Aim 3: Characterizing CD5RA as a Potential Primiitive T-Cell Lineage Marker

In this final notebook, we will be analyzing Days 7-13 HSCs that were differentiated in LEM according to FW's populations. We will specifically be working on characterizing CD45RA as a potential marker for T-lineage priming, contrasting it's performance with that of CD45RA+CD7+. As such, the Research Questions (RQ) for this notebook would be:

1. How Do CD45RA and CD7 Expression Patterns Interact Over Time, and When Does the CD45RA^{+}CD7^{+} State First Emerge?

2. What Transcription Factors and Pathways Are Upregulated in CD45RA+7+ Compared to CD45RA+7- Cells?

3. Do CD45RA+7+ Cells Show Higher T-Lineage Gene Module Scores Than Just CD7+ Cells? Does the Acquisition of CLEC12A Affect This?

4. What Are the Transcriptional Profiles and Phenotypes of CD45RA^{+}CD7^{+} Subclusters With Elevated T-Lineage Scores (if any)?

5. How Does the New T-Lineage Primed Cluster Fit Within Our Population in Psuedotime?

Let's begin with Pre-Processing the Data first below:

Pre-Processing Workflow

Let's begin by loading the .RData objects into our environment, and naming all the relevant populations:

In [1]:

```r
# Loading the necessary libraries

library(BiocSingular) # We need this to use the BioConductor libraries that work on
library(SingleCellExperiment) # We need this to use the SingleCellExperiment data s
library(ggplot2) # we need this to make ggplot visualizations #nolint
library(tidyr) # we need this to manipulate data #nolint
library(dplyr) # we need this to manipulate data #nolint
library(patchwork) # to display plots side by side. #nolint
library(ggforce) # Allows me to display circles on ggplots. #nolint
library(limma) # helps with differential expression analysis #nolint
library(IRdisplay) # lets me display JPEGs in the notebook #nolint
library(org.Hs.eg.db) # lets me do gene annotation #nolint
library(clusterProfiler) # lets me do gene set enrichment analysis #nolint
library(broom) # lets me manipulate data #nolint
library(enrichplot) # lets me visualize gene set enrichment analysis #nolint
library(scales) # for percent_format()
library(tidyverse) # lets me manipulate data #nolint
library(ggrepel) # for non-overlapping labels
library(pheatmap) # for heatmaps #nolint
library(scran) # for clustering and dimensionality reduction #nolint
library(igraph) # for graph analysis #nolint
library(slingshot) # for trajectory analysis #nolint
```

```
Loading required package: SummarizedExperiment

Loading required package: MatrixGenerics

Loading required package: matrixStats

Loading required package: MatrixGenerics

Loading required package: matrixStats


Attaching package: 'MatrixGenerics'


The following objects are masked from 'package:matrixStats':

    colAlls, colAnyNAs, colAnys, colAvgsPerRowSet, colCollapse,
    colCounts, colCummaxs, colCummins, colCumprods, colCumsums,
    colDiffs, colIQRDiffs, colIQRs, colLogSumExps, colMadDiffs,
    colMads, colMaxs, colMeans2, colMedians, colMins, colOrderStats,
    colProds, colQuantiles, colRanges, colRanks, colSdDiffs, colSds,
    colSums2, colTabulates, colVarDiffs, colVars, colWeightedMads,
    colWeightedMeans, colWeightedMedians, colWeightedSds,
    colWeightedVars, rowAlls, rowAnyNAs, rowAnys, rowAvgsPerColSet,
    rowCollapse, rowCounts, rowCummaxs, rowCummins, rowCumprods,
    rowCumsums, rowDiffs, rowIQRDiffs, rowIQRs, rowLogSumExps,
    rowMadDiffs, rowMads, rowMaxs, rowMeans2, rowMedians, rowMins,
    rowOrderStats, rowProds, rowQuantiles, rowRanges, rowRanks,
    rowSdDiffs, rowSds, rowSums2, rowTabulates, rowVarDiffs, rowVars,
    rowWeightedMads, rowWeightedMeans, rowWeightedMedians,
    rowWeightedSds, rowWeightedVars


Loading required package: GenomicRanges

Loading required package: stats4

Loading required package: BiocGenerics


Attaching package: 'BiocGenerics'


The following objects are masked from 'package:stats':

    IQR, mad, sd, var, xtabs


The following objects are masked from 'package:base':

    anyDuplicated, aperm, append, as.data.frame, basename, cbind,
    colnames, dirname, do.call, duplicated, eval, evalq, Filter, Find,
    get, grep, grepl, intersect, is.unsorted, lapply, Map, mapply,
    match, mget, order, paste, pmax, pmax.int, pmin, pmin.int,
    Position, rank, rbind, Reduce, rownames, sapply, saveRDS, setdiff,
    table, tapply, union, unique, unsplit, which.max, which.min
```

```
Loading required package: S4Vectors


Attaching package: 'S4Vectors'


The following object is masked from 'package:utils':

    findMatches


The following objects are masked from 'package:base':

    expand.grid, I, unname


Loading required package: IRanges


Attaching package: 'IRanges'


The following object is masked from 'package:grDevices':

    windows


Loading required package: GenomeInfoDb

Loading required package: Biobase

Welcome to Bioconductor

    Vignettes contain introductory material; view with
    'browseVignettes()'. To cite Bioconductor, see
    'citation("Biobase")', and for packages 'citation("pkgname")'.



Attaching package: 'Biobase'


The following object is masked from 'package:MatrixGenerics':

    rowMedians


The following objects are masked from 'package:matrixStats':

    anyMissing, rowMedians



Attaching package: 'tidyr'
```

```
The following object is masked from 'package:S4Vectors':

    expand


Attaching package: 'dplyr'

The following object is masked from 'package:Biobase':

    combine

The following objects are masked from 'package:GenomicRanges':

    intersect, setdiff, union

The following object is masked from 'package:GenomeInfoDb':

    intersect

The following objects are masked from 'package:IRanges':

    collapse, desc, intersect, setdiff, slice, union

The following objects are masked from 'package:S4Vectors':

    first, intersect, rename, setdiff, setequal, union

The following objects are masked from 'package:BiocGenerics':

    combine, intersect, setdiff, union

The following object is masked from 'package:matrixStats':

    count

The following objects are masked from 'package:stats':

    filter, lag

The following objects are masked from 'package:base':

    intersect, setdiff, setequal, union
```

```

Attaching package: 'limma'


The following object is masked from 'package:BiocGenerics':

    plotMA


Loading required package: AnnotationDbi


Attaching package: 'AnnotationDbi'


The following object is masked from 'package:dplyr':

    select





clusterProfiler v4.14.4 Learn more at https://yulab-smu.top/contribution-knowledge-mining/

Please cite:

G Yu. Thirteen years of clusterProfiler. The Innovation. 2024,
5(6):100722


Attaching package: 'clusterProfiler'


The following object is masked from 'package:AnnotationDbi':

    select


The following object is masked from 'package:IRanges':

    slice


The following object is masked from 'package:S4Vectors':

    rename


The following object is masked from 'package:stats':

    filter

```

```
enrichplot v1.26.6 Learn more at https://yulab-smu.top/contribution-knowledge-minin
g/

Please cite:

Qianwen Wang, Ming Li, Tianzhi Wu, Li Zhan, Lin Li, Meijun Chen, Wenqin
Xie, Zijing Xie, Erqiang Hu, Shuangbin Xu, Guangchuang Yu. Exploring
epigenomic datasets by ChIPseeker. Current Protocols. 2022, 2(10): e585

── Attaching core tidyverse packages ──────────────────────── tidyverse 2.0.0 ──
✓ forcats   1.0.0     ✓ readr     2.1.5
✓ lubridate 1.9.4     ✓ stringr   1.5.1
✓ purrr     1.0.2     ✓ tibble    3.2.1
── Conflicts ────────────────────────────────────────── tidyverse_conflicts() ──
✖ lubridate::%within%()     masks IRanges::%within%()
✖ readr::col_factor()       masks scales::col_factor()
✖ dplyr::collapse()         masks IRanges::collapse()
✖ dplyr::combine()          masks Biobase::combine(), BiocGenerics::combine()
✖ dplyr::count()            masks matrixStats::count()
✖ dplyr::desc()             masks IRanges::desc()
✖ purrr::discard()          masks scales::discard()
✖ tidyr::expand()           masks S4Vectors::expand()
✖ clusterProfiler::filter() masks dplyr::filter(), stats::filter()
✖ dplyr::first()            masks S4Vectors::first()
✖ dplyr::lag()              masks stats::lag()
✖ ggplot2::Position()       masks BiocGenerics::Position(), base::Position()
✖ purrr::reduce()           masks GenomicRanges::reduce(), IRanges::reduce()
✖ clusterProfiler::rename() masks dplyr::rename(), S4Vectors::rename()
✖ lubridate::second()       masks S4Vectors::second()
✖ lubridate::second<-()     masks S4Vectors::second<-()
✖ clusterProfiler::select() masks AnnotationDbi::select(), dplyr::select()
✖ purrr::simplify()         masks clusterProfiler::simplify()
✖ clusterProfiler::slice()  masks dplyr::slice(), IRanges::slice()
ℹ Use the conflicted package (<http://conflicted.r-lib.org/>) to force all conflicts
to become errors
Loading required package: scuttle


Attaching package: 'igraph'


The following objects are masked from 'package:lubridate':

    %--%, union


The following objects are masked from 'package:purrr':

    compose, simplify


The following object is masked from 'package:tibble':

    as_data_frame
```

```
The following object is masked from 'package:clusterProfiler':

    simplify


The following objects are masked from 'package:dplyr':

    as_data_frame, groups, union


The following object is masked from 'package:tidyr':

    crossing


The following object is masked from 'package:GenomicRanges':

    union


The following object is masked from 'package:IRanges':

    union


The following object is masked from 'package:S4Vectors':

    union


The following objects are masked from 'package:BiocGenerics':

    normalize, path, union


The following objects are masked from 'package:stats':

    decompose, spectrum


The following object is masked from 'package:base':

    union


Loading required package: princurve

Warning message:
"package 'princurve' was built under R version 4.4.3"
Loading required package: TrajectoryUtils


Attaching package: 'TrajectoryUtils'


The following object is masked from 'package:scran':
    createClusterMST
```

In [2]:

```
load("data/phenotype_with_ID.RData")
load("data/merge2.RData")

# Adding Phenotype data to the SCE object
pheno.d7 <- rep("CD34+CD45RA-CLEC12A-", 3039)
names(pheno.d7) <- colnames(merge2)[1:3039]

pheno.merge2 <- c(pheno.d7, pheno.d10, pheno.d13)


colData(merge2)$Phenotype <- pheno.merge2


# Cleaning Up the Phenotype Data so it belongs to the 3 populations

# Define phenotype groups
phenotype_groups <- list(
    Cneg = c("CD34+CD45RA-CLEC12A-", "CD34-CD45RA-CLEC12A-", "CD34+CD45RA+CLEC12A-"
    Cpos = c("CD34-CD45RA-CLEC12A+", "CD34+CD45RA-CLEC12A+", "CD34+CD45RA+CLEC12A+"
    Other = c("CD10+", "CD14CD15+") # Pro -B #Pro-NM #FW Gating from a flow cytomet
)

# Assign group labels to phenotypes
group_labels <- sapply(pheno.merge2, function(phenotype) {
    group <- names(phenotype_groups)[sapply(phenotype_groups, function(g) phenotype
    if (length(group) > 0) group else "Other"
})

# Add group labels to colData of the SCE object
colData(merge2)$Group <- group_labels
```

Great! Now let's add a table labelling which day each table belongs to:

In [3]:

```
# Extract the day information from cell names
colData(merge2)$Day <- gsub(".*Day_([0-9]+).*", "\\1", rownames(colData(merge2)))
# Convert to a factor (optional, for better categorical handling)
colData(merge2)$Day <- factor(colData(merge2)$Day, levels = sort(unique(colData(mer


# Define columns to keep
cols_to_keep <- c("Group", "Day", "Phenotype")

# Create a lighter version of the SCE object
merge2_light <- merge2
colData(merge2_light) <- colData(merge2_light)[, cols_to_keep]

# Checking characteristics of the new light version
merge2_light
colnames(colData(merge2_light))
```

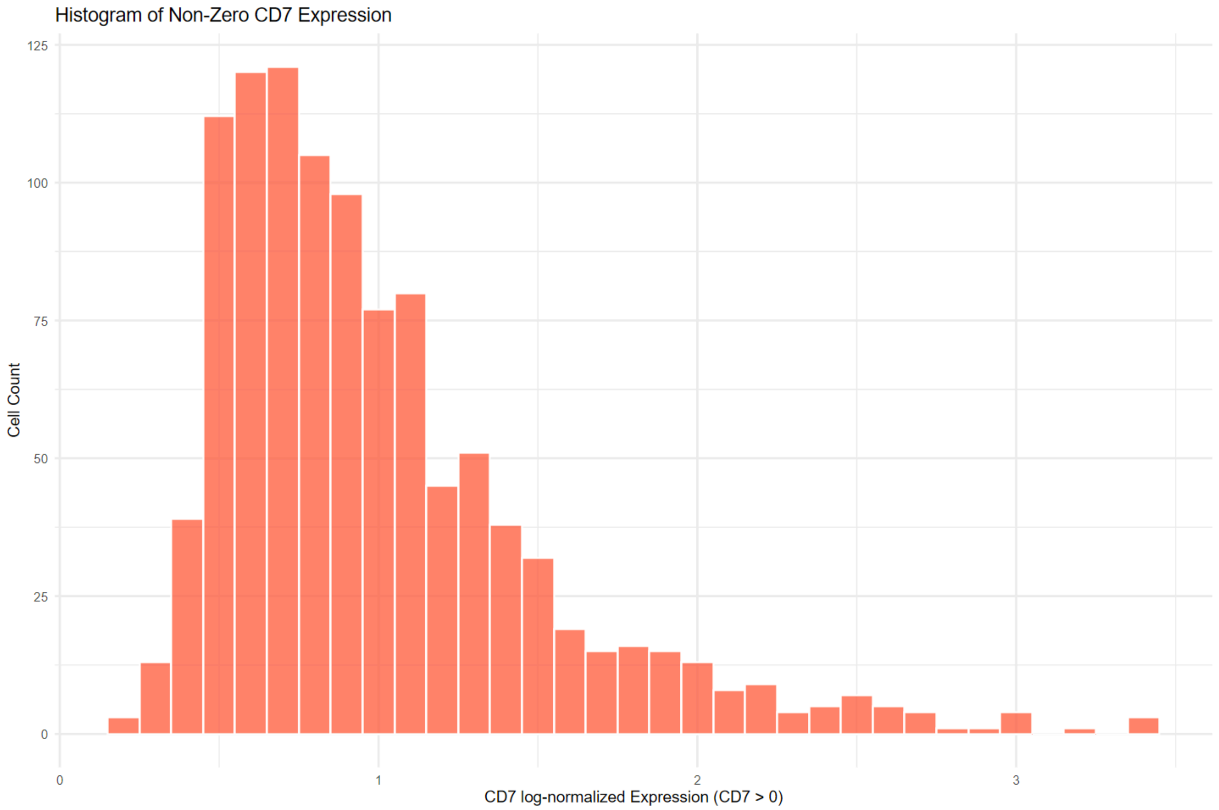
```
class: SingleCellExperiment
dim: 36601 12181
metadata(12): Samples scDblFinder.stats ... scDblFinder.stats
  scDblFinder.threshold
assays(2): counts logcounts
rownames(36601): MIR1302-2HG FAM138A ... AC007325.4 AC007325.2
rowData names(3): ID Symbol Type
colnames(12181): cell1Day_7 cell2Day_7 ... cell5137Day_13
  cell5138Day_13
colData names(3): Group Day Phenotype
reducedDimNames(9): PCA.cc UMAP.cc ... PCA TSNE
mainExpName: Gene Expression
altExpNames(1): Antibody Capture
```

'Group' · 'Day' · 'Phenotype'

Great! Let's now create a gate for CD7 based on gene expression:

In [4]:

```r
# Creating a Violin Plot to Generate CD7 Distribution Across All Cells Overall

options(repr.plot.width = 12, repr.plot.height = 8)

# 1. Extract CD7 expression from the RNA assay
cd7_expr <- logcounts(merge2_light)["CD7", ]


# 2. Add CD7 expression to metadata
colData(merge2_light)$CD7_gene <- cd7_expr

# Filter to cells with CD7_expr > 0
# becuase otherwise the plot becomes right skewed and useless
nonzero_df <- as.data.frame(colData(merge2_light)) %>%
  filter(CD7_gene > 0)

# Plot histogram of non-zero CD7 expression
ggplot(nonzero_df, aes(x = CD7_gene)) +
  geom_histogram(binwidth = 0.1, fill = "tomato", color = "white", alpha = 0.8) +
  labs(
    title = "Histogram of Non-Zero CD7 Expression",
    x = "CD7 log-normalized Expression (CD7 > 0)",
    y = "Cell Count"
  ) +
  theme_minimal()


colData(merge2_light)$CD7_geneStat <- case_when(
  merge2_light$CD7_gene == 0 ~ "CD7_0",
  merge2_light$CD7_gene >= 1 ~ "CD7_hi",
  TRUE ~ "CD7_lo" # Optional middle category
)
```



Great! Based on the visualization, this is the gating strategy I implemented:

CD7 Status	Expression Range (CD7_expr)	Rationale
CD7_0	≤ 0	Truly negative
CD7-lo	> 0 & < 1	Weak expression; transitional or noise
CD7-hi	≥ 1	Stronger, confident expression

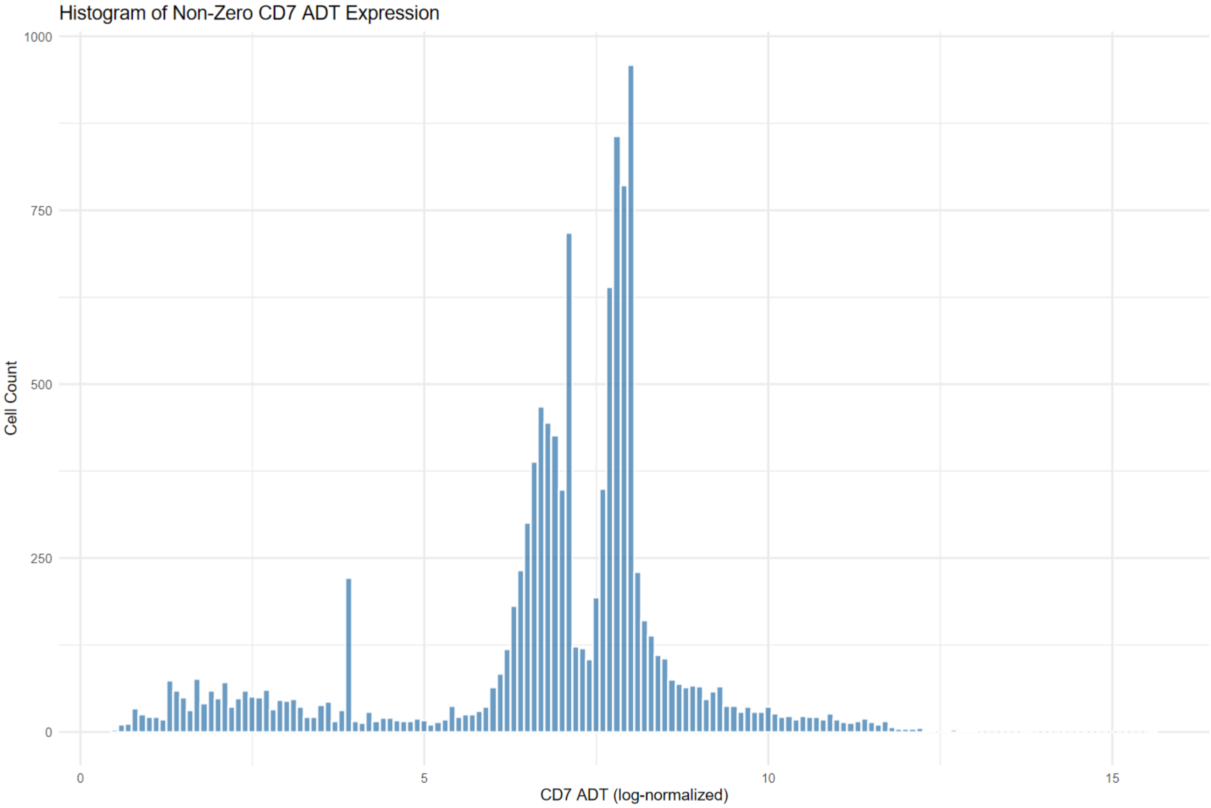
Now let's work on gating the same, using the ADT assay:

In [5]:

```r
# Extract CD7 ADT signal from Antibody Capture assay
cd7_adt <- logcounts(altExp(merge2_light, "Antibody Capture"))["TOTALSEQB_CD7", ]
colData(merge2_light)$CD7_adt <- cd7_adt

# Filter to non-zero ADT values to avoid heavy skew
adt_nonzero <- as.data.frame(colData(merge2_light)) %>%
    filter(CD7_adt > 0)

# Plot histogram
ggplot(adt_nonzero, aes(x = CD7_adt)) +
    geom_histogram(binwidth = 0.1, fill = "steelblue", color = "white", alpha = 0.8
    labs(
        title = "Histogram of Non-Zero CD7 ADT Expression",
        x = "CD7 ADT (log-normalized)",
        y = "Cell Count"
    ) +
    theme_minimal()

colData(merge2_light)$CD7_adtStat <- case_when(
    merge2_light$CD7_adt <= 1 ~ "CD7-",
    merge2_light$CD7_adt >= 6 ~ "CD7+",
    TRUE ~ "CD7~"
)

colnames(colData(merge2_light))

# Identify which cells have CD7_gene == 0
zero_gene_idx <- which(colData(merge2_light)$CD7_gene == 0)

# Set their ADT expression to 0
colData(merge2_light)$CD7_adt[zero_gene_idx] <- 0

# Set their ADT status to "CD7-"
colData(merge2_light)$CD7_adtStat[zero_gene_idx] <- "CD7-"

# Check the updated data frame
table(CD7_gene = merge2_light$CD7_gene > 0, CD7_adt = merge2_light$CD7_adt > 0)
```

'Group' · 'Day' · 'Phenotype' · 'CD7_gene' · 'CD7_geneStat' · 'CD7_adt' · 'CD7_adtStat'

```
        CD7_adt
CD7_gene FALSE  TRUE
   FALSE 11117     0
   TRUE     24  1040
```



Cool! The gating strategy I used for this (reminder, all this is just a repeat of the process in Aim 2) is shown below:

Gating Strategy for CD7 ADT:

Gate	Range (CD7_adt)	Rationale
CD7-	`≤ 1`	Undetectable expression (log-space 0)
CD7~	`> 1 & < 6`	Ambiguous or transitional zone
CD7+	`≥ 6`	Robust protein-level CD7 expression

I have also already fixed the stickyness issue in the code above.

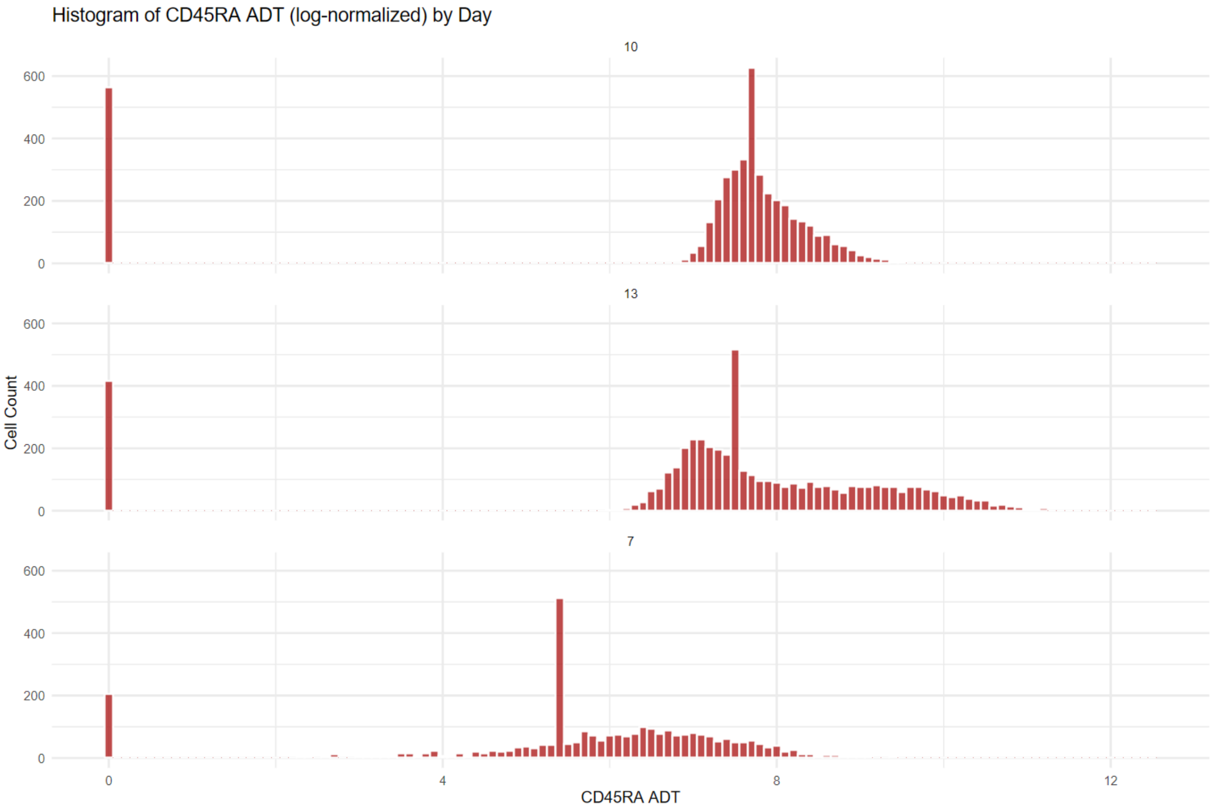
Now, let's repeat this process with CD45RA. Note that CD45RA is an isoform of CD45, which is a protein coded by the `PTPRC` gene, which is commong for all isoforms. So I **can not simply gate based on gene expression**, since the gene is not specific to 45RA at all, and this would make any of the results I find worthless.

As such, I will be establishing a work-around by gating based on ADT only, and forcing cells with 0 PTPRC expression to be CD45RA-, and then applying some statistical filters to gate out some more of the non-specific binding. Note however, this would still be a major hit to the statistical rigour of these findings.

Let's code this in below:

In [6]:

```r
# Step 1: Extract CD45RA ADT values
cd45ra_adt <- logcounts(altExp(merge2_light, "Antibody Capture"))["TOTALSEQB_CD45RA
colData(merge2_light)$CD45RA_adt <- cd45ra_adt

# Step 2: Filter out non-hematopoietic cells using PTPRC
ptprc_expr <- logcounts(merge2_light)["PTPRC", ]
ptprc_cutoff <- 0.25
ptprc_positive <- ptprc_expr > ptprc_cutoff

# Optional: Set CD45RA ADT to 0 for PTPRC- cells, and track them
colData(merge2_light)$CD45RA_adt[!ptprc_positive] <- 0
colData(merge2_light)$CD45RA_stat <- ifelse(!ptprc_positive, "CD45RA-", NA)

# Step 3: Visualize CD45RA ADT by Day (post-filtering)
library(ggplot2)
cd45ra_df <- as.data.frame(colData(merge2_light)) %>%
  filter(!is.na(CD45RA_adt))

ggplot(cd45ra_df, aes(x = CD45RA_adt)) +
  geom_histogram(binwidth = 0.1, fill = "firebrick", color = "white", alpha = 0.8)
  facet_wrap(~Day, scales = "fixed", ncol = 1) +
  labs(
    title = "Histogram of CD45RA ADT (log-normalized) by Day",
    x = "CD45RA ADT", y = "Cell Count"
  ) +
  theme_minimal()
```



Here’s how I would interpret these results:

- **Very sharp peak at 0**: likely true CD45RA$^-$ cells and/or background noise.
- **Clear separation** begins around log-normalized ADT ≈ 2.5–3.
- **Robust, unimodal peak** around 7.5: strong CD45RA$^+$ signal.
- A spike near ~5 is likely technical (common in CITE-seq) — potentially aggregates or residual antibody binding.

Here's the gating thresholds I will be implementing:

Gate	CD45RA ADT Range	Rationale
`CD45RA−`	≤ **2.5**	No expression / noise
`CD45RA~`	> 2.5 & < 6	Ambiguous or low-level staining
`CD45RA+`	≥ **6**	Clear, biologically meaningful surface signal

In [7]:

```
# Extract CD45RA ADT and PTPRC expression vectors from colData
cd45ra_adt <- colData(merge2_light)$CD45RA_adt
ptprc_expr <- logcounts(merge2_light)["PTPRC", ]
ptprc_cutoff <- 0.25

# Generate gating status
colData(merge2_light)$CD45RA_stat <- case_when(
  ptprc_expr <= ptprc_cutoff ~ "CD45RA-", # Force CD45RA- for PTPRC- cells
  cd45ra_adt <= 2.5 ~ "CD45RA-",
  cd45ra_adt >= 6 ~ "CD45RA+",
  TRUE ~ "CD45RA~"
)
```

Let's now do some gating for CD5 based on gene expression:

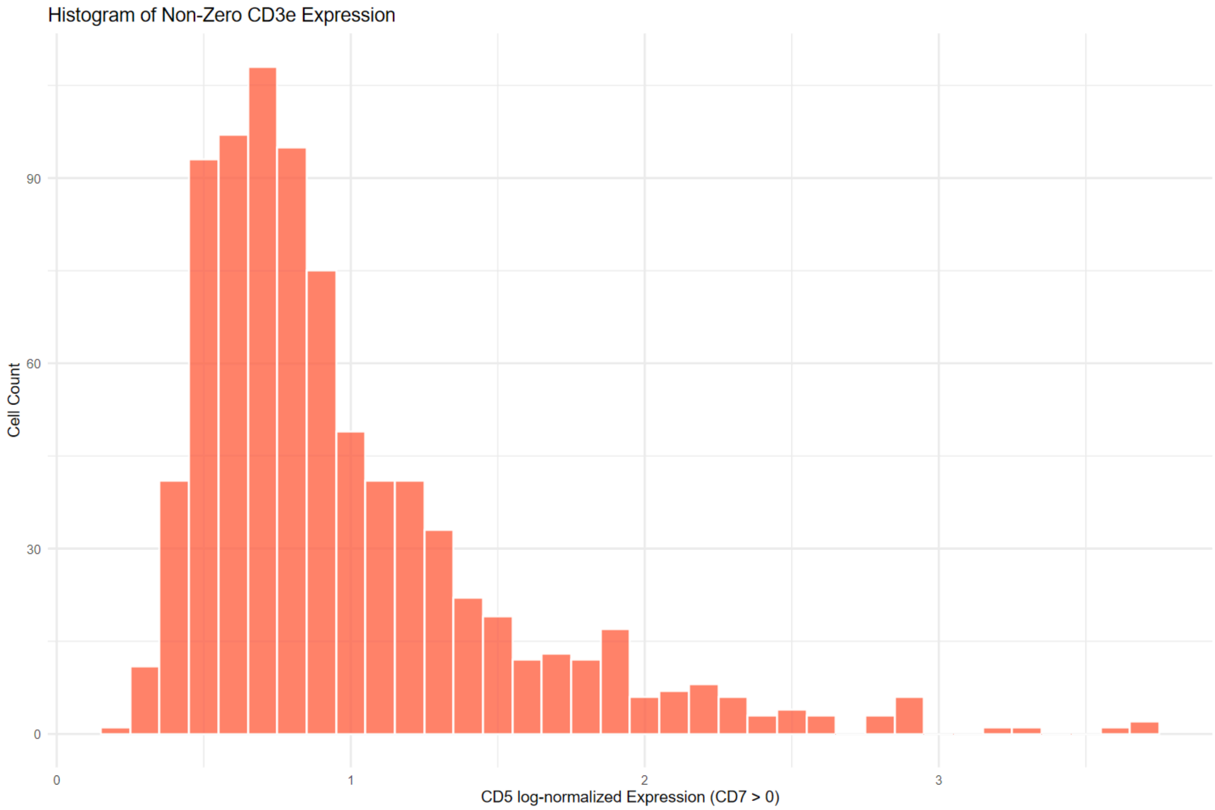
In [8]:

```
"CD3E" %in% rownames(merge2_light)
```

TRUE

In [9]:

```
# Creating a Violin Plot to Generate CD5 Distribution Across All Cells Overall

options(repr.plot.width = 12, repr.plot.height = 8)

# 1. Extract CD5 expression from the RNA assay
cd3e_expr <- logcounts(merge2_light)["CD3E", ]


# 2. Add CD5 expression to metadata
colData(merge2_light)$CD3e_gene <- cd3e_expr

# Filter to cells with CD5_expr > 0
# becuase otherwise the plot becomes right skewed and useless
nonzero_df <- as.data.frame(colData(merge2_light)) %>%
  filter(CD3e_gene > 0)

# Plot histogram of non-zero CD5 expression
ggplot(nonzero_df, aes(x = CD3e_gene)) +
  geom_histogram(binwidth = 0.1, fill = "tomato", color = "white", alpha = 0.8) +
  labs(
    title = "Histogram of Non-Zero CD3e Expression",
    x = "CD5 log-normalized Expression (CD7 > 0)",
    y = "Cell Count"
  ) +
  theme_minimal()


colData(merge2_light)$CD3e_geneStat <- case_when(
  merge2_light$CD3e_gene == 0 ~ "CD3e-",
  merge2_light$CD3e_gene >= 1 ~ "CD3e_hi",
  TRUE ~ "CD3e_lo" # Optional middle category
)
```



Great! We are now ready to dive into the analysis workflow!

Analysis Workflow

RQ1: Do CD45RA+CD7- Cells Exist At Earlier Timepoints, and How Does Their Proportion Compare to Earlier Timepoints

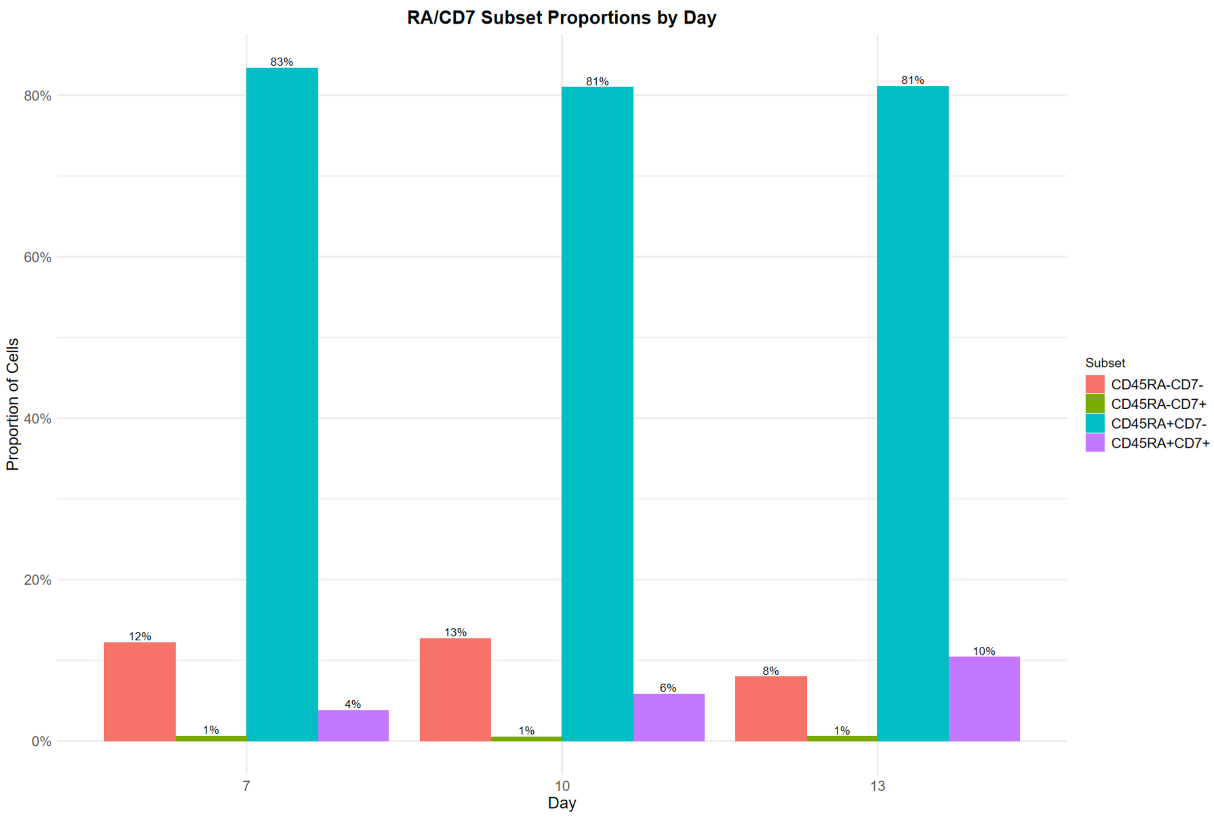
To evaluate whether CD45RA can serve as an early indicator of T-lineage priming, it is important to determine when CD45RA^{+}CD7^{-} cells first emerge and how their abundance changes over time. If CD45RA^{+}CD7^{-} cells are detectable at earlier timepoints—before robust CD7 expression is established—this would support the hypothesis that CD45RA marks a priming event that precedes or operates independently of CD7. Tracking their proportional dynamics across Days 7–13 enables us to assess whether CD45RA expression anticipates CD7 and thereby holds greater utility as an early marker of T-lineage bias.

Let's begin by visualizing the proportion of all CD45RA+/- & CD7+/- cells over time, from all populations:

In [10]:

```
options(repr.plot.width = 15, repr.plot.height = 10)

# Step 1: Extract metadata into a dataframe
meta_df <- as.data.frame(colData(merge2_light))


# Step 1: Define combo categories across all cells
```

```r
meta_df <- meta_df %>%
  mutate(RA7_combo_full = case_when(
    CD45RA_stat == "CD45RA+" & CD7_adtStat == "CD7+" ~ "CD45RA+CD7+",
    CD45RA_stat == "CD45RA+" & CD7_adtStat == "CD7-" ~ "CD45RA+CD7-",
    CD45RA_stat == "CD45RA-" & CD7_adtStat == "CD7+" ~ "CD45RA-CD7+",
    CD45RA_stat == "CD45RA-" & CD7_adtStat == "CD7-" ~ "CD45RA-CD7-",
    TRUE ~ "Other"
  ))

# Step 2: Count by day and combo
combo_df <- meta_df %>%
  filter(RA7_combo_full != "Other") %>%
  group_by(Day, RA7_combo_full) %>%
  summarise(N = n(), .groups = "drop") %>%
  group_by(Day) %>%
  mutate(Prop = N / sum(N))

# Reorder Day as a factor with levels in desired order
combo_df$Day <- factor(combo_df$Day, levels = c("7", "10", "13"))


# Plot side-by-side bars (position = "dodge") with percentage labels
prop_plot_AllHSCs <- ggplot(combo_df, aes(x = Day, y = Prop, fill = RA7_combo_full)
  geom_col(position = position_dodge(width = 0.9)) +
  geom_text(aes(label = scales::percent(Prop, accuracy = 1)),
    position = position_dodge(width = 0.9),
    vjust = -0.25, size = 3.5
  ) +
  scale_y_continuous(labels = percent_format()) +
  labs(
    title = "RA/CD7 Subset Proportions by Day",
    x = "Day", y = "Proportion of Cells",
    fill = "Subset"
  ) +
  theme_minimal() +
  theme(legend.position = "right") +
  theme(
    plot.title = element_text(hjust = 0.5, size = 16, face = "bold"),
    axis.title = element_text(size = 14),
    axis.text = element_text(size = 12),
    plot.subtitle = element_text(size = 15, face = "italic"),
    strip.text = element_text(size = 12),
    legend.text = element_text(size = 12),
  )

print(prop_plot_AllHSCs)
```



Here's my interpretation of these results:

CD45RA$^+$CD7$^-$ (Teal: 83% on Day 7, 81% on Days 10/13)

- Present at very **high** levels from Day 7 onward, indicating that CD45RA expression precedes CD7 during early T-lineage priming.
- Their dominance at the earliest timepoint strongly supports the hypothesis that CD45RA$^+$CD7$^-$ cells represent an early-primed, lymphoid-biased state — prior to CD7 induction.
  - These cells are not expressing CD7 — a known **pan-T marker** — yet they're already **expressing CD45RA**, which is often re-expressed on **committed lymphoid progenitors**.
- The relatively **stable frequency** of this population suggests that **CD45RA$^+$ priming is initiated early and maintained**, aligning with a gradual or stepwise model of commitment.

CD45RA$^+$CD7$^+$ (Purple: 4% on Day 7, increasing to 10% by Day 13)

- This population emerges gradually over time, consistent with the idea that **CD7 turns on later**, within a **CD45RA$^+$-primed background**.
- Suggests progression from CD45RA$^+$CD7$^-$ → CD45RA$^+$CD7$^+$, matching models of T-cell specification (e.g., in the Yale paper or Edgar's pseudotime analysis).
  - This implies that **CD45RA expression is a prerequisite** for T-lineage activation — i.e., **CD7 expression only occurs in CD45RA$^+$ cells**.

CD45RA$^-$CD7$^-$ (Red: ~12–13% on Day 7/10, decreasing to 8% by Day 13)

- Likely includes **non-responding or latent HSCs** that remain unprimed for lymphoid-lineage, possibly retained from the Ra$^-$C$^-$ gate.
- Their **declining proportion over time** reflects selection against unprimed cells under T-inductive conditions (i.e., LEM cytokines).

CD45RA$^-$CD7$^+$ (Green: ~1% at all timepoints)

- Very rare population, likely representing **transient noise**, **technical artifacts**, or cells with **non-hematopoietic CD7 activation**.
- Critically, their frequency **does not increase over time**, and **remains negligible** despite lymphoid-lineage stimulation.

Biological Model Emerging fromThis

I'm building towards a model where:

Undifferentiated HSC → CD45RA$^-$CD7$^-$ (latent/unprimed) -> CD45RA$^+$CD7$^-$ (primed) → CD45RA$^+$CD7$^+$ (T-lineage Initial Priming)

Takeaways:

Given our Aim 3 hypothesis:

> **CD45RA, particularly in the CD45RA$^+$CD7+ state, is a better marker of early T-lineage priming than CD7+ alone.**

- CD45RA expression is **present and dominant by Day 7**, while CD7$^+$ cells are scarce.
- CD7$^+$ cells emerge **only within the CD45RA$^+$ population**, suggesting that **CD45RA marks an upstream primed state**.
- CD45RA$^-$CD7$^+$ cells are **rare and not expanding**, weakening the case for CD7 as an early standalone marker.
- But by being upstream, CD45RA is also not inspiring confidence in it's role as a more selective T-lineage marker at this stage.

> In my opinion, this doesn't mean that 45RA+ is a better/worse marker than CD7+ - we haven't tested that yet. Instead, these plots mean that 45RA+ is an earlier (possibly earliest?) detectable marker of Lymphoid/T-lineage commitment, and exploring subsets of CD45RA+ for T-lineage genes would be key to finding a more restricted population.

RQ2: What Transcription Factors and Pathways Are Upregulated in CD45RA+7+ Compared to CD45RA+7- Cells?

In this section, we are going to try to characterize the transcriptional differences that occur with the acquisition of CD7 within the CD45 subpopulation. We will do so by completing some

In [11]:

```
run_dge_dataframe <- function(expr_matrix, group_vector) {
  stopifnot(length(group_vector) == ncol(expr_matrix))

  # Sanitize group names (e.g., CD7+ → CD7pos, CD7- → CD7neg)
  group_vector_clean <- recode(group_vector, "CD7+" = "CD7pos", "CD7-" = "CD7neg")

  # Create design matrix with clean names
  group_factor <- factor(group_vector_clean, levels = c("CD7neg", "CD7pos"))
  design <- model.matrix(~ 0 + group_factor)
  colnames(design) <- levels(group_factor)

  # Fit model and apply contrast
  fit <- lmFit(expr_matrix, design)
  contrast.matrix <- makeContrasts(CD7pos_vs_CD7neg = CD7pos - CD7neg, levels = des
  fit2 <- contrasts.fit(fit, contrast.matrix)
  fit2 <- eBayes(fit2)

  # Extract results
  top_genes <- topTable(fit2, coef = 1, number = Inf, sort.by = "logFC")
  cd_genes <- top_genes[grep("^CD\\d+", top_genes$ID), ]

  return(list(top_genes = top_genes, top_cd_genes = cd_genes))
}
```

In [12]:

```
# Suppose you’ve already subsetted your data to CD45RA+ cells:
sce_ra_pos <- merge2_light[, merge2_light$CD45RA_stat == "CD45RA+" & merge2_light$C

# Run function
result <- run_dge_dataframe(
  expr_matrix = logcounts(sce_ra_pos),
  group_vector = colData(sce_ra_pos)$CD7_adtStat
)
```

```
Warning message in asMethod(object):
"sparse->dense coercion: allocating vector of size 2.6 GiB"
Warning message:
"Zero sample variances detected, have been offset away from zero"
```

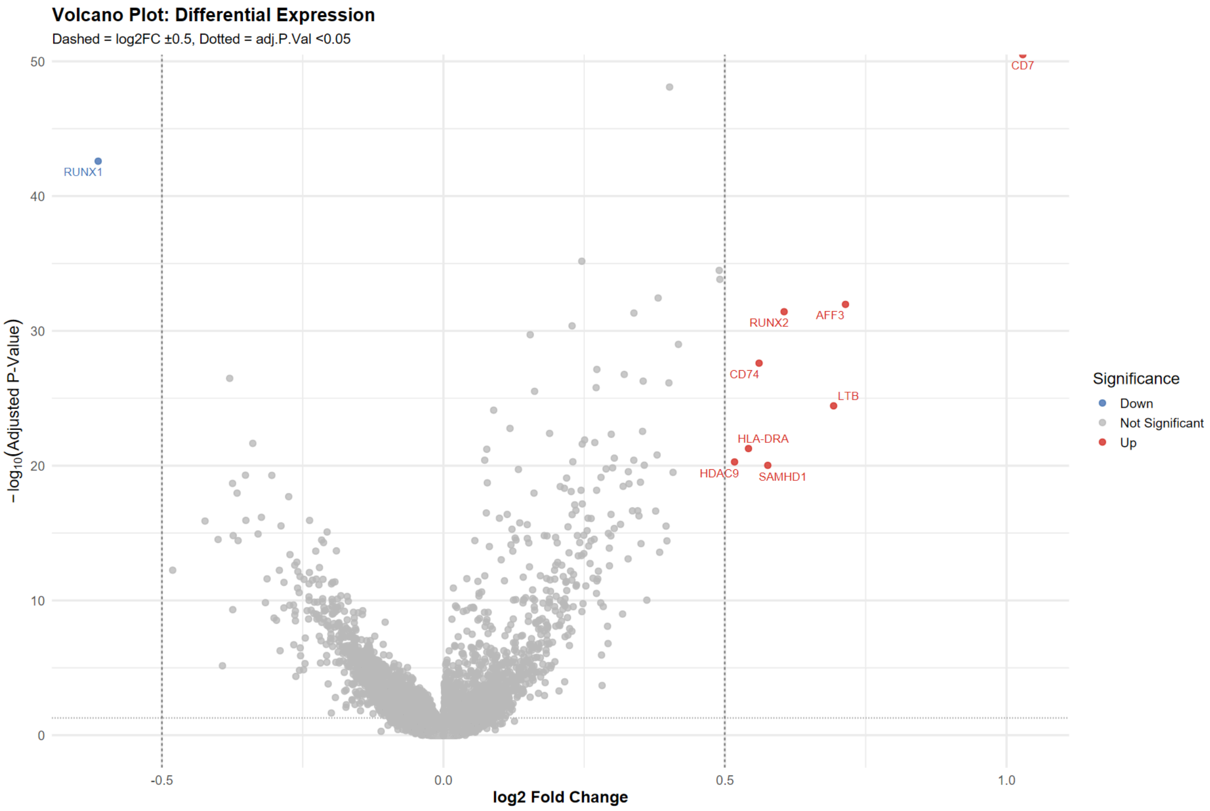
Great! Let's visualize these results using a volcano plot and heatmap:

In [13]:

```
draw_volcano <- function(result) {
  # --- Assume result$top_genes already exists ---
  df <- result$top_genes

  # Define thresholds
  fc_thresh <- 0.5
  pval_thresh <- 0.05

  # Annotate significance
  df <- df %>%
    mutate(
      Significant = case_when(
        adj.P.Val < pval_thresh & logFC > fc_thresh ~ "Up",
        adj.P.Val < pval_thresh & logFC < -fc_thresh ~ "Down",
        TRUE ~ "Not Significant"
      )
    )

  # Get top 10 genes for labeling
  top_labels <- df %>%
    filter(Significant != "Not Significant") %>%
    arrange(adj.P.Val) %>%
    slice_head(n = 10)

  # Make volcano plot
  ggplot(df, aes(x = logFC, y = -log10(adj.P.Val), color = Significant)) +
    # Highlight thresholds
    geom_vline(xintercept = c(-fc_thresh, fc_thresh), linetype = "dashed", color =
    geom_hline(yintercept = -log10(pval_thresh), linetype = "dotted", color = "gray

    # Points
    geom_point(alpha = 0.8, size = 2) +

    # Labels
    geom_text_repel(
      data = top_labels,
      aes(label = ID),
      size = 3.5,
      max.overlaps = 15,
      box.padding = 0.3,
      segment.color = "gray40",
      show.legend = FALSE
    ) +

    # Aesthetics
    scale_color_manual(values = c("Up" = "#d73027", "Down" = "#4575b4", "Not Signif
    labs(
      title = "Volcano Plot: Differential Expression",
      subtitle = paste0("Dashed = log2FC ±", fc_thresh, ", Dotted = adj.P.Val <", p
      x = "log2 Fold Change",
      y = expression(-log[10]("Adjusted P-Value")),
      color = "Significance"
    ) +
    theme_minimal(base_size = 14) +
    theme(
      plot.title = element_text(face = "bold", size = 16),
      plot.subtitle = element_text(size = 12),
      axis.title = element_text(face = "bold"),
      legend.position = "right"
    )
}
```

In [14]:

```
draw_volcano(result)
```



Here's my interpretation of these results:

- T-lineage markers and functions (CD7, HLA-DRA, CD74) are upregulated in CD7$^+$ cells.
- RUNX1 and RUNX2, key transcription factors with known hematopoietic roles, are more abundant in CD7$^+$, suggesting transcriptional priming.
- AFF3 and HDAC9 point to epigenetic and transcriptional activation pathways being engaged.
- SAMHD1 and LTB suggest additional regulation of cell fate and immune signaling.

> Overall, this suggests that CD45RA$^+$CD7$^+$ cells exhibit transcriptional features consistent with early lymphoid/T-lineage priming relative to CD45RA$^+$CD7$^-$ cells. However, the absence of definitive T-lineage transcription factors (e.g., GATA3, TCF7, BCL11B) indicates that commitment is not yet fully established.

RQ3: Do CD45RA+7+ Cells Show Higher T-Lineage Gene Module Scores Than Just CD7+ Cells? Does the Acquisition of CLEC12A Affect This?

In this section, I will be evaluating the performance of Different populations against generating T-cell scores. We already answered the question "Is CD7 statistically correlated with T-Cell Program Scores -> It's not". This analysis was conducted over all HSCs, as typically in literature, people use CD7+ as a standalone marker, or in conjunction with CD5+ to indicate T-lineage bias.

Now, I'm (hoping) to improve the score (and achieve statistical correlation) by subsetting on the CD45RA+ population, and comparing if having DP CD45RA+7+ cells improves the score compared to the Aim 2 results (Just CD7+ cells), and if the acquisition of CLEC changes this behaviour.

In Essence, we are going to complete this analysis in two parts:

1. CD45RA^{+}CD7^{+} vs. CD7^{+} (i.e. does CD45RA status enhance T-lineage priming?)

2. The influence of CLEC12A expression on any of the above patterns.

Let's begin by defining the scoring program:

Now, let's begin with Part 1:

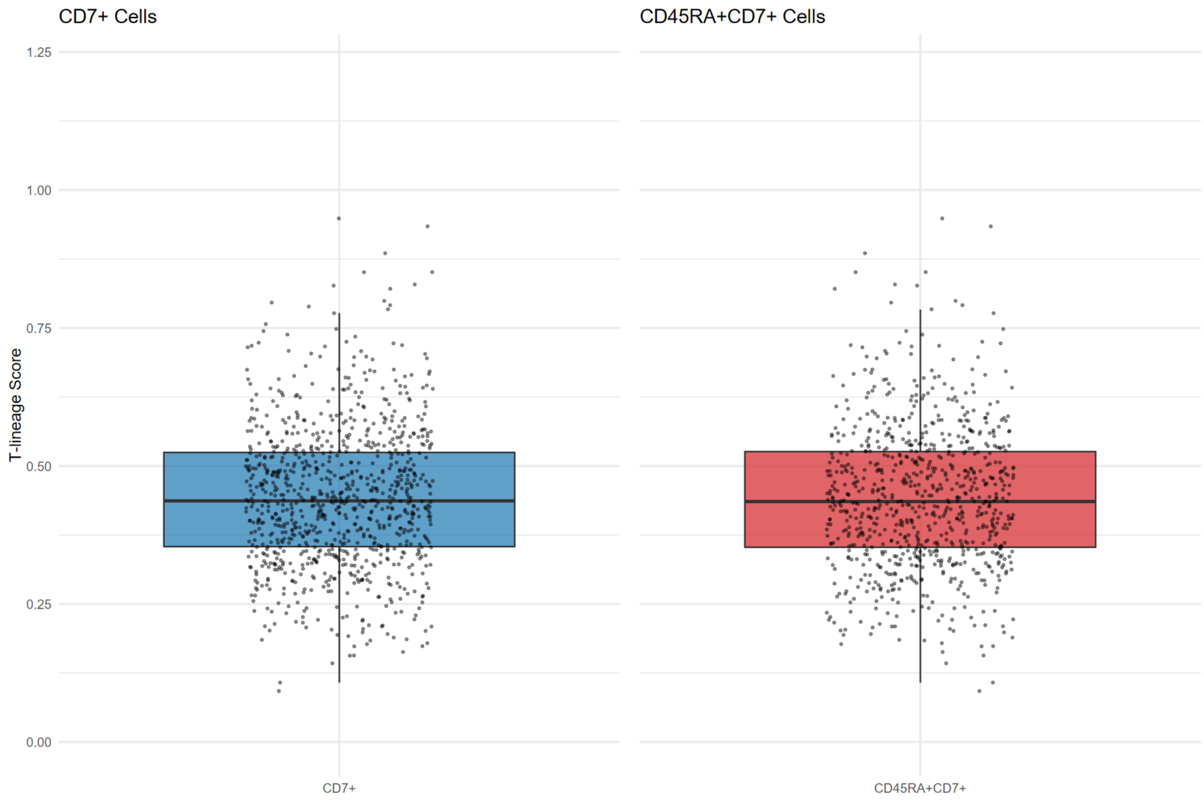
In [15]:

```r
# 1. Define your T-lineage program gene set
tcell_genes <- c(
  "RAG2", "NOTCH1", "CD3D", "CD3E", "CD3G", "TCF7", "GATA3", "BCL11B",
  "RAG1", "DTX1", "IL7R", "PTCRA", "LEF1", "SPI1", "RUNX1",
  "BCL11A", "IKZF1", "ZBTB16"
)

# 2. Filter to genes present in the SCE object
tcell_genes_present <- intersect(tcell_genes, rownames(merge2_light))

# Optional: print missing genes
missing_genes <- setdiff(tcell_genes, rownames(merge2_light))
cat("Missing genes:", paste(missing_genes, collapse = ", "), "\n")

# 3. Score each cell by average expression of T-cell genes
tcell_scores <- colMeans(logcounts(merge2_light)[tcell_genes_present, , drop = FALS

# 4. Store scores in metadata
colData(merge2_light)$Tcell_score <- tcell_scores

# Get the metadata
meta_df <- as.data.frame(colData(merge2_light)) %>%
  mutate(cell = colnames(merge2_light))

# 1. Subset: CD7+ cells (all)
cd7_pos_df <- meta_df %>%
  filter(CD7_adtStat == "CD7+")

# 2. Subset: CD45RA+CD7+ cells only
cd7_ra_pos_df <- meta_df %>%
  filter(CD7_adtStat == "CD7+", CD45RA_stat == "CD45RA+")

# 3. Determine shared y-axis scale range
y_min <- min(meta_df$Tcell_score, na.rm = TRUE)
y_max <- max(meta_df$Tcell_score, na.rm = TRUE)
```

```r
# 4. Make the two plots
p1 <- ggplot(cd7_pos_df, aes(x = "CD7+", y = Tcell_score)) +
  geom_boxplot(fill = "#1f77b4", alpha = 0.7, outlier.shape = NA) +
  geom_jitter(width = 0.2, size = 0.6, alpha = 0.4) +
  ylim(y_min, y_max) +
  labs(
    title = "CD7+ Cells",
    y = "T-lineage Score",
    x = NULL
  ) +
  theme_minimal(base_size = 14)

p2 <- ggplot(cd7_ra_pos_df, aes(x = "CD45RA+CD7+", y = Tcell_score)) +
  geom_boxplot(fill = "#d62728", alpha = 0.7, outlier.shape = NA) +
  geom_jitter(width = 0.2, size = 0.6, alpha = 0.4) +
  ylim(y_min, y_max) +
  labs(
    title = "CD45RA+CD7+ Cells",
    y = NULL,
    x = NULL
  ) +
  theme_minimal(base_size = 14) +
  theme(axis.text.y = element_blank(), axis.ticks.y = element_blank())

# 5. Combine plots side-by-side
p1 + p2 + plot_layout(ncol = 2)
```

```
Missing genes:
```

```
Warning message:
"Removed 1 row containing missing values or values outside the scale range
(`geom_point()`)."
Warning message:
"Removed 1 row containing missing values or values outside the scale range
(`geom_point()`)."
```



Big Oof. It looks like There isn't much difference here between the two populations. Let's do some statistical testing to see if there's a difference:

In [16]:
```
# 1 - Let's look at a SLR to see if we can improve scores

cd7_model <- lm(Tcell_score ~ CD7_adt, data = meta_df) # baseline model
tidy(cd7_model)

cd7cd45ra_model <- lm(Tcell_score ~ CD7_adt + CD45RA_adt, data = meta_df) # model w
tidy(cd7cd45ra_model)
```

A tibble: 2 × 5

term <chr>	estimate <dbl>	std.error <dbl>	statistic <dbl>	p.value <dbl>
(Intercept)	0.4387958724	0.001169412	375.227787	0.000000
CD7_adt	0.0006293015	0.000481905	1.305862	0.191624

A tibble: 3 × 5

term <chr>	estimate <dbl>	std.error <dbl>	statistic <dbl>	p.value <dbl>
(Intercept)	3.946758e-01	0.0031890687	123.7589521	0.000000e+00
CD7_adt	8.396653e-05	0.0004790292	0.1752848	8.608588e-01
CD45RA_adt	6.583985e-03	0.0004433590	14.8502351	1.875887e-49

Very surprising given the boxplots!

Interpreting Results From Model 1 (CD7 Only):

There is not enough evidence to make the claim that CD7_adt expression is associated with T-cell score ($p = 0.19$). There is no statistically significant evidence that CD7_adt alone predicts T-lineage gene module scores.

Interpreting Results from Model 2 (CD7 & CD455RA):

Holding CD7 constant, there is statistically significant evidence ($p < 0.001$) that CD45RA$^+$ expression is associated with an increase in T-lineage scores. The effect size is small but precise, suggesting CD45RA adds marginal predictive value to the model.

This suggests that while CD7$^+$ alone is not predictive, adding CD45RA$^+$ expression significantly improves the model's ability to predict T-lineage priming.

Takeaway:

> Based on the SLR model output, the addition of CD45RA_adt as a predictor significantly improves the model's fit to Tcell_score, as evidenced by a p-value < 0.001. This suggests that CD45RA$^+$ is a statistically significant predictor of T-lineage program scores when controlling for CD7 expression.

This is indicating that CD45RA$^+$CD7$^+$ cells may indeed reflect stronger or more consistent T-lineage priming, even if the effect is subtle on raw distributions.

Great! Let's now look at how the acquisition of CLEC affects this dynamic!:

In [17]:

```r
# Fit a model with interactions between CD7_adt, CD45RA_adt, and CLEC12A Group
interaction_df <- meta_df %>%
    filter(Group %in% c("Cneg", "Cpos")) # Filter to only Cneg and Cpos groups

interaction_model <- lm(Tcell_score ~ CD7_adt * Group + CD45RA_adt * Group, data =

# View model summary
tidy(interaction_model)
```

A tibble: 6 × 5

term	estimate	std.error	statistic	p.value
<chr>	<dbl>	<dbl>	<dbl>	<dbl>
(Intercept)	0.3808898720	0.0038586702	98.710139	0.000000e+00
CD7_adt	0.0009765631	0.0006105230	1.599552	1.097271e-01
GroupCpos	0.0373864464	0.0077038466	4.852958	1.233210e-06
CD45RA_adt	0.0097120363	0.0005460187	17.787005	8.617545e-70
CD7_adt:GroupCpos	-0.0017328004	0.0010174216	-1.703129	8.857245e-02
GroupCpos:CD45RA_adt	-0.0075479172	0.0010388983	-7.265309	3.976460e-13

Here's what the coefficients mean:

`(Intercept)` There is enough statistical evidence to make the claim that the **mean T-cell score** for **Cneg cells** when both **CD7_adt and CD45RA_adt = 0** is approximately **0.381**, and this is statistically significant at p < 0.05.

`CD7_adt` There is **not enough statistical evidence** to make the claim that CD7_adt is associated with T-cell score in Cneg cells (p = 0.110).

`GroupCpos` There is enough statistical evidence to make the claim that **Cpos cells** have, on average, a **T-cell score 0.0374 higher** than Cneg cells when CD7_adt and CD45RA_adt are both 0 (p < 0.001).

`CD45RA_adt` There is enough statistical evidence to make the claim that, among **Cneg cells**, for every 1 unit increase in **CD45RA_adt**, the mean **T-cell score increases by 0.0097**, which is statistically significant at p < 0.001.

`CD7_adt:GroupCpos` (Interaction) There is **not enough statistical evidence** to make the claim that the effect of CD7_adt on T-cell score is different between **Cpos** and **Cneg** cells (p = 0.0886).

`GroupCpos:CD45RA_adt` (Interaction) There is enough statistical evidence to make the claim that the effect of CD45RA_adt on T-cell score is different between Cpos and Cneg groups. Specifically, the **marginal effect of CD45RA_adt is reduced by 0.0075** in Cpos cells compared to Cneg (p < 0.001).

Interpretation

- CD45RA_adt is a **strong and significant predictor** of T-cell score in Cneg cells.
- GroupCpos cells overall have higher T-cell scores than Cneg cells when marker levels are held at 0.

- However, the strength of association between CD45RA_adt and T-cell score is **weaker** in Cpos cells.
- CD7_adt does **not appear to have a significant effect** on T-cell scores in either group.
- This supports the idea that **CD45RA**, not CD7, is more reliably associated with **T-lineage transcriptional activity** — particularly in Cneg cells.

Alignment with Fangwu's Paper These results are consistent with the biological interpretation expected from Fangwu Wang's CLEC paper, where **CLEC12A$^+$ (Cpos) cells are proposed to be myeloid-biased (especially neutrophil/monocyte)**, while **CLEC12A$^-$ (Cneg) cells** are more lymphoid-permissive.

According to Fangwu:

- CLEC12A$^+$ cells are **monocyte/neutrophil-biased** and **lose lymphoid competence** early.
- CLEC12A$^-$ cells retain **T-lineage priming potential**.
- CD45RA and CD7 are used as early T-cell–associated surface markers in the transition from HSC → T-lineage.

According to my results:

1. **CD45RA_adt** was a **strong predictor** of higher T-cell scores in **Cneg** (CLEC12A$^-$) cells. This aligns with FW, since Cneg is supposed to be more T-permissive.
2. The **CD45RA_adt × GroupCpos interaction** was **significantly negative**, meaning the effect of CD45RA expression on T-cell scores is **reduced in Cpos (CLEC12A$^+$) cells**, consistent with **a loss of lymphoid potential**.
3. **CD7_adt** was not a strong predictor — aligning with previous Aim 2 findings that **CD7 expression is not reliably correlated with transcriptional T-priming**.
4. **GroupCpos had a higher intercept**, but this is only meaningful at CD45RA = 0, which might not be biologically relevant — so it does **not contradict the overall directionality** of CLEC function.

Conclusion: This model, and these results, fit very well with the known lineage trajectory:

- **CD45RA$^+$ Cneg cells** show strong T-lineage transcriptional activity.
- **CD45RA$^+$ Cpos cells** do **not** show the same benefit — the **CD45RA marker does not convey T-lineage meaning in Cpos cells**.

This supports the idea that **Cpos cells are “biased” into a non-lymphoid fate**, even when they express CD45RA.

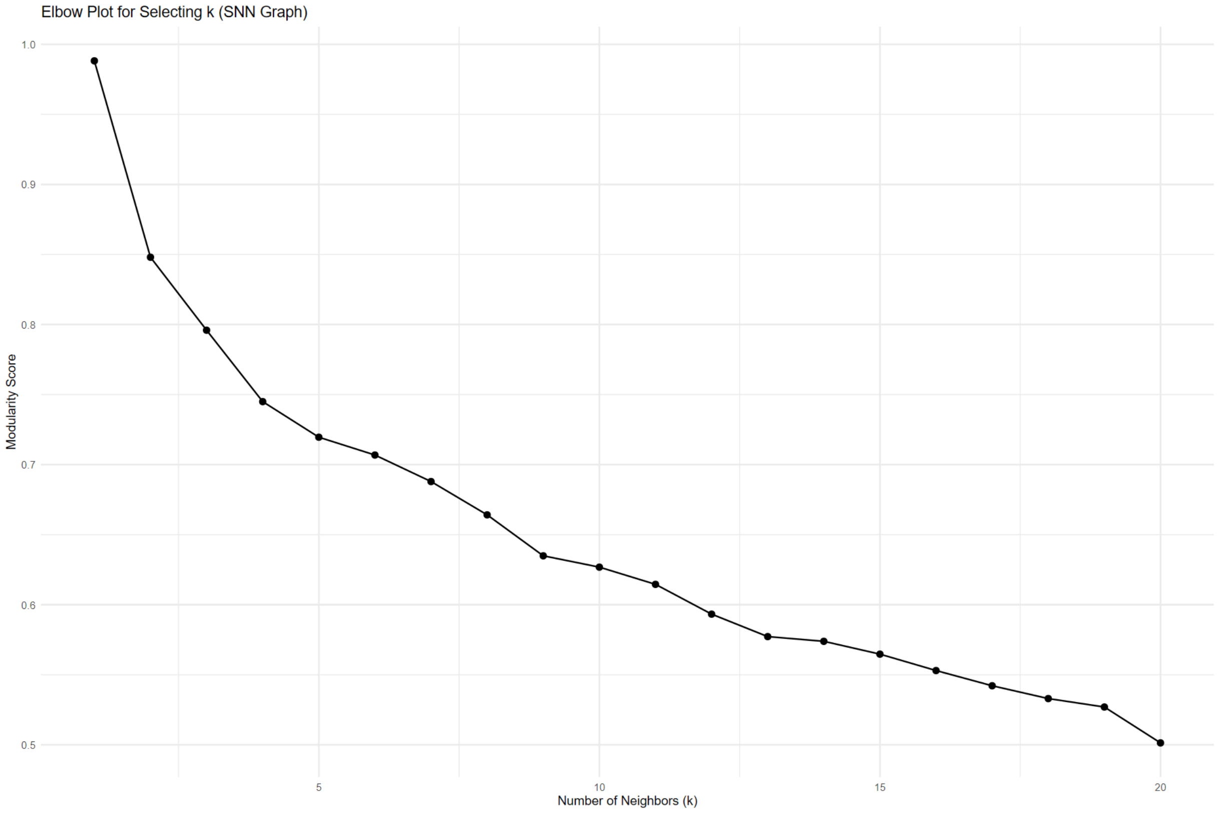
RQ4: What Are the Transcriptional Profiles and Phenotypes of CD45RA$^+$CD7$^+$ Subclusters With Elevated T-Lineage Scores (if any)?

Ok, now that we have some statistical support indicating that CD45RA+7+ is better than just CD7+ as a standalone marker for T-cell scoring, I wonder if there will be clustering within this double positive population that have especially elevated scores? Then, we could conduct DGE analysis on that cluster, look at the highly expressed CD markers, and perhaps add that as a predictor to our linear model as well - hopefully further improving T-cell scoring.

Let's begin by creating an SCE object with the relevant cells (that is, CD45RA+7+ DP cells). We will begin by subsetting our SCE object, clustering on T-sne, and then plotting an elbow plot to find the optimal number of clusters:

In [18]:

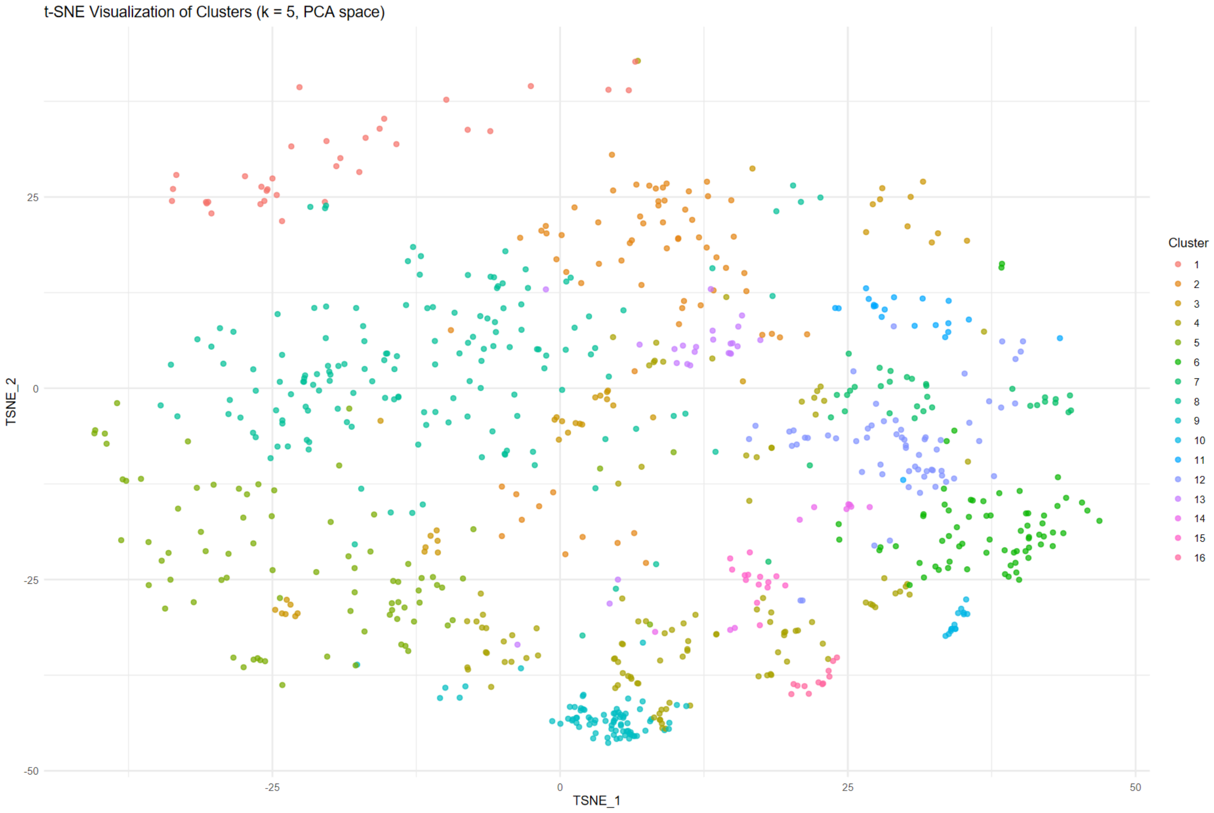
```
# Subsetting DP cells

sce_dp <- merge2_light[
  ,
  colData(merge2_light)$CD45RA_stat == "CD45RA+" &
    colData(merge2_light)$CD7_adtStat == "CD7+"
]

# Score Each Cell with T-lineage Program
tcell_genes_present <- intersect(tcell_genes, rownames(merge2_light))

dp_tcell_score <- colMeans(logcounts(sce_dp)[tcell_genes_present, , drop = FALSE])
colData(sce_dp)$Tcell_score <- dp_tcell_score


# Values of k to test
k_values <- seq(1, 20, by = 1)

# Placeholder for modularity scores
modularity_scores <- numeric(length(k_values))

# Loop through k and calculate modularity of the clusters
for (i in seq_along(k_values)) {
  k <- k_values[i]
  g <- buildSNNGraph(sce_dp, use.dimred = "PCA", k = k)
  clust <- cluster_walktrap(g)
  modularity_scores[i] <- modularity(clust)
}


elbow_df <- tibble(k = k_values, Modularity = modularity_scores)

ggplot(elbow_df, aes(x = k, y = Modularity)) +
  geom_point(size = 2) +
  geom_line() +
  theme_minimal() +
  labs(
    title = "Elbow Plot for Selecting k (SNN Graph)",
    x = "Number of Neighbors (k)",
    y = "Modularity Score"
  )
```



I would say the elbow here is at 4, so let's go with that. Especially since I am trying to find a likely elusive population.

In [19]:

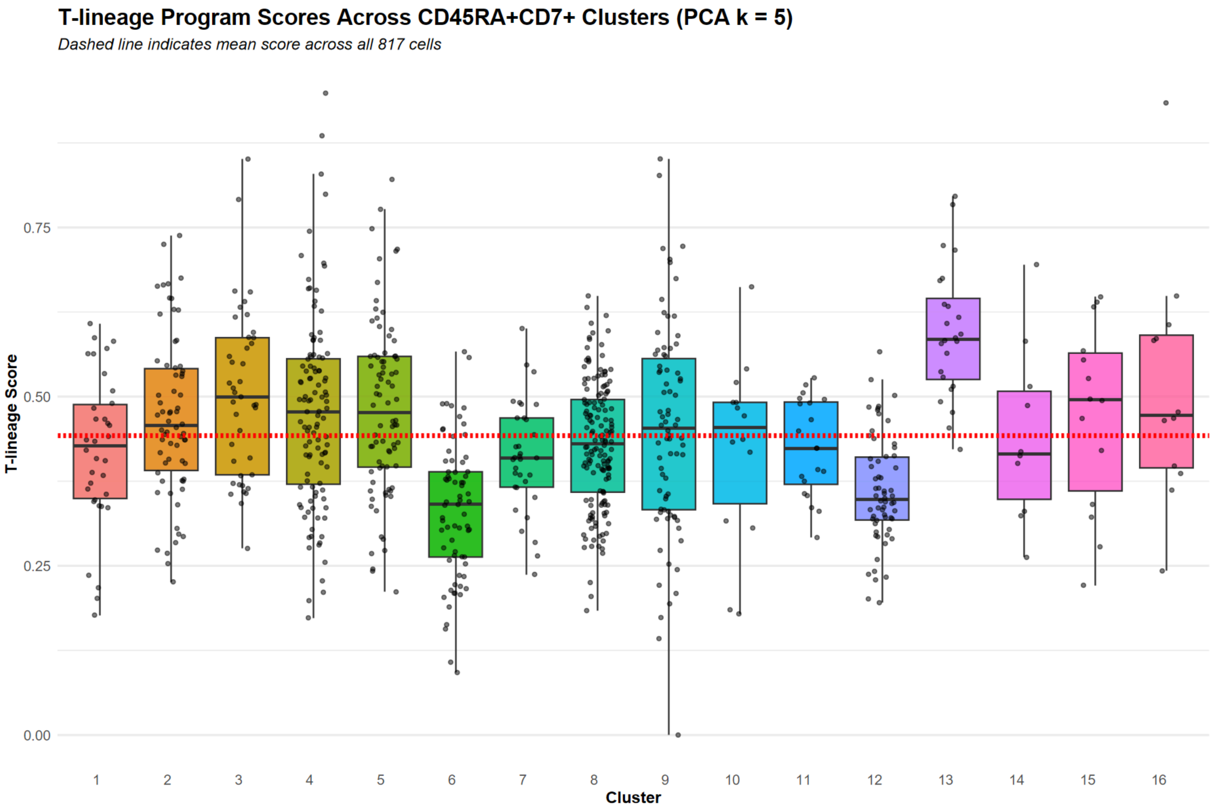
```r
# Build SNN graph using PCA
g_pca <- buildSNNGraph(sce_dp, use.dimred = "PCA", k = 4)

# Cluster using Walktrap algorithm
pca_clusters <- igraph::cluster_walktrap(g_pca)$membership

# Save to colData
colLabels(sce_dp) <- factor(pca_clusters)

# Extract t-SNE coordinates (for plotting only)
tsne_coords <- reducedDim(sce_dp, "TSNE")

# Create plotting dataframe
tsne_df <- data.frame(
  TSNE_1 = tsne_coords[, 1],
  TSNE_2 = tsne_coords[, 2],
  Cluster = as.factor(colLabels(sce_dp))
)

# Plot
ggplot(tsne_df, aes(x = TSNE_1, y = TSNE_2, color = Cluster)) +
  geom_point(alpha = 0.7, size = 1.5) +
  theme_minimal() +
  labs(
    title = "t-SNE Visualization of Clusters (k = 5, PCA space)",
    color = "Cluster"
  )
```



Amazing! Let's now look at the T-lineage score of each cluster. I pray we find something that's elevated within this population:

In [20]:

```r
# Create plotting dataframe
boxplot_df <- data.frame(
  Cluster = colLabels(sce_dp),
  TlineageScore = colData(sce_dp)$Tcell_score
)

# Compute global mean
mean_score <- mean(boxplot_df$TlineageScore, na.rm = TRUE)

# Plot
ggplot(boxplot_df, aes(x = Cluster, y = TlineageScore, fill = Cluster)) +
  geom_boxplot(outlier.shape = NA, alpha = 0.85) +
  geom_jitter(width = 0.2, size = 1.2, alpha = 0.5, color = "black") +
  geom_hline(yintercept = mean_score, linetype = "dashed", color = "red", linewidth
  scale_fill_manual(values = scales::hue_pal()(length(unique(boxplot_df$Cluster))))
  labs(
    title = "T-lineage Program Scores Across CD45RA+CD7+ Clusters (PCA k = 5)",
    subtitle = paste("Dashed line indicates mean score across all", nrow(boxplot_df
    x = "Cluster",
    y = "T-lineage Score"
  ) +
  theme_minimal(base_size = 14) +
  theme(
    axis.text.x = element_text(hjust = 1),
    legend.position = "none",
    panel.grid.major.x = element_blank(),
    title = element_text(size = 16, face = "bold"),
    plot.subtitle = element_text(size = 14, face = "italic"),
    axis.title = element_text(size = 14),
    axis.text = element_text(size = 12)
  )
```


T-lineage Program Scores Across CD45RA+CD7+ Clusters (PCA k = 5)

Dashed line indicates mean score across all 817 cells

This looks very promising! Let's do a statistical test comparing the T-lineage score of each cluster against the population mean:

In [21]:

```
# Step 1: Compute overall mean T-lineage score
overall_mean <- mean(boxplot_df$TlineageScore, na.rm = TRUE)

# Step 2: Apply a one-sample t-test for each cluster vs. the overall mean
cluster_tests <- boxplot_df %>%
  group_by(Cluster) %>%
  summarise(
    n = n(),
    mean_score = mean(TlineageScore),
    t_test = list(t.test(TlineageScore, mu = overall_mean, alternative = "greater"))
  ) %>%
  mutate(
    p_value = sapply(t_test, function(x) x$p.value),
    significance = case_when(
      p_value < 0.001 ~ "***",
      p_value < 0.01 ~ "**",
      p_value < 0.05 ~ "*",
      TRUE ~ "ns"
    )
  ) %>%
  select(Cluster, n, mean_score, p_value, significance) %>%
  filter(significance != "ns") %>% # Filter out non-significant results
  arrange(desc(mean_score)) # Sort by scoring value
```

```
# View results
(cluster_tests)
```

A tibble: 5 × 5

Cluster	n	mean_score	p_value	significance
<fct>	<int>	<dbl>	<dbl>	<chr>
13	24	0.5951306	4.593790e-08	***
3	41	0.5037379	1.543730e-03	**
5	80	0.4825490	3.126881e-03	**
4	108	0.4771422	6.174880e-03	**
2	66	0.4692697	3.558985e-02	*

Great! There is enough evidence to claim from a One-Sided T.test result that clusters 13, 3, 5 have very significantlu higher T-cell scores, while Clusters 2 and 4 also have some significance!

Amazing! Let's see what genes are differentially expressed in each of these clusters compared to the remaining DP population. I am going to start by looking at the top 20 significant genes overall by logFC, but also characterize the Top CD genes that are expressed in that cluster. I will also create a list of "Jackpot genes" - that are a lot more specific to T-lineage commitment than the general scoring function, and see if those genes are anywhere in the DGE list, and where they are positioned.

I am particularly interested in CD genes, since I'm curious if I can find a more restricted T-lineage population within this DP population. |

Lets begin by conducting DGE here:

In [22]:
```
run_dge_for_cluster <- function(expr_matrix, cluster_labels, target_cluster) {
  stopifnot(length(cluster_labels) == ncol(expr_matrix))

  # Create binary group vector (target cluster vs rest)
  group_vector <- ifelse(cluster_labels == target_cluster, "Target", "Other")

  # Create design matrix
  group_factor <- factor(group_vector, levels = c("Other", "Target")) # "Other" is
  design <- model.matrix(~group_factor)

  # Fit model
  fit <- lmFit(expr_matrix, design)
  fit <- eBayes(fit)

  # Get top DE genes (coef=2 because 'Target' is the second factor level)
  top_genes <- topTable(fit, coef = 2, number = Inf, sort.by = "logFC")

  # Get CD genes only (e.g., CD3, CD7, CD45, etc.)
  cd_genes <- top_genes[grep("^CD\\d+", top_genes$ID), ]

  return(list(
    top_genes = top_genes,
    top_cd_genes = cd_genes
  ))
}
```

Now, let's run all the DGE on all of our clustering:

In [23]:

```
# Make sure your sce_dp object and clusters are defined
cluster_vector <- colLabels(sce_dp)

# Run for cluster 6:
dge_cluster13 <- (run_dge_for_cluster(
    expr_matrix = logcounts(sce_dp),
    cluster_labels = cluster_vector,
    target_cluster = "13"
)
)



dge_cluster3 <- (run_dge_for_cluster(
    expr_matrix = logcounts(sce_dp),
    cluster_labels = cluster_vector,
    target_cluster = "3"
)
)

dge_cluster5 <- (run_dge_for_cluster(
    expr_matrix = logcounts(sce_dp),
    cluster_labels = cluster_vector,
    target_cluster = "5"
)
)

dge_cluster2 <- (run_dge_for_cluster(
    expr_matrix = logcounts(sce_dp),
    cluster_labels = cluster_vector,
    target_cluster = "2"
)
)

dge_cluster4 <- (run_dge_for_cluster(
    expr_matrix = logcounts(sce_dp),
    cluster_labels = cluster_vector,
    target_cluster = "4"
)
)
```

```
Warning message:
"Zero sample variances detected, have been offset away from zero"
Warning message:
"Zero sample variances detected, have been offset away from zero"
Warning message:
"Zero sample variances detected, have been offset away from zero"
Warning message:
"Zero sample variances detected, have been offset away from zero"
Warning message:
"Zero sample variances detected, have been offset away from zero"
```

Finally, let's look at the results from our Clustering. We will go through each Cluster one by one, and generate volcano plots. Let's first write a function to automate the task:

In [24]:

```r
draw_cluster_volcano <- function(result_df) {
  # Return early if input is NULL
  if (is.null(result_df)) {
    message("No DEGs to plot. Input dataframe is NULL.")
    return(NULL)
  }

  # Jackpot genes
  jackpot_genes <- c(
    "RAG2", "NOTCH1", "CD3D", "CD3E", "CD3G",
    "TCF7", "GATA3", "BCL11B", "RAG1", "DTX1"
  )

  # Thresholds
  fc_thresh <- 0.5
  pval_thresh <- 0.05

  # Annotate significance
  df <- result_df %>%
    mutate(
      Significant = case_when(
        adj.P.Val < pval_thresh & logFC > fc_thresh ~ "Up",
        adj.P.Val < pval_thresh & logFC < -fc_thresh ~ "Down",
        TRUE ~ "Not Significant"
      ),
      IsJackpot = ID %in% jackpot_genes
    )

  # Labels: top DEGs + any jackpot genes
  top_labels <- df %>%
    filter(Significant != "Not Significant") %>%
    arrange(desc(logFC)) %>%
    slice_head(n = 50)

  jackpot_labels <- df %>% filter(IsJackpot)
  label_df <- bind_rows(top_labels, jackpot_labels) %>% distinct(ID, .keep_all = TR

  # Volcano plot
  ggplot(df, aes(x = logFC, y = -log10(adj.P.Val))) +
    geom_vline(xintercept = c(-fc_thresh, fc_thresh), linetype = "dashed", color =
    geom_hline(yintercept = -log10(pval_thresh), linetype = "dotted", color = "purp
    geom_point(data = subset(df, !IsJackpot), aes(color = Significant), alpha = 0.8
    geom_point(data = subset(df, IsJackpot), color = "green", alpha = 0.8, size = 2
    ggrepel::geom_text_repel(
      data = subset(label_df, IsJackpot),
      aes(label = ID),
      color = "#139a13",
      size = 4, box.padding = 0.3, show.legend = FALSE
    ) +
    ggrepel::geom_text_repel(
      data = subset(label_df, !IsJackpot),
      aes(label = ID),
      color = "black",
      size = 4, box.padding = 0.3, show.legend = FALSE, max.overlaps = Inf
    ) +
    scale_color_manual(values = c("Up" = "#d73027", "Down" = "#4575b4", "Not Signif
    labs(
      title = "Volcano Plot: Differential Expression Between Cluster and Remaining
      subtitle = paste0("Dashed = log2FC ±", fc_thresh, ", Dotted = adj.P.Val <", p
      x = "log2 Fold Change",
      y = expression(-log[10]("Adjusted P-Value")),
      color = "Significance"
    ) +
    theme_minimal(base_size = 14) +
    theme(
      plot.title = element_text(face = "bold", size = 16),
      plot.subtitle = element_text(size = 12),
      axis.title = element_text(face = "bold"),
      legend.position = "right"
    )
}
```

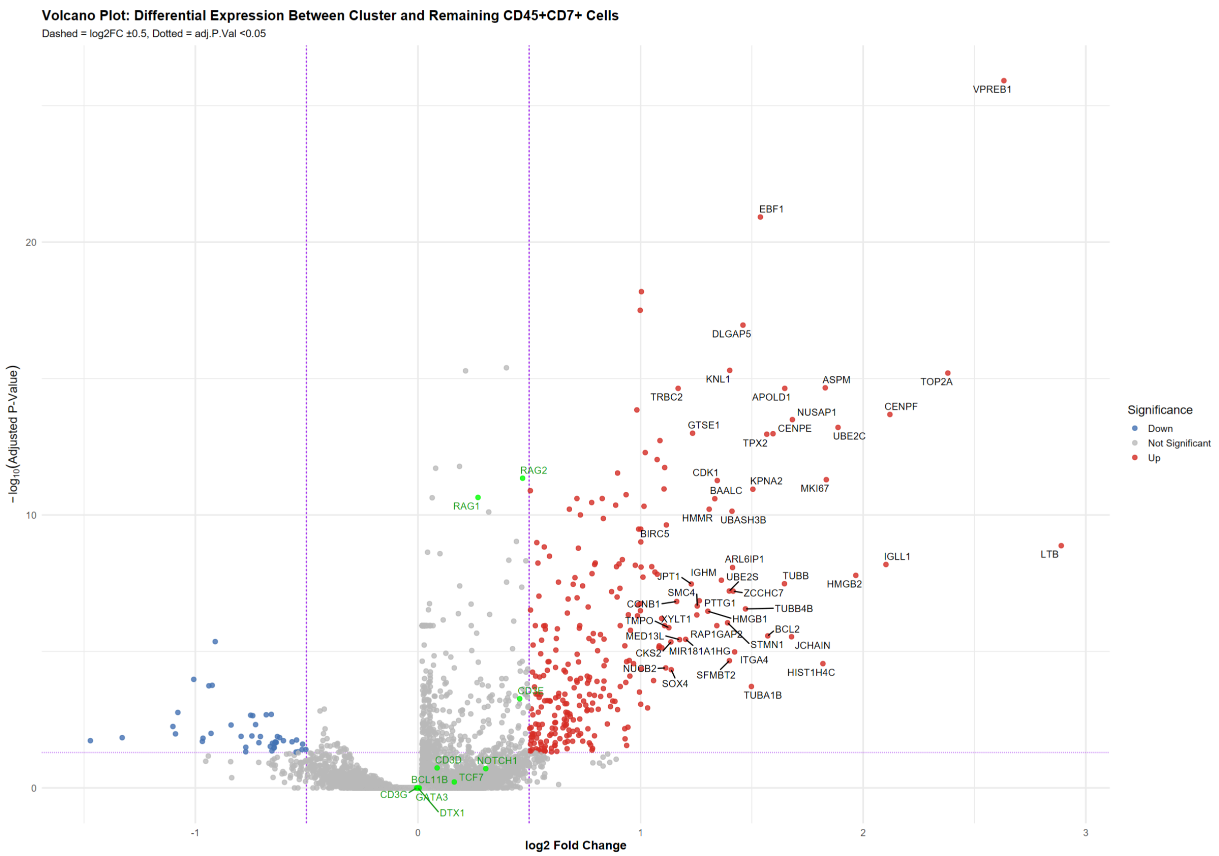
Characterizing Cluster 13 (Best Scoring Cluster by a Really Wide Margin)

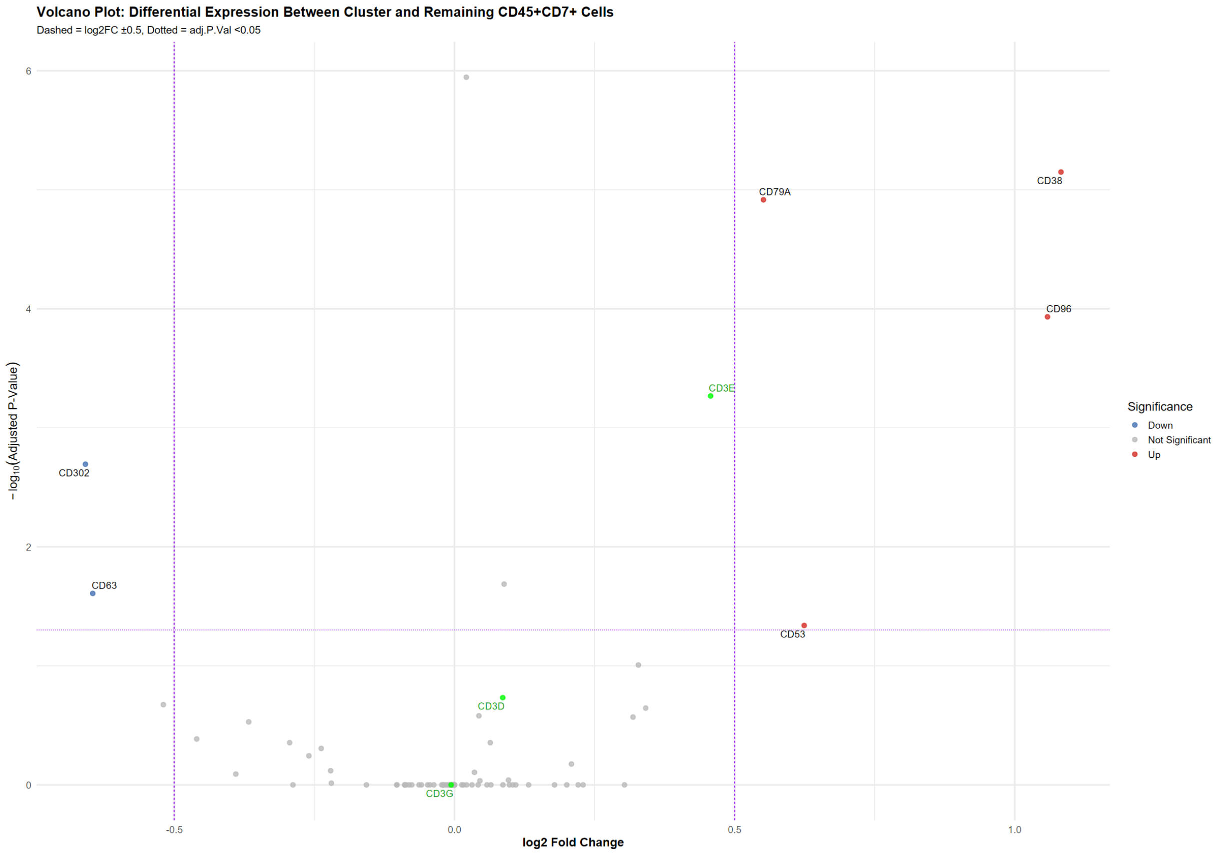
Now, let's begin with Cluster 13. Let's generate a figure that combines both Volcano plots:

In [25]:

```
options(repr.plot.width = 20, repr.plot.height = 14)

clust13_geneVolcano <- draw_cluster_volcano(dge_cluster13$top_genes)
clust13_cdVolcano <- draw_cluster_volcano(dge_cluster13$top_cd_genes)


print(clust13_geneVolcano)
print(clust13_cdVolcano)
```

```
Warning message:
"ggrepel: 5 unlabeled data points (too many overlaps). Consider increasing max.overl
aps"
```





Very Interesting!!!!!!

On the **first plot (differentially expressed genes)**, we see two genes that are unique **only to the T-lineage compartment of the lymphoid subset**, and not to B-cells. These are:

1. `TRBC2` : Encodes TCR β-chain constant region. T-cell receptor component — exclusively in T cells. UniProt: P01850 (TRBC2)

2. `UBASH3B (Sts-1)` : Regulator of TCR signaling, specifcially CD3 complex assembly. Expressed primarily in T cells (and NK to a much lesser extent), but not in B cells. KEGG/UniProt: Q9H1A4 (UBASH3B)

Additionally, we see strong upregulation of `HMMR` (Hyaluron Mediated Motility Receptor), which is as proliferation associated regulator often seen in early thymocytes, specifically in the double negative thymocyte stage. We also see `BAALC`, which is expressed in immature thymocytes, mostly during early T-cell development. It is enriched in T-cells (but not unique to it), and is also enriched in AML.

Finally, we see that two key Jackpot Genes (RAG1 and RAG2)

On the **second plot (differentially expressed CD Markers)**, we see two very important results. Specifically, very very strong upregulation of `CD96`. CD96 is a Adhesion molecule involved in immune synapse formation. Its expression is mostly in NK cells, some T cell subsets (e.g., CD8+), especially in cytotoxic contexts. It is highly enriched in T cells and NK cells, but not exclusive to it. Stil, a very promising result.

However, if we look at the JackPot Gene CD3E, it is very very close to being accepted as substantially logFC upregulated. CD3E (Epsilon) is a pan-T-cell marker that reacts with an antigen present at the surface and cytoplasm of both immature and mature T lymphocytes, including NK/T cells. CD3e is also expressed in almost all T-cell lymphomas and leukaemias, and can therefore be used to distinguish them from B-cell and myeloid neoplasms.

Overall, it does look like this cluster is uniquely T-cell primed, and is therefore scoring substantially higher than the remaining clusters.

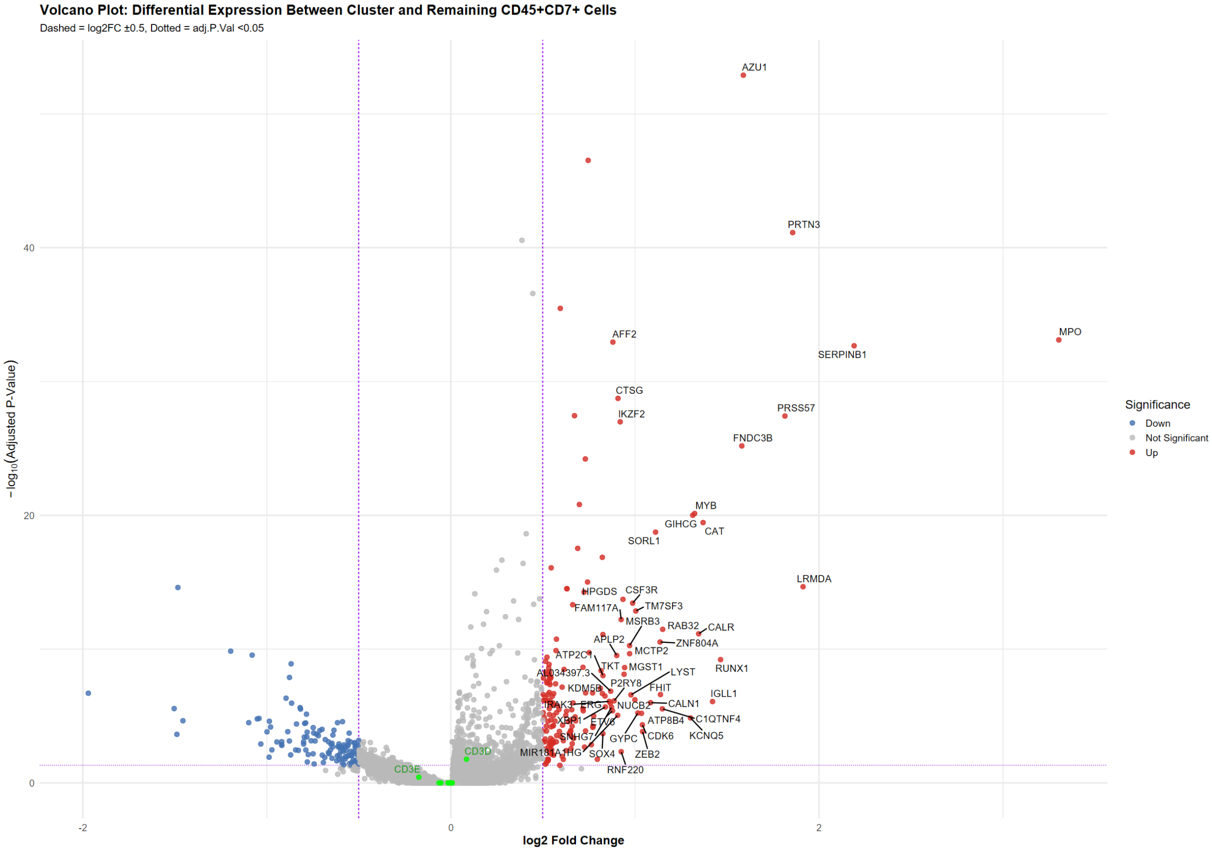
> Specifically, this raises the question: Can CD96 or CD3E be used as predictors to further improve T-lineage tracking.

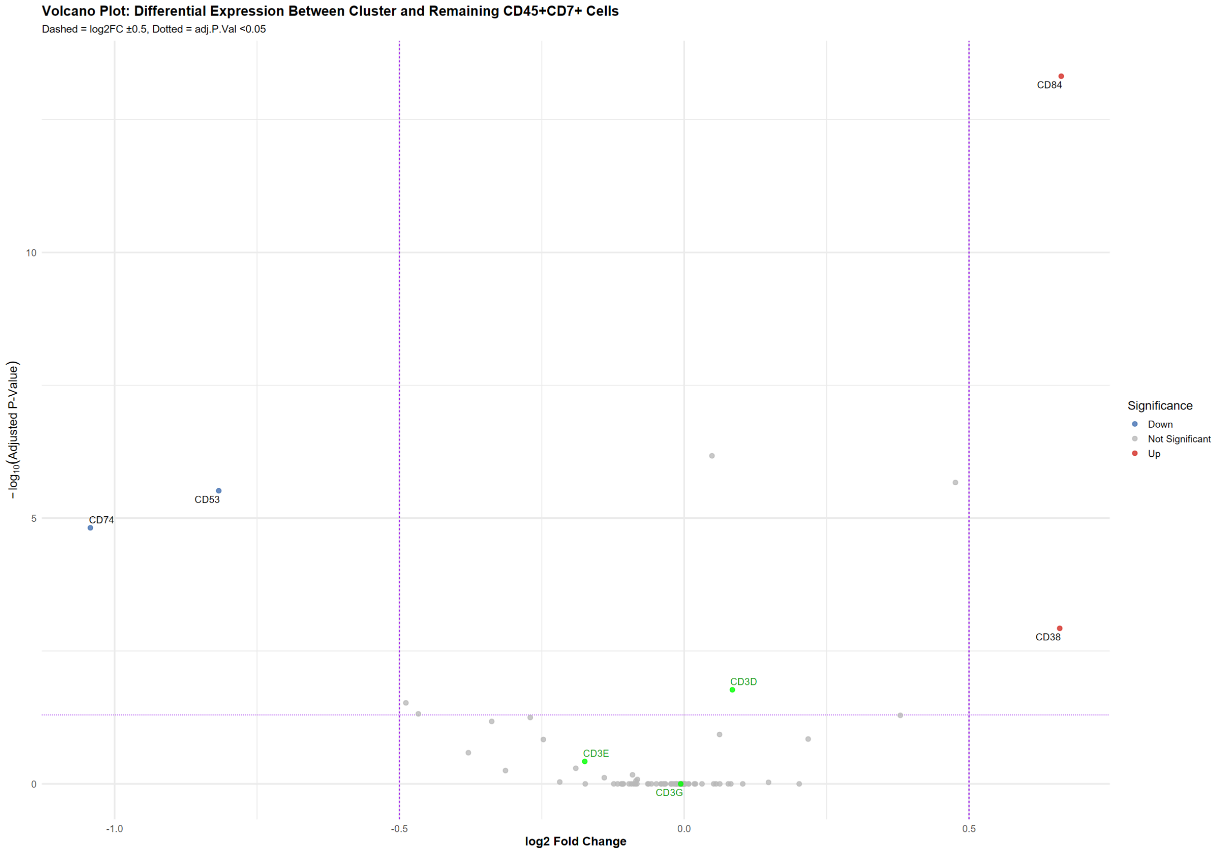
Characterizing Cluster 3

In [26]:

```r
options(repr.plot.width = 20, repr.plot.height = 14)

clust3_geneVolcano <- draw_cluster_volcano(dge_cluster3$top_genes)
clust3_cdVolcano <- draw_cluster_volcano(dge_cluster3$top_cd_genes)


print(clust3_geneVolcano)
print(clust3_cdVolcano)
```

```
Warning message:
"ggrepel: 9 unlabeled data points (too many overlaps). Consider increasing max.overl
aps"
Warning message:
"ggrepel: 8 unlabeled data points (too many overlaps). Consider increasing max.overl
aps"
```





This is very interesting, and a little concering. Among the upregulated genes:

- `AZU1` : Azurocidin, stored in neutrophil granules.
- `PRTN3` : Proteinase 3, another neutrophil granule enzyme.
- `CTSG` : Cathepsin G, strongly enriched in neutrophil granules.
- `MPO` : Myeloperoxidase, gold-standard for neutrophil lineage.

These together strongly suggest a granulocytic/neutrophil signature. Then how is this the second best T-cell scoring cluster?

Well, for starters, cluster 3 is only marginally performing better than the mean population score. So it is expected to have some non-specific leakiness. As such, my interpretation of this cluster is that it may represent:

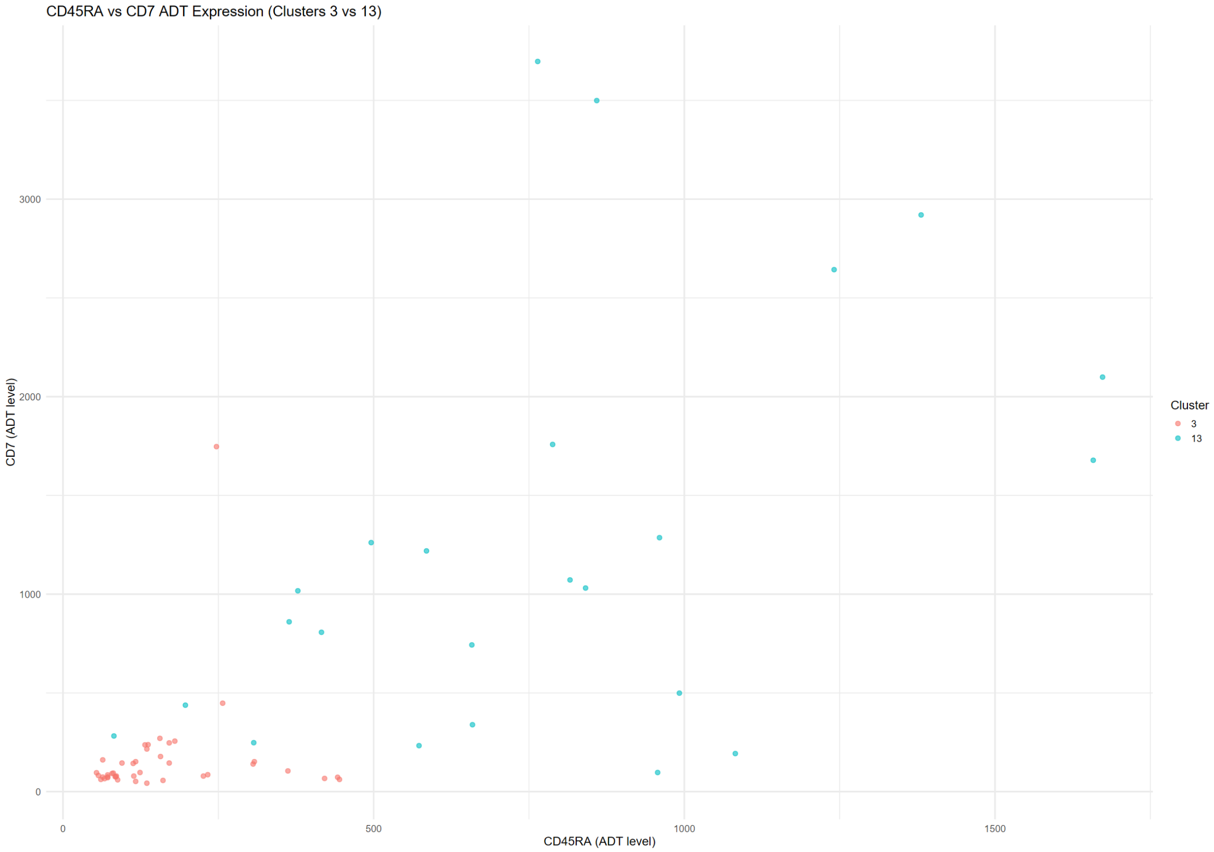
1. A contaminating neutrophil-primed population that co-expresses CD45RA and CD7 at low/moderate levels (CD7 can be expressed at low levels in some myeloid contexts).
2. A bipotent myelo-lymphoid progenitor still capable of limited T-program expression (i.e. CD3D/CD3G), but biased toward myeloid fate.
3. A cell-state artifact, where residual expression of T-program genes remains as cells commit toward granulocyte fate.

This also means there's no point checking for clusters below this - there's likely to be non-T lineage leakiness in clusters than scored below this one, and as such, I will not be evaluating cluster 5, 2 and 4.

As a sanity check, let's compare the CD7 and CD45RA expression levels of Cluster 13 and Cluster 3. I'm hoping the Cluster 13 cells will be a lot higher overall: -->

In [27]:

```
# Extract ADT matrix
adt <- assay(altExp(sce_dp, "Antibody Capture"))

# Extract values (note transposition to get values per cell)
cd7_vals <- as.vector(adt["TOTALSEQB_CD7", ])
cd45ra_vals <- as.vector(adt["TOTALSEQB_CD45RA", ])


adt_df <- data.frame(
  CD45RA = cd45ra_vals,
  CD7 = cd7_vals,
  Cluster = as.factor(colData(sce_dp)$label)
)

# Subset to clusters 3 and 13
adt_subset <- adt_df[as.numeric(as.character(adt_df$Cluster)) %in% c(3, 13), ]

# Plot
Cluster13_Gating_Plot <- ggplot(adt_subset, aes(x = CD45RA, y = CD7, color = Cluste
  geom_point(alpha = 0.6, size = 2) +
  labs(
    title = "CD45RA vs CD7 ADT Expression (Clusters 3 vs 13)",
    x = "CD45RA (ADT level)",
    y = "CD7 (ADT level)"
  ) +
  theme_minimal(base_size = 14) +
  theme(legend.position = "right")

print(Cluster13_Gating_Plot)
```



CD45RA vs CD7 ADT Expression (Clusters 3 vs 13)

Aha! A very Clear boundary between Cluster 3 (Neutrophil biased) and Cluster 13 (T-Cell Biased) cells within the CD45RA+CD7+ subset. While both clusters are positive, the T-lineage subset is a lot more positive!

Let's do a quick classifier logistic regression model clustering decision more:

In [28]:

```
# Create binary outcome: 1 = Cluster 13, 0 = Cluster 3
adt_subset$Label <- ifelse(adt_subset$Cluster == "13", 1, 0)

# Fit logistic regression model
model <- glm(Label ~ CD45RA + CD7, data = adt_subset, family = "binomial")

# Summarize model
tidy(model, exponentiate = TRUE)
```

A tibble: 3 × 5

term	estimate	std.error	statistic	p.value
<chr>	<dbl>	<dbl>	<dbl>	<dbl>
(Intercept)	0.00851711	1.167023158	-4.083619	4.433966e-05
CD45RA	1.00949650	0.003226377	2.929506	3.395011e-03
CD7	1.00227470	0.001220653	1.861396	6.268825e-02

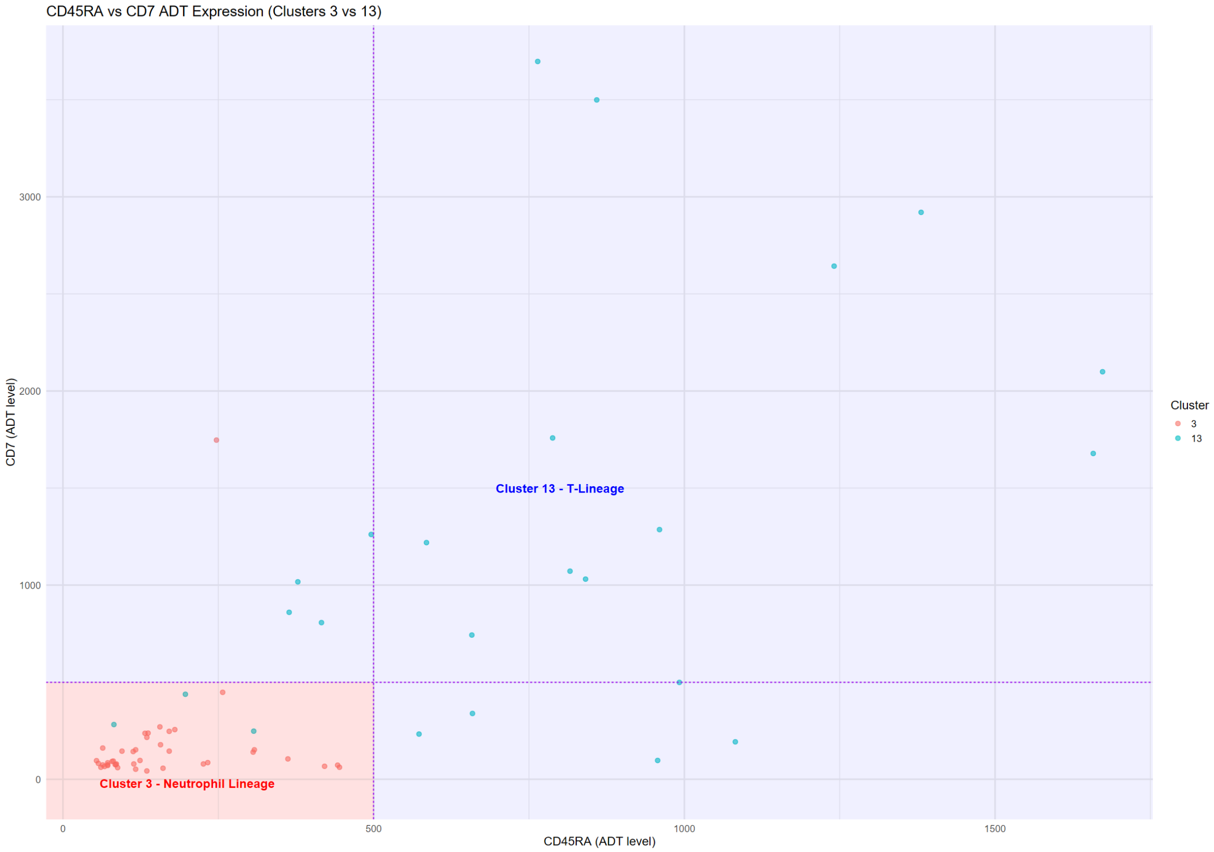
Here's my interpretation of these results:

- There is enough statistical evidence to make the claim that when CD45RA and CD7 expression is 0, the odds of being assigned to Cluster 13 (Lymphoid/T Cell Fate) are 0.0009.
- There is enough statistical evidence to make the claim that, while holding CD7 constant, increasing CD45RA ADT expression by one unit increases the odds of being assigned to Cluster 13 are 1.0095, or increased by 0.9%
- There is not enough eviednce to make the claim that whilel holding CD45RA constant, increasing CD7 affects the log-odds of being assigned to Cluster 13.

Neat - this also quantifies something we can already see in the graph - The decision is primarily being driven by CD45RA expression, rather than the CD7 expression

In [29]:

```
Lymphoid_Decision_Boundary <- ggplot(adt_subset, aes(x = CD45RA, y = CD7, color = C
    # Scatter points
    geom_point(alpha = 0.6, size = 2) +

    # Shaded red quadrant: bottom-left
    annotate("rect",
        xmin = -Inf, xmax = 500, ymin = -Inf, ymax = 500,
        fill = "red", alpha = 0.1
    ) +

    # Shaded blue quadrants: everything else
    annotate("rect",
        xmin = 500, xmax = Inf, ymin = -Inf, ymax = Inf,
        fill = "blue", alpha = 0.05
    ) +
    annotate("rect",
        xmin = -Inf, xmax = 500, ymin = 500, ymax = Inf,
        fill = "blue", alpha = 0.05
    ) +

    # Quadrant labels
    annotate("text",
        x = 200, y = -20, label = "Cluster 3 - Neutrophil Lineage",
        color = "red", size = 5, fontface = "bold"
    ) +
    annotate("text",
        x = 800, y = 1500, label = "Cluster 13 - T-Lineage",
        color = "blue", size = 5, fontface = "bold"
    ) +

    # Guide lines
    geom_hline(yintercept = 500, linetype = "dashed", color = "purple") +
    geom_vline(xintercept = 500, linetype = "dashed", color = "purple") +

    # Labels and theme
    labs(
        title = "CD45RA vs CD7 ADT Expression (Clusters 3 vs 13)",
        x = "CD45RA (ADT level)",
        y = "CD7 (ADT level)"
    ) +
    theme_minimal(base_size = 14) +
    theme(legend.position = "right")

Lymphoid_Decision_Boundary
```



RQ5 How Does the New T-Lineage Primed Cluster Fit Within Our Population in Psuedotime?

Amazing results. For the final RQ5, we want to characterize where Cluster 13 fits within our developmental model in psuedotime. Our goal is to establish the psuedotemporal order of Cluster 13 cells, specifically to characterize their position relative to CD45RA positivity and CD7 positivity.

We will answer this question with the following outline:

1. Computing the Psuedotime
2. Subsetting Clusters to Make Combinations of Interest
3. Visualizing Results

Let's begin by computing psuedotime first:

```
In [30]: colnames(colData(merge2_light))
```

'Group' · 'Day' · 'Phenotype' · 'CD7_gene' · 'CD7_geneStat' · 'CD7_adt' · 'CD7_adtStat' · 'CD45RA_adt' · 'CD45RA_stat' · 'CD3e_gene' · 'CD3e_geneStat' · 'Tcell_score'

In [31]:

```
# breakpoint breakpoint breaker breakpoint

library(scran)
library(igraph)

snn <- buildSNNGraph(merge2_light, use.dimred = "PCA", k = 15)
clusters <- igraph::cluster_walktrap(snn)$membership
colData(merge2_light)$pseudo_cluster <- factor(clusters)
```

In [32]:

```
merge2_light <- slingshot(merge2_light,
    clusterLabels = "pseudo_cluster",
    reducedDim = "PCA.cc"
)
```

In [33]:

```
colnames(colData(merge2_light))
```

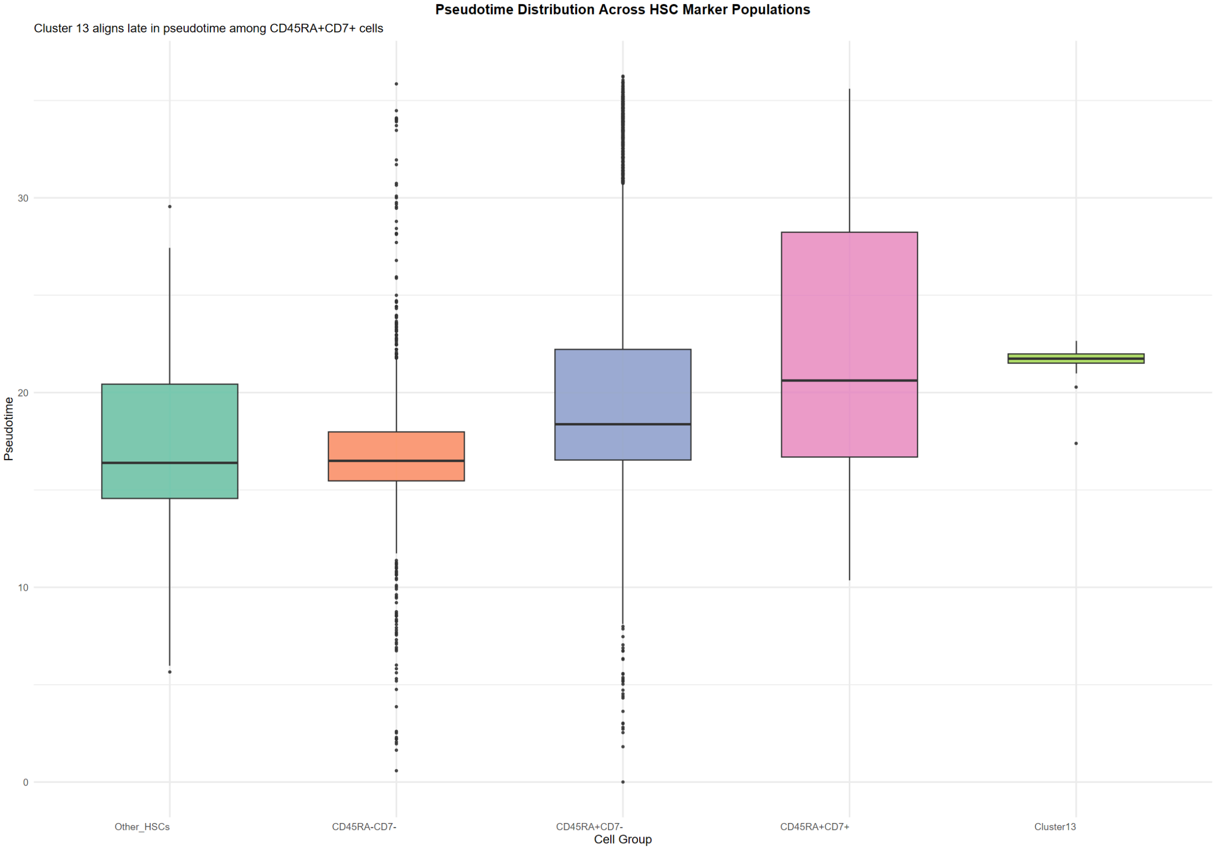
```
'Group' · 'Day' · 'Phenotype' · 'CD7_gene' · 'CD7_geneStat' · 'CD7_adt' · 'CD7_adtStat' ·
'CD45RA_adt' · 'CD45RA_stat' · 'CD3e_gene' · 'CD3e_geneStat' · 'Tcell_score' · 'pseudo_cluster' ·
'slingshot' · 'slingPseudotime_1' · 'slingPseudotime_2' · 'slingPseudotime_3' ·
'slingPseudotime_4' · 'slingPseudotime_5' · 'slingPseudotime_6' · 'slingPseudotime_7' ·
'slingPseudotime_8'
```

In [34]:

```
# Labelling cells that were in Cluster 13
colData(merge2_light)$is_cluster13 <- colnames(merge2_light) %in% colnames(sce_dp[,

plot_df <- data.frame(
  pseudotime = colData(merge2_light)$slingPseudotime_1,
  Cluster13 = colData(merge2_light)$is_cluster13,
  CD7 = colData(merge2_light)$CD7_adtStat,
  CD45RA = colData(merge2_light)$CD45RA_stat
)

plot_df$Group <- dplyr::case_when( # make sure you go from more specific to less sp
  plot_df$Cluster13 ~ "Cluster13",
  plot_df$CD45RA == "CD45RA+" & plot_df$CD7 == "CD7+" ~ "CD45RA+CD7+",
  plot_df$CD45RA == "CD45RA+" & plot_df$CD7 == "CD7-" ~ "CD45RA+CD7-",
  plot_df$CD45RA == "CD45RA-" & plot_df$CD7 == "CD7-" ~ "CD45RA-CD7-",
  TRUE ~ "Other_HSCs"
)

plot_df$Group <- factor(plot_df$Group, levels = c("Other_HSCs", "CD45RA-CD7-", "CD4

plot_df$Group %>%
  as.data.frame() %>%
  table()

psuedotime_plot <- ggplot(plot_df, aes(x = Group, y = pseudotime, fill = Group)) +
  geom_boxplot(alpha = 0.85, outlier.size = 0.8, width = 0.6) +
  scale_x_discrete(labels = group_labels) +
  scale_fill_brewer(palette = "Set2") + # Nice pastel colors
  labs(
    title = "Pseudotime Distribution Across HSC Marker Populations",
    subtitle = "Cluster 13 aligns late in pseudotime among CD45RA+CD7+ cells",
    x = "Cell Group",
    y = "Pseudotime"
  ) +
  theme_minimal(base_size = 14) +
  theme(
    axis.text.x = element_text(angle = 0, hjust = 1),
    plot.title = element_text(face = "bold", size = 16, hjust = 0.5),
    legend.position = "none"
  )


print(psuedotime_plot)
```

```
.
 Other_HSCs CD45RA-CD7- CD45RA+CD7- CD45RA+CD7+   Cluster13
       1419        1135        8810         793          24
```

```
Warning message:
"Removed 3413 rows containing non-finite outside the scale range
(`stat_boxplot()`)."
```



This is phenomeal! Here's an interpretation of my results. The pseudotime plot from RQ5 shows the distribution of pseudotime values across different HSC marker populations, including the newly identified **Cluster 13** (CD45RA^{+}CD7^{+} T-lineage primed cells). We see:

1. **Cluster 13 aligns late in pseudotime**:

    - Cluster 13 cells are positioned significantly later in pseudotime compared to other populations, including the broader CD45RA^{+}CD7^{+} population.

   - This suggests that Cluster 13 represents a more advanced or committed state within the T-lineage trajectory.

2. **CD45RA^{+}CD7^{+} cells span a wide pseudotime range**:
   - The broader CD45RA^{+}CD7^{+} population spans a large range of pseudotime, indicating heterogeneity within this group.
   - Cluster 13 appears to represent a subset of these cells that are further along the T-lineage differentiation pathway.
3. **CD45RA^{+}CD7^{-} cells precede CD45RA^{+}CD7^{+} in pseudotime**:
   - CD45RA^{+}CD7^{-} cells are positioned earlier in pseudotime compared to CD45RA^{+}CD7^{+} cells.
   - This supports the hypothesis that CD45RA^{+}CD7^{-} cells are an intermediate state that transitions into CD45RA^{+}CD7^{+} cells during T-lineage priming.
4. **CD45RA^{-}CD7^{-} cells are the earliest in pseudotime**:
   - CD45RA^{-}CD7^{-} cells are positioned at the earliest pseudotime values, consistent with their role as unprimed or undifferentiated HSCs.
5. **Cluster 13 as a distinct late-stage T-lineage population**:
   - The distinct pseudotime positioning of Cluster 13 suggests it represents a specialized subset of T-lineage primed cells, potentially closer to full T-lineage commitment.

This supports the notion that:

- **Cluster 13** likely represents a critical stage in T-lineage differentiation, where cells exhibit strong T-lineage transcriptional activity and surface marker expression (e.g., CD45RA^{+}CD7^{+}).
- The pseudotime progression aligns with the hypothesis that CD45RA expression precedes CD7 expression, and Cluster 13 cells are the culmination of this trajectory.
- This reinforces the utility of CD45RA^{+}CD7^{+} as a marker for identifying T-lineage primed cells, with Cluster 13 being a particularly enriched subset.