NicheNet's ligand activity analysis on a gene set of interest: predict active ligands and their target genes ========== Alberto Valdeolivas: alberto.valdeolivas@bioquant.uni-heidelberg.de; Date: 22/06/2020


## Abstract

This vignette follows the steps of the original vignette, available in the NicheNetr repository:

https://github.com/saeyslab/nichenetr/blob/master/vignettes/ligand_activity_geneset.md

In our particular case, we use sets of interactions availabe in the **Omnipath** database. We will study potential ligand-targets influence upon SARS-CoV-2 infection.


## Introduction

A NicheNet analysis can help one to generate hypotheses about an intercellular communication process of interest for which you have bulk or single-cell gene expression data. Specifically, NicheNet can predict 1) which ligands from one cell population ("sender/niche") are most likely to affect target gene expression in an interacting cell population ("receiver/target") and 2) which specific target genes are affected by which of these predicted ligands.

Because NicheNet studies how ligands affect gene expression in neighboring cells, you need to have data about this effect in gene expression you want to study. So, you need to have a clear set of genes that are putatively affected by ligands from one of more interacting cells.

The pipeline of a basic NicheNet analysis consist mainly of the following steps:

- 1. Define a "sender/niche" cell population and a "receiver/target" cell population present in your expression data and determine which genes are expressed in both populations
- 2. Define a gene set of interest: these are the genes in the "receiver/target" cell population that are potentially affected by ligands expressed by interacting cells (e.g. genes differentially expressed upon cell-cell interaction)
- 3. Define a set of potential ligands: these are ligands that are expressed by the "sender/niche" cell population and bind a (putative) receptor expressed by the "receiver/target" population
- 4) Perform NicheNet ligand activity analysis: rank the potential ligands based on the presence of their target genes in the gene set of interest (compared to the background set of genes)
- 5) Infer top-predicted target genes of ligands that are top-ranked in the ligand activity analysis

This vignette guides you in detail through all these steps. We are going to use expression data after SARS-CoV-2 infection to try to dissect which ligands
expressed by infected cells can have an influence on the expression of target genes in the same cell lines (Autocrine view). In particular, we will focus on the inflamatory response potentially induced by this ligands.

## Step 0: NicheNet's ligand-target prior model and expression data of interacting cells

We first loaded the required packages

```
library(nichenetr)
library(tidyverse)
library(VennDiagram)
library(fgsea)
```

Then, we read the prior ligand-target model. This model denotes the prior potential that a particular ligand might regulate the expression of a specific target gene.

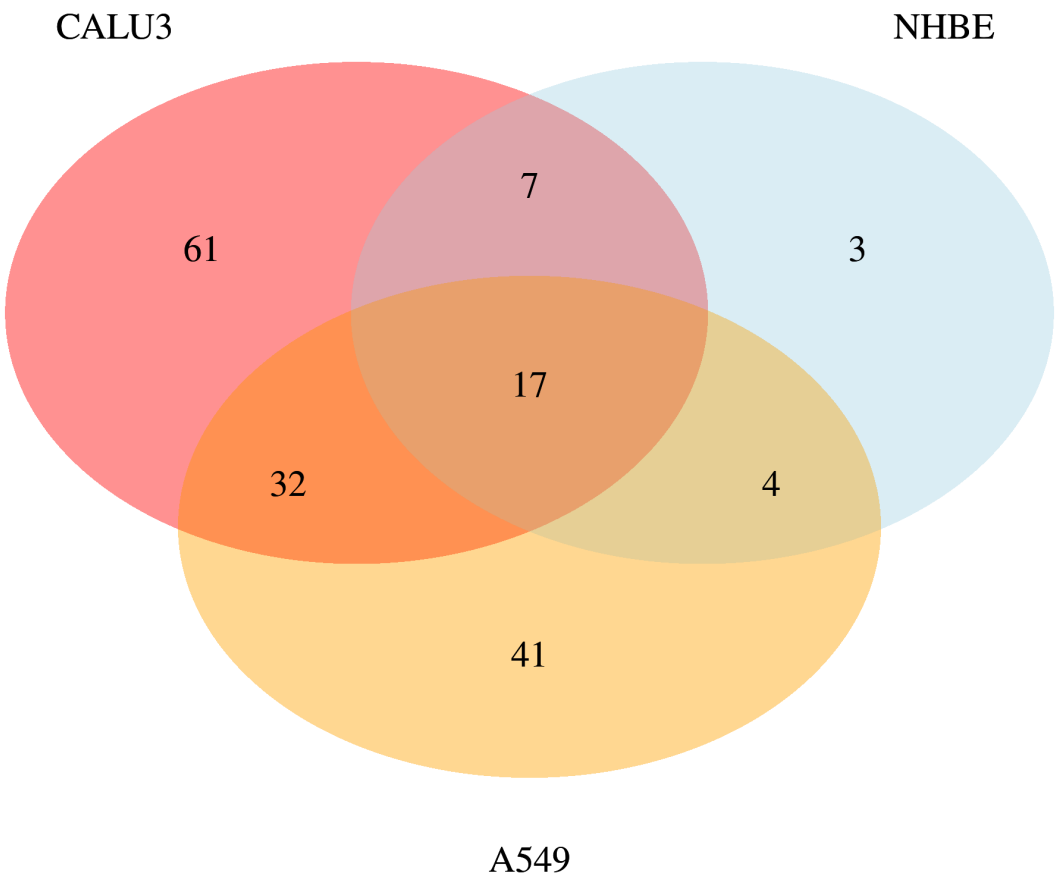
```
ligand_target_matrix = readRDS("Results/ligand_target_matrixWithweights.rds")
# target genes in rows, ligands in columns
dim(ligand_target_matrix)
## [1] 12547   840
ligand_target_matrix[1:5,1:5]
##               CALM1        WNT5A       CXCL16       CCL3L3      TNFSF10
## A1BG   0.0000000000 0.0000000000 0.000000e+00 0.000000e+00 0.0000000000
## A1CF   0.0000000000 0.0000000000 0.000000e+00 0.000000e+00 0.0000000000
## A2M    0.0011027517 0.0004845514 2.936421e-03 5.441192e-03 0.0017391820
## A2ML1  0.0000000000 0.0000000000 0.000000e+00 0.000000e+00 0.0000000000
## A4GALT 0.0002105736 0.0001070804 5.825834e-05 9.488076e-05 0.0001410451
```

We read the differential expression analysis results from several cell lines upon SARS-CoV-2 infection. We are going to explore which ligands are overexpressed after infection in different cell lines belonging to the following dataset: GSE147507 (https://www.biorxiv.org/content/10.1101/2020.03.24.004655v1)

```
padj_tres <- 0.1
log2FoldChange_tres <- 1

## We take our ligands in the network
ligands <-
    readRDS("OmniNetworks_NNformat/lr_Network_Omnipath.rds") %>%
    dplyr::pull(from) %>%
    unique()

DDS_NHBE_ligands <-
    readRDS("Results/dds_results_NHBEvsCOV2.rds") %>%
    as.data.frame() %>%
    tibble::rownames_to_column(var = "Gene") %>%
    dplyr::filter(padj < padj_tres,
                  log2FoldChange > log2FoldChange_tres,
                  Gene %in% ligands) %>%
    dplyr::pull(Gene)

DDS_CALU3_ligands <-
    readRDS("Results/dds_results_CALU3vsCOV2.rds") %>%
    as.data.frame() %>%
    tibble::rownames_to_column(var = "Gene") %>%
    dplyr::filter(padj < padj_tres,
                  log2FoldChange > log2FoldChange_tres,
                  Gene %in% ligands) %>%
    dplyr::pull(Gene)

DDS_A549_ligands <-
    readRDS("Results/dds_results_A549vsCOV2.rds") %>%
    as.data.frame() %>%
    tibble::rownames_to_column(var = "Gene") %>%
    dplyr::filter(padj < padj_tres,
                  log2FoldChange > log2FoldChange_tres,
                  Gene %in% ligands)  %>%
    dplyr::pull(Gene)
```

After checking the overlap between over-expressed ligands in the different cell lines, we decided to continue with the analysis using CALU3, since it has the larger number of over-expressed ligands.

```
Venn_plot <- draw.triple.venn(length(DDS_NHBE_ligands),
    length(DDS_CALU3_ligands),
    length(DDS_A549_ligands),
    n12 = length(intersect(DDS_NHBE_ligands,
        DDS_CALU3_ligands)),
    n23 = length(intersect(DDS_CALU3_ligands,
        DDS_A549_ligands)),
    n13 = length(intersect(DDS_NHBE_ligands,
        DDS_A549_ligands)),
    n123 = length(intersect(intersect(DDS_NHBE_ligands,
        DDS_CALU3_ligands),
        DDS_A549_ligands)),
    category = c("NHBE", "CALU3","A549"),
    lty = rep("blank", 3), fill = c("light blue", "red","orange"),
    alpha = rep(0.25, 3), euler.d = TRUE, scaled=TRUE,
    rotation.degree = 0, reverse=TRUE, cex=1.25, cat.pos = c(330, 30 , 180),
    cat.dist = rep(0.075, 3), cat.cex = 1.25)
grid.draw(Venn_plot)
```




## Step 1: Define expressed genes in sender and receiver cell populations

Our research question is to prioritize which ligands overexpressed upon SARS-CoV-2 in the CALU-3 cell line have an effect in the inflamatory response in this very same cell line. This can be considered as an example of autocrine signaling.

Now, we will take again the overexpressed ligands after infection and we will define as a background all the genes expressed by the CALU3 cells.

```
expressed_genes_receiver <-
    readRDS("Results/dds_results_CALU3vsCOV2.rds") %>%
    as.data.frame() %>%
    tibble::rownames_to_column(var = "Gene") %>%
    dplyr::filter(!is.na(stat)) %>%
    dplyr::pull(Gene)

## Check the number of ligands and background genes
length(ligands)
## [1] 840
length(expressed_genes_receiver)
## [1] 16818
```

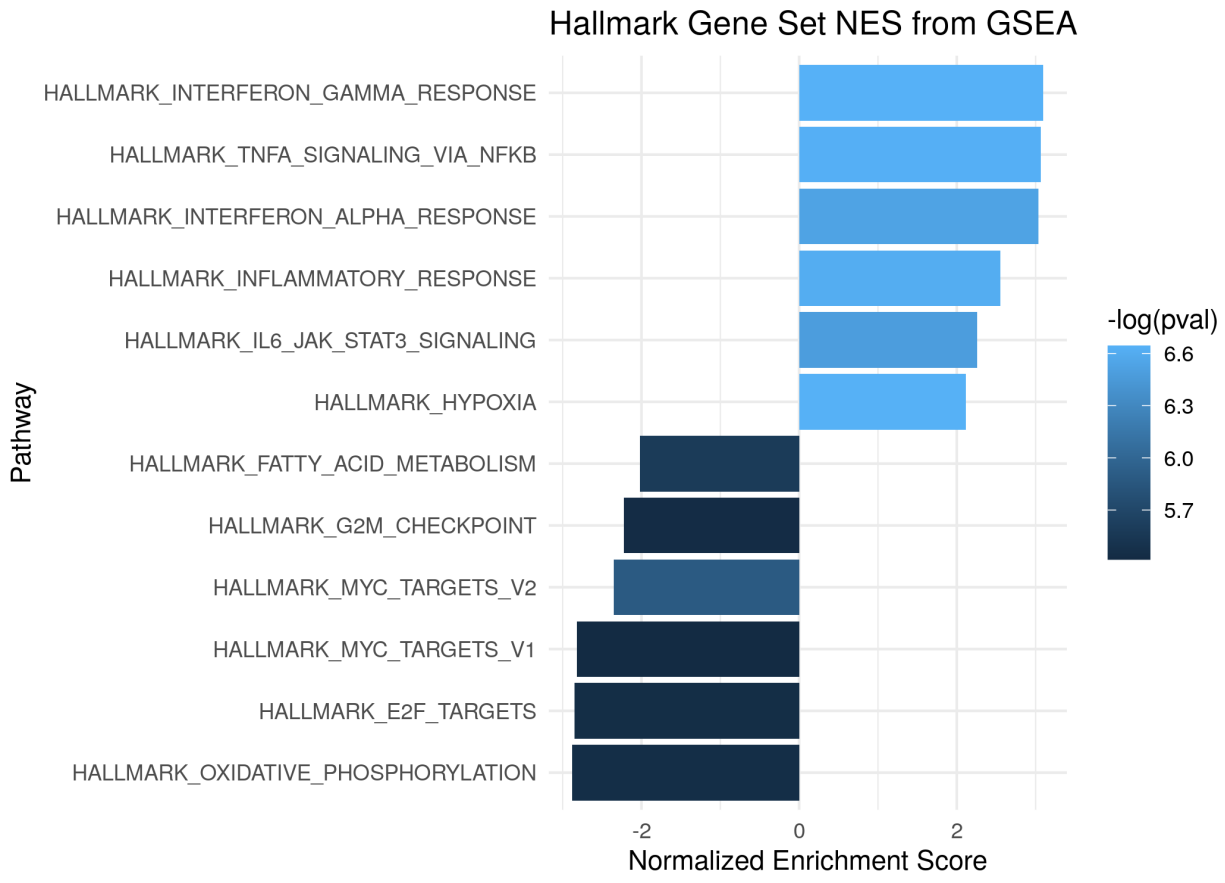
## Step 2: Define the gene set of interest and a background of genes

To establish a gene set of interest, we perform a Gene set Enrichment analysis (GSEA) and we check among the most appealing overrepresanted signatures upon SARS-CoV-2 infection. We remove the differentially expressed ligands from this comparison.

```
ranks <- readRDS("Results/dds_results_CALU3vsCOV2.rds") %>%
    as.data.frame() %>%
    tibble::rownames_to_column(var = "Gene") %>%
    dplyr::filter(!(Gene %in% DDS_CALU3_ligands)) %>%
    dplyr::filter(!is.na(stat)) %>%
    dplyr::pull(stat, name=Gene)

# immunologic_signatures <- gmtPathways("NicheNet_Omnipath/c7.all.v7.1.symbols.gmt")
hallmarlk_signatures <- gmtPathways("h.all.v7.1.symbols.gmt")
# go_signatures <- gmtPathways("NicheNet_Omnipath/c5.bp.v7.1.symbols.gmt")

fgseaRes <- fgsea(hallmarlk_signatures, ranks,  nperm=1000)
# Testing only one pathway is implemented in a more efficient manner

SignificantResults <- fgseaRes %>%
    dplyr::filter(padj < 0.01) %>%
    dplyr::arrange(desc(NES)) %>%
    dplyr::top_n(12, abs(NES))
SignificantResults
##                              pathway        pval        padj         ES
##  1: HALLMARK_INTERFERON_GAMMA_RESPONSE 0.001302083 0.005199667  0.8627654
##  2:   HALLMARK_TNFA_SIGNALING_VIA_NFKB 0.001319261 0.005199667  0.8608318
##  3: HALLMARK_INTERFERON_ALPHA_RESPONSE 0.001461988 0.005199667  0.9172465
##  4:     HALLMARK_INFLAMMATORY_RESPONSE 0.001347709 0.005199667  0.7274370
##  5:   HALLMARK_IL6_JAK_STAT3_SIGNALING 0.001533742 0.005199667  0.7126008
##  6:                   HALLMARK_HYPOXIA 0.001307190 0.005199667  0.5893036
##  7:     HALLMARK_FATTY_ACID_METABOLISM 0.003787879 0.007407407 -0.5010564
##  8:            HALLMARK_G2M_CHECKPOINT 0.004385965 0.007407407 -0.5370578
##  9:            HALLMARK_MYC_TARGETS_V2 0.002793296 0.007407407 -0.6827875
## 10:            HALLMARK_MYC_TARGETS_V1 0.004444444 0.007407407 -0.6785459
## 11:               HALLMARK_E2F_TARGETS 0.004329004 0.007407407 -0.6829123
## 12: HALLMARK_OXIDATIVE_PHOSPHORYLATION 0.004310345 0.007407407 -0.6946604
##           NES nMoreExtreme size                                leadingEdge
```

```
##  1:  3.097636           0  174     OAS2,IFIT1,RSAD2,IFIT2,IFIT3,TNFAIP3,...
##  2:  3.062342           0  161 IFIT2,TNFAIP3,ATF3,PPP1R15A,NFKBIA,IFIH1,...
##  3:  3.037484           0   89        RSAD2,IFIT2,IFIT3,MX1,IFIH1,TXNIP,...
##  4:  2.555097           0  148       NFKBIA,IRF1,LAMP3,IFITM1,KLF6,RTP4,...
##  5:  2.256719           0   67         IRF1,STAT2,MAP3K8,STAT1,JUN,PIM1,...
##  6:  2.114591           0  173  TNFAIP3,ATF3,PPP1R15A,TIPARP,DUSP1,STC2,...
##  7: -2.019464           0  146        ACAT2,DHCR24,NSDHL,FASN,NTHL1,MIF,...
##  8: -2.225125           0  190         KPNA2,MCM5,SQLE,HSPA8,MCM6,LMNB1,...
##  9: -2.352049           0   58         TMEM97,MCM5,PHB,DCTPP1,PLK1,MCM4,...
## 10: -2.821150           0  193           KPNA2,MCM5,PHB,MCM6,SRSF2,NME1,...
## 11: -2.847626           0  195       KPNA2,MCM5,MXD3,SPAG5,NCAPD2,POLD1,...
## 12: -2.876772           0  184      MAOB,POLR2F,COX8A,LDHB,VDAC3,NDUFB2,...
```

```
plot_enrichment <- ggplot(SignificantResults, aes(reorder(pathway, NES), NES)) +
  geom_col(aes(fill=-log(pval))) +
  coord_flip() +
  labs(x="Pathway", y="Normalized Enrichment Score",
       title="Hallmark Gene Set NES from GSEA") +
  theme_minimal()
plot_enrichment
```




```
saveRDS(SignificantResults,file = "Results/Enrichment_Significant_Results.rds")
```

One of the most interesting results is inflamatory response. So, we define the leading edge genes involved in the inflamatory response as the target genes, i.e. we want to see how likely is that the secreted ligands have an effect in this inflamatory response.

```
## I am going to check with Inflamatory genes
InflamatoryGenes <- SignificantResults %>%
    dplyr::filter(pathway == "HALLMARK_INFLAMMATORY_RESPONSE") %>%
    dplyr::pull(leadingEdge) %>% unlist()

## We chech that there are no upregulated ligands here.
intersect(DDS_CALU3_ligands,InflamatoryGenes)
## character(0)

geneset_oi <- InflamatoryGenes[InflamatoryGenes %in% rownames(ligand_target_matrix)]

head(geneset_oi)
## [1] "NFKBIA" "IRF1"   "IFITM1" "KLF6"   "RTP4"   "IRAK2"
background_expressed_genes <- expressed_genes_receiver %>%
    .[. %in% rownames(ligand_target_matrix)]
head(background_expressed_genes)
## [1] "SAMD11"   "NOC2L"    "ISG15"    "AGRN"     "TNFRSF18" "SDF4"
```

### Step 3: Define a set of potential ligands

As potentially active ligands, we will use ligands that are 1) Over-expressed in CALU3 after SARS-CoV-2 infection and 2) can bind a (putative) receptor expressed by malignant cells. Putative ligand-receptor links were gathered from Omnipath ligand-receptor data sources.

```
receptors <- unique(lr_network$to)
expressed_receptors <- intersect(receptors,expressed_genes_receiver)

lr_network_expressed <- lr_network %>%
    filter(from %in% DDS_CALU3_ligands & to %in% expressed_receptors)
head(lr_network_expressed)
## # A tibble: 6 x 4
##   from  to    source         database
##   <chr> <chr> <chr>          <chr>
## 1 CXCL1 CXCR2 kegg_cytokines kegg
## 2 CXCL2 CXCR2 kegg_cytokines kegg
## 3 CXCL3 CXCR2 kegg_cytokines kegg
## 4 CXCL5 CXCR2 kegg_cytokines kegg
## 5 CCL20 CCR6  kegg_cytokines kegg
## 6 CCL17 CCR4  kegg_cytokines kegg
```

This ligand-receptor network contains the expressed ligand-receptor interactions. As potentially active ligands for the NicheNet analysis, we will consider the ligands from this network.

```
potential_ligands <- lr_network_expressed %>% pull(from) %>% unique()
head(potential_ligands)
## [1] "CXCL1" "CXCL2" "CXCL3" "CXCL5" "CCL20" "CCL17"
```

### Step 4: Perform NicheNet's ligand activity analysis on the gene set of interest

In this section, we calculate the ligand activity of each ligand, or in other words, we will assess how well each over-expressed ligand after viral infection can predict the inflatmatory response gene set compared to the background of expressed genes (predict whether a gene belongs to the inflatmatory response program or not).

```
ligand_activities <- predict_ligand_activities(
    geneset = geneset_oi,
```

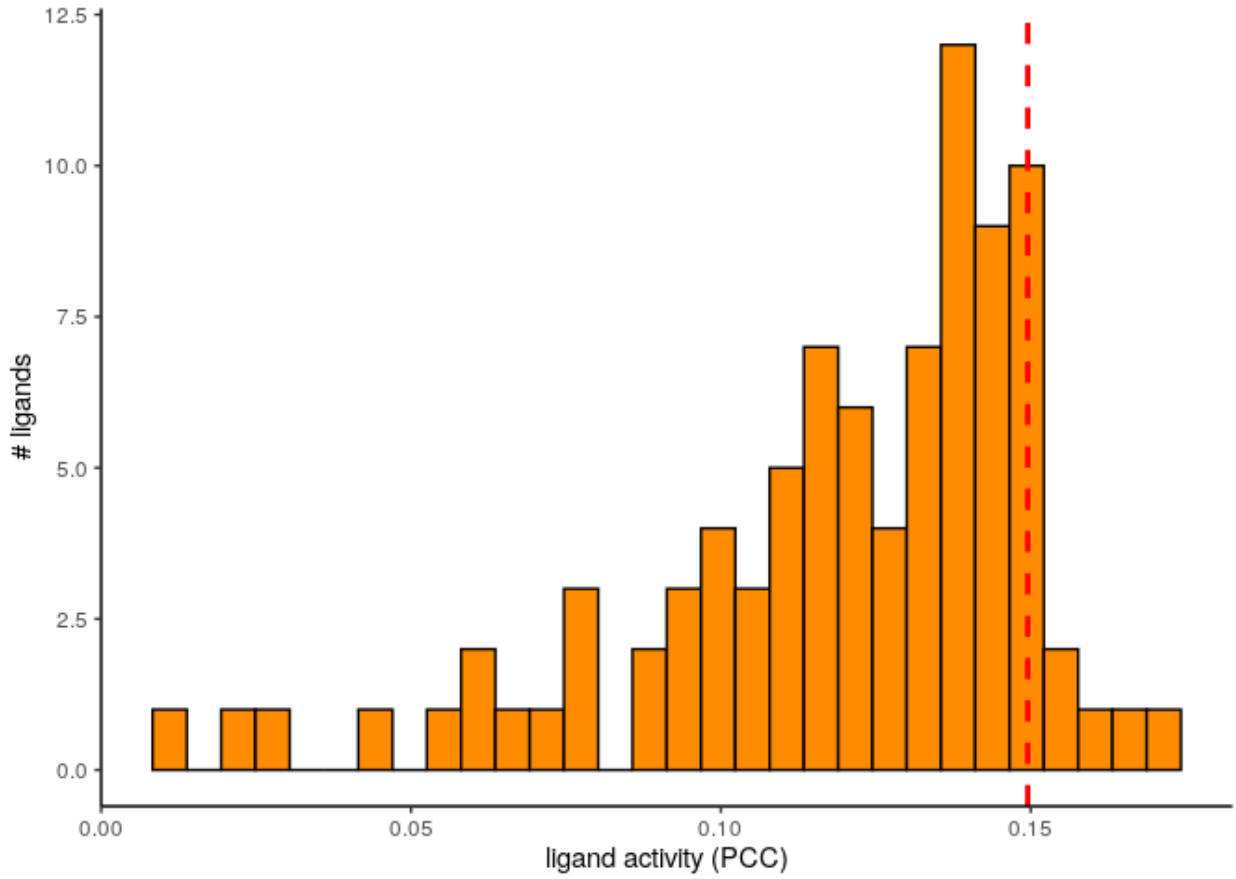
```
    background_expressed_genes = background_expressed_genes,
    ligand_target_matrix = ligand_target_matrix,
    potential_ligands = potential_ligands)
```

We will rank the ligands based on their pearson correlation coefficient. This allows us to prioritize inflamory response-regulating ligands.

```
ligand_activities %>% arrange(-pearson)
## # A tibble: 89 x 4
##    test_ligand auroc   aupr pearson
##    <chr>       <dbl>  <dbl>   <dbl>
##  1 IL23A       0.742 0.0693   0.173
##  2 TNF         0.753 0.0604   0.165
##  3 TNFSF13B    0.732 0.0568   0.159
##  4 IL1A        0.712 0.0532   0.155
##  5 LAMA2       0.740 0.0597   0.152
##  6 ICAM4       0.731 0.0645   0.151
##  7 L1CAM       0.735 0.0645   0.151
##  8 CXCL9       0.742 0.0771   0.151
##  9 NPPB        0.724 0.0721   0.151
## 10 INHBA       0.677 0.0591   0.150
## # ... with 79 more rows
best_upstream_ligands <- ligand_activities %>%
    top_n(12, pearson) %>%
    arrange(-pearson) %>%
    pull(test_ligand)
head(best_upstream_ligands)
## [1] "IL23A"    "TNF"      "TNFSF13B" "IL1A"     "LAMA2"    "ICAM4"
```

We see here that the performance metrics indicate that the 12 top-ranked ligands can predict the inflamatory genes reasonably, this implies that ranking of the ligands might be accurate as shown in our study. However, it is possible that for some gene sets, the target gene prediction performance of the top-ranked ligands would not be much better than random prediction. In that case, prioritization of ligands will be less trustworthy.

```
# show histogram of ligand activity scores
p_hist_lig_activity = ggplot(ligand_activities, aes(x=pearson)) +
  geom_histogram(color="black", fill="darkorange")  +
  # geom_density(alpha=.1, fill="orange") +
  geom_vline(aes(xintercept=min(ligand_activities %>% top_n(12, pearson) %>%
    pull(pearson))), color="red", linetype="dashed", size=1) +
  labs(x="ligand activity (PCC)", y = "# ligands") +
  theme_classic()
p_hist_lig_activity
```




```
saveRDS(ligand_activities,file = "Results/LigandActivityScoreDistribution.rds")
```

### Step 5: Infer target genes of top-ranked ligands and visualize in a heatmap

Now we will show how you can look at the regulatory potential scores between ligands and target genes of interest. In this case, we will look at links between top-ranked ligands regulating inflamatory response genes. In the ligand-target heatmaps, we show here regulatory potential scores for interactions between the 12 top-ranked ligands and following target genes: genes that belong to the gene set of interest and to the 250 most strongly predicted targets of at least one of the 12 top-ranked ligands (the top 250 targets according to the general prior model, so not the top 250 targets for this dataset). Consequently, genes of your gene set that are not a top target gene of one of the prioritized ligands, will not be shown on the heatmap.

```
active_ligand_target_links_df <- best_upstream_ligands %>%
    lapply(get_weighted_ligand_target_links,
           geneset = geneset_oi,
           ligand_target_matrix = ligand_target_matrix,
           n = 250) %>%
    bind_rows()
nrow(active_ligand_target_links_df)
## [1] 179
head(active_ligand_target_links_df)
## # A tibble: 6 x 3
##   ligand target weight
##   <chr>  <chr>   <dbl>
## 1 IL23A  CD69   0.0239
## 2 IL23A  CDKN1A 0.0549
```

```
## 3 IL23A  F3     0.0314
## 4 IL23A  IFITM1 0.0185
## 5 IL23A  IL18   0.0232
## 6 IL23A  IL4R   0.0197
```

For visualization purposes, we adapted the ligand-target regulatory potential matrix as follows. Regulatory potential scores were set as 0 if their score was below a predefined threshold, which was here the 0.10 quantile of scores of interactions between the 10 top-ranked ligands and each of their respective top targets (see the ligand-target network defined in the data frame).

```
active_ligand_target_links <- prepare_ligand_target_visualization(
    ligand_target_df = active_ligand_target_links_df,
    ligand_target_matrix = ligand_target_matrix,
    cutoff = 0.10)
nrow(active_ligand_target_links_df)
## [1] 179
head(active_ligand_target_links_df)
## # A tibble: 6 x 3
##   ligand target weight
##   <chr>  <chr>   <dbl>
## 1 IL23A  CD69   0.0239
## 2 IL23A  CDKN1A 0.0549
## 3 IL23A  F3     0.0314
## 4 IL23A  IFITM1 0.0185
## 5 IL23A  IL18   0.0232
## 6 IL23A  IL4R   0.0197
```

The putatively active ligand-target links will now be visualized in a heatmap. The order of the ligands accord to the ranking according to the ligand activity prediction.

```
order_ligands <-
    intersect(best_upstream_ligands, colnames(active_ligand_target_links)) %>%
    rev()
order_targets <- active_ligand_target_links_df$target %>%
    unique()
vis_ligand_target <- active_ligand_target_links[order_targets,order_ligands] %>%
    t()
p_ligand_target_network <- vis_ligand_target %>%
    make_heatmap_ggplot("Prioritized ligands","Inflamatory Related genes",
        color = "blue",legend_position = "top", x_axis_position = "top",
        legend_title = "Regulatory potential") +
        scale_fill_gradient2() +
            # ) +
    theme(axis.text.x = element_text(face = "italic"))
p_ligand_target_network
```


```
saveRDS(vis_ligand_target,file = "Results/Ligand_Target_Matrix.rds")
```

Note that the choice of these cutoffs for visualization is quite arbitrary. We recommend users to test several cutoff values.

If you would consider more than the top 250 targets based on prior information, you will infer more, but less confident, ligand-target links; by considering less than 250 targets, you will be more stringent.

If you would change the quantile cutoff that is used to set scores to 0 (for visualization purposes), lowering this cutoff will result in a more dense heatmap, whereas highering this cutoff will result in a more sparse heatmap.

## Follow-up analysis 1: Ligand-receptor network inference for top-ranked ligands

One type of follow-up analysis is looking at which receptors can potentially bind to the prioritized ligands.

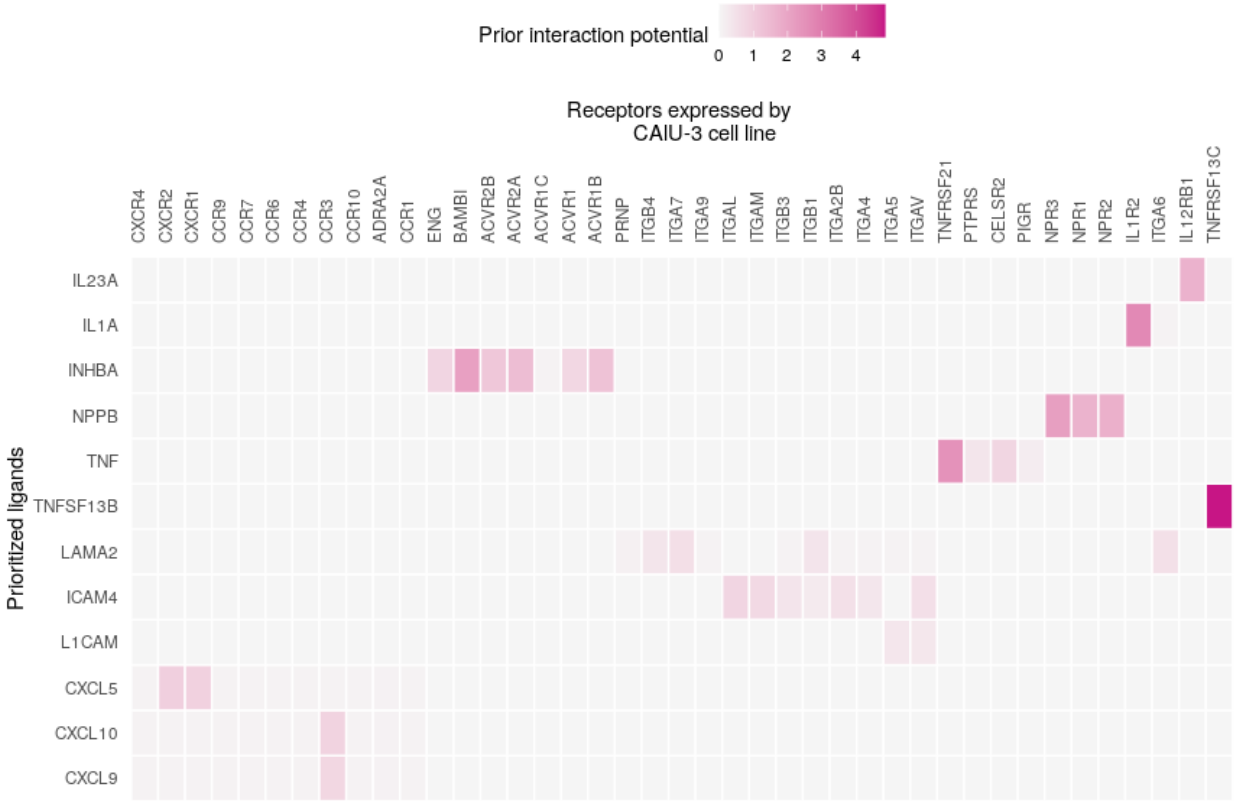
So, we will now infer the predicted ligand-receptor interactions of the top-ranked ligands and visualize these in a heatmap.

```
## get the ligand-receptor network of the top-ranked ligands
lr_network_top <- lr_network %>%
    filter(from %in% best_upstream_ligands & to %in% expressed_receptors) %>%
    distinct(from,to)

best_upstream_receptors <- lr_network_top %>% pull(to) %>% unique()
```

```
## get the weights of the ligand-receptor interactions as used in the NicheNet model
weighted_networks <- readRDS("Results/weighted_networksWithSourceWeights.rds")

lr_network_top_df <- weighted_networks$lr_sig %>%
    filter(from %in% best_upstream_ligands & to %in% best_upstream_receptors)

## convert to a matrix
lr_network_top_df <- lr_network_top_df %>%
    spread("from","weight",fill = 0)
lr_network_top_matrix <- lr_network_top_df %>%
    select(-to) %>%
    as.matrix() %>%
    magrittr::set_rownames(lr_network_top_df$to)

## perform hierarchical clustering to order the ligands and receptors
dist_receptors <- dist(lr_network_top_matrix, method = "binary")
hclust_receptors <- hclust(dist_receptors, method = "ward.D2")
order_receptors <- hclust_receptors$labels[hclust_receptors$order]
dist_ligands <- dist(lr_network_top_matrix %>% t(), method = "binary")
hclust_ligands <- hclust(dist_ligands, method = "ward.D2")
order_ligands_receptor <- hclust_ligands$labels[hclust_ligands$order]
```

Show a heatmap of the ligand-receptor interactions

```
vis_ligand_receptor_network <-
    lr_network_top_matrix[order_receptors, order_ligands_receptor]
p_ligand_receptor_network <- vis_ligand_receptor_network %>%
    t() %>%
    make_heatmap_ggplot("Prioritized ligands","Receptors expressed by
        CAlU-3 cell line", color = "mediumvioletred", x_axis_position = "top",
        legend_title = "Prior interaction potential")
p_ligand_receptor_network
```




```
saveRDS(vis_ligand_receptor_network,file = "Results/Ligand_Receptor_Matrix.rds")
```

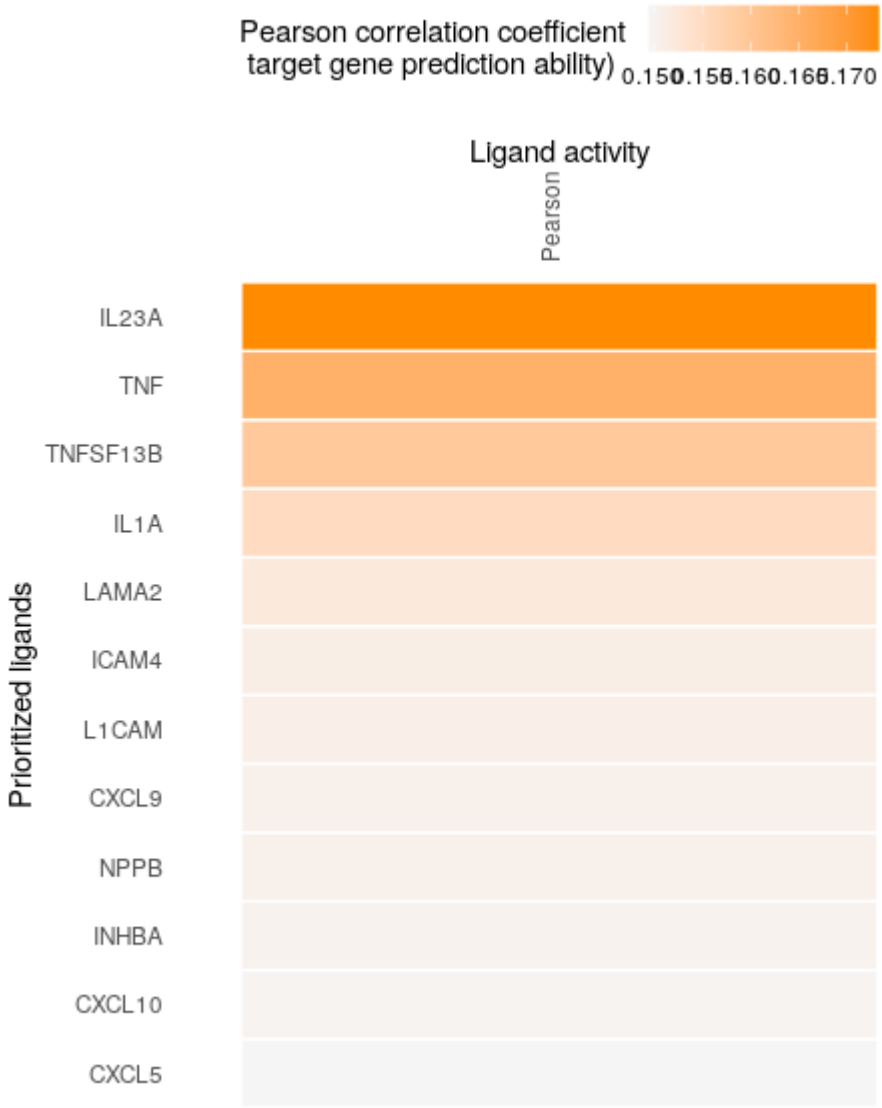
## Follow-up analysis 2: Visualize expression of top-predicted ligands and their target genes in a combined heatmap

NicheNet only considers expressed ligands of sender cells, but does not take into account their expression for ranking the ligands. The ranking is purely based on the potential that a ligand might regulate the gene set of interest, given prior knowledge. Because it is also useful to further look into expression of ligands and their target genes, we demonstrate here how you could make a combined figure showing ligand activity, ligand expression, target gene expression and ligand-target regulatory potential.

```
library(RColorBrewer)
library(cowplot)
library(ggpubr)
```

```
ligand_pearson_matrix <- ligand_activities %>%
    select(pearson) %>%
    as.matrix() %>%
    magrittr::set_rownames(ligand_activities$test_ligand)

vis_ligand_pearson <- ligand_pearson_matrix[order_ligands, ] %>%
    as.matrix(ncol = 1) %>%
    magrittr::set_colnames("Pearson")
```

```
p_ligand_pearson <- vis_ligand_pearson %>%
    make_heatmap_ggplot("Prioritized ligands","Ligand activity",
    color = "darkorange",legend_position = "top", x_axis_position = "top",
    legend_title = "Pearson correlation coefficient \n target gene prediction ability)")
p_ligand_pearson
```




**Prepare the ligand activity matrix**

```
saveRDS(vis_ligand_pearson, file = "Results/ligand_Pearson.rds")
```

## References


Browaeys, R., Saelens, W. & Saeys, Y. NicheNet: modeling intercellular communication by linking ligands to target genes. Nat Methods (2019) doi:10.1038/s41592-019-0667-5

Puram, Sidharth V., Itay Tirosh, Anuraag S. Parikh, Anoop P. Patel, Keren Yizhak, Shawn Gillespie, Christopher Rodman, et al. 2017. "Single-Cell Transcriptomic Analysis of Primary and Metastatic Tumor Ecosystems in Head and Neck Cancer." Cell 171 (7): 1611–1624.e24. https://doi.org/10.1016/j.cell.2017.10.044.