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WEB SERVER ISSUE

CausalCCC: a web server to explore intracellular causal pathways enabling cell-cell communication

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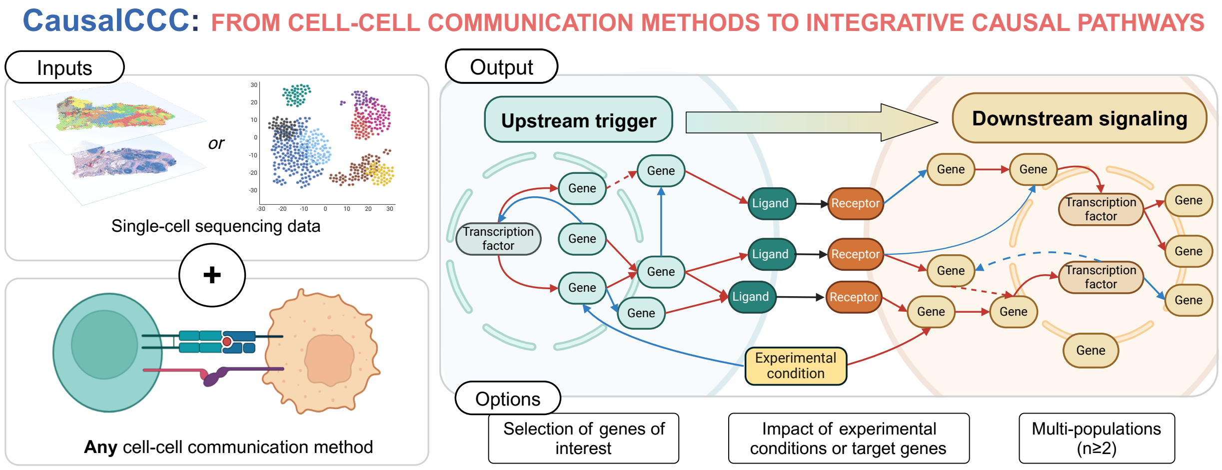
Abstract

Understanding cell-cell communication (CCC) pathways from single-cell or spatial transcriptomic data is key to unraveling biological processes. Recently, multiple CCC methods have been developed but primarily focus on refining ligand-receptor (L-R) interaction scores. A critical gap for a more comprehensive picture of cellular crosstalks lies in the integration of upstream and downstream intracellular pathways in the sender and receiver cells. We report here CausalCCC, `https://miic.curie.fr/causalCCC.php`, an interactive web server, which addresses this need by reconstructing gene-gene interaction pathways across two or more interacting cell types from single-cell or spatial transcriptomic data. CausalCCC includes a graphical introduction and a demo dataset within the workbench page as well as a comprehensive tutorial. CausalCCC methodology integrates a robust and scalable causal network reconstruction method, MIIC, with internally computed ligand-receptor pairs using LIANA+ (including CellphoneDBv5, SingleCellSignalR, Connectome, NATMI, Log2FC). Alternatively, user-defined ligand-receptor pairs from any CCC methods can also be uploaded. We showcase here CausalCCC on different single cell and spatial transcriptomic datasets from three original CCC methods (NicheNet, CellChat and Misty). CausalCCC web server offers unique interactive visualization tools dedicated to single-cell data practitioners seeking to go beyond L-R scores and explore extended CCC pathways across multiple interacting cell types.

Graphical abstract



Introduction

Cell-cell communication (CCC) is a fundamental process in multicellular organisms, enabling coordination between individual cells and ensuring an intricate signaling network of complex biological processes. High-throughput technologies such as single-cell RNA sequencing (scRNAseq), spatial transcriptomics and proteomics have revolutionized the study of CCC, providing unprecedented insights into the heterogeneity of cellular interactions. Decoding ligand-receptor (L-R) interactions and mapping associated signaling pathways are critical for understanding how dysregulation of communication networks contributes to disease, particularly in autoimmune disease and cancer. Over the past decade, CCC methods have become increasingly sophisticated, moving beyond reliance on curated ligand-receptor (L-R) databases or gene regulatory network (GRN) repositories to identify and rank interacting L-R pairs [1].

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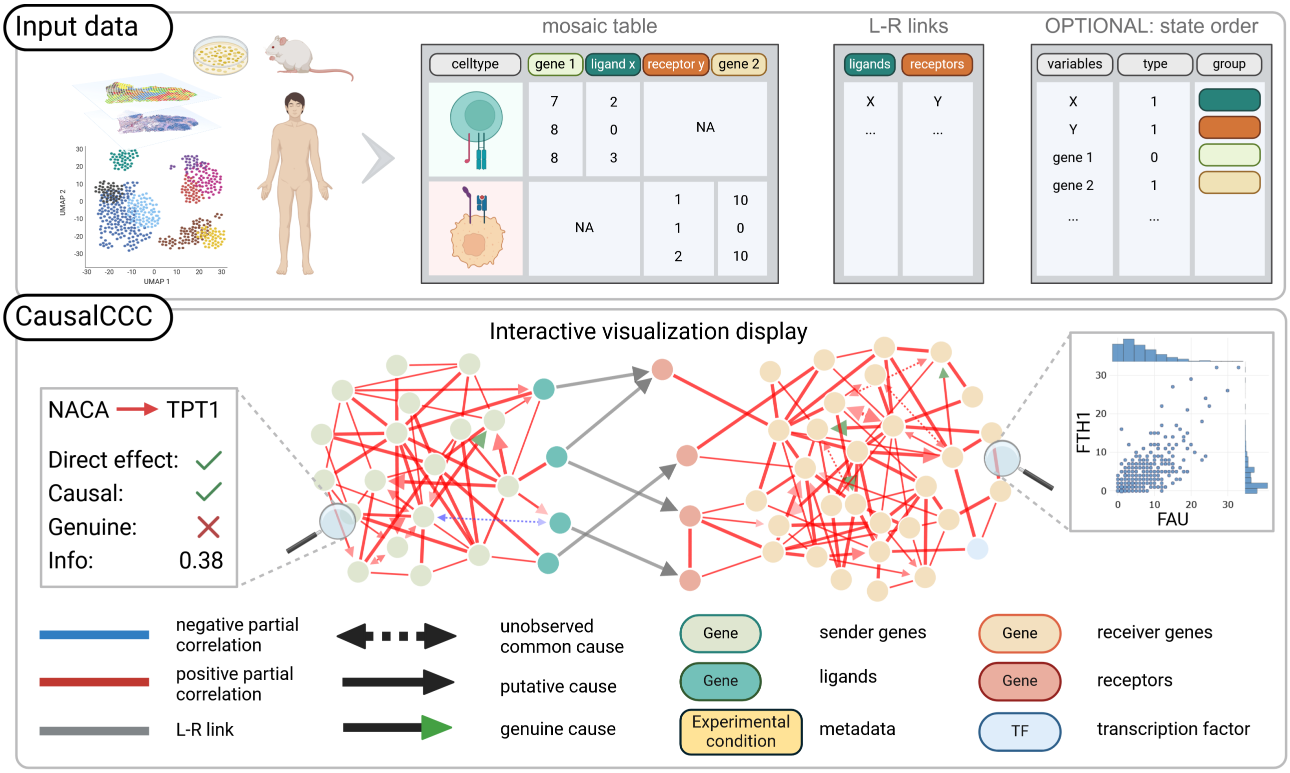

Fig. 1. Overview of CausalCCC web server's workflow to infer, visualize and interprete CausalCCC networks across two or more cell populations, starting from single-cell or spatial transcriptomic data and internally computed or user-defined ligand-receptor (L-R) links from any CCC method.

However, while curated L-R databases [2, 3, 4, 5, 6, 7, 8] and integrative CCC methods [5, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23] provide an informative starting point, inferring upstream and downstream intracellular signals remains a complex task and CCC network inference methods face significant challenges. Existing approaches often rely on correlation-based methodologies, inferring many spurious associations, which limit the accuracy of predicted intracellular pathways and lack the ability to uncover causal relations. Another issue concerns the selection of genes included in the inferred CCC networks. CCC methods typically rely on differentially expressed gene (DEG) analysis or on GRN-based approaches, which estimate regulatory gene activity based on known GRN interactions. While DEG analysis between cell populations may overlook important genes in practice, GRN-based approaches are more robust but somewhat indirect and effectively reverse the cause-effect relation between regulatory genes and their known targets. In addition, both methodologies lack the ability to include other important features of interest, such as experimental conditions (*e.g.* control *versus* perturbation) or patient specific information. Finally, CCC studies integrating information from gene interaction pathways are typically restricted to cell-cell interactions between two populations only, overlooking the complexity of molecular interactions across multiple populations or molecular niches.

To address these limitations, we report CausalCCC, an interactive web server, which reconstructs gene-gene interaction pathways across two or more interacting cell types from single-cell or spatial transcriptomic data, Figure 1. CausalCCC methodology integrates a robust causal network reconstruction method, MIIC, with internally computed or user-defined L-R pairs from any available CCC methods. CausalCCC provides also a customable selection of relevant genes based on a robust information theoretic measure of gene expression variations in single cell populations, rather than DEGs across populations. Finally, CausalCCC web server provides a user-friendly platform with comprehensive online documentation, including demo datasets, and a unique interactive visualization tool, which facilitates the exploration and interpretation of complex communication pathways across multiple cell types.

Materials and methods

Workflow implementation

CausalCCC computational workflow consists of 3 main steps:

First, the L-R links of interest between sender and receiver populations are retrieved using with internally computed L-R pairs using LIANA+ [23] (including CellphoneDBv5 [4], Connectome [8], SingleCellSignalR [12], NATMI [13], Log2FC [23]). Alternatively, user-defined L-R pairs from any CCC methods (such as NicheNet [10], CellChat [14], COMMOT [24], MISTy [25]) can also be uploaded. The case studies, presented in Results, showcase CausalCCC on three single cell and spatial transcriptomic datasets from three original CCC methods.

Second, an optional feature selection step recovers upstream and downstream genes of interest in sender and receiver cells, based on their high mutual information (MI) scores with the selected ligand or receptor genes, respectively, or with any other experimental features or metadata of interest.

Third, the reconstruction of the upstream and downstream intracellular networks is performed on the web server using the robust and scalable causal network reconstruction method, MIIC [26, 27, 28, 29, 30] (Multivariate Information-based Inductive Causation), already successfully applied to single cell transcriptomic data [26, 27, 31].

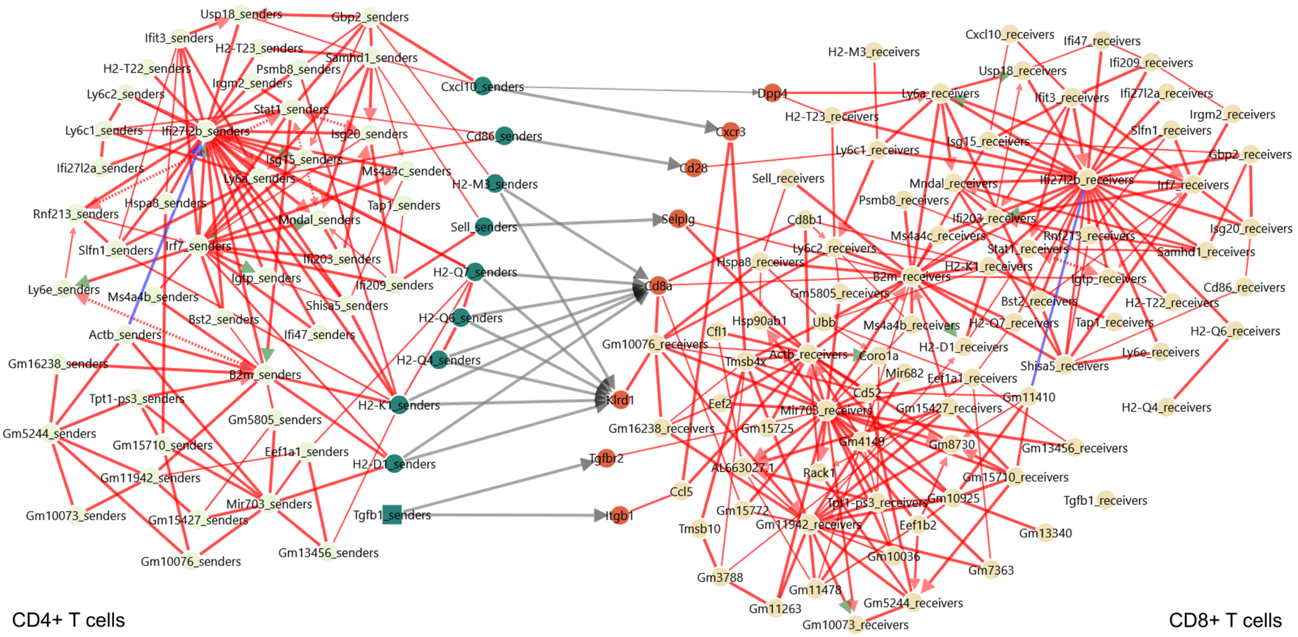

Fig. 2. CausalCCC network between CD4$^+$ (sender) and CD8$^+$ (receiver) T cells in the inguinal lymph nodes [32] using L-R pairs inferred by NicheNet [10]. Nodes represent genes, with ligands in dark green and their paired receptors in dark orange. Upstream and downstream genes were selected using CausalCCC MI-based selection. Edges indicate direct association (non-oriented), putative causation (oriented) and genuine causation (green arrowhead). Edge thickness reflects the strength or confidence scores of intracellular interactions (MI) and L-R links (optional). Key ligands, H2-K1 and H2-D1, connect to MHC-I pathways, essential for antigen presentation.

The final CausalCCC result is a global network highlighting how upstream intracellular signals lead to downstream intracellular effects through specific L-R interactions.

Results

The CausalCCC web server

CausalCCC offers an interactive web server solution to reconstruct gene interaction pathways across interacting cell types, downstream of the usual processing of single-cell RNAseq objects. This allows users to keep a total control of their single-cell object. CausalCCC includes a **Graphical introduction** and a demo dataset downsized from a classical T Cell-B Cell interaction network, as well as, a comprehensive **Tutorial** with detailed optional R bookdowns. CausalCCC network outputs provide a unique integration of upstream and downstream pathways across interacting cell types, which cannot be obtained by intracellular pathway analysis or CCC methods alone.

Input: Users need to provide the raw count expression matrices for the sender and receiver cells as a text table (csv, tsv, txt, etc) with columns corresponding to selected genes and rows to single cells. Large input files can be compressed (zip) to facilitate upload. A list of ligand-receptor (L-R) pairs of interest with (optional) strength or confidence scores should also be provided to integrate CCC analysis. If needed, CausalCCC offers a wrapper function to seamlessly prepare all input files from a single-cell object (Seurat or Anndata) and a selection of CCC methods. This function also offers an MI-based selection of relevant genes. Step-by-step guidance is provided in the **Quick start** and **Advanced mode** Tutorials.

Output: The result is a causal network which elucidates how intracellular signaling upstream of ligand expression in sender cells leads to receptor activation and downstream signaling in receiver cells. Edge thickness reflects the strength or confidence scores of the intracellular interactions (MI) and the internally computed or user-defined L-R links (optional). Compared to an R package, CausalCCC web server offers enhanced computational power along with a unique interactive visualization tool within the browser, allowing users to explore their CausalCCC networks and underlying data in detail. For guidance on the interpretation of CausalCCC network results, visit the **How to interpret your network** section of the Tutorial. We showcase below three examples of CausalCCC networks reconstructed from single-cell and spatial transcriptomic datasets using different CCC methods and two or more cell populations. Additional biological interpretation is provided in the Supplementary Note.

Case study 1: CausalCCC network between two immune cell populations in lymph nodes

The first case study infers intracellular pathways between CD4$^+$ (sender) and CD8$^+$ (receiver) T cell populations in the inguinal lymph nodes under steady-state condition and after viral infection [32]. The resulting CausalCCC network, Figure 2, highlights expected intracellular pathways across these two interacting T cell populations, using L-R pairs inferred by Nichenet [10]. In the CD4$^+$ sender network, top predicted ligands are associated to antigen presentation to CD8+ T cells via MHC class I *H2* proteins, with first-degree neighbors including *Psmb8, Ifi27l2b, Slfn1, Shisa5, Irf7, Mir703* and *B2m*. The potential upstream trigger *Stat1*, known to mediate the MHC-I pathway and Type I and II interferon (IFN) signaling in T cells [33], is found to be a genuine cause of *B2m* sharing direct or indirect associations with IFN-inducible genes (*e.g., Ifi27l2b, Ifi27l2a, Ifi47*) and IFN-stimulated genes (*Isg15, Isg20*). During viral infection, these pathways are indeed expected to be upregulated as part of a broad antiviral immune response with active antigen presentation to CD8$^+$ T cells. In the CD8$^+$ receiver network, the *Cd8a*

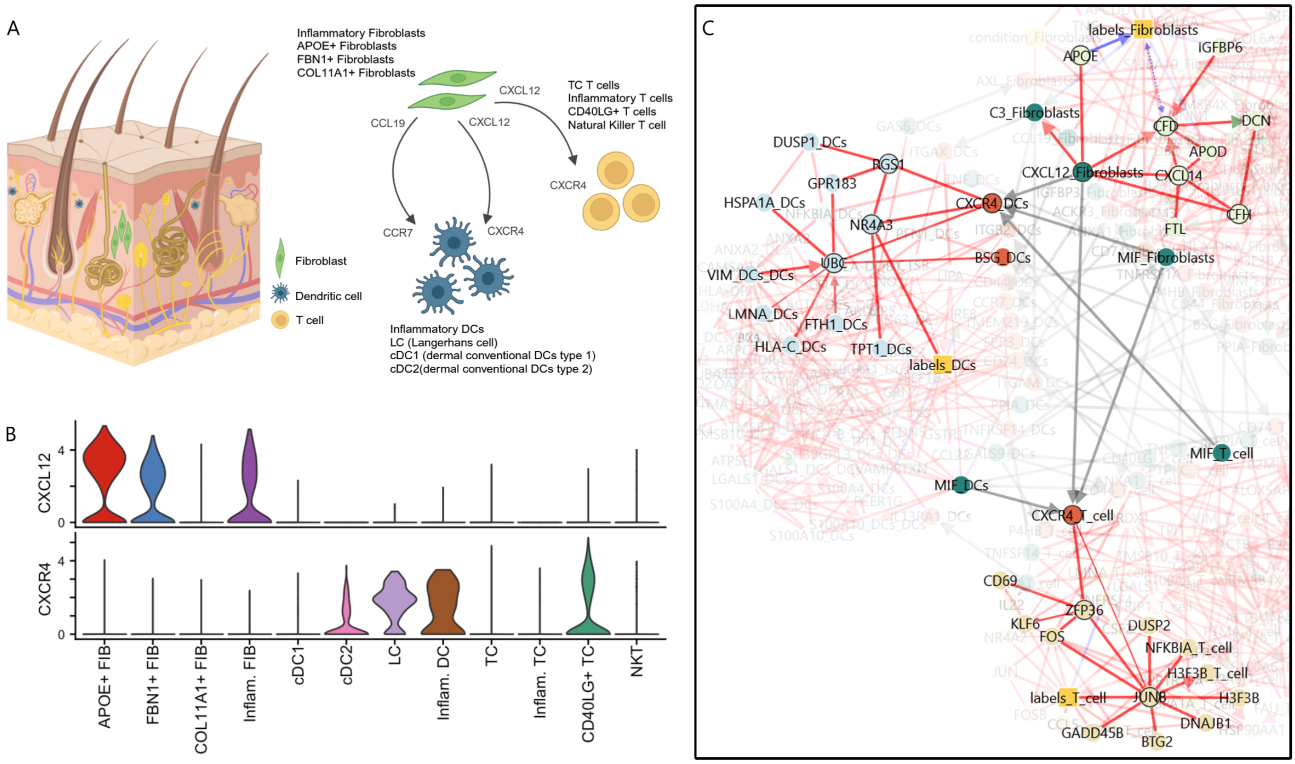

Fig. 3. CausalCCC network between fibroblasts, dendritic cells and T cells in atopic dermatitis [35], using L-R pairs inferred by CellChat [14]. **A**: Key cell populations in atopic dermatitis. **B**: violin plot showing CXCL12 and CXCR4 expression levels in lesional skin. **C**: CausalCCC second-degree neighbourhood subnetwork of CXCL12→CXCR4 L-R links. This network reveals the role of CXCL12, secreted by fibroblasts, in immune cell recruitment and inflammation, highlighting its important contribution to atopic dermatitis pathogenesis. The full causalCCC network is shown in Figure S1.

and *Klrd1* receptors of the MHC class I ligands are critical for the activation of CD8$^+$ T cell cytotoxicity. Downstream interactions with *Cd8a* and *Klrd1* include another subunit of the CD8 complex (*Cd8b1*) and genes known to transmit signals for T-cell activation and cytokine production, and found in high levels in virus-specific memory CD8+ T cells (*e.g. Ly6c2*, *Ly6c1* and *Ly6ca* [34]).

Case study 2: CausalCCC network between three cell populations in atopic dermatitis

The second case study concerns fibroblast and immune cell interactions in atopic dermatitis in the human skin [35]. Using L-R pairs inferred in the original CellChat study [14], we explored the crosstalks between fibroblasts (FIB), dentric cells (DC), and T cells (TC), Figure 3A, and reconstructed a three cell population causalCCC network, Figure S1, where each cell type is both sender and receiver. Focusing on the CXCL12-CXCR4 L-R pairs (Figure 3B) between FIB and either DC or TC, Figure 3C highlights CXCL12's upstream interactions in FIB and CXCR4's downstream interactions in either DC or TC. These CausalCCC predictions uncover pathways consistent with immune cell recruitment, migration, and amplification of inflammatory signals in the skin. In particular, upstream associations of CXCL12 in FIB include fibroblastic markers, such as DCN, and adipose-related genes, such as APOD and CFD, reported in disease-associated fibroblast profiles and tissue repair regulation [36, 37]. In turn, downstream targets of CXCR4 activation in DCs include pathways involved in cell migration (GPR183 [38]), cytoskeletal remodeling, and immune regulation (NR4A3 [39], VIM [40]) crucial for antigen presentation and immune activation. Finally, CXCR4 activation in T cells is central for migration into inflamed tissue and subsequent inflammatory and immune responses. Notably, AP-1 activity (FOS, JUNB) supports the hypothesis of sustained presence and activation of T cells in dermatitis.

Case study 3: CausalCCC network from spatial transcriptomic data in Myocardial infarction

The third case study concerns a spatial transcriptomic dataset on human myocardial infarction [41], Figure 4A. Using L-R pairs inferred with Misty [25, 41], we explored the crosstalks between cardiomyocytes (sender) and myelofibroblasts (receiver), Figure 4B, under hypoxia stress one hour post-myocardial infarction, Figure 4C. The reconstructed causalCCC network, Figures 4D & Figure S2, captures many of the known intracellular processes post-myocardial infraction (see zooms in Figure 4D and Supplementary Note). First, CausalCCC identifies: early biological responses caused by hypoxia and calcic stress (zoom I); structural protein instability and rescue by heat shock proteins in cardiomyocytes (zoom II); cell-cell interactions through collagen-integrin interactions (zoom III). Then, despite reduced time-frame (data taken just one hour post-infraction), causalCCC successfully captures the activation of TGFB signaling and some downstream effects (zoom IV). In particular, the network recovers the main findings of the original study, including the POSTN+/COL1A1+/FN1+ fibroblast profile (Fib2), while pointing also toward other potential markers of the myofibroblast transition, such as ASPN (Asporin) (zooms IV & V and Supplementary Note). Finally, causalCCC identifies a causal role of SPP1 for the differentiation of SPP1+ macrophages (zoom V), despite the low number (151) of myeloid cells in the analyzed dataset (Patient 9).

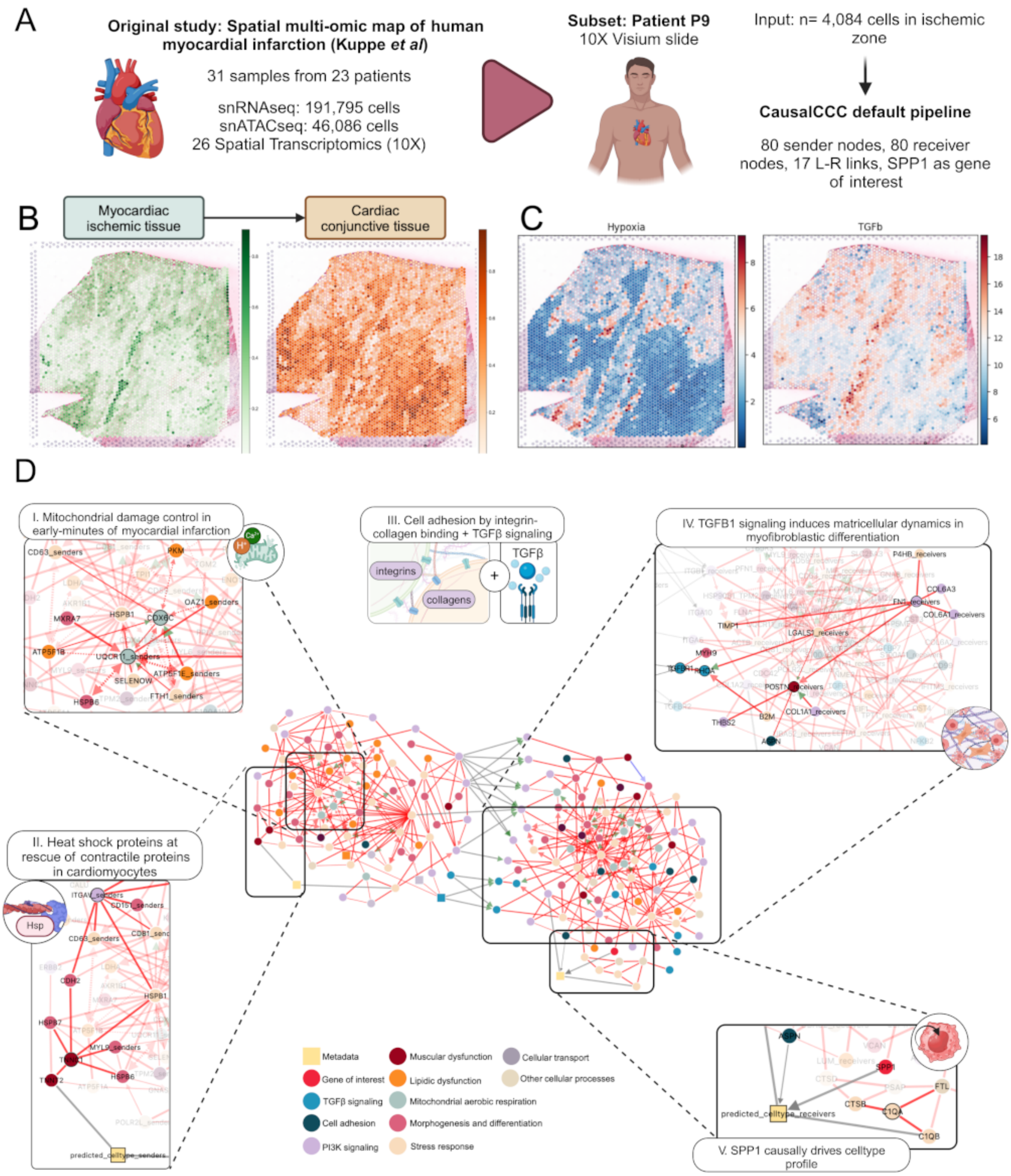

Fig. 4. CausalCCC analysis of myocardial infarction. **A**: Schematic overview of the analysis. **B**: Spatially resolved sender-receiver cell populations in the cardiac tissue (Patient P9 in [41]). Senders (left): regions of myocardial ischemia (green), including cardiomyocytes, pericytes, endothelial cells, vSMCs, and myocytes. Receivers (right): cardiac conjunctive tissue (orange) including fibroblasts and myeloid cells. **C**: Progeny pathway enrichment maps displaying hypoxia and TGFβ signaling intensities (blue-to-red). **D**: CausalCCC network reconstruction, using L-R links inferred by Misty [25], with highlighted regions of interest. Functional annotation from Human Phenotype Ontology. The full causalCCC network is shown in Figure S2.

Discussion

CausalCCC web server offers a user-friendly platform (with demo datasets, detailed documentation and tutorials) based on a robust causal discovery method (MIIC) to reconstruct data-inferred intracellular pathways across interacting cell types. As cell-cell communication analysis continues to face significant methodological challenges in capturing the complexity of cellular interactions, causalCCC provides a unique and data-driven framework accepting L-R pairs from any available CCC method as input. As different biological questions may require different CCC analysis strategies, single cell data practitioners should be able to select a CCC methodology that best suits their specific research contexts and limitations.

We showcase here CausalCCC on different single cell and spatial transcriptomic datasets from three original CCC methods (NicheNet, CellChat and Misty). These use cases highlight causalCCC's possibilities of integration with a broad range of CCC methods and demonstrate its ability to reconstruct data-inferred intracellular networks across multiple interacting cell types.

Most alternative graph-reconstructing CCC methods offer intercellular (rather than intracellular) networks[7, 14, 42, 43], while CCC methods returning intracellular networks are knowledge-based [10, 21, 44, 45] or do not offer a web server[16]. Hence, CausalCCC provides a powerful and flexible web server platform for a comprehensive data-inferred analysis of cell-cell communication in single cell or spatial transcriptomic data.

Data availability

`https://miic.curie.fr/causalCCC.php`

Supplementary Data

Supplementary files are available at NAR Online.

Conflict of interest statement

The authors have no relevant financial or nonfinancial interests to disclose.

Author contributions statement

LD, OD, HI designed the research, and LD, OD, FS implemented the research. LD, OD, HI wrote and reviewed the manuscript.

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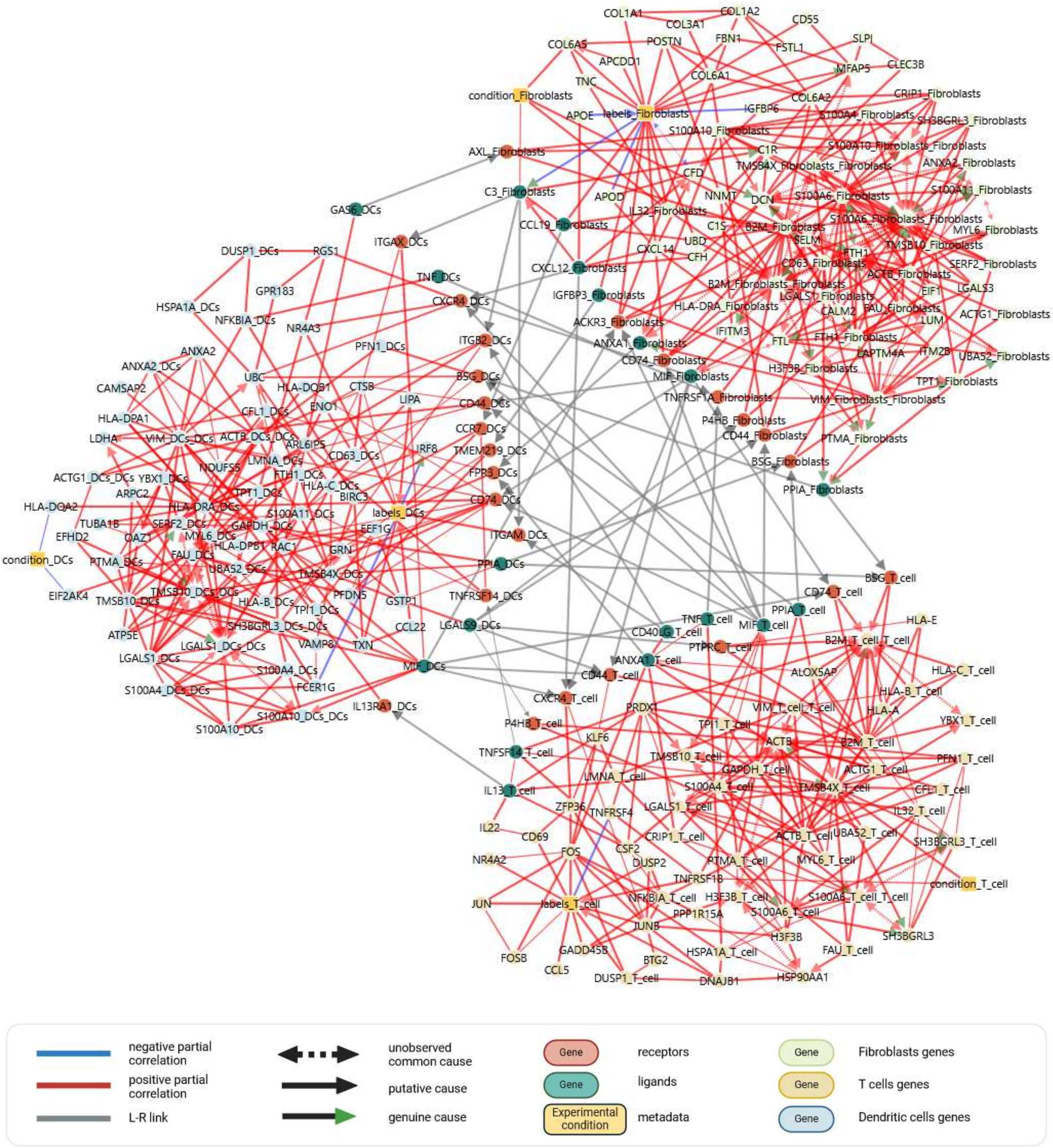

Fig. S1. CausalCCC full network of immune interactions in atopic dermatitis

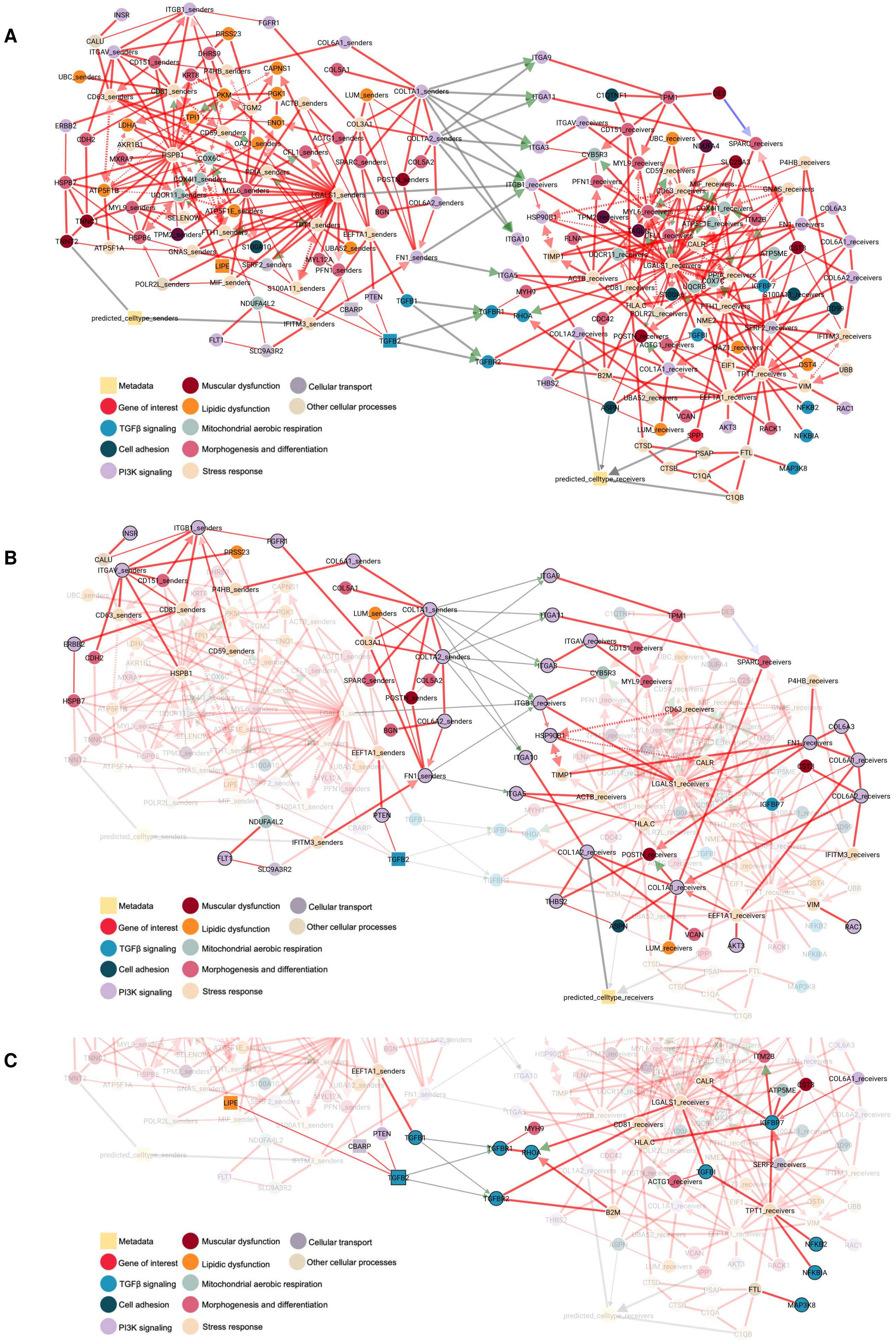

Fig. S2. Myocardiac Infraction CausalCCC network. **A.** Full CausalCCC networks. **B.** ECM-Integrin pathways. **C.** TGFB pathways.

SUPPLEMENTARY NOTES

Case study 1: CausalCCC network between two immune cell populations within lymph nodes

Immune niches within lymph nodes

The dataset generated in the study "Spatial reconstruction of immune niches by combining photoactivatable reporters and scRNA-seq" (Medaglia et al., 2017 [1]) serves as an important resource for studying immune cell interactions within lymphoid tissues. Using their NICHE-seq method, Medaglia et al. [1] analyzed immune cell composition in the T cell area of inguinal lymph nodes, both in steady-state conditions and after infection with lymphocytic choriomeningitis virus (LCMV). NICHE-seq combines photoactivatable fluorescent reporters, two-photon microscopy, and scRNA-seq to map cellular and molecular components of immune niches with high spatial resolution. The NicheNet CCC method [2] has been applied to this scRNA-seq data to uncover ligand-receptor interactions, revealing key communication pathways between immune cells during infection. The scRNA-seq data included 3,900 photoactivated cells, identifying five major clusters dominated by B cells and subsets of $CD4^+$ and $CD8^+$ T cells.

NicheNet analysis

Following NicheNet **vignette**, we studied the communication interactions received by $CD8^+$ T cells. We focused on the signals sent by $CD4^+$ T cells for their statistical robustness and the significant number of $CD4^+$ T cells in the dataset. We reconstructed a CausalCCC network with $CD4^+$ T cells as senders and $CD8^+$ T cells as receivers, following default CausalCCC pipeline with upstream and downstream genes selected using CausalCCC MI-based selection with respect to the top 18 L-R pairs from NicheNet.

CausalCCC results

During NicheNet analysis, the *Tgfb1* ligand standed out for its high regulatory potential, despite its low expression level, and was ranked among the top predicted ligands. However, we found that *Tgfb1* was not connected to any other selected genes in the sender compartement of CausalCCC network, Figure 2. This reflects the lack of information shared between *Tgfb1* and all the other nodes in the sender cells, and highlights causalCCC robustness against inferring spurious associations in single cell transcriptomic data.

By contrast, examples of predicted ligands with high activity and expression are *H2-K1* and *H2-D1*, which show strong associations in the sender $CD4^+$ CausalCCC network, Figure 2, as well as, through their receptors, with the downstream effects in the receiver $CD8^+$ T cells, as discussed in more details below.

On the sender side, *H2-K1* and *H2-D1* are known to enable MHC class I (MHC-I) protein binding activity and peptide antigen binding activity to mediate antigen presentation to CD8+ T cells. *H2-K1* has a lot of direct neighbors in the CausalCCC $CD4^+$ T cells network: *Psmb8, Ifi27l2b, Slfn1, Shisa5, B2m* and *Gm13456*, while *H2-D1* is directly linked to *B2m*, *Irf7* and *Mir703*. Although *B2m* was found as a potential ligand by NicheNet, it was not retained amongst the top ligands based on the default setting for the number of selected L-R pairs. Yet, *B2m* was subsequently retained as an informative gene by causalCCC unsupervized MI-based selection of relevant genes and found to be regulated by *Stat1* (genuine causal edge) in the $CD4^+$ network, Figure 2. *Stat1* is known to mediate expression of the MHC-I pathway and genes related to Type I and II interferon (IFN) signaling in T cells [3]. In the CausalCCC $CD4^+$ T cells network, *Stat1* is a second degree neighbor of *H2-K1*, with *Ifi27l2b* mediating the indirect effect between *Stat1* and *H2-K1*. In fact, causalCCC network is consistent with the reported direct or indirect effects of *Stat1* [3] on genes related to the MHC-I pathways, such as MHC-I genes *H2-K1, H2-D1 and B2m*, genes involved in MHC-I antigen presentation (*Tap1 and Psmb8)* but also Type I and II IFN signaling like Interferon regulatory factor 7 *Irf7*, IFN-inducible genes *Ifi203, Ifi209, Ifi27l2a, Ifi27l2b, Ifi47, Ifit3,* and IFN-stimulated genes *Isg15* and *Isg20*, Figure 2. During viral infection, like LCMV, it is expected that these pathways are upregulated as part of a broader antiviral immune response with active antigen presentation to $CD8^+$ T cells.

On the receiver side, *Klrd1* and *Cd8a*, the receptors of both ligands *H2-K1* and *H2-D1* (as well as other *H2*-genes), are known to be critical for the activation of $CD8^+$ T cells cytotoxicity, a key mechanism in clearing viral infections like LCMV. In the CausalCCC $CD8^+$ T cells network, the direct neighbor of *Klrd1* is *Gm10076* and the direct neighbours of *Cd8a* include *Gm10076, Cd8b1, B2m,* and *Gm15772. Cd8b1* is expected as *Cd8a* and *Cd8b1* are subunits of the heterodimer CD8 complex. *Gm10076* is a transcript with a biotype annotation conflict (Ensembl: lncRNA; NCBI: pseudogene) and no known function, which has been found to be highly and specifically expressed within the injury locus in a spatial total RNA-sequencing approach applied to study the skeletal muscle regeneration after injury [4]. Other downstream interactions with *Cd8a* and *Klrd1* include *Hspa8, Ly6c2, Tmsb4x, Actb, Mir703, Gm11942 Gm16238* and *Gm15772.* Although their function is not completely understood, *Ly6C* genes are known to transmit signals for T-cell activation and cytokine production, and have be found highly expressed in virus-specific memory CD8+ T cells studied ex vivo. *Ly6c2* (and its neighbor *Ly6c1* and second degree neighbor *Ly6ca*) could support viral response of the CD8+ T cells [5]. *Tmsb4x* and *Actb* are cytoskeletal-associated proteins important for the motility and function of immune cells, possibly influencing CD8+ T cells migration to infection sites. *Tmsb4x* is also involved in proliferation and could represent a marker of a CD8+ T expansion phase, as CD8+ T cells must proliferate, differentiate, and acquire cytotoxic functions during viral infection [6].

Case study 2: CausalCCC network between three cell populations in atopic dermatitis

Immune interactions in atopic dermatitis

The second case study is based on the paper "Single-cell transcriptome analysis of human skin identifies novel fibroblast subpopulations and enrichment of immune subsets in atopic dermatitis" (He et al., 2020 [7]). Atopic Dermatitis (AD) is a prevalent inflammatory skin disease with a complex pathogenesis involving immune cell and epidermal manifestation. The original study generated transcriptomic profiles for 39,042 AD (lesional and nonlesional) and healthy skin cells and identified novel fibroblast subpopulations unique to lesional AD and expressing CCL2 and CCL19 cytokines. Dendritic cell (DC) subpopulations were identified as receptors of this fibroblast signaling (validated using immunofluorescence staining), specific to the AD lesions, illustrating a potential role for fibroblast signaling to immune cells. The proportions of T cell subtypes were different in lesional AD and nonlesional AD. AD lesions were characterized by expanded subtypes of T cells and inflammatory DCs, and by a unique inflammatory fibroblast, possibly interacting with immune cells to regulate lymphoid cell organization and inflammation.

CellChat analysis

CellChat [8] was showcased on this dataset to investigate signaling changes between lesional and nonlesional AD skins and study the intercellular communication among fibroblasts (four subpopulations: APOE + FIB, FBN1 + FIB, COL11A + FIB, and Inflam.FIB), DCs (four subpopulations: cDC1, cDC2, LC, and Inflam.DC), and TCs (four subpopulations: TC, Inflam.TC, CD40LG + TC and NKT), Figure 3. CellChat inferred separate intercellular communication networks for the nonlesional (NL) and lesional (LS) skin, and then jointly mapped them to identify functional similarities. Common pathway groups such as VEGF, GAS, LIGHT, CD40, and MIF, suggested that these pathways likely did not critically contribute to disease pathogenesis. Signaling pathways were highly active in LS, including 9 pathways involved in inflammatory and immune response, such as CXCL, LIGHT, GALECTIN, COMPLEMENT, MIF, CSF, IL4, CCL, and TNF. Concerning CCL signaling, CellChat identified the L-R pair CCL19-CCR7 as the most significant link contributing to the communication from Inflam.FIB to Inflam.DC, in agreement with the reported experimental finding [7]. Interestingly, ligand CXCL12 and its receptor CXCR4 were also found to be highly active in LS, in particular, in the signaling from Inflam.FIB to cDC2 and Inflam.DC, and CellChat presented this analysis in details in their **vignette**.

CausalCCC results

Using CellChat L-R results, we reconstructed a CausalCCC network following the default pipeline. Most of the times in CCC analysis, the focus is on specific sender and receiver populations but we wanted to showcase the possibility of CausalCCC to analyze crosstalks between more than two populations by reconstructing a triangular network, where Fibroblasts, DCs and T cells are both senders and receivers, giving an overview of all interactions in this complex cellular system, Figure S1. We further focused on the L-R pair presented as the interaction of interest in the CellChat **vignette**: CXCL12(in fibroblasts)-CXCR4(in DCs or TCs), see Figure 3A&B. We highlighted the second degree neighbourhood of these genes in Figure 3C. A non-exhaustive analysis highlights the upstream interactions with CXCL12 in fibroblasts and the downstream targets of CXCR4 in both DCs and T cells, and reveals implicated pathways such as immune cell recruitment, migration, and the amplification of inflammatory signals during the disease.

CXCL12 production in fibroblasts is regulated by a range of genes that are primarily involved in inflammation, lipid metabolism, extracellular matrix remodeling, and immune surveillance. Notable upstream regulators in the CausalCCC network include APOE, for its role in regulation of cytokine production that could enhance CXCL12 secretion. In fact, this direct link is consistent with APOE+FIB being the subpopulation of fibroblasts with the highest expression of CXCL12 (see Figure 3B). CXCL12 is linked to fibroblastic markers such as DCN (and third degree neighbour LUM, linked to DCN) as well as adipose-related genes, such as APOD and CFD, related to disease-associated fibroblast profiles and tissue repair regulation [9, 10]. CFD, CFH and C3 are components of the complement system, known to be expressed in fibroblasts, and C3 activation leads to chemoattractants C3a and C5a production, pro-inflammatory mechanisms potentially mediating CXCL12 production and CXCR4 recruitment [11]. While CXCL14 receptors remain unclear, cutaneous CXCL14 (such as expressed by fibroblasts) has been shown to target DCs, assigning new roles to CXCL14 from epidermal tissue, as attractant and niche of differentiation, respectively, in the renewal of DCs under steady-state conditions [12].

In turn, CXCR4 activation in DCs triggers pathways involved in cell migration, cytoskeletal remodeling, and immune regulation, crucial for antigen presentation and immune activation. Downstream targets of CXCR4 in DCs include RGS1 which is associated with G-protein coupled receptor signaling which is an essential part of chemokines receptors. DUSP1 modulates the inflammatory response by attenuating cytokine production and is part of the MAPK pathway. The heat shock protein HSPA1Awhich has shown co-expression with CXCR4 regulation before. GPR183, also known as EBI2, is a receptor involved in DC migration, particularly towards oxysterol gradients in tissues has shown co-expression with CXCR4 before [13]. NR4A3 is a transcription factor involved in TLR-mediated activation and gene expression of DCs [14]. NR4A3 was also found to explain the indirect effect between subpopulations of DCs and expression of CXCR4 (together with RGS1), meaning the separating set used to remove the direct link between CXCR4 and the subpopulations annotation labels_DCs consists of NR4A3 and RGS1. Vimentin (VIM) is a second degree neighbor to CXCR4, known to modulate human DCs activation [15] by decreasing secretion of pro-inflammatory cytokines while increasing secretion of the anti-inflammatory cytokine in LPS-activated DCs[15]. VIM expression is increased in non-lesional DCs versus lesional DCs, and dicreased in inflammatory DCs versus other DCs.

Similarly, CXCR4 activation in T cells is central to the migration of these immune cells into inflamed tissue and the subsequent activation of inflammatory and adaptive immune responses. Downstream targets of CXCR4 in T Cells in the causalCCC network (Figures 3C & S1) are consistent with these immune responses. In particular, ZFP36, from a family of RNA binding proteins, regulates homeostatic and autoreactive T cell responses and CD69 is an early activation marker on T cells. FOS and JUNB are essential components of the AP-1 transcription factor complex, which regulates the expression of pro-inflammatory cytokines and is critical in T cell activation and proliferation. CXCR4 activation driving AP-1 activity supports the hypothesis of a sustained presence and activation of T cells in dermatitis. Finally, DUSP2 is a phosphatase that regulates MAPK signaling, similar to DUSP1 in DCs.

Overall, cellular cross-talk in AD involves CXCL12 production by fibroblasts, influenced by APOE and CXCL14 upstream expression and genes featured in disease-associated fibroblast profiles. Then, CXCL12 binds to CXCR4 on both DCs and TCs, and leads to downstream signaling cascades such as MAPK and NF-κB pathways, that can be pro-inflammatory when dysregulated.

Together, these reconstructed intracellular networks across three cell populations (Figures 3C & S1) illustrate the potential of CausalCCC by highlighting how fibroblasts, DCs, and TCs might interact through chemokine and receptor pathways to coordinate immune responses in acute dermatitis, with potential implications for both tissue damage and repair during skin inflammation.

Case study 3: CausalCCC network from spatial transcriptomic data in Myocardial infarction

The third case study concerns spatial transcriptomic data from the "Spatial multi-omic map of human myocardial infarction" study [16], which employed multiple sequencing technologies across nearly half a million cells to characterize cardiac remodeling post-infarction, Figure 4A. The main result of this study was to identify subtypes of fibroblasts and macrophages and to understand their roles in cardiac tissue remodeling, inflammation, and scar formation upon myocardial infarction. Kuppe *et al.* [16] identified novel specific fibroblast subtypes, including Fibroblasts type 2 (Fib2) marked by POSTN+/COL1A1+/FN1+ in ischemic zones. Macrophages were characterized by the SPP1+ subtype, predominantly found in ischemic zones and marked by SPP1 (osteopontin), C1QA, CD68, and TGFB1 expression. These macrophages were shown to drive inflammatory responses, phagocytosis of necrotic tissue, and the activation of fibroblasts, particularly myofibroblasts, through pathways such as SPP1-integrin and TGFB1 signaling.

We reconstructed a CausalCCC network on this spatial transcriptomic dataset, focusing on Patient P9's data sampled one hour post-infarction. This significantly reduced dataset (∼4,000 cells in total, including 151 myeloid cells) represents a more typical user case and Patient P9 was chosen as it presents some interesting specificities, frequently observed in this type of data. In particular, at just one hour post-infarction, gene expression changes are subtle as secondary signaling cascades are only beginning to emerge. Additionally, the sequential activation (Hypoxia - TGFB - NFkB) creates a particular challenge, as TGFB serves as both an intermediate signal and an effector in both sending and receiving cell populations. Traditional correlation-based methods struggle to capture such intermediate signaling events.

CausalCCC processing

To investigate the cross-talk between ischemic myocardial cells (including cardiomyocytes, vascular smooth muscle cells, endothelial cells, pericytes taken as senders) and connective tissue (including fibroblasts and myeloid cells taken as receivers), Figure 4B, we used MISTy and Progeny to retrieve ligand-receptor pairs and pathway enrichment scores, following the **vignette** of the original paper [16]. As expected in P9's ischemic region, hypoxia signaling was restricted to the sender compartment, while TGFB showed a gradient across both populations, Figure 4B & C. We then ran CausalCCC's default pipeline using MISTy's top 17 ligand-receptor links and CausalCCC's mutual information (MI) based feature selection, after excluding housekeeping genes, such as GAPDH and ribosomal genes, as they tend to share substantial mutual information with other genes while not being relevant to the biological question of interest. For the MI-based feature selection, we combined genes of interest, including TGFB1/2, ERBB2, FGFR1, FLT1, INSR, TGFBR1/2, RHOA, RAC1, MAP3K8, CDC42, AKT3, NFKB2, and NFKBIA, with SPP1 as our target genes. We then selected in each compartment the top 60 genes sharing the highest mutual information with these targets.
The dataset is freely accessible at `https://cellxgene.cziscience.com/e/32513e13-8f4a-4418-b4d5-faac85fa430d.cxg/`.

CausalCCC results

Biological context

Cardiomyocytes–myelofibroblasts crosstalks in Myocardial infarction usually follow classical steps. First, hypoxia induces cardiomyocytes necrosis and consequent calcic release in the environment. Hypoxic/calcic stress and contractile protein uncoupling is a major stress for both senders and receivers, as it increases mitochondrial calcic uptake through mitochondrial calcic uniporter (MCU) that triggers mitochondrial permeability transition pore (mPTP) opening, leading to mitochondrial inner membrane rupture, and ultimately to (cytochrome-C mediated) cell death. Cardiomyocytes outer cell membrane disruption leads to cell adhesion molecules (cadherin, integrins and collagens) to recruit fibroblasts, which drive early ECM deposition and scar formation following Myocardial infarction. Fibroblasts concomitantly transition from a pro-inflammatory phenotype (associated to received TGFB and PI3K signals) to myofibroblasts, which produce and organize the ECM, and recruit inflammatory myeloid cells, both ensuring structural support and preventing further heart damage. Of note, several studies highlighted that some biomarkers were commonly shared by cardiomyocytes and myofibroblasts in early-stage of Myocardial infarction ([17], [18], [19], [20], [21]).

1. Hypoxic, calcic stress and mitochondrial damage are hallmarks of early-minute of myocardial infarction.

CausalCCC effectively uncovers some known triggers of intercellular crosstalk happening in the very first minutes of Myocardial infarction as it captures direct elements (CBARP, PGK1, LDHA) and indirect elements denoting hypoxic and calcic stress. CausalCCC predicts a consistent hub (zoom I in Figure 4D) including 9 genes handling mitochondrial damage, such as complexes III (UQCR11, ubiquinol-cytochrome C reductase) and IV (COX6C, COX4I1, cytochrome C oxidase subunits), both preventing cytochrome C release and subsequent cell death. Of note, causalCCC estimates a strong mutual information of 0.68 bits between COX6C and its predicted genuine cause ATP5F1E. ATP5F1E/B (ATP synthase components) upregulation due to Ca^{2+}/H^{+} cycle inversion, PKM, OAZ1, SELENOW, and FTH1, all contribute to oxidative stress regulation. This hub involves a number of latent variables (*i.e.* bidirected dashed edges), consistent with the fact that metabolites at play are not experimentally quantified in this study (Ca^{2+}, H^{+}, Fe^{3+}, organic metabolites). The identification of such latent relationships from unobserved metabolite dynamics illustrates CausalCCC's ability to uncover the consequences of unobserved common causes, solely based on observed gene expression patterns.

2. Cardiomyocytes structural protein maintenance and contractibility damage-control

In an attempt to retain cellular integrity and function, cardiomyocytes under stress try to maintain proper folding and interaction of their contractile proteins by expressing heat shock proteins (HSPs). Cytoskeleton contractile proteins ACTG1/ACTB, MYL9 and TNNC1/TNNT2, found in the network, share strong direct or indirect positive associations (0.5-1 bit) with heat shock proteins HSPB1, HSPB6 and HSPB7, consistent with these genes being upregulated under ischemic stress [22, 23] (Figure 4D, zoom II). Moreover, HSPB1, a major hub in the sender network (Figure S2), is known to be central to different functions, such as morphogenesis, differenciation, lipidic metabolism (Figure 4D, annotated following Human Phenome Annotation).

Interestingly, TNNC1 — CDH2 — ITGAV constitutes an informative path. Elevated TNNC1 levels are strongly and directly associated with CDH2 activation (0.55 bits), which is known to play a key role in the quiescence-to-activation transition of skeletal stem cells [24] and to enhance the reparative potency of cardiac myocytes post-Myocardial infarction ([25]). Additionnally, recent studies suggest that cadherins could orchestrate fibroblastic activation and modeling in the context of cell-matrix interaction [26], which seems well supported by CDH2 and ITGAV sharing 0.65 bits of mutual information in the CausalCCC network.

3. Cell-Cell and Cell-ECM interactions through collagen-integrin L-R interactions.

As recalled in the biological context above, Myocardial infarction rapidly leads to Cell-Cell and Cell-ECM interactions through collagen-integrin L-R interactions between myocardiac ischemic tissue and conjunctive tissue (zoom III in Figure 4D). In fact, collagens and integrins make the vast majority of top ligand-receptors pairs inferred by Misty for this dataset [16].

Yet, while the present analysis takes the viewpoint of cardiomyocytes as senders and myelofibroblasts as receivers, both cell types actually express collagen ligands and integrin receptors, as recovered by causalCCC relying only on MI-based selection. In fact, other ligand receptor pairs, like collagen-integrin pairs, are also found in both sender and receiver networks, although they have not been linked by L-R links in the present network (Figure S2A). In particular, CD81, which is a cell surface glycoprotein known to complex with integrins is found both in senders and receivers. This protein has been reported to promote muscle cell fusion and support myotube maintenance [27]. Likewise, LGALS1 (Galectin-1), which is implicated in modulating cell-cell and cell-matrix interactions, is a central hub both in senders and receivers (a mean mutual information of 0.80 bits towards its neighbors in senders, 0.43 bits for neighbors in receivers). In parallel, cardiomyocytes activate PI3K-AKT and TGFB pathways, Figures S2B & S2C, which promote fibroblast activation and differentiation in the receiver compartment.

4. Downstream effects of TFGB signaling in receivers

TGFB downstream signaling classically operates through SMAD2/3 operators (transcription factors, secondary signaling), observed only after 60-90 minutes of TGFBR activation. In a more reduced time-frame, corresponding to the P9 dataset analyzed here, TGFBR can lead to non-SMAD pathways, including the MAP kinase pathways (ERK, JNK, p38), PI3K/AKT, Rho GTPases, and NFKB (zoom IV in Figure 4D and Figure S2C). While TGFB1 showed high expression levels, TGFBR1 was only expressed by 734 cells and TGFB-TGFBR signaling was partially imputed by targeting this pathway in the mutual information-based selection list. This focus successfully captured TGFB signaling as a sequential process in causalCCC network (Figure S2C).

Specifically in fibroblasts, TGFB signaling through TGFBR1 was shown to induce the expression of MYH9 and ACTB (1st and 2nd degree nodes of TGFBR1), which are crucial for fibroblast activation (Figure S2A). Activated fibroblasts transform into myofibroblasts. This differentiation is critical for the myocardial infarction response and is triggered by canonical signaling involving TGFBR1 and non-canonical signaling via RhoA, a first-degree node of TGFBR1. Of note, 44 out of 87 TGFBR1 mediated effects in the receiver cells are partially explained by RhoA, underlying its significance in the network, even though RhoA is not highly connected. In particular, RhoA explains 84% of the weak mutual information between TGFBR1 and MYH9 (0.03 bits). TGFB also theoretically upregulates FN1 (fibronectin) and induces TIMP1, which inhibits matrix metalloproteinases, although only weak signals and no links are present between TGFBR1 and FN1 or TIMP1 (zoom IV in Figure 4D). However, FN1 and TIMP1 share substantial MI (0.50 bits), while TIMP1 is conditioned by RhoA (84%) and MYH9 (16%).

Looking at the synergy between TGFB and PI3K-AKT signaling pathways, an interesting finding of CausalCCC concerns the early regulation of matricellular proteins such as FN1, POSTN (Periostin), THSB2 (Thrombospondin-2), and ASPN (Asporin), Figure S2B. In the original article [16], the Fib2 subtype, highly present in ischemic zones, was marked with FN1+, COL1A1+ and POSTN+ high expressions - informative genes automatically selected by CausalCCC. Here, THSB2 and POSTN shares substantial information (0.49 bits, mainly explained by COL1A1), and POSTN is a genuine consequence of COL1A1 (0.59 bits, mainly explained by FN1) and a strong putative consequence of FN1 (0.93 bits). This is consistent with recent reports showing that ITGA10 - COL * is regulating POSTN levels [28, 29]. These results are well in line with the original paper's findings on the POSTN+/COL1A1+/FN1+ myofibroblastic transition. Yet, this gene trio POSTN+/COL1A1+/FN1+ is not found to be directly connected to the "predicted celltype" in the receiver network. Instead, myofibroblasts seem to be more directly associated to COL1A2 and ASPN (Figure S2B). As ASPN and POSTN are also strongly associated (they share 0.46 bits of information), ASPN emerges as an interesting candidate marker to further characterize these myofibroblasts. This hypothesis is in fact supported by recent findings showing that ASPN constitutes a cardioprotective marker which inhibits TGFB1 signaling [30, 31].

5. SPP1 and macrophage differenciation

Finally, a strinking finding of CausalCCC concerns the gene of interest *SPP1*, which is predicted to be causally linked to the "predicted celltype" node in the receiver compartment (zoom V in Figure 4D). This edge, central to the original publication and validated using multiple independent methods [16], highlights CausalCCC precision even with a small sample size (*i.e.* only 151 myeloid cells in P9 dataset). Additionally, CausalCCC's MI-based gene selection approach identified *C1QA* and *C1QB* in the receiver compartment with *C1QB* being even linked to the "predicted celltype" node. This is consistent with the original paper [16], which reported *C1QA* and *C1QB* as markers of SPP1+ macrophages.

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