.7      29
## [10] digest_0.6.29            foreach_1.5.1     htmltools_0.5.2      30
## [13] fansi_0.5.0              checkmate_2.0.0   magrittr_2.0.1      31
## [16] tensor_1.5                cluster_2.1.0    ROCR_1.0-11        32
## [19] limma_3.46.0              tzdb_0.2.0        readr_2.1.1        33
## [22] recipes_0.1.17           globals_0.14.0   gower_0.2.2        34
## [25] matrixStats_0.61.0       spatstat.sparse_2.0-0 jpeg_0.1-9        35
## [28] colorspace_2.0-2          ggrepel_0.9.1    textshaping_0.3.6    36
## [31] xfun_0.28                 crayon_1.4.2     jsonlite_1.7.2      37
## [34] spatstat.data_2.1-0       survival_3.2-7   zoo_1.8-9         38
## [37] iterators_1.0.13          glue_1.5.1       polyclip_1.10-0     39
## [40] gtable_0.3.0              ipred_0.9-12    leiden_0.3.9        40
## [43] car_3.0-12                future.apply_1.8.1 abind_1.4-5        41
## [46] scales_1.1.1              DBI_1.1.1        rstatix_0.7.0      42
## [49] miniUI_0.1.1.1            Rcpp_1.0.7       htmlTable_2.3.0     43
## [52] viridisLite_0.4.0          xtable_1.8-4     reticulate_1.22     44
## [55] spatstat.core_2.3-2        foreign_0.8-81   proxy_0.4-26       45
## [58] Formula_1.2-4              lava_1.6.10     prodlim_2019.11.13 46
## [61] htmlwidgets_1.5.4           httr_1.4.2      DiagrammeR_1.0.6.1 47
## [64] ellipsis_0.3.2              ica_1.0-2       pkgconfig_2.0.3      48
## [67] farver_2.1.0               nnet_7.3-14     uwot_0.1.11        49
## [70] deldir_1.0-6               utf8_1.2.2     caret_6.0-90       50
## [73] tidyselect_1.1.1            labeling_0.4.2   rlang_0.4.12       51
## [76] reshape2_1.4.4              later_1.3.0     visNetwork_2.1.0     52
## [79] munsell_0.5.0               tools_4.0.3     generics_0.1.1      53
## [82] broom_0.7.10                ggridges_0.5.3   fdrtool_1.2.17     54
## [85] evaluate_0.14                stringr_1.4.0   fastmap_1.1.0      55
## [88] yaml_2.2.1                  ragg_1.2.1     goftest_1.2-3       56
## [91] ModelMetrics_1.2.2.2        knitr_1.36     fitdistrplus_1.1-6 57

```

## [94] caTools_1.18.2	randomForest_4.6-14	purrrr_0.3.4	58
## [97] RANN_2.6.1	pbapply_1.5-0	future_1.23.0	59
## [100] nlme_3.1-153	mime_0.12	rstudioapi_0.13	60
## [103] compiler_4.0.3	plotly_4.10.0	png_0.1-7	61
## [106] e1071_1.7-9	ggsignif_0.6.3	spatstat.utils_2.2-0	62
## [109] tibble_3.1.6	stringi_1.7.6	highr_0.9	63
## [112] RSpectra_0.16-0	lattice_0.20-41	Matrix_1.3-4	64
## [115] vctrs_0.3.8	pillar_1.6.4	lifecycle_1.0.1	65
## [118] spatstat.geom_2.3-0	lmtest_0.9-39	RcppAnnoy_0.0.19	66
## [121] bitops_1.0-7	data.table_1.14.2	irlba_2.3.5	67
## [124] httpuv_1.6.3	patchwork_1.1.1	latticeExtra_0.6-29	68
## [127] R6_2.5.1	promises_1.2.0.1	KernSmooth_2.23-20	69
## [130] gridExtra_2.3	parallelly_1.29.0	codetools_0.2-18	70
## [133] MASS_7.3-53	assertthat_0.2.1	withr_2.4.3	71
## [136] sctransform_0.3.2	hms_1.1.1	mgcv_1.8-33	72
## [139] grid_4.0.3	rpart_4.1-15	nichenetr_1.0.0	73
## [142] timeDate_3043.102	tidyr_1.1.4	class_7.3-17	74
## [145] rmarkdown_2.11	carData_3.0-4	Rtsne_0.15	75
## [148] pROC_1.18.0	base64enc_0.1-3	shiny_1.7.1	76
## [151] lubridate_1.8.0			77