Reconstruction of Intercellular Interaction Network in *Mus musculus* Immune System

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Abstract

Intercellular interactions through receptor and ligand molecules play an indelible role in regulating communications of cells with each other, especially in multicellular organisms. So far, extensive studies have been done with both experimental and computational approaches in this field. One of the advanced experimental methods is constructing a three-dimensional medium similar to *in vivo* conditions, but due to the existing limitations, it cannot be investigated on a large scale. Therefore, in order to investigate and analyze the intercellular interaction, use of computational methods such as network reconstruction has attracted the attention of many researchers recently.

In this study, the first draft of intercellular interaction network in *mus musculus* immune system were reconstructed and analyzed through gene expression data analysis related to the first phase of the immunological genome project. In this reconstructed network, there are 9,271 communications between 162 cells which were created through 460 different receptor-ligand interactions. The results indicate that cells belonging to hematopoietic lineages use fewer communication pathways for interacting with each other and the most network communications belong to non-hematopoietic stromal cells followed by macrophages. The investigation of expression specificity of receptors and ligands revealed the high specificity of all involved genes and also high specificity of interactions between them.

Keywords: Intercellular interaction; Signaling network reconstruction; Immunological genome project; Cell-cell interaction; Mus musculus immune system

Introduction

Of the important features of unicellular organisms, and multicellular organisms in particular, is their ability to respond to external stimuli through cellular signaling process. Cellular signaling occurs during two main steps of signal transmission and transduction. In the signal transmission step, first messenger sent from the source of the signal- cells or environment of the target cell- are received by the target cell receptors through transmission in the extracellular matrix. In the signal transduction stage, the receptor-ligand complex, triggers intracellular signaling pathways by secondary messenger molecules and ultimately, by affecting the expression level of related genes, causing cellular responses in the target cell [1]. Cellular signaling results in adaptation of cell to different conditions, maintaining cellular hemostasis, growth, differentiation, migration, survival, apoptosis, cellular motility, etc.[2]–[7]. Cellular signaling if occurs between cells, is called intercellular signaling, which is a highly regulated process. In this signaling a cell as the sender of the signal, the signal carrier molecule (first messenger or ligand), an environment through which the messenger molecule is transmitted, and a receptor of signal recipient cell are essential [8].

Intercellular communication, which is established through interactions between ligand and receptor molecules, plays an inevitably important role in the behavior of cells and regulates their communications with each other and with the environment, particularly in multicellular beings. In multicellular organisms, cellular behavior is much more complicated than that of a unicellular organism and their ability to communicate with adjacent cells is the basis of their coordinated activity, and formation and maintenance of their integrity [9]. Thus, the necessity of studying different aspects of this process is essential. So far extensive studies have been done with both experimental and computational approaches in this field. Due to the difficulty of studying this processes in a large scale manner in the presence of different types of cells, the use of computational systems biology approaches has recently attracted the attention of many scholars in order to investigate and analyze the intercellular signaling process [10]. Currently, most of the reconstructed networks have been linked to the intracellular signaling networks, and intercellular interactions that trigger the intracellular signaling have been underestimated. In most of the reconstructed networks of intercellular interactions, a limited number of cells have been examined. Only in two studies, an intercellular interconnection network has been investigated in a variety of cells [11], [12]. In this study, the intercellular interaction network between the immune cells and stromal cells of *Mus musculus* is being reconstructed and analyzed.

Methods

Reconstruction of the intercellular interaction network takes place during two stages of data extraction and, integration and filtering of data. *Figure 1* shows the general stages of our network reconstruction.

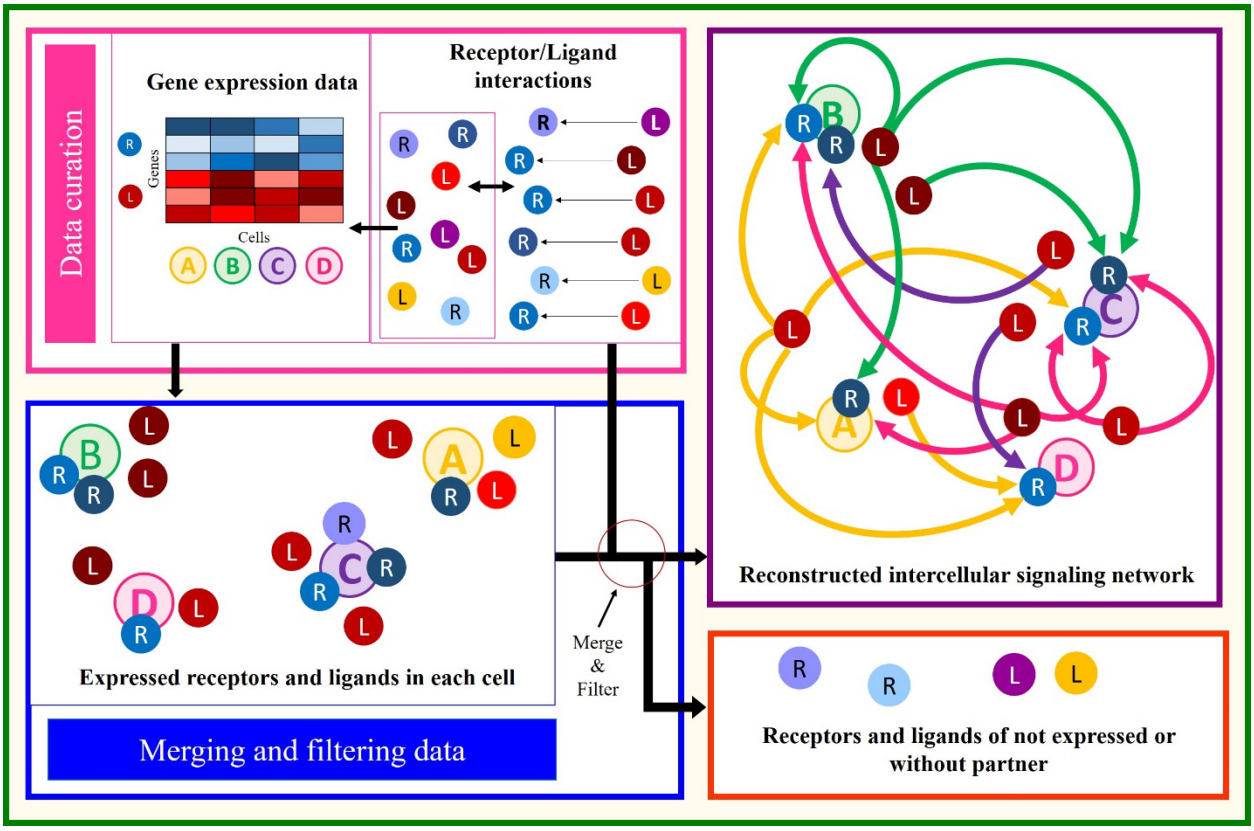

Figure 1: *Schematic workflow for intercellular interaction network reconstruction.*

Receptor–ligand interaction dataset

To create this dataset, we first extracted receptor-ligand genes from the molecular databases such as the KEGG, GPCRdb, Uniprot, and IUPHAR [13]–[16]. In order to detect receptor- ligand interactions in the proteins interactions databases, the iRefWeb interface [17] was used and the receptor-ligand interactions (receptor-ligand pairs) among protein-protein interactions of the mouse organism were extracted, and a dataset was created for the receptor-ligand interactions.

In addition to extracting receptor-ligand interactions from the iRefWeb database, interactions of IUPHAR and KEGG database were also extracted. The data obtained from these sources was added to the interactions in previous related articles [18], [19]. After removing the repetitive interactions, the benchmark dataset was created for reconstructing the network, which included 958 interactions between 340 receptors and 374 ligands *(Fig. 2)*.

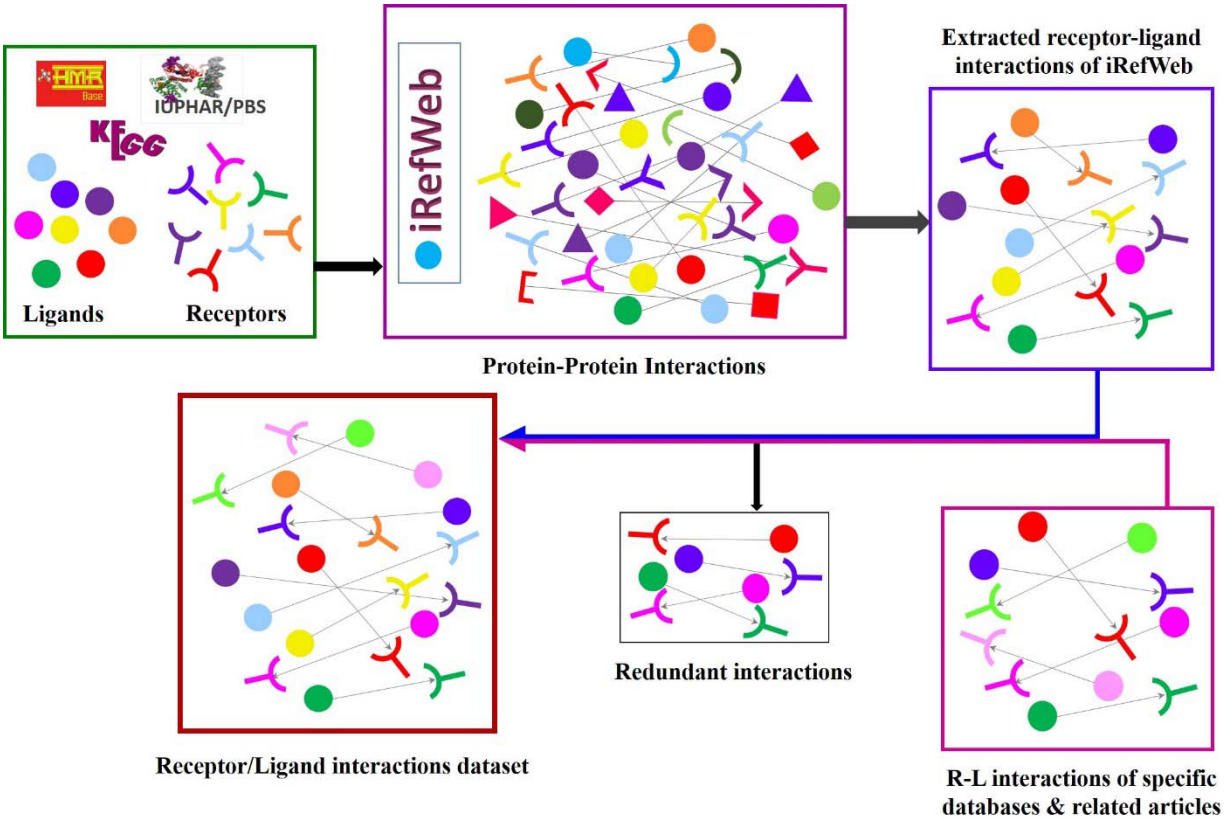

Figure 2: *Creating a receptor-ligand interaction dataset*

Receptor-ligand gene expression dataset

The microarray expression data deposit in the Gene Expression Omnibus (NCBI/GEO) (http://www.ncbi.nlm.nih.gov/geo/) [20] under accession number GSE15907, which is related to the first phase of the immunological genome project (http://www.immgen.org/Protocols/ImmGen) [21]. The samples studied in this project (ImmGen) are from adult male B6 mouse and from 24 different tissues. The Affymetrix 1.0 ST MuGene arrays platform was used to profile the expression of genes, based on criteria such as sensitivity, noise, different expression and reliability or diagnostic validity for each platform, using a collection of the common RNAs of two $CD4^+$ and $CD19^+$ cells, selected from among the several platforms used in the microarray analysis. This dataset consists of 653 samples related to 212 cell types, which include all major hematopoietic lineages [21].

These lineages are divided into 8 main lineages based on the hematopoietic lineage tree created in this project. The eight main hematopoietic lineages include stem and progenitor cell (S&P), granulocytes, monocytes, macrophages (MF), dendritic cells (DC), natural killer cells (NK), B cell and T cells. The T cells are divided into four subgroups of gamma and delta T cells (γδT), alpha and beta T cells (αβT), regulatory T cells (T_{regs}) and natural killer T cells (NKT). In

addition to the hematopoietic lineages, the stromal cells which are categorized as non-hematopoietic cells lineage, exist in this project [22].

Of the 653 samples examined in the first phase of ImmGene immune Project, 25 samples from $CD4^+$ and $CD19^+$ cells were used as control and test samples to select the type of platform used for fine-array analysis of these samples, and the rest of the samples included 29 embryonic and 599 adult samples [21]. These samples were derived from tissues such as lymph nodes, spleen, liver, kidney, bone marrow, lung, pancreas, skin and some other tissues. For analyzing this dataset, R-software and packages Limma were used, and the method of normalizing the mean of several precise arrays (RMA) was used to normalize the data [23], [24].

After normalizing the data, Ensemble Biomart tool was used to map the probe identifier to gene names [24], [25]. After the removal of probes lacking gene names and probes with more than one gene name [26], a collection of 25751 probes, each mapped to a single gene name, was created. For 3726 gene names that had more than one probe on the arrays, i.e. more than one probe identifier, the probe identifier was assigned to the gene name which had the highest amount of expression between the probes which were attributed to that gene [22].

Subsequently, using 730 gene identifiers for ligands and receptors collected in the receptor-ligand interactions dataset, the probes belonging to the genes encoding receptor and ligand proteins were extracted from this collection *(Fig. 3 a)*. After identifying the genes of the receptor and ligand in each cell sample, the number of which after removing the genes that did not have the probe identifier exceeded 695 genes, the threshold defined in equation 1, which represented the expressed genes of receptors and ligands was used in each cell. Because there were more than one sample from each cell type, before the threshold was applied, the average expression of the same cell samples was obtained and finally the threshold was applied on 199 samples which were representatives of each cell type. In order to reconstruct the network, those genes of the receptor and ligand, whose, according to the threshold defined in equation 1, expressions were above their mean expression (X_i) plus three times the standard deviation of their expression (σ_i) in all samples, was considered as the expressed genes in each cell [12]. If the expression level of each gene is greater than the defined threshold, then the number (1) and otherwise (0) are assigned to them. *Figure 3b* shows how to obtain the expressed genes of the receptor and the ligand in 199 cell samples.

$$\bar{X}_i + 3\sigma_i < expression\ level\ of\ each\ gene \qquad \textbf{(1)}$$

Reconstruction of intercellular communications

According to the dataset of the receptor-ligand interactions and the list of receptor-ligand genes expressed in all the samples, only those receptor-ligand pairs or interactions were selected in which the expression of both the ligand and receptor was above the defined threshold. By identifying these interactions, communication between the cells is also indicated. For example, if the expression of a pair of receptor-ligands in two cells is higher than the specified threshold, obviously those two cells, one of which expresses the ligand gene and the other receptor gene,

have intercellular communication with one another [12], [27]. Next, the network reconstructed interactions were visualized and analyzed by open source software of Cytoscape *(Fig. 3 c)* [28].

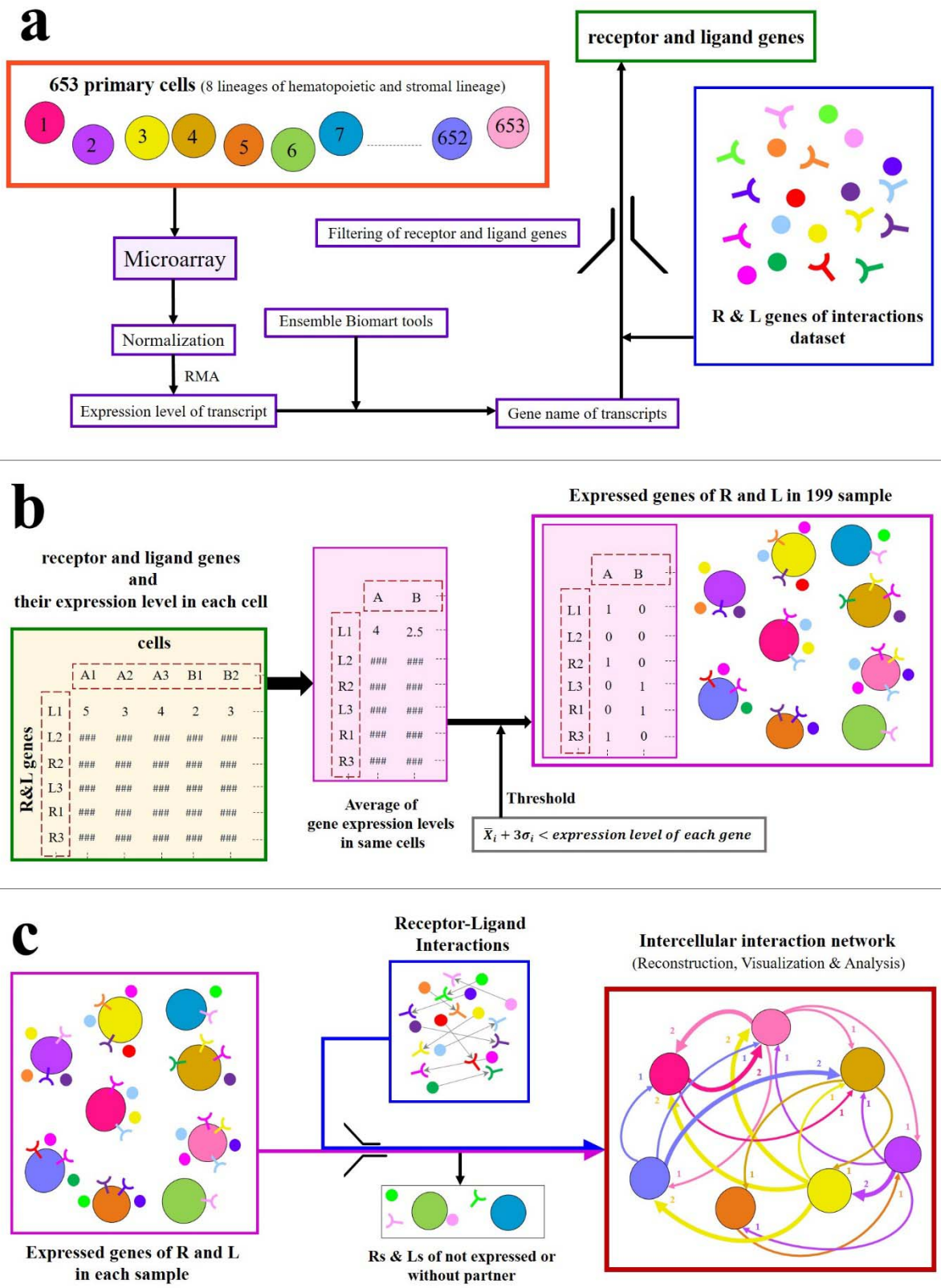
a
653 primary cells (8 lineages of hematopoietic and stromal lineage)
1
2
3
4
5
6
7
652
653
Microarray
Normalization
RMA
Expression level of transcript
Ensemble Biomart tools
Gene name of transcripts
Filtering of receptor and ligand genes
receptor and ligand genes
R & L genes of interactions dataset
b
receptor and ligand genes and their expression level in each cell
cells
A1 A2 A3 B1 B2
R&L genes
L1 5 3 4 2 3
L2 ### ### ### ### ###
R2 ### ### ### ### ###
L3 ### ### ### ### ###
R1 ### ### ### ### ###
R3 ### ### ### ### ###
A B
L1 4 2.5
L2 ### ###
R2 ### ###
L3 ### ###
R1 ### ###
R3 ### ###
Average of gene expression levels in same cells
Threshold
$\bar{X}_i + 3\sigma_i < expression\ level\ of\ each\ gene$
Expressed genes of R and L in 199 sample
A B
L1 1 0
L2 0 0
R2 1 0
L3 0 1
R1 0 1
R3 1 0
c
Expressed genes of R and L in each sample
Receptor-Ligand Interactions
Rs & Ls of not expressed or without partner
Intercellular interaction network
(Reconstruction, Visualization & Analysis)

Figure 3: *Creating a dataset of expressed receptor and ligand genes.* ***a)*** *Identification of receptor and ligand genes and their expression level in each sample.* ***b)*** *Determining the receptor and ligand genes expressed in each sample by applying the threshold.* ***c)*** *Reconstructing, visualizing and analyzing the intercellular interconnection network by integrating the receptor-ligand interaction dataset with the dataset of receptors and ligands expressed in each cell sample.*

Results

After integrating two data sets, based on the threshold defined, of 199 cell samples, 162 cells had receptors and ligands that were linked through 9271 communication paths. Of these cells, 102 cells, were both sender and recipient of the signal, while 14 were sender and 46 cells were recipient of signals. As shown in *Figure 4a*, more than half of the interactions between the receptors and the ligands were discarded using this threshold, and this volume of communications on the network was only created by 460 interactions between 202 receptors and 226 ligands.

Non-hematopoietic stromal lineage has the highest number of expressed receptor and ligand genes among other lineages

In this network, the non-hematopoietic stromal lineage expresses the most number of receptor and ligand genes, with expressions of 125 ligands and 102 receptors. Among the immune lineages, the macrophage lineage has the highest expression of receptor and ligand genes compared to other immune lineages, and in the remaining cell lineages, the lowest amount of gene expression is that of NK cells with expression of 3 receptor genes and 5 ligand genes *(Fig. 4b)*. The results of this reconstruction indicate that cells belonging to the immune systems use less pathways to communicate with each other than cells that are associated with non-hematopoietic lineages. These results confirm the results of previous study in this area where hematopoietic lineage cells had more limited network communications than other lineages, such as the parenchymal, epithelial and neural cell lineages [11].

Thymus tissue has the highest number of expressed receptor and ligand genes among other tissues

The dispersion diagram was used to examine the tissue distribution of the cells of the network and to determine the extent to which the cells of each tissue express the receptor and ligand genes and what lineage they belong to. According to *Figure 4c*, which is related to the distribution of receptor and ligand expression, mesenteric and thymic cells with expression of 162 genes (79 ligand genes and 83 receptors genes) and 173 genes (90 ligand genes and 83 receptors genes) respectively, had the highest expression of these genes compared to other tissues. In this network, cells of kidney, and pancreas tissue exhibited the least expression receptor and ligand genes respectively, compared to other tissues.

In *Figure 4d*, the number of cells, and lineages, as well as the number of receptor and ligand genes expressed in each tissue are shown. This chart is arranged based on the number of cells in each tissue from the tissues with the smallest number of cells to the tissues with the highest number of cells. As could be seen, tissues with a larger number of cells do not necessarily have more receptors and ligands. As an example, in a tissue like the spleen with 40 types of cells, the number of receptors and ligands is significantly less than the thymus gland with 32 types of cells. So, as seen in these two tissues, the number of genes expressed in each of these, is not only not increasing, but decreasing with the increase in the number of cells and lineages.

This can be due to the different type of cell lineages that exists in each of these two tissues. Spleen tissue cells consist of five lineages of B lymphocytes, dendritic cells, macrophages, natural killer cells and T lymphocytes, whereas in the thymus, the cells belong to the T lymphocyte, dendritic cell, and stromal cell lineages. Here again, the effect of stromal cells could be seen in the number of communications that may exist in each tissue, so that in tissues that have these types of cells (skin, thymus gland, skin lymph nodes, subcutaneous lymph nodes and mesentery), the expression of the receptor and ligand genes is much higher than those without them.

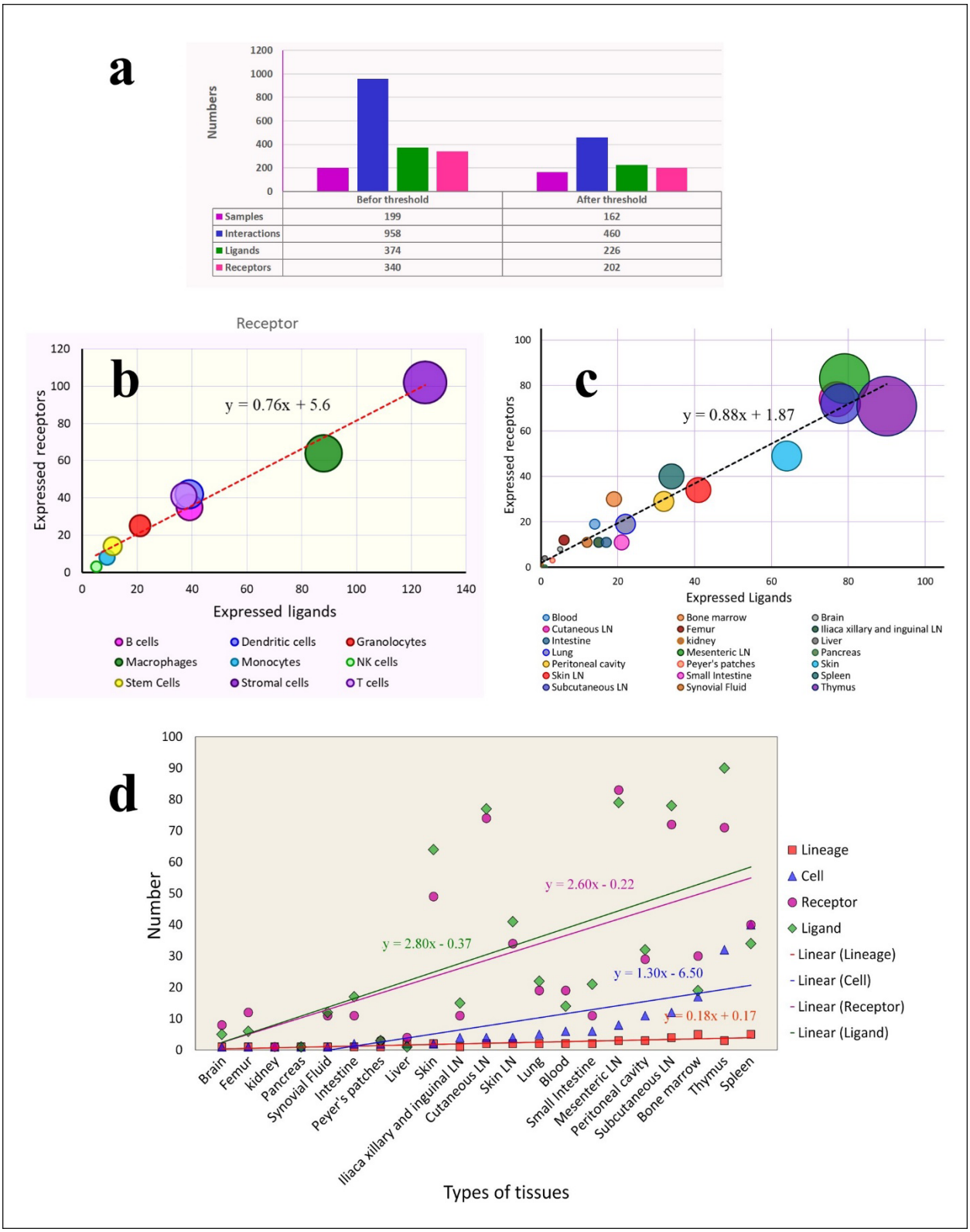

Figure 4: *Initial results of network reconstruction.* ***a)*** *Number of cells, number of interactions and number of receptors and ligands involved in the interactions before and after the threshold* ***b)*** *Level of expression of receptor and ligand genes separated by different lineages of the network* ***c)*** *Level of expression of receptor and ligand genes*

separated by extraction cell source ***d)*** *Number of cells, lineages, and the number of genes expressed by the receptor and the ligand, separated by the tissue from which they were extracted from (each of the lineages, cells, receptors, and ligands are marked with a distinct color).*

There is a correlation between the frequency of receptors and ligands, and their degree of specificity.

To characterize the cell type-specific expression of receptor and ligand genes, the degree of specificity of each, as well as the degree of specificity of each of the 460 receptor-ligand interactions in the network, was investigated. The results of this study indicate the specificity of the receptor and ligand genes expressed in 162 cell samples, such that, of a total of 226 ligands and 202 receptors expressed in 162 cell samples, 52 ligand genes and 45 receptor genes had a cell type specific expression. The Ccl2 ligand and the Ccr1 receptor, with expression in 15 and 14 cell samples, respectively, had the lowest degree of specificity compared to other receptors and ligands. In *Figure 5a*, the amount of cell specificity of the ligand with the number of ligand genes expressed in each of the specificity degrees is shown. For example, at the specificity level of seven, 16 ligands were expressed in seven different cell types of 162 cell samples. In *Figure 5b*, which is related to the cell specificity of the expressed receptor genes, indicates a high specificity of the expression of the genes. Therefore, a decreasing trend could be seen in the number of receptors expressed simultaneously with the decrease in the level of specificity in the range of 1 to 14, such that the expression level of the receptors, ranges from 36 receptor genes with the specificity degree of 1, to one receptor gene with the specificity degree of 14.

Receptors and ligands with high degree of specificity are expressed in stromal cells and macrophages.

Ligands with the highest degree of specificity were expressed in 21 different cell types, and thus some cells expressed more than a ligand with a specificity of one, so that the samples of stromal cells extracted from the thymus gland with expression of 12 specific ligands and macrophage cells in the spleen tissue with the expression of 9 specific ligands had the highest expression of specific ligands. In other words, the total number of ligands, which consisted of 21 ligands, is only expressed in these two cell type. In *Figure 5c*, the number of ligands expressed by each of these 21 cell types is displayed.

In 12 cell samples, which account for more than half of the studied cells, only one specific ligand was expressed. *Figure 5d*, represents the number of specific receptors in each of the cell samples, indicating that 36 specific receptors are expressed in 29 cell samples, of which 9 cells have more than one specific receptor, whereas 20 samples synthesize only one receptor gene. The macrophage cells located in spleen tissue and stromal cells of thymus tissue, with expressions of 6 and 4 receptor genes respectively, have a higher number of specific receptors than other cells.

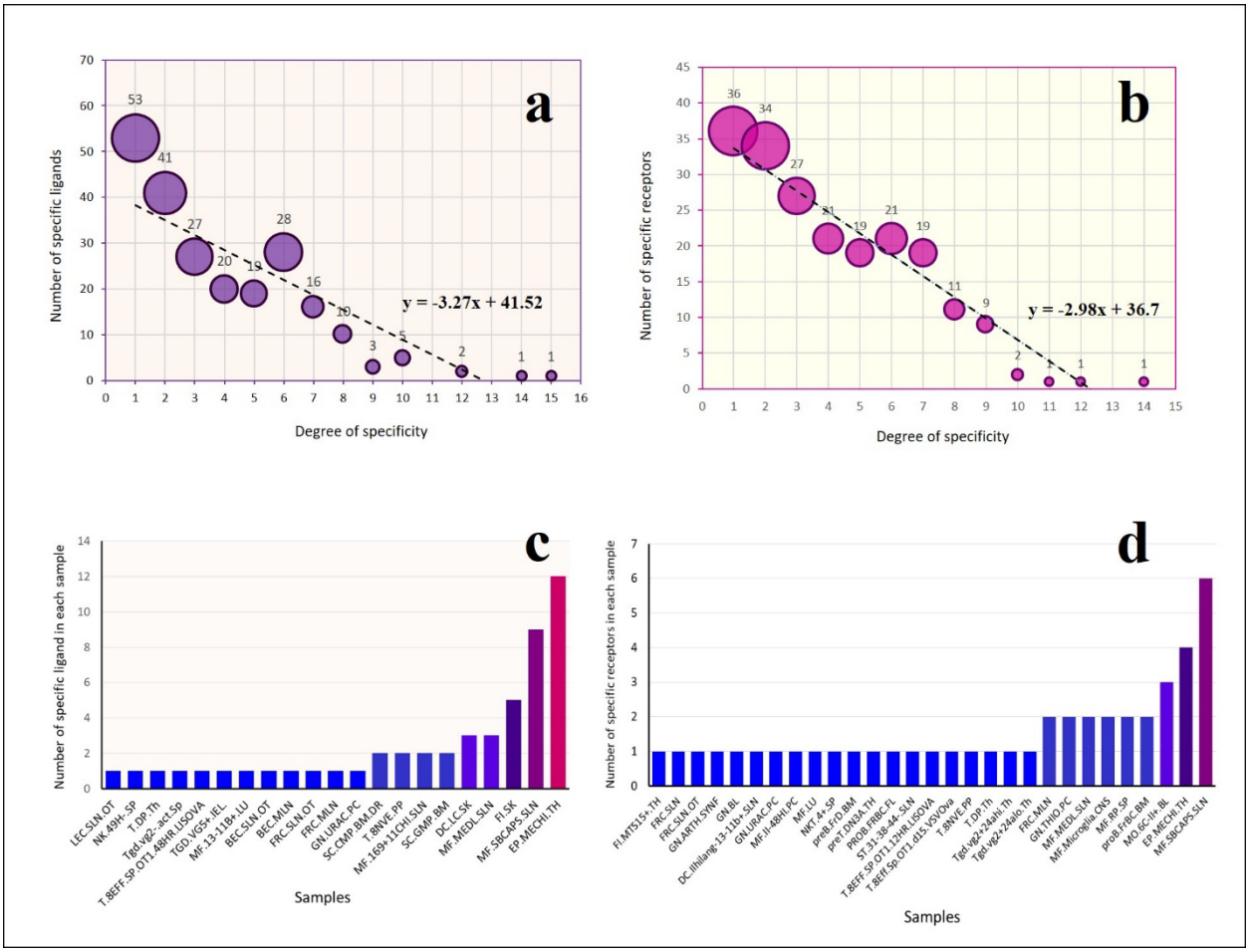

Figure 5: *Specificity of cell expression of receptors and ligands* ***(a*** *and* ***b)****. The number of receptors and ligands with the highest specificity in each cell type* ***(c*** *and* ***d)****: In the horizontal axis, each column represents a cell type, and the vertical axis of the graph shows the number of receptors and ligands with specificity of one in each of these cells.*

The results obtained from reviewing the specificity of the interactions between the receptor and ligands, and the 9271 communication links that exist in the network among 162 cells, are shown in *Figure 6* in form of two graphs. In graph A of *Figure 6*, the distribution of the specificity of receptor and ligand interactions among 162 cells has been investigated, which indicates the high specificity of these interactions in this reconstructed network, such that, interactions with the specificity for ligand 15 and receptor 14 are interactions with lowest specificities, whereas the lowest level of interaction specificity that could exist in this network with 162 cells, is the interaction with the receptor and ligand specificity of 162 which is the number of cells in the network.

As shown in the diagram above, the range of the specificity of the interactions between the number 1 to 14 for the receptors and 1 to 15 for the ligands in the 9271 interactions in the network has been reconstructed; in other words, the interactions of the network have a specificity of 1-1 to 14-15. *Figure 6* shows the frequency of interactions occurring at each of these specificity levels. As could be seen in this chart, the frequency of interactions has a specificity of

6 for the ligands, and 8, 7, and 6 for the receptors, which means the specificity degree of 6→6, 6→7 and 6→8 is high. The frequency of interactions with a specificity of 1 (1→1) for both the receptor and the ligand genes are very low in the reconstructed network and equal to 17. The most frequent interactions, are for the specificity of 8→6, which is 432 interactions, and the lowest frequency is related to the specificity of 1→10 and 1→2, each with 10 interactions. Of the 9271 interactions, the frequency of interactions in which the receptor specificity is lower than the ligand is 4331. The interactions in which the specificity of the ligand is less than the receptor are 3750, in 1190 of which, the specificity of the receptor and the ligand are equal.

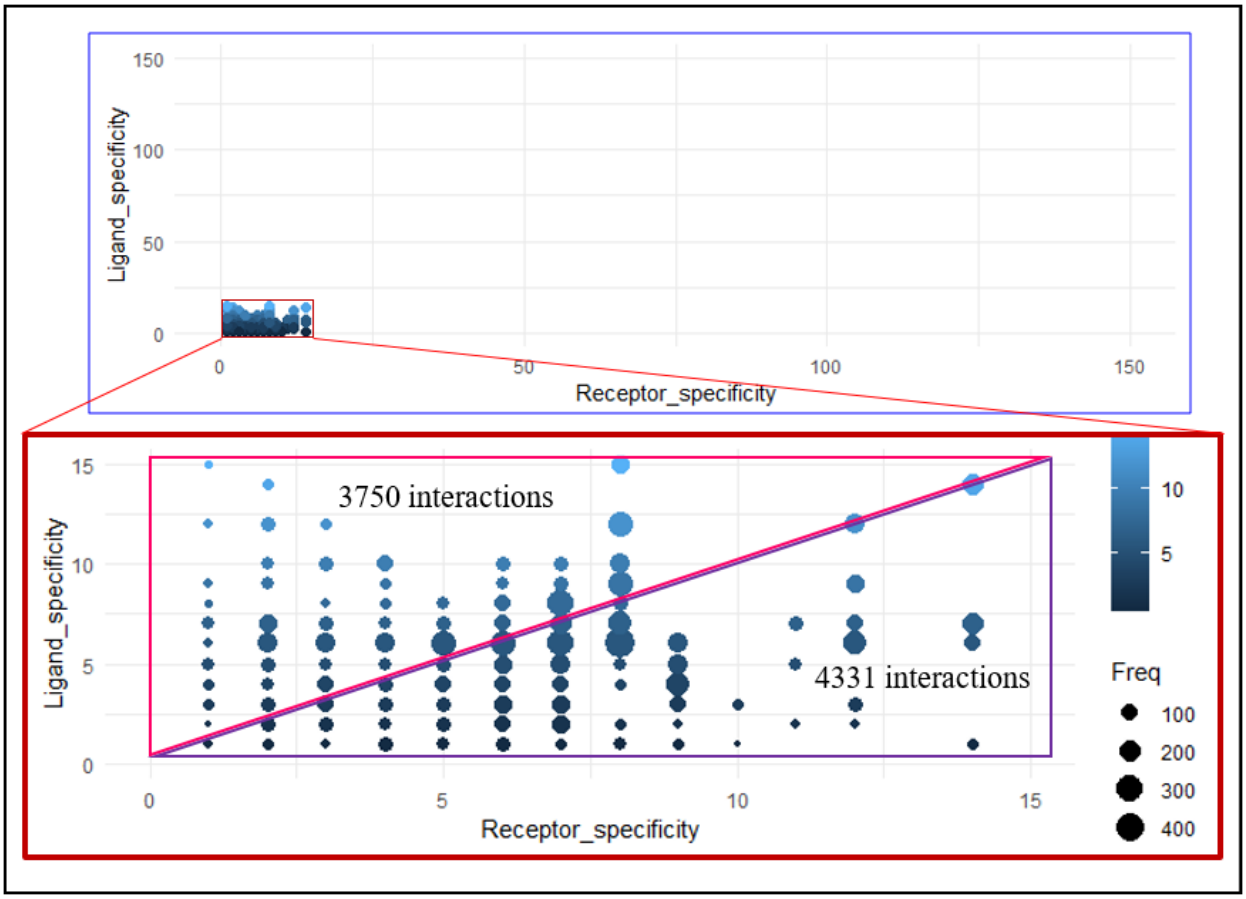

Figure 6: *Specificity of the receptor-ligand interactions: Each circle represents an interaction specificity, which is represented by two numbers, including the specificity of the receptor and ligand involved in each interaction. The frequency of each level of specificity in the network is determined by size.*

Stromal cells have the highest number of communication in the network

In the intercellular communication network, the number of nodes displayed was 162, representing each of the cell types in the reconstructed network. The results of the network analysis indicate that the cells with the most network communications are related to the non-hematopoietic stromal cell lineage and also among the hematopoietic lineages the lineage with the most communication is the B lymphocyte, and T lymphocytes in this network have the least communication with other cells. Two stromal cells called FRC.SLN and FRC.MLN of lymph nodes of the skin and mesentery have, respectively, an output node degree of 651 and an input degree of 381, having the most communication as the sender and as the recipient of the signal in the network. Examining other network measurement criteria indicates that the stromal cells associated with the thymus and lymph nodes and subcutaneous lymph nodes have the highest degree of closeness centrality in the network. Measuring the amount of betweenness centrality indicates that all the cells studied in the network demonstrate low values in terms of interstitial centrality. The highest clustering coefficient, equal to 1, belongs to the T and B lymphocytes and the dendritic cells.

Stromal cells have the most autocrine communication

In order to investigate the existence of autocrine communications in the network, and determine what cells are involved in this very important process of signaling, the network representation was examined based on the presence or absence of the loop. In this network, there were 35 nodes or cells that had a loop showing an autocrine signaling in these cells (*Fig. 7a*). The size of the nodes is based on their degree, high-degree cells or hub nodes of the network are all cells that have autocrine signaling paths. The stromal cells in this network have the most autocrine communications in comparison with the other cells.

Natural killer cells have a small share of network communications

In *Figure* 7*b*, the cells involved in the interaction are presented on the basis of the lineage to which they belong. Non-hematopoietic stromal cells, although small in number compared to immune cells and according to the results of the analysis, have the most communications with themselves and with other cells. NK cells, despite being more than monocyte lineage cells, have the smallest amount of communications in the network and have a very small share of the network communications. The network representation of the tissues from which the cells have been extracted, according to *Figure 7c*, are mainly the spleen, thyroid gland, bone marrow, and subcutaneous lymph nodes and some tissues such as the brain, kidney and liver have only one sample in the reconstructed network.

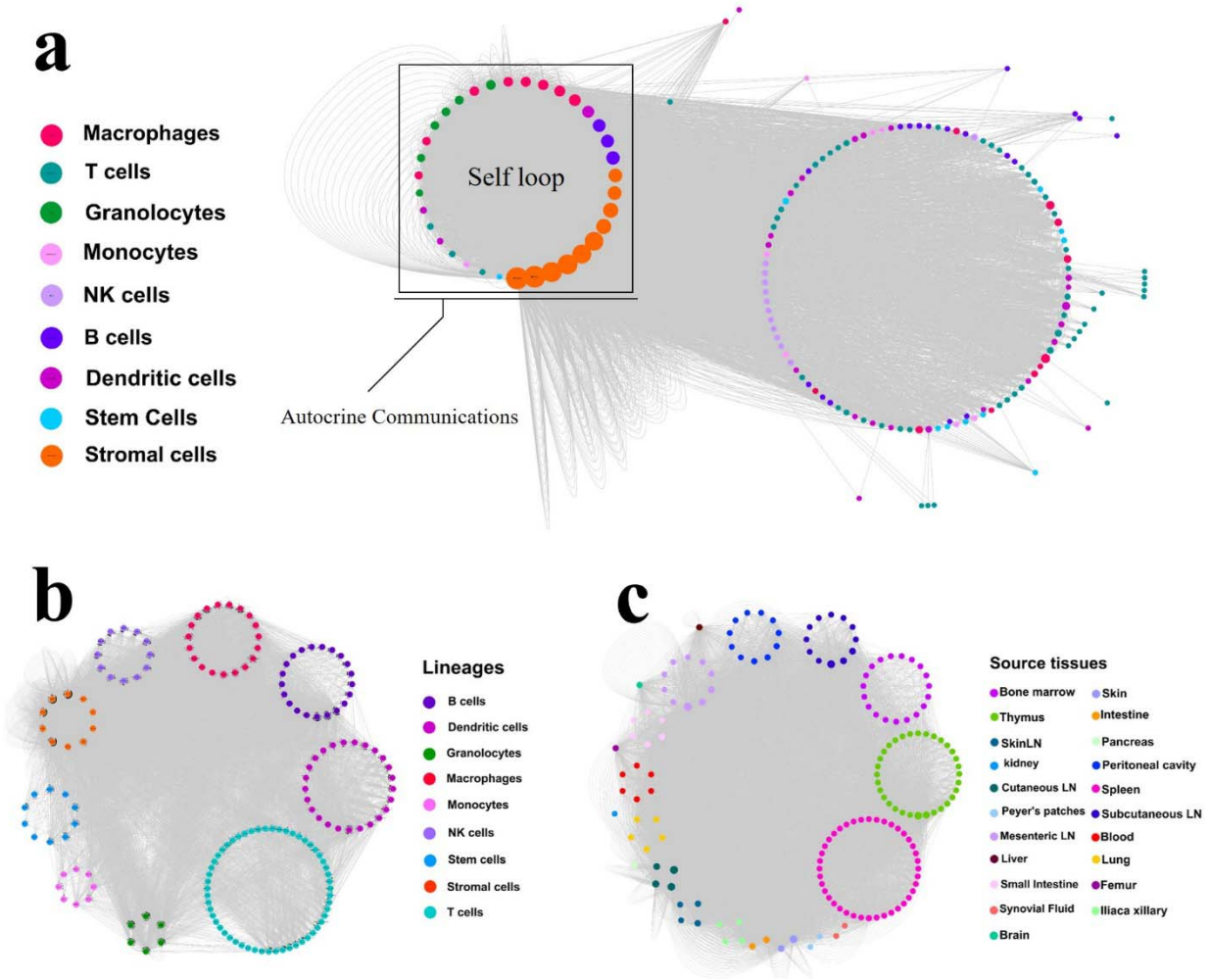

Figure7: *Intercellular communications network.* ***a)*** *Intercellular interaction network, determining the existence of autocrine interactions.* ***b)*** *Intercellular interaction network separated by different lineages* ***c)*** *and different tissues.*

Macrophages have the most intra-lineage communications compared to other immune lineages.

Figure 8 is a view of the network visualized by the Cytoscape software, which, for ease of distinguishing the lineage and existence of a number of different communication pathways between all the lineages, nodes and edges are represented by various sizes and colors. In the inter-lineage interaction network, there are 9 nodes, each representing a cell lineage. The linking edges between the lineages are weighted, which indicates the difference in the number of signals that are exchanged between different network lineages.

In this network, the stromal cells have the highest degree of connectivity with other lineages, and the (NK) lineage also has the least interaction in network. The stromal cell lineage has the most intra-lineage communications in comparison to other lineages. In this network, the stromal cell lineage sends the most signals to other lineages, and the macrophage lineage receives the most

signals. The NK lineage has no intra-lineage communications and also lacks inter-lineage communications with monocytes and hematopoietic stem cells.

Examining the degree distribution parameter in this network indicates that the stromal cells, dendritic cells, granulocytes, and macrophages, have the highest degree in this network. In this network, due to the fact that, removal of any of the nodes does not cause much damage in the stability and existence of the network and the loss of communication between two cells or two lineages and most lineages are directly related to each other and there is no need for an intermediate cell to interact, the parameter of betweenness centrality of these nodes is low. In this network, the parameter of the clustering coefficient calculated for all nodes is high and the values of 1 and close to 1 for all nodes have been reported. The reason for this parameter to be high in the network is that the neighboring lineages of each lineage, are intra-connected themselves.

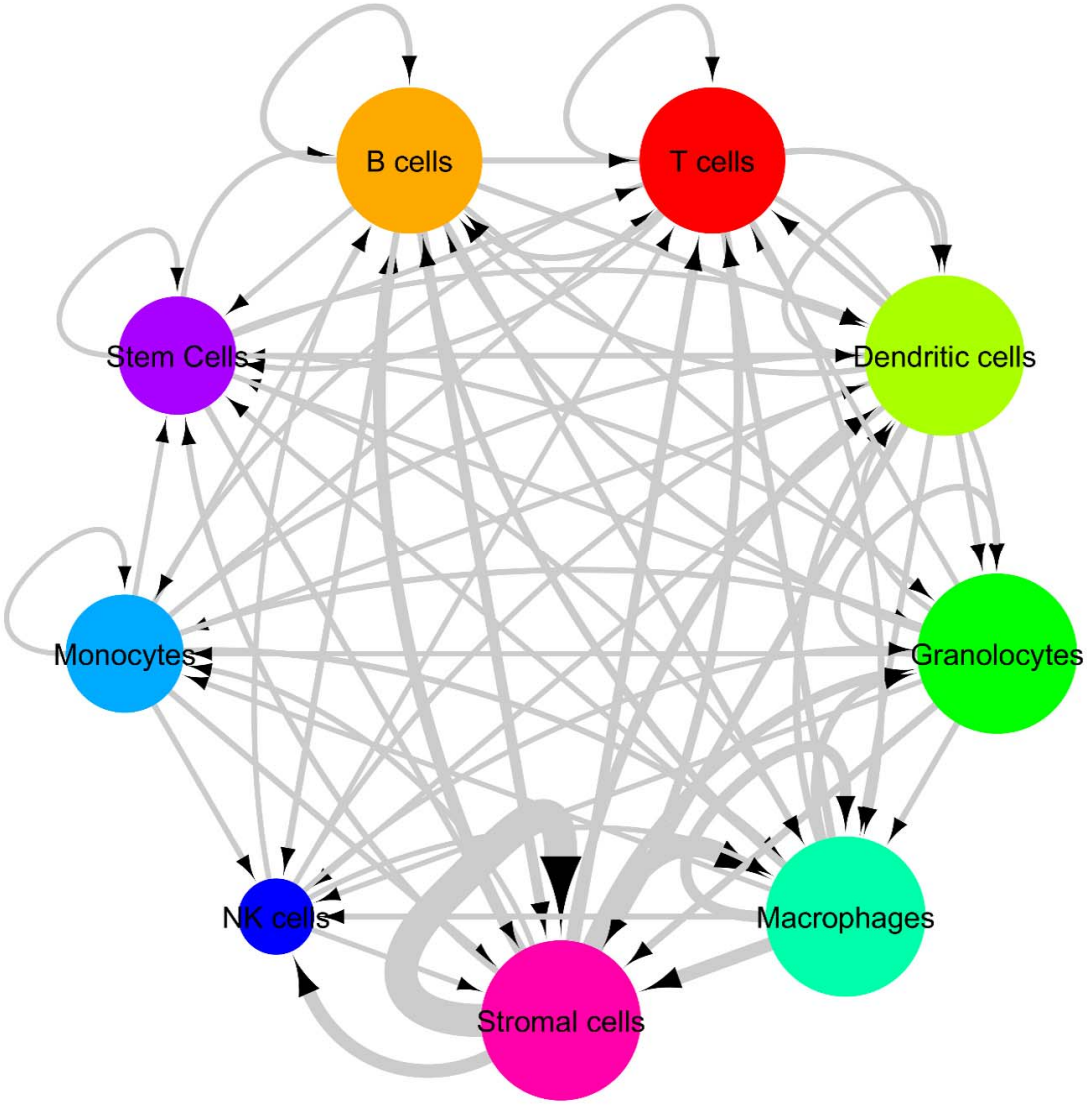

Figure 8: *Inter-lineage communication network*

Figure 9 is a demonstration of an intercellular communication network with a three component pattern in which besides cells, receptors and ligands are also displayed as network nodes, so that more information can be displayed visually from the network [1]. In this tripartite network, the number of cells that only send the message is less than the other cells, and the cells that act on the network as the sender and recipient of the signal are more abundant than the rest of the cells. The betweenness centrality parameter for all components of the network including cells, receptors, and ligands is very low. In this network, integrin receptors and also chemokine receptors have the highest degree of communication with the ligand molecules.

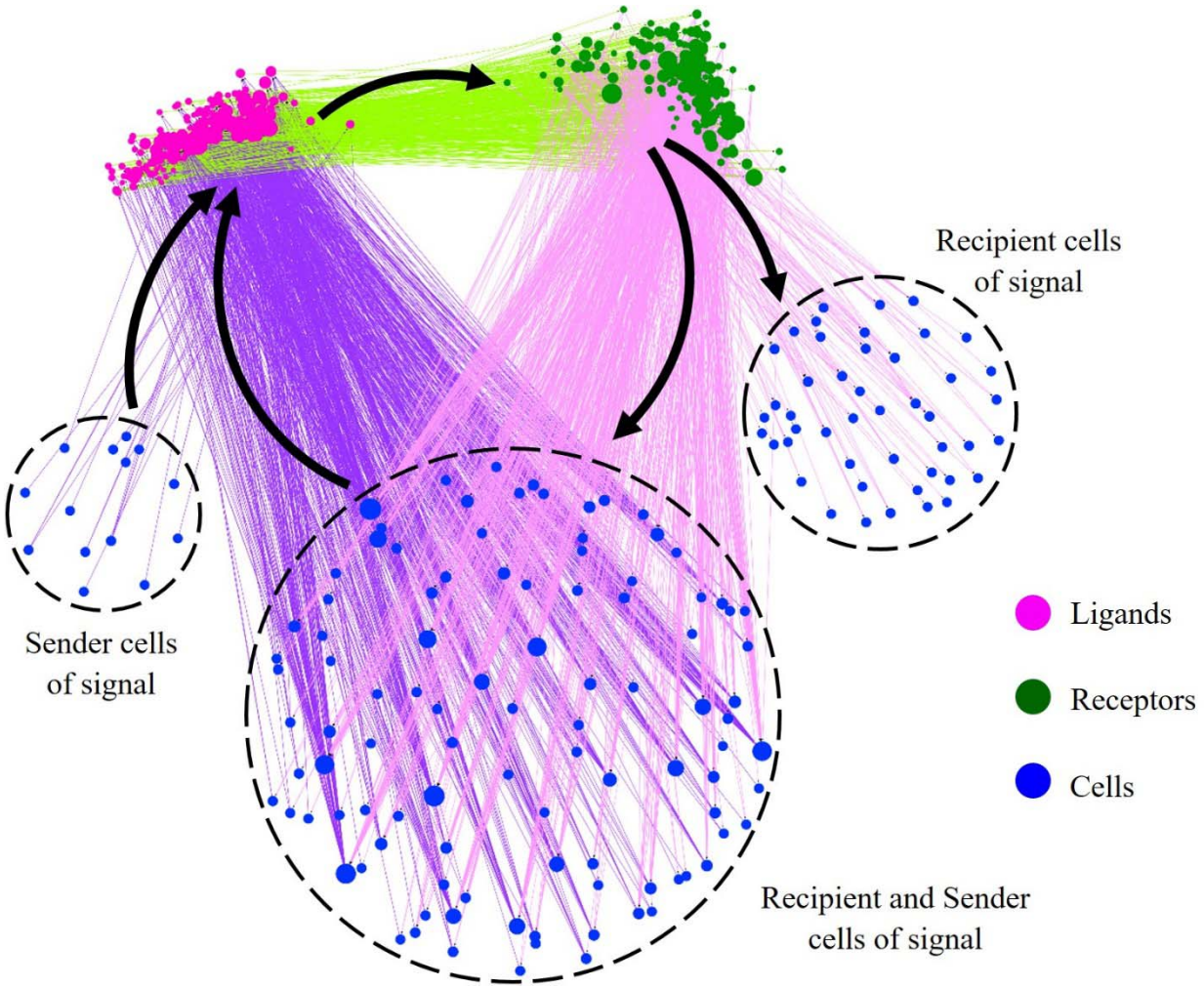

Figure 9: *Visualization of the intercellular interactions tripartite pattern: The blue nodes represent the types of cells in the network and the pink and green nodes represent the ligands and receptors in the network, respectively.*

Discussion

In this study, the intercellular interaction network was reconstructed using receptor and ligand interactions and using gene expression data. The expression dataset used for this reconstruction included immune cells from different hematopoietic lineages and non-hematopoietic stromal cells. Finally, using the receptor-ligand interaction dataset, communications between the remaining 162 cells expressing receptor and ligand genes were determined and the network was reconstructed.

A large number of receptor and ligand genes in this study, were expressed in stromal cells and macrophages. This suggests that the type of cell involved in cellular communication has a great influence on the level of the expression of these genes. Stromal cells are non-hematopoietic stem cells and are present in most organs of the body, such as skin. Kidney, pancreas, bone marrow, lymph nodes, and the like. Tissue -resident stromal cells play an important role in the differentiation and maturation of the tissues cells and their progenitors [29]. These cells have a supporting role in the function of cells and also participate in hematopoiesis and its regulation [30]. One of the reasons for the high expression of receptor and ligand genes in these cells can be due to different roles that play in each body organ, which requires expression of different types of receptor and ligand molecules to interact with cells located in each organ. In the lymph nodes (LNs), the interactions between the stromal cells, T cells, B cells and dendritic cells lead to the initiation and maintenance of the adaptive immune response process, and on the other hand, these cells also organize the function of the lymph nodes [31], [32].

Researches have shown that markers of skin-resident stromal cells are very similar to markers of mesenchymal stem cells and can be differentiated to adipogenic and osteogenic cells [33]. T cell development depends on a variety of thymic stromal cells which are located in a specialized microenvironment [34] In this network, receptor and ligand genes of stromal cells are expressed in thymus, skin and lymph nodes. Most of the ligands synthesized by stromal cells are cytokines, growth factors, and ECM proteins, and the receptors expressed by these cells also include RTK and RSTK receptors which categorized as enzyme-linked receptors. Gene enrichment analysis of ligands and receptors expressed of stromal cells that were carried out by the DAVID tool [35] shows that these genes are present in biological processes such as the development of multicellular organisms, tissues and organs development, positive regulation of cell division, immune responses, morphogenesis of organs, and anatomical structures and cellular differentiation.

Among the stromal lineage cells, there is a strong tendency to intra-lineage communications, in which the interactions between the wnt5a ligands with receptors of the Ror1/2 and Fzd5a play a very important role. Activating the Fzd1 and Ror1/2 by Wnt5, triggers pathways associated with cell proliferation, differentiation, polarity, and apoptosis and have a positive effect on inflammatory diseases [36]–[38]. In this network,

the FRC.MLN stromal cell of mesenteric lymph nodes, has 32 loops with the most autocrine communication pathways, of which 32 communication pathways are only due to two interacting receptor-ligands, one of which is the interaction between the angiotensin-converting peptide hormone and its specific receptor. Macrophages exist in various tissues such as brain, lung, liver, spleen, bone marrow and lymph nodes. This cells are involved in immune and anti-tumoral responses to pathogens, maintenance of tissue homeostasis, and tissue repair [39], [40].

According to the results, the stromal cells have the most communication with other cells especially macrophages *(Fig. 7)*. In addition, bone marrow stromal cells play an important role in differentiating these cells by creating a suitable environment for growth and differentiation of hematopoietic cells. One of the most important immune cells that forms such an environment is macrophages [41]. Therefore, one of the reasons that the stromal cells in the network have the most communication with macrophages can be the role of these two cells in the process of hematopoietic cell differentiation.

A part of this study was assessing the specificity of cellular expression of the receptor and ligand genes in the regenerated network, which indicates that their specificity is high. The specificity of the expression of receptor and ligand genes and, consequently, the interactions they have with each other, can be attributed to the fact that the transmission of information between cells in a porous tissue is not a random process without planning. Therefore, the coordinated behavior of cells to maintain the stable state of multicellular organisms, which is largely dependent on intercellular communication, is that the transmission of messages between cells is selective. This is possible only if the expression of the components involved in the transmission of the message, i.e. receptors and ligand molecules, is specific to the type of sender and recipient cells [11], [42]

In one study on the specificity of cell-dependent expression of receptor and ligand genes, the cell specificity of various proteins expressed in different positions of a cell was evaluated. The results of this study showed that the secreted and membrane proteins, receptors and ligands included, have significantly higher cell-specific expression than that of the intracellular proteins such as cytoplasmic proteins and proteins present in nuclei and organelles.

Conclusion

One of the most challenging steps in this study was the choice of the type of data needed to determine the receptor and the ligand used in each of our cells, such that, selection of each causes loss of some information needed for thorough assessment of this process. For example, the use of transcriptomics data or proteomics information needs to ignore some receptor and ligand interactions, where the ligand molecule is non-protein such as steroid hormones. Similarly, the use of metabolomics data requires the exclusion of molecules from proteins. On the other hand, data on the interactions between metabolites and proteins are very small in the database.

Because of the limited amount of proteome data, transcriptomics data is more frequently used to study and investigate intercellular communication networks and if any research has been done in this area with proteomics data, it has been in small scales and with experimental data from the same research.

One of the ways to overcome this limitation can be the simultaneous use of AMIC data such as transcriptomics, proteomics, and metabolomics which have been derived from a cell. By combining several OMICS data, complex biological processes such as intercellular communication can be more effectively analyzed. Needless to say, due to the lack of data obtained from the simultaneous analysis of several omics, which are also referred to as "multiomics analysis", the use of this method to restructure the network is subject to limitations [43].

Proposed strategies to involve interactions between receptors and metabolic ligands in a network reconstructed by transcript information could be examining the expression of genes involved in the synthesis of these metabolites. For example, the enzyme which catalyzes the last reaction of the pathway of a metabolite synthesis or the gene associated with the restriction enzyme in that metabolic pathway, both of which could be thought of as a representative of that metabolite, and their expression level is considered as the level of expression of that metabolite.

Another constraint in the procedure of messaging network reconstruction and generally any bio-network reconstruction, which makes interpreting the results of the reconstruction difficult, is the fact that biological processes are affected by various parameters and conditions in a dynamic bio system. For instance, in the case of the intercellular signaling process, the distance parameter plays a very critical role in communication between two cells. The distance at which the two cells with a pair of expressed receptor and ligand genes are located from one another, and whether the two cells are located in the same tissue or in two separate tissues, affect the communication between the two cells through these receptor and ligand.

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