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Elucidating Human Disease Mechanisms Using Innovative Open Microfluidic Platforms

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Abstract

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Open microfluidic technologies are innovative research tools that offer unique advantages in chemical and biomedical research. This thesis explores the diverse biomedical applications of open microfluidic platforms in understanding the complex mechanisms underlying human diseases. Chapter 1 introduces the concept of open microfluidic systems, discussing their design principles, fabrication methods, and distinctive advantages over traditional macroscale systems or closed microfluidic systems. Chapter 2 presents the application of a novel open microfluidic coculture device for investigating mechanisms of airway inflammation by coculturing fibroblasts and eosinophils. The results show the induction of a proinflammatory phenotype of primary human lung fibroblasts following the coculture with degranulating eosinophils. This model enables the discovery of new analytes in the fibroblast-eosinophil signaling that were not found

in previous conditioned media studies. Chapter 3 discusses another application of the same coculture device for examining injury-induced paracrine effects on the podocyte's transcriptome in glomerular disease. Transcriptomic analysis revealed shared and unique pathways between three common forms of podocyte injury inducers. This model is the first to allow direct measurements of the paracrine signals derived from injured podocytes and their effects on a healthy podocyte population. Chapter 4 describes the application of homeRNA, a remote blood collection and stabilization device, as a flexible and responsive approach to assess the effects of wildfire smoke exposure. The flexibility of the homeRNA kits allows participants to collect blood samples throughout the year, even during emergency evacuations. Ongoing transcriptomic analysis will reveal information on the overall health of participants from a mechanistic perspective, particularly on systemic inflammation. Chapter 5 concludes the thesis by proposing future directions of the work presented and discussing exciting opportunities in biomedical research with open microfluidic technologies. Through these chapters, this thesis demonstrates the pivotal role of open microfluidic technologies in advancing biomedical research by offering unique insights into disease mechanism studies.

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Chapter 1 | Introduction

Portions of this chapter were excerpted or paraphrased from Chapters 2-4 of this dissertation.

1.1 Open microfluidics for bioanalytical research

Reproduced in part from Y. Zeng*, J. W. Khor*, T. L. van Neel, W.-c. Tu, J. Berthier, S. Thongpang, E. Berthier, and A. B. Theberge. “Miniaturizing chemistry and biology using droplets in open systems.” *Nat. Rev. Chem.*, Apr. 2023, doi: 10.1038/s41570-023-00483-0. (*equal contribution)

Open microfluidic systems manipulate fluids with at least one dimension (i.e., width, depth, or length) at the micrometer (submillimeter) scale in an open environment.¹ In these systems, at least one component (i.e., the floor, ceiling, or walls) of the microfluidic system is exposed to an external environment, typically through one or more air-liquid or liquid-liquid interfaces. Fluids were controlled using material properties such as wetting, capillary force, or microscale geometries (e.g., channels or microstructured surfaces).

Research in the field of open microfluidics is multidisciplinary. The field was started by analytical chemists and engineers as a method of manipulating small volumes of liquid in an open environment. In the past decade, this miniaturized technology has advanced and branched out into numerous applications in chemistry and biology.² The small volumes needed in microfluidic systems make them desirable for biomedical researchers who use precious samples such as rare cell types or expensive reagents. Moreover, these miniaturized systems offer mobility and portability, which is important for point-of-care and at-home sampling applications.³⁻⁶ Lastly, the versatility of microfluidic system design allows bioengineers to construct complex microphysiological systems, such as organ-on-a-chip.^{7,8}

Compared to conventional microfluidic systems which are often closed,^{9,10} open systems offer many advantages, including accessibility, ease of fabrication, and ease of use. As closed microfluidic systems have often become more complex, open microfluidic systems generally take the opposite approach and simplify the mechanisms of complex tasks and functions by using passive forces such as capillarity. Openness allows direct access to the liquid sample (e.g., using a pipette) instead of relying on complicated fluidic manipulation through a network of external pumps and tubing. Moreover, the openness of open droplet systems naturally allows gas exchange (such as the exchange of oxygen and carbon dioxide during cell culture incubation), whereas closed systems rely heavily on the gas permeability properties of the channel material(s) to allow this exchange.

However, there are also challenges associated with open microfluidic systems, such as evaporation management, relatively low throughput, and the need to carefully consider the material properties of the device (such as the wettability). Although many solutions have been proposed over years of careful development,^{11–14} more innovations are required to fully overcome these challenges. Overall, when used for the right application, open microfluidic systems offer a simple yet effective solution with great potential in bioanalytical research.

1.2 Microfluidic coculture tools for cell-cell signaling

Physiological functions in the human body involve seamless coordination between multiple organs, which can be attributed to the signaling among different cell types. There are four major categories of cell-cell signaling: endocrine signaling, paracrine signaling, autocrine signaling, or juxtacrine signaling.¹⁵ In paracrine signaling, the signaling molecules—typically chemokines or cytokines—are released by one type of cells to the extracellular matrix and reach their target cells in the same local microenvironment via diffusion.¹⁶ These signaling ligands, as

well as their receptors and signaling pathways, are highly conserved across different organs and species, thus becoming the focus of many cell signaling studies.¹⁷

A common method to study cell-cell signaling in vitro is via conditioned media, where the media in which the first type of cell was cultured was used to culture the second type of cell. Cell coculture is an increasingly popular approach with the advantages of real-time, two-way, and continuous signal exchange.¹⁸ In coculture systems, two or more biologically relevant types of cells are cultured closely together. For mixed coculture, different cell types are in physical contact with one another. For example, endothelial cells are often seeded with fibroblasts as the latter promote functional vascularization in engineered tissues.^{19,20} For segregated coculture, different cell types are physically separated by a permeable membrane (e.g., Transwell) or half wall, where only soluble factors can travel across.^{21,22}

Microfluidic coculture offers several unique advantages for studying cell-cell signaling over conventional approaches, such as conditioned media or Transwell® inserts.^{23,24} Besides its ability to provide bidirectional communication, microfluidic coculture can be achieved with a higher degree of flexibility such as culture configuration, cell seeding ratios, or microenvironment control, enabling more accurate recapitulation of the physiological model.²⁵ Additionally, microfluidic devices operate with less volume, which minimizes the use of expensive reagents and precious primary samples isolated from patients.²⁶ Lastly, compared to closed microfluidics, the emerging open microfluidic technology allows easy accessibility and can be adopted in the workflow of a standard biological laboratory without specialized equipment.^{27–32}

Our group has previously developed an open microfluidics coculture device, where two populations of cells can be cultured in adjacent chambers while physically separated by a half-

wall.^{33,34} Different types of cells can be seeded and treated independently using a standard pipet. To connect both chambers, additional culture media is added to overflow the half wall and thus initiate the soluble factor signaling. Besides the initial proof-of-concept study on kidney endothelial and epithelial cells,³⁴ the device can be applied to many other organ models and provide critical insights into intercellular crosstalk in disease development in vitro. In this dissertation, I describe two disease models established using the open microfluidic coculture device on airway inflammation³³ (Chapter 2) and glomerular disease³⁵ (Chapter 3).

1.3 Microfluidic sampling tools for remote blood collection

Blood sample collection is a standard procedure in medical research, clinical trials, and healthcare practice. With an increasing demand for decentralized clinical trials³⁶ and telemedicine³⁷, there has been a significant shift from clinic-based blood draws to remote blood sampling, especially during the COVID-19 pandemic³⁸. Typically, a clinic-based blood draw requires participants to travel physically to a nearby medical center or healthcare facility and have their blood drawn in person by a licensed phlebotomist. While it facilitates standard procedures, the time commitment, inflexible schedules, and cost burden associated with an in-person blood draw remain a barrier to healthcare access or clinical trial participation, especially in rural or low-income communities.³⁹

On the other hand, self-administered blood collection has numerous advantages. Remote blood sampling enables a broader reach of participants in rural or underserved communities who lack access to nearby clinics or are unable to travel due to work schedules, caregiving responsibilities, or mobility challenges. It also allows for the capture of acute and transient biomarker fluctuations immediately after unexpected events, such as an acute exposure, asthma attack, or autoimmune disease flare. Moreover, for longitudinal studies requiring frequent sample

collections, it provides convenience, flexibility, and comfort for the participants, improving their study experiences.

Blood sampling in disaster research,⁴⁰ such as wildfire smoke exposure, is particularly challenging due to the unpredictable nature of wildfires and limited healthcare accessibility in rural areas, where most wildfires occur. Conducting a clinic-based blood draw study on the general population experiencing wildfire smoke exposure would require either (1) relying on historical data to choose a location with the necessary infrastructure for clinic-based blood draws (usually in an urban or suburban environment) and a high prevalence of wildfire (usually in rural areas), (2) setting up a multi-site study to increase the chances that at least one site has a wildfire smoke event, or (3) relying on retrospective studies or capturing smoke exposure incidentally in a different study where longitudinal blood samples are collected.^{41,42} These factors make designing studies where blood is collected at multiple time points in response to wildfire smoke exposure both expensive and logistically challenging.

To address this challenge, in Chapter 4, I describe a remote and flexible study design using homeRNA: a self-sampling kit made of a commercially available blood collection device, Tasso SST, and a custom-engineered RNA stabilization tube we previously developed.⁶ The homeRNA kit allows study participants to self-collect and stabilize blood by themselves in their own homes for shipment back to a centralized lab for downstream analysis.

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Chapter 2 | An Open Microfluidic Coculture Model of Fibroblasts and Eosinophils to Investigate Mechanisms of Airway Inflammation

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YZ, SE, KB, and ABT conceptualized the research. KB and ABT supervised the research. YZ, XS, SE, KB, and ABT planned the experiments. JWP and SJS advised on cell culture in the microfluidic coculture device. FYL, LCD, NNJ, SKM, NS, EB, SE, KB, and ABT provided expertise in the isolation and culture of eosinophils and fibroblasts, overall microfluidic device design/assay development, and the clinical significance of the work. YZ and MGT fabricated the devices. UNL optimized the solvent bonding process. PSF isolated the blood eosinophils. YZ, XS, and MGT conducted experiments and collected data. YZ analyzed the data. YZ, SE, KB, and ABT interpreted the results of experiments. YZ, MGT, UNL, KB, and ABT drafted the manuscript. YZ prepared the figures. YZ, FYL, NNJ, SKM, SE, KB, and ABT edited the manuscript. All authors reviewed and approved the manuscript.

Abstract: Interactions between fibroblasts and immune cells play an important role in tissue inflammation. Previous studies have found that eosinophils activated with interleukin-3 (IL-3) degranulate on aggregated immunoglobulin G (IgG) and release mediators that activate fibroblasts in the lung. However, these studies were done with eosinophil-conditioned media that have the capacity to investigate only one-way signaling from eosinophils to fibroblasts. Here, we demonstrate a coculture model of primary normal human lung fibroblasts (HLFs) and human blood eosinophils from patients with allergy and asthma using an open microfluidic coculture device. In our device, the two types of cells can communicate via two-way soluble factor signaling in the shared media while being physically separated by a half wall. Initially, we assessed the level of eosinophil degranulation by their release of eosinophil-derived neurotoxin (EDN). Next, we analyzed the inflammation-associated genes and soluble factors using reverse transcription quantitative polymerase chain reaction (RT-qPCR) and multiplex immunoassays,

respectively. Our results suggest an induction of a proinflammatory fibroblast phenotype of HLFs following the coculture with degranulating eosinophils, validating our previous findings. Additionally, we present a new result that indicate potential impacts of activated HLFs back on eosinophils. This open microfluidic coculture platform provides unique opportunities to investigate the intercellular signaling between the two cell types and their roles in airway inflammation and remodeling.

2.1 Introduction

Airway inflammation is associated with a number of pulmonary diseases, including asthma^{1,2}. In particular, eosinophilic airway inflammation is found in up to 80% of people with asthma and 40% with chronic obstructive pulmonary disease, indicated by an increased eosinophil count in their sputum³. Eosinophils are immune cells that play an essential role in tissue inflammation and remodeling⁴. These cells can be activated to undergo degranulation and cytotoxicity, releasing cytotoxic granule proteins and a variety of proinflammatory soluble mediators that impact surrounding cells, including fibroblasts⁵⁻⁷. Fibroblasts are mesenchymal cells often found in connective tissues that provide structural support in various organs⁸. They play a key role in the immune response and wound healing via the uptake and secretion of various inflammatory signals^{9,10}.

In asthma, chronic airway inflammation is accompanied by the activation and differentiation of lung fibroblasts, which eventually leads to enhanced subepithelial thickness, fibrosis, and irreversible airway obstruction¹¹⁻¹³. Eosinophils release cytokines that promote fibroblast activation, including transforming growth factor β (TGF- β) and interleukin-1 β (IL-1 β)¹⁴⁻¹⁷. Previous work has shown that eosinophil-mediated signaling could lead to either pro-inflammatory or pro-fibrotic fibroblast phenotypes^{18,19}. However, these studies did not employ

ex vivo coculture of primary human eosinophils with primary human lung fibroblasts (HLFs), which may exhibit important differential signaling processes compared to these previous studies. Thus, coculture methods of these primary cells provide a unique advantage in the investigation of the potential mechanisms of airway inflammation via fibroblast activation.

We previously reported an *ex vivo* eosinophil degranulation model, where primary human blood eosinophils activated with interleukin-3 (IL-3) can robustly degranulate and lyse on well plates coated with heat aggregated immunoglobulin G (HA-IgG) ²⁰. To study the impact of the degranulation products and cellular content from eosinophils on HLFs, we previously isolated supernatants from eosinophils in that model, and exposed them to primary lung fibroblasts, including HLFs and human bronchial fibroblasts (HBFs) ^{19,21,22}. In these previous studies, we observed a proinflammatory fibroblast phenotype after the treatment with eosinophil supernatants ^{19,21,22}. However, these studies were performed using eosinophil-conditioned media collected from these cells after they had degranulated and lysed, which only allowed one-way signaling from eosinophils to fibroblasts. This approach lacked signaling from fibroblasts to eosinophils, as well as the real-time assessment of cell-cell soluble factor bidirectional signaling.

Microfluidic coculture offers several advantages for studying cell-cell signaling over conventional approaches, such as conditioned media or Transwell® inserts ^{23,24}. Besides its ability to realize bidirectional communication in coculture, the versatile configurations of microfluidic coculture platforms provide more accurate recapitulation of the microenvironment ²⁵; the flexibility in designs also enables different cell seeding ratios, culture chamber dimensions and numbers with a potential for triculture. Additionally, microfluidic devices operate with less volume, which minimizes the use of expensive reagents and conserves precious primary samples isolated from patients ²⁶. Moreover, the emerging open microfluidic technology provides easy

accessibility and can be operated with a pipette in a standard biological laboratory without specialized equipment ^{27–32}. In light of this, we recently developed a novel open microfluidic coculture device using the common cell culture material polystyrene, where two culture chambers are separated by a half wall, the connection of which can be temporally controlled to allow soluble factor signaling via a liquid bridge ³³ (**Figure 2.1**). The current study builds on our prior work using this device to study cell signaling in the kidney ³³; and extends its application to include immune cell-fibroblast interactions, thus, establishing a new biological model. Further, in this work we expand the readouts possible with this device beyond fluorescence microscopy and gene expression reported in Zhang et al. 2020, demonstrating the ability to conduct phase contrast microscopy to study eosinophil morphology changes upon degranulation/cytolysis and monitor HLFs in real time (facilitated by both culture chambers being in the same focal plane). Further, the open nature of our microfluidic device (in contrast to conventional closed channels) allows for easy sampling and measurement of soluble factors using multiplexed immunoassays.

In this study, we adapt the open microfluidic coculture platform to establish a novel coculture model of degranulating eosinophils and primary HLFs to investigate the mechanisms of airway inflammation. We validate the model by characterizing eosinophil degranulation (release of eosinophil-derived neurotoxin (EDN)), HLF proinflammatory gene expression (interleukin 6 (*IL6*), C-X-C motif chemokine ligand 8 (*CXCL8*), and intercellular adhesion molecule 1 (*ICAM1*)), as well as soluble factors released in the culture media (interleukin-6 (IL-6) and interleukin-8 (IL-8)) that were previously found in the eosinophil-conditioned media studies ^{19,21,22}. Additionally, we present a new analyte (granulocyte-macrophage colony-stimulating factor (GM-CSF)) found in our coculture system which has not been characterized by our group before. In the future, we will continue using this validated model to discover novel

bidirectional signals and the consequences on the biology of both cell types that are conferred in cocultures. Although the present study uses eosinophils derived from participants with allergy and asthma, in the future, we envision that this coculture model system could be more broadly applied to other immune cells and organs (*e.g.*, liver, breast, prostate, gastrointestinal tract). Taken together, we demonstrate that our open microfluidic coculture device can be effectively used to dissect soluble factor signaling mechanisms between immune cells and fibroblasts.

2.2 Results and Discussion

2.2.1 Design and workflow of the open microfluidic coculture device

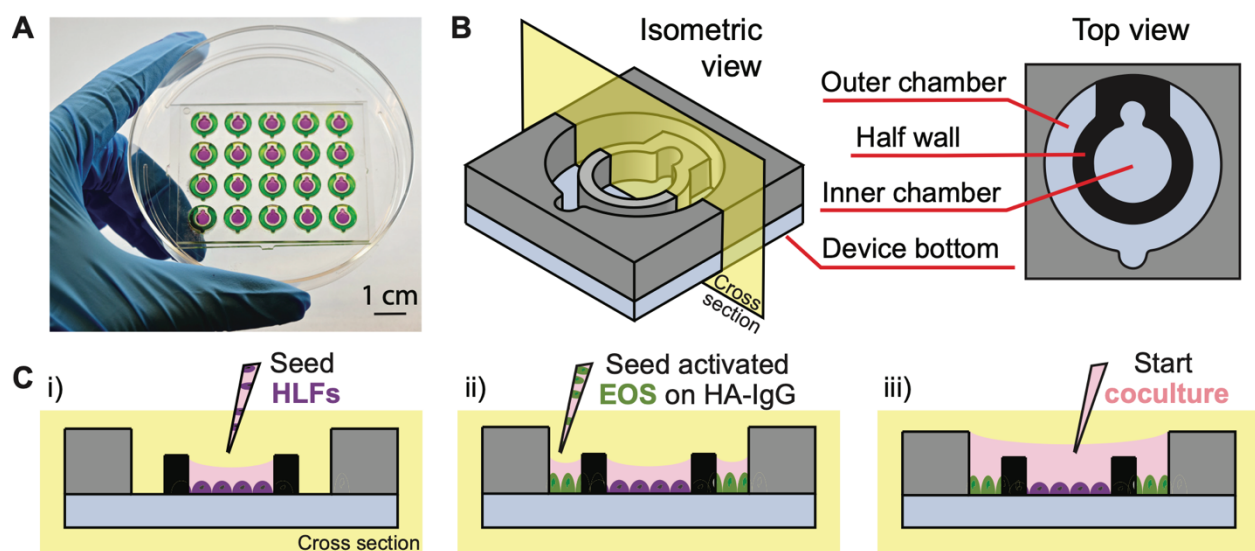


Figure 2.1. Open microfluidic coculture device description. **A)** A device chip containing 20 coculture devices in a 4×5 array fitted in a 10-cm petri dish³³. Reproduced from Zhang et al. 2020 as allowed per journal policy. Inner (purple) and outer (green) chambers are loaded with food coloring dyes for visualization. **B)** An isometric and top view of the device design. **C)** A cross-sectional view of the device and simplified workflow. (i) HLFs are seeded to the inner chambers on day 1; (ii) interleukin-3 (IL-3)-activated eosinophils (EOS) are seeded to the outer chambers precoated with heat-aggregated immunoglobulin G (HA-IgG) on day 3; (iii) inner and outer chambers are connected via the addition of coculture medium after 1.5 h to initiate coculture.

In this study, we present an open microfluidic coculture model to investigate the bidirectional signaling between HLFs and blood eosinophils and their roles in airway inflammation. To establish this *in vitro* model, we adapted a previously developed open microfluidic coculture device made of polystyrene, where two culture chambers are separated by a half wall but can be later connected to start coculture³³. Our device is purposefully designed to be simple and easy to use, which are factors that are increasingly desired in microfluidic devices to enable adoption by biology labs (in contrast to devices with complicated pumps and peripheral equipment)²⁶. The coculture devices are fabricated in an array to allow for testing of multiple experimental conditions in one chip, and a chip is designed to fit in a standard 10-cm petri dish, which can be used as a primary container for culture sterility and evaporation control (**Figure 2.1A**). The petri dish (and a secondary container) can then be fitted easily into an incubator and imaged with phase contrast or fluorescence microscopy directly using existing microscope holders.

Each of the microfluidic coculture devices is composed of an inner and an outer chamber, separated by a half wall (**Figure 2.1B**). The chambers are designed to be operated simply with a standard pipette; the pipette tip (up to the size of a P200) can be anchored to the notch, and the liquid will fill the entire culture chamber with a single dispensing step due to the spontaneous capillary flow^{28,29,33}. Also, because the chambers are on the same focus plane (unlike Transwell[®] inserts where the membrane is higher than the well plate bottom), the two cell types in coculture can be easily imaged at the same time using a standard microscope. The compatibility of our coculture devices with traditional workflows (*e.g.*, use of petri dishes, pipettes, and microscopes) allows them to be used conveniently by most biologists.

To demonstrate the use of coculture devices in the study, a simplified workflow of one of the experimental conditions ("HLF + EOS (IL-3 IgG)") is shown (**Figure 2.1C, Supplementary Figure S2.2**). Briefly, HLFs were first seeded to the inner chamber of the coculture device, allowing them to attach and spread out. Next, eosinophils activated by interleukin-3 (IL-3) were seeded to the outer chambers which were previously coated with heat-aggregated immunoglobulin (HA-IgG). After the eosinophils attached, the inner and outer chambers were connected with additional coculture medium to overflow the half wall and initiate coculture. The two cell types were allowed to communicate via soluble factors for 72 h.

2.2.2 Eosinophils degranulate on HA-IgG in the microfluidic coculture device

Eosinophil degranulation starts with the release of soluble factor contents, such as toxic proteins (*e.g.*, EDN), from intracellular granules while eosinophils are alive ⁶. Adhesion of activated eosinophils typically triggers degranulation ^{34,35}. In contrast, following eosinophil cell death (*e.g.*, apoptosis or cytolysis), cell-free granules and other cellular contents are released as the cell membrane ruptures ⁷. Additionally, it has been shown that extracellular cell-free granules also contain surface receptors that allow them to be activated and to further degranulate even when outside of cells ³⁶; thus, cell-free granules from eosinophils can also respond to soluble factors secreted by neighboring cells (*e.g.*, fibroblasts).

We previously developed an *ex vivo* eosinophil degranulation model that recapitulates the responses of activated airway eosinophils to a biologically relevant extracellular cue found in the human lung airways ²⁰. Briefly, freshly isolated blood eosinophils were primed with IL-3 and seeded to well plates coated with or without HA-IgG. Eosinophil degranulation occurred as soon as 30 min after adhesion to HA-IgG, and significant cytolysis was observed after 4 h ⁷. To translate the eosinophil degranulation model established on well plates to the microfluidic

coculture device, the first step is to validate that primary eosinophils will behave similarly in the coculture devices.

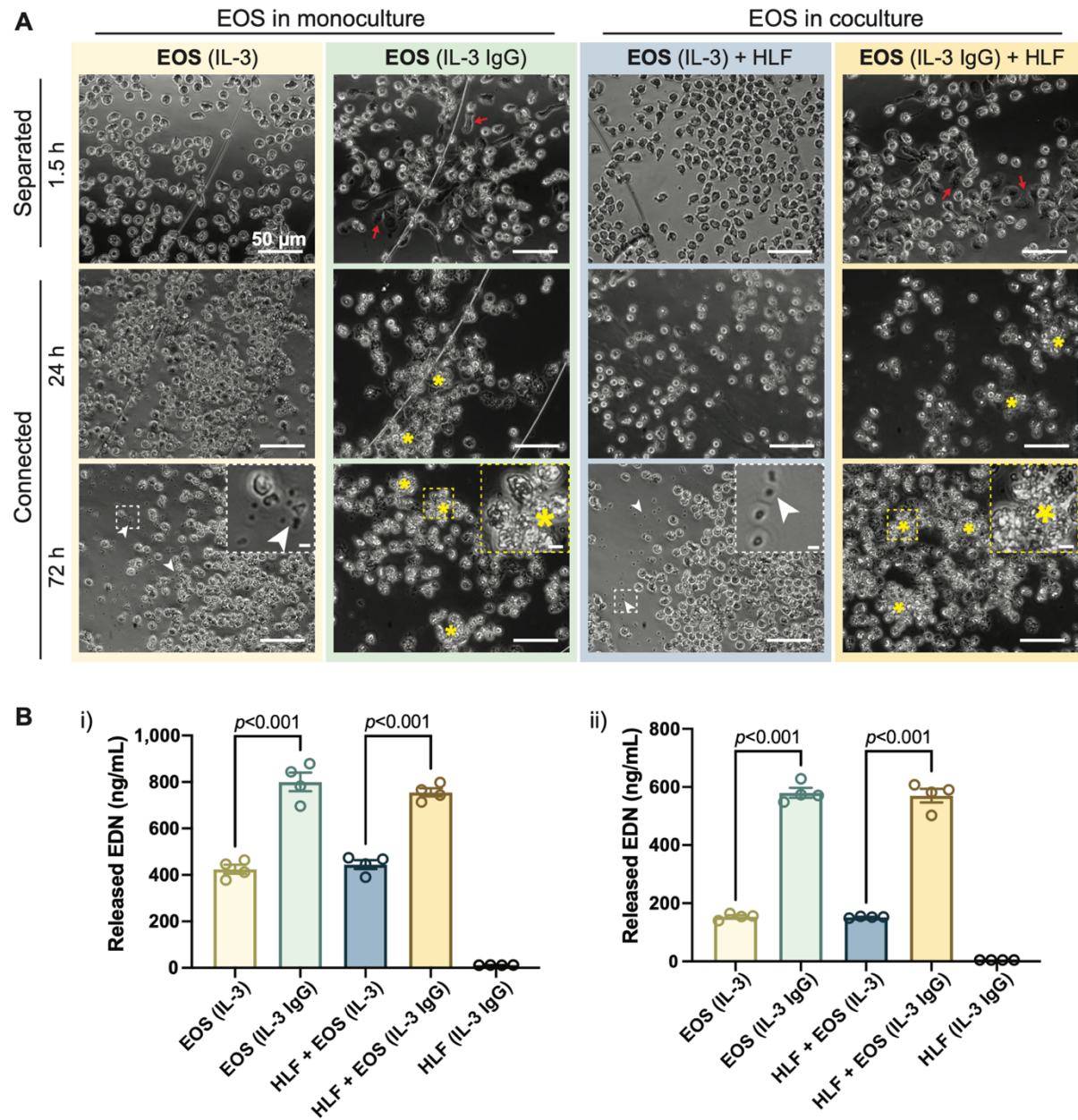


Figure 2.2. IL-3-activated eosinophils degranulate on HA-IgG in the coculture device. **A)** Phase contrast images of eosinophils (EOS) activated with IL-3 (“IL-3”) and in some conditions also seeded onto HA-IgG (“IL-3 IgG”) in coculture devices at the indicated times. The chambers remained separated for 1.5 h and then were connected for up to 72 h. Red arrows show eosinophils spreading on HA-IgG. Yellow asterisks show aggregates of eosinophil cell debris. White arrowheads show eosinophil cell-free granules. Magnified inserts included for improved visualization of cell debris and cell-free granules (scale bars: 50 μ m). Images are representative of four culture replicates, as well as three eosinophil donors and two HLF donors. **B)** Levels of

released eosinophil-derived neurotoxin (EDN) in culture media after 24 h measured by ELISA. Results from two different representative eosinophil/HLF donor pairs are shown in i) and ii). Data are expressed as mean $\pm$ SEM (n=4); each point is one culture replicate. Data were analyzed using one-way ANOVA, followed by Holm-Šidák's multiple comparisons test.

In accordance with our previous work ⁷, after being seeded to the outer chambers for 1.5 h, eosinophils pre-activated by IL-3 displayed apparent adhesion and spreading on HA-IgG, while still maintaining their cellular membrane integrity (red arrows, **Figure 2.2A**: “EOS (IL-3 IgG)” or “EOS (IL-3 IgG) + HLF”). In contrast, eosinophils seeded to devices without HA-IgG appeared in suspension and some displayed polarized morphology due to the prior activation with IL-3 (**Figure 2.2A**: “EOS (IL-3)” or “EOS (IL-3) + HLF”) ^{20,37}. After the chambers were connected for 24 h and as expected according to our previous work ⁷, IL-3 IgG-activated eosinophils had likely undergone significant cytolysis, leaving aggregates of cell debris (yellow asterisks, **Figure 2.2A**). At 72 h, control eosinophils not exposed to HA-IgG also released some cell-free granules (white arrowheads, **Figure 2A**), suggesting eosinophil death after prolonged IL-3-free culture *ex vivo* ^{38,39}. Images from eosinophil donor 1/HLF donor 2 are shown and are representative of a total of 6 donor pairs (eosinophil donor 1-3, HLF donor 1 and 2); images from all donor pairs can be found in **Supplementary Figure S2.4**.

Because both chambers of the open microfluidic coculture device are on the same focus plane, we were able to monitor HLFs together with eosinophils in culture at the same time. Across different culture conditions, we did not see obvious morphological dissimilarities of HLFs (**Supplementary Figure S2.5**); the HLFs maintained a spindle-like morphology (characteristic of fibroblasts) in both monoculture and coculture conditions. Further, we observed a consistent increase in cell density as the experiment progressed, indicating growth and expansion of HLFs in our microfluidic device.

Conditioned media from the connected chambers was collected after 24 h, and the EDN content measured using ELISA. Eosinophils in IL-3 IgG conditions (with or without HLF) released significantly more EDN than control eosinophils in IL-3 conditions (**Figure 2.2B**). Data from eosinophil donor 1/HLF donor 1 (**Figure 2.2Bi**) and eosinophil donor 2/HLF donor 2 (**Figure 2.2Bii**) were shown and are representative of a total of 4 donor pairs (eosinophil donor 1 and 3, HLF donor 1 and 2); data from all available donor pairs can be found in **Supplementary Figure S2.6**. We note that there is some donor-to-donor variation; importantly, the trends hold across all donor pairs and are statistically significant.

Taken together, our results show that IL-3-activated eosinophils further degranulate on HA-IgG-coated outer chambers, suggesting we were able to recapitulate the previously developed eosinophil degranulation model ²⁰ in the open microfluidic coculture device.

2.2.3 CXCL8, IL6, and ICAM1 are upregulated in HLFs cocultured with degranulating eosinophils

After the validation of the eosinophil degranulation model in the microfluidic coculture device, we wished to investigate the response of HLFs to degranulating eosinophils in coculture. First, we aimed to determine the changes of HLFs at the mRNA expression level. To achieve this goal, HLFs from the inner chambers of the coculture device were lysed after 72 h and analyzed using RT-qPCR. Notably, in HLF monoculture conditions (“HLF (IL-3 IgG)”), the coculture devices were treated the same way as in “HLF + EOS (IL-3 IgG)” conditions minus the presence of eosinophils (*i.e.*, outer chambers were coated with HA-IgG and loaded with IL-3 treated medium before connection) to account for the possible impact of residual IL-3 and released HA-IgG from the process on HLFs.

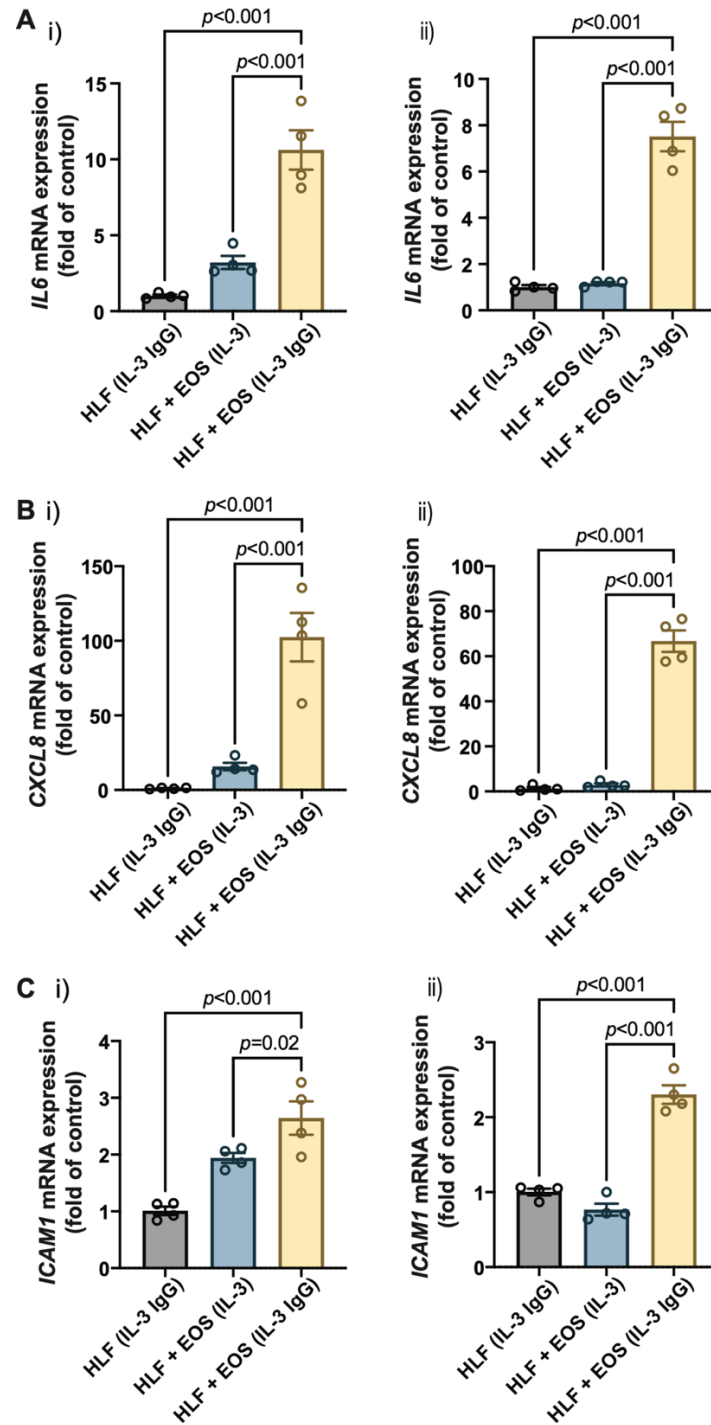


Figure 2.3. Proinflammatory genes are upregulated in HLFs cocultured with degranulating eosinophils. HLFs in coculture with degranulating eosinophils (“HLF + EOS (IL-3 IgG)”) for 72 h showed the highest mRNA level upregulation of **A)** *IL6*, **B)** *CXCL8*, and **C)** *ICAM1*. Results from two representative eosinophil/HLF donor pairs are shown in i) and ii). Data are expressed as mean $\pm$ SEM (n=4); each point is one culture replicate. Data were analyzed using one-way ANOVA, followed by Holm-Šidák's multiple comparisons test.

Our previous RNA-sequencing analyses identified several genes that were upregulated in HLFs cultured with conditioned media from degranulated eosinophils, which had downstream functions on inflammation, tissue remodeling, and lipid synthesis ²¹. Among those genes, we selected 3 representative genes that promote granulocytic inflammation to measure in our study: interleukin 6 (*IL6*), C-X-C motif chemokine ligand 8 (*CXCL8*), and intercellular adhesion molecule 1 (*ICAM1*). As a result, HLFs in coculture with degranulating eosinophils (“HLF + EOS (IL-3 IgG)”) showed the highest expression of *IL6* (**Figure 2.3A**), *CXCL8* (**Figure 2.3B**), and *ICAM1* (**Figure 2.3C**), significantly higher than control HLFs, those in monoculture (“HLF (IL-3 IgG)”) or in coculture with non-degranulating eosinophils (“HLF + EOS (IL-3)”). These trends were consistent with our previous RNA expression results using conditioned media ^{19,21}. Data from eosinophil donor 1/HLF donor 1 **i**) and eosinophil donor 2/HLF donor 2 **ii**) were shown and are representative of a total of 4 donor pairs (eosinophil donor 1 and 2, HLF donor 1 and 2); data from all available donor pairs can be found in **Supplementary Figure S2.7**.

Together, our results demonstrate that HLFs were activated by the coculture with degranulating eosinophils in the microfluidic coculture device, as indicated by the upregulation of proinflammatory genes, *IL6*, *CXCL8*, and *ICAM1*.

2.2.4 Coculture of HLFs with degranulating eosinophils leads to increased secretion of IL-6 and IL-8 than in either HLF or EOS monoculture

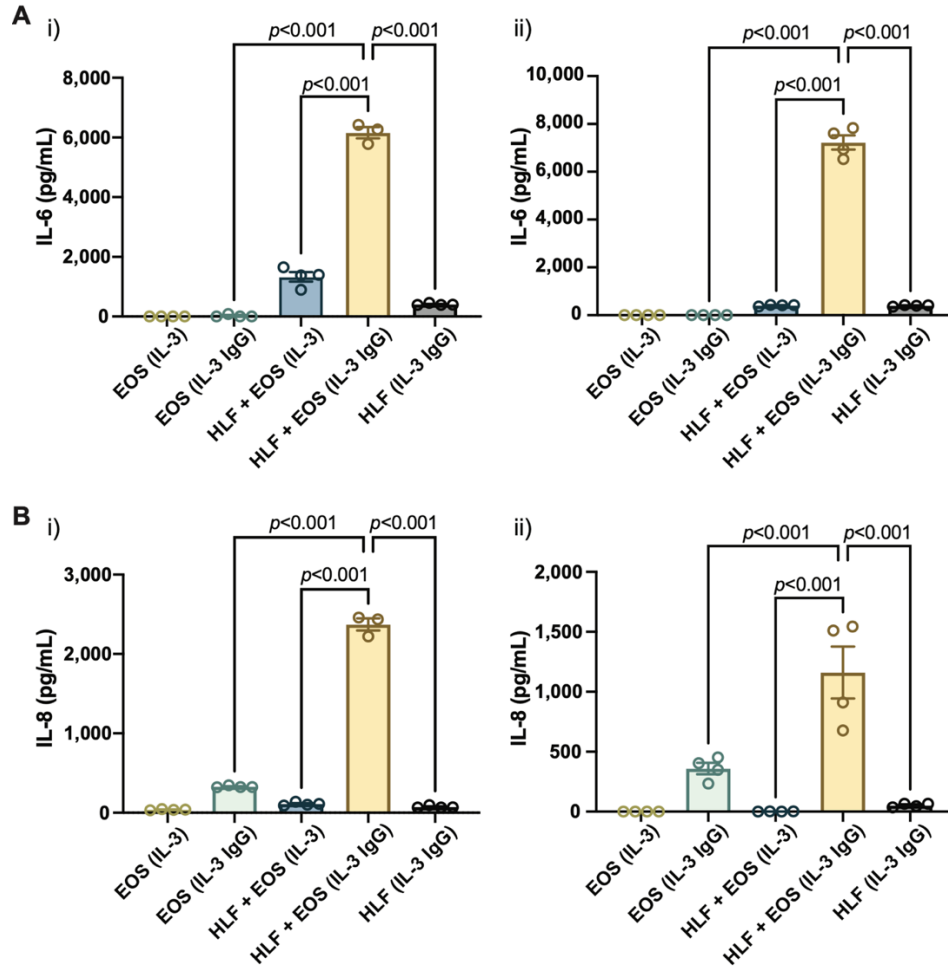


Figure 2.4. The coculture of HLFs with degranulating eosinophils releases the highest levels of proinflammatory cytokines compared to HLF and eosinophil monocultures and other control conditions. Levels of soluble factors, **A)** IL-6 and **B)** IL-8, were measured in the conditioned media after 72 h in culture. Results from two representative eosinophil/HLF donor pairs are shown in **i)** and **ii)**. Data are expressed as mean $\pm$ SEM (n=3-4); each point is one culture replicate. Data were analyzed using one-way ANOVA, followed by Holm-Šidák's multiple comparisons test.

Next, we sought to assess the cellular changes at the protein expression level, specifically the level of secreted (extracellular) proteins. After the eosinophils and HLFs were cultured in the microfluidic coculture device for 72 h, we collected the culture supernatants and analyzed their contents of IL-6 and IL-8, proteins encoded by the two genes characterized above: *IL6* and *CXCL8*, respectively. IL-6 and IL-8 are important proinflammatory cytokines as they play vital

roles in neutrophil regulation, with IL-8 as a neutrophil chemoattractant ⁴⁰ and IL-6 as a neutrophil activator ⁴¹.

Similar to the results reported previously ^{19,22}, we observed a significant increase of both IL-6 (**Figure 2.4A**) and IL-8 (**Figure 2.4B**) in the coculture conditions of HLFs with degranulating eosinophils (“HLF + EOS (IL-3 IgG)”) compared to the control conditions (*i.e.*, monoculture or non-degranulating eosinophils). Notably, the trends of IL-6 and IL-8 release among the three conditions with HLFs were similar to the trends of *IL6* (**Figure 2.3A**) and *CXCL8* (**Figure 2.3B**) mRNA expression, suggesting the changes of the two markers took place at both transcription and translation levels in HLFs. Data from eosinophil donor 1/HLF donor 1 **i)** and eosinophil donor 3/HLF donor 2 **ii)** were shown and are representative of a total of 6 donor pairs (eosinophil donor 1-3, HLF donor 1 and 2); data from all donor pairs can be found in **Supplementary Figure S2.8**.

Together, our results suggest that the coculture of HLFs with degranulating eosinophils induced a proinflammatory microenvironment in the microfluidic coculture device, as indicated by the increased levels of IL-6 and IL-8 compared to either cells in monoculture or the coculture with non-degranulating eosinophils.

2.2.5 GM-CSF expression and secretion are increased in the coculture of HLFs with degranulating eosinophils

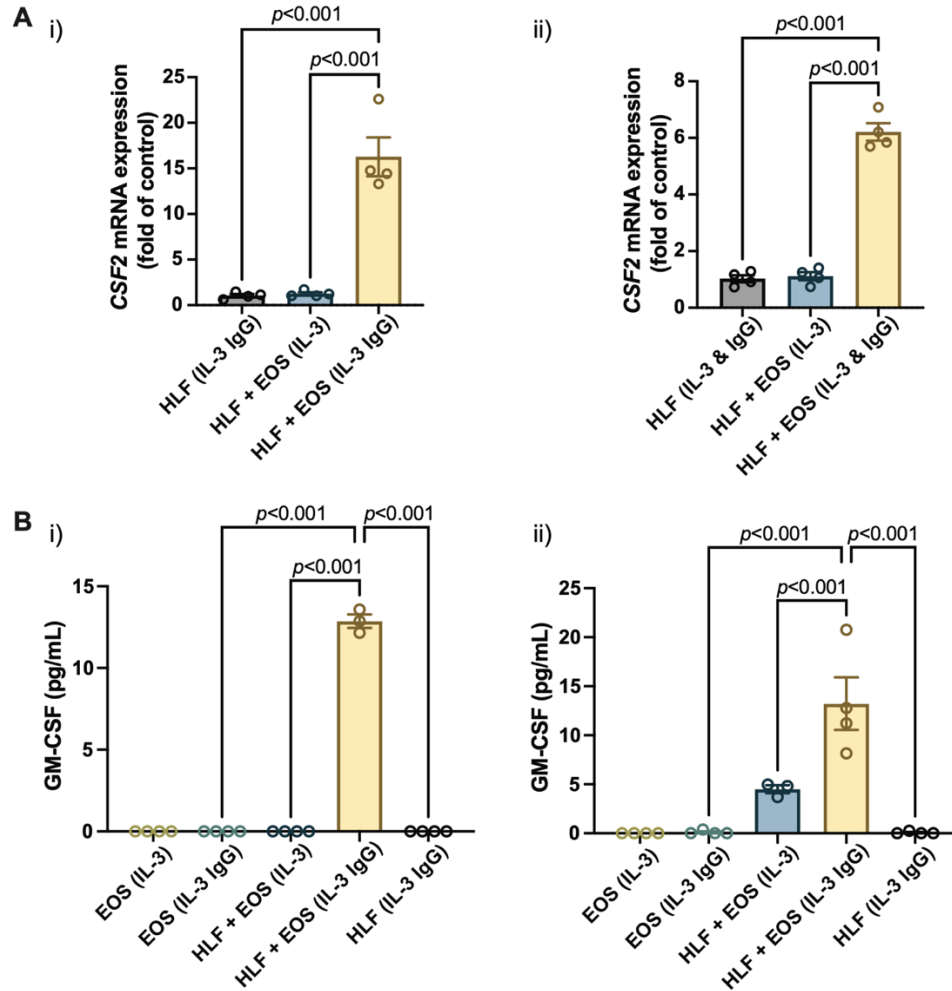


Figure 2.5 The coculture of HLFs with degranulating eosinophils induces the highest levels of **A)** mRNA expression of *CSF2* in HLFs and **B)** protein secretion of GM-CSF in the conditioned media after 72 h in culture. Results from two representative eosinophil/HLF donor pairs are shown in **i)** and **ii)**. Data are expressed as mean $\pm$ SEM (n=3-4); each point is one culture replicate. Data were analyzed using one-way ANOVA, followed by Holm-Šidák's multiple comparisons test.

Having validated our coculture model with previously reported analytes from degranulating eosinophils that activate HLFs, we then sought to discover additional soluble factors that could have more bidirectional effects on the eosinophil-HLF crosstalk. GM-CSF has been found to enhance eosinophil survival *in vitro* and accumulation *in vivo*, even at a minute level^{42,43}. Research shows that fibroblasts can promote eosinophil survival and adherence via the

increased secretion of GM-CSF, but the studies were done in direct coculture systems (with cell-cell contact)^{39,44,45}. Further, the primary cell sources used in the previous studies were different from ours: we used normal HLFs and blood eosinophils from donors with allergy and asthma; Vancheri (1989) used normal HLFs and blood eosinophils from donors with allergic rhinitis; Zhang (1996) used bronchial myofibroblasts and blood eosinophils from donors with high eosinophil counts; Solomon (2000) used conjunctival fibroblasts and blood eosinophils from donors with mild atopy. Herein, we aimed to study if GM-CSF could be upregulated in our segregated coculture system (without cell-cell contact) with our HLFs and eosinophils.

Similar to other analytes presented in the study, we first characterized the mRNA expression of colony stimulating factor 2 (*CSF2*), the protein encoding gene of GM-CSF, in HLFs that were in coculture or monoculture for 72 h in the microfluidic device. According to the results (**Figure 2.5A**), the expression of *CSF2* was upregulated the most in HLFs cocultured with degranulating eosinophils (“HLF + EOS (IL-3 IgG)”). Then, we measured the levels of soluble GM-CSF secreted into the culture supernatants after 72 h and observed a similar trend (**Figure 2.5B**): the release of GM-CSF is significantly higher in the coculture conditions of HLFs with degranulating eosinophils than in monocultures or cocultures with non-degranulating eosinophils. Data from eosinophil donor 2/HLF donor 1 **i**) and eosinophil donor 2/HLF donor 2 **ii**) were shown and are representative of up to 6 donor pairs; data from all available donor pairs can be found in **Supplementary Figure S2.9**.

Jointly, the results suggest that the GM-CSF expression was upregulated in HLFs when in coculture with degranulating eosinophils. The increased levels of GM-CSF in coculture media could in turn prolong the survival of eosinophils in our *ex vivo* coculture system as a bidirectional signaling effect from HLFs. This new finding, to our knowledge, is the first to show

GM-CSF upregulation in a segregated coculture system of eosinophils and fibroblasts; it could provide us with further directions in studying the impacts from HLFs to eosinophils, given the importance of GM-CSF in eosinophil activation *in vitro* and *in vivo*.

Despite many benefits (enabling bidirectional signaling, *etc*), we acknowledge the limitation of the shared media mechanism of our microfluidic coculture devices: as with other coculture systems, the shared media can lead to ambiguity when determining the source of the soluble factors in coculture. Consequently, complementary studies that characterize only one type of cells (*e.g.*, conditioned media, qPCR, microscopy) are needed to fully investigate the signaling mechanisms. For example, in our study, because the culture media was shared between the degranulating eosinophils and HLFs in coculture, we cannot definitively pinpoint the source of the elevated levels of soluble factors (*i.e.*, IL-6, IL-8, GM-CSF) in coculture, since either cells could be responsible for this effect. However, considering the findings in previous conditioned media studies^{19,21,22,39} and our RT-qPCR data as above (*i.e.*, *IL6*, *CXCL8*, *CSF2*), which explicitly analyzed HLFs only, we suspect that the HLFs are likely the predominant source of the additional IL-6, IL-8, and GM-CSF in our coculture model. Still, more experiments are needed (*e.g.*, transcriptomic studies of eosinophils) before a definitive conclusion is drawn.

Taken together, our mRNA (**Figure 2.3 and 2.5A**) and secreted protein results (**Figure 2.4 and 2.5B**), in conjunction with previous publications^{19,21,22,39}, suggest that the HLFs in coculture with degranulating eosinophils likely secrete increased levels of IL-6, IL-8, and GM-CSF, compared to the coculture with non-degranulating eosinophils or either cell type in monoculture. This suggests the induction of HLFs into a proinflammatory phenotype in the presence of degranulating eosinophils in the microfluidic coculture device, which could in turn further activate eosinophils and amplify the inflammatory response.

2.3 Materials and Methods

2.3.1 Device fabrication

The open microfluidic coculture device (**Figure 2.1**) was designed with Solidworks (Solidworks, Waltham, MA). An engineering drawing of the device with dimensions and original design files are included in **Supplementary Figure S2.1** and the Supplemental Materials of the previous work ³³. The coculture devices were fabricated using a DATRONneo (Datron Dynamics, Milford, NH) Computer Numerical Control (CNC) mill. Device top and bottom pieces were milled from 2-mm and 1.2-mm thick polystyrene (PS) sheets (Goodfellow USA, Coraopolis, PA), respectively. After milling, the pieces were cleaned thoroughly with pressurized air to remove most of the plastic particles. The top and bottom pieces were then further cleaned via sonication and solvent bonded together to form a complete device chip using previously established protocols ^{33,46} with a few modifications to improve the bonding efficiency as described in the following paragraphs.

Modifications to the solvent bonding procedure to improve on prior protocol in Zhang et al. 2020: The inner wall of the coculture device is prone to faulty solvent bonding because it is not flush with the top surface, thus, when the weight is added the inner wall is not pressed firmly to the bottom layer for solvent bonding. A PDMS layer was designed to apply downward pressure to the inner wall during the solvent bonding process and ensure its bonding. A mold was milled from 4.5 mm poly(methyl methacrylate) on a DATRONneo. PDMS (Sylgard™ 184, Dow) base and curing agent was mixed in a 8:1 ratio and degassed in a vacuum chamber. The mixture was poured over the mold in an even layer. The mold with PDMS was degassed and cured at room temperature for 24 h. The PDMS piece, along with the 2-mm and 1.2-mm milled

PS pieces, were then sonicated with 70% ethanol for 30 minutes, then was dried with pressurized air prior to solvent bonding.

For solvent bonding, a hot plate (HP60, Torrey Pines Scientific) was preheated to 65 °C. The sonicated PS and PDMS pieces and an aluminum bar (McMaster-Carr, Los Angeles, CA) were placed on the hot plate to completely dry off excess ethanol from sonication and heat each item. Once the preheating was complete, a device bottom piece was placed on a Kimwipe (Kimberly-Clark Professional™), and acetonitrile (ACN; A998-4, Fisher Chemicals) was pipetted dropwise onto the bottom piece until it covered most of the surface. The top piece was tilted at an angle such that its top edge was in contact with the longer edge of the bottom piece and was gently lowered onto the bottom piece to align the two pieces. The Kimwipe previously placed underneath the device was removed and used to gently wipe around the newly bonded device to absorb excess ACN. The heated PDMS piece was then stacked onto the device and pressure was applied to the stack with the palm of a gloved hand for about 1 min; more Kimwipes were used to absorb any excess ACN in the process. While leaving the PDMS stacked onto the device, a heated aluminum bar was placed on top to continue applying pressure for 10 min at 65 °C to facilitate the reaction between ACN and the PS interface.

After bonding, the device was sonicated again with 70% ethanol for 30 min then dried completely with pressurized air. Before cell culture use, the device was plasma treated for 5 min at 0.25 mbar and 70 W in a Zepto LC PC Plasma Treater (Diener Electronic, Ebhausen, Germany) using oxygen followed by 10 min of ultraviolet sterilization.

2.3.2 Human subjects and cell preparations

Normal HLFs were isolated as described previously^{21,47} using deidentified tissue samples from thoracic surgical resection specimens. These samples were collected and characterized by

the Carbone Cancer Center Translational Science BioCore at the University of Wisconsin-Madison, under Institutional Review Board approval (IRB #2016-0934) and fibroblast subsequently derived under IRB #2011-0521. To obtain non-fibrotic fibroblasts, we utilized adjacent (uninvolved) lung from lobectomy or biopsy specimens from patients undergoing lung resection for pulmonary nodules and who did not have any identifiable lung disease by history or histologic assessment. All specimens used for fibroblast isolation were examined by a pathologist to ensure that no underlying lung disease (emphysema, idiopathic lung diseases, etc.) was present in histology. HLF cultures were derived from two different donors.

As previously, to isolate fibroblasts, tissue specimens were placed in fibroblast growth media (Dulbecco's Modified Eagle's Medium (DMEM; Corning 10-013-CV) with 100 units/mL streptomycin, 250 ng/mL amphotericin B, 100 units/mL penicillin (Corning 30-004-CI), and 10 µg/mL ciprofloxacin (Corning 61-277-RF))^{13,48}. Alveolar lung tissue was minced and plated onto 10-cm plates (Falcon 353003) in HLF growth medium containing DMEM supplemented with 10% fetal bovine serum (FBS; HyClone, Cytiva SH30396.03), 2 mM L-glutamine (Sigma-Aldrich G7513), and antibiotics as above. Expanded populations of fibroblasts were subsequently subcultured after 4–5 days, resulting in the development of a homogenous fibroblast population. All primary cultures were used from passages 5–10.

Eosinophil isolation was performed under a study protocol approved by the University of Wisconsin-Madison Health Sciences Institutional Review Board (IRB #2013-1570). Informed written consent was obtained from subjects prior to participation. Peripheral blood eosinophils were obtained from subjects with diagnoses of allergic rhinitis and asthma (n = 3). Subjects using low doses of inhaled corticosteroids did not use their corticosteroids on the day of the blood draw.

Eosinophils were purified by negative selection as previously described^{20,21} from three different donors. Briefly, 200 ml of heparinized blood was diluted 1:1 in Hank's Balanced Salt Solutions (HBSS; Corning 21-022-CM) and was overlaid above Percoll (1.090 g/ml; GE). After centrifugation at 700 xg for 20 min, at room temperature, the mononuclear cells were removed from the plasma/Percoll interface and erythrocytes were eliminated from the cell pellet by hypotonic lysis. The remaining pellet was resuspended in 2% new calf serum (Sigma-Aldrich; N4762) in HBSS. Cells were then incubated with anti-CD16, anti-CD3, anti-CD14 and anti-Glycophorin-A beads from Miltenyi (San Diego, CA), and run through an AutoMACS (Miltenyi). Eosinophil purity was determined by a manual count of a cytospin slide with eosin stain under the microscope. Eosinophil preparations with purity $\geq 99\%$ were shipped on the same day, ~5 h after the blood draw. Fresh cells were shipped overnight on ice in eosinophil culture medium containing RPMI 1640 (Corning 10041CV) supplemented with 10% FBS, 2 mM L-glutamine, and antibiotics as above using a Petaka®G3 FLAT cell culture device (Celartia, OH).

2.3.3 Cell culture

On day 1, HLFs were seeded on the inner chambers (surface area: 13.8 mm², volume: 16 μ L) of the coculture device at a density of 200 cells/mm² (a total of 2760 fibroblasts per inner chamber) in HLF growth medium and allowed to attach and spread overnight (**Supplementary Figure S2.2**).

On day 2, HLF media was replaced with starvation medium containing DMEM supplemented with 0.1% bovine serum albumin (BSA; Fisher BioReagents BP9706100), 2 mM L-glutamine, and antibiotics as above, and allowed to serum starve overnight. On the same day, peripheral blood eosinophils were received, retrieved, and activated in eosinophil culture medium with 2 ng/mL interleukin-3 (IL-3; BD Pharmingen 554604) at 1×10^6 cells/mL for 24 h

in a well plate (Corning). Human immunoglobulin G (IgG; Sigma-Aldrich I2511) was heat aggregated (HA-IgG) in phosphate buffered saline (PBS; Gibco 10010049) for 30 min at 63 °C^{7,21} and used to coat outer chambers (15 µg/mL; 36 µL/chamber) of the coculture device overnight.

On day 3, HLF media was replaced with serum-free medium containing DMEM supplemented with 2 mM L-glutamine and antibiotics as above. HA-IgG was removed and outer chambers were saturated with 0.1% gelatin (Sigma-Aldrich G1890) in PBS for 30 min at 37 °C. Activated eosinophils were washed and resuspended at 2.6×10^6 cells/mL in fresh eosinophil culture medium (no cytokines), then seeded to the outer chambers (surface area: 29.3 mm², volume: 36 µL) at a density of 3125 cells/mm² (a total of 91563 eosinophils per outer chamber). After eosinophils were seeded and allowed to attach for 1.5 h, the inner and outer chambers were connected using additional 50 µL coculture medium (1:1 ratio of HLF serum-free medium and eosinophil culture medium) to start coculture (**Supplementary Figure S2.2**). The coculture was allowed for 72 h without media change. In control conditions (*i.e.*, monoculture or without HA-IgG), all procedures were performed the same way minus the one component, as indicated in figures.

The coculture period was determined based on previous studies where eosinophil-conditioned media was added to HLFs for 72 h^{19,21,22}. Moreover, 72 h in coculture allows sufficient time for the signaling factors from the degranulating eosinophils to reach the HLF compartment and for HLFs to express and release cytokines to the coculture media. The seeding density of eosinophils in the microfluidic device was also translated from previous studies by keeping the surface area seeding density consistent (**Supplementary Table S2.1**)²⁰. Fibroblast seeding densities (157 cells/mm²) utilized in previous publications^{19,22} were modestly expanded

(200 cells/mm²) to increase the output for assays in our microfluidic coculture devices and was examined by visual inspection after 72 h in culture.

Throughout culturing, the devices were kept in a primary container (OmniTray, Thermo Scientific 264728), where sacrificial water droplets (about 1 mL) were pipetted around the device chip to mitigate evaporation. The primary container was placed in a secondary container (BioAssay dishes, Thermo Scientific 240835), in which the peripheral of the primary container was wrapped tightly with Kimwipes (Kimberly-Clark Professional 34256) soaked with water (about 100 mL) (**Supplementary Figure S2.3**). The secondary container was then placed on the bottom shelf of an incubator at 37 °C and 5% CO₂. As an additional precaution, we also alternated condition layouts for each presented experiment to mitigate potential edge effect.

2.3.4 Microscopy

For live-cell imaging, the coculture devices were placed into an OmniTray with a 50 × 61 mm rectangle cut out of the bottom and resealed with 48 × 60 mm No. 1 glass coverslips (Gold Seal® Cover Glasses, thickness 0.13-0.17 mm, Thermo Scientific 48×60-1-002G). Phase-contrast images of eosinophils were acquired using a ZEISS Primovert inverted microscope (40× objective, LD Plan-ACHROMAT 40×/0,50 Ph1 ∞/1,0; Carl Zeiss) mounted with an MU1403B Microscope Digital Camera (AmScope).

2.3.5 Enzyme-linked immunosorbent assay

After chambers were connected for 24 h, 7 µL of conditioned media from each device were harvested. Cells were removed by centrifugation at 1,500 rpm (about 1,000 g) for 10 min at 4 °C, and 5 µL of each supernatant were collected and stored at -80 °C until use. The human EDN enzyme-linked immunosorbent assay (ELISA) kit (MBL International 7630, Woburn, MA)

has a minimum detection limit of 0.62 ng/mL. Samples were diluted 1:30~1:50 with the Assay diluent and centrifuged again before use according to the manufacturer's protocol. The absorbance was read at 450 nm (reference at 620 nm) using a Cytation 5 Cell Imaging Multi-Mode Reader (Agilent Biotek).

2.3.6 Reverse transcription quantitative PCR

HLFs were lysed at the end of experiments (72 h in connection) and the cell lysates were stored in RNase-free buffer at -80 °C until use. HLF mRNA was extracted from lysates using the Dynabeads™ mRNA DIRECT™ Micro Purification Kit (Invitrogen™ 61021). The reverse transcription reaction was performed using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems™ 4387406). Gene expression levels were determined by qPCR using SsoAdvanced SYBR Green Supermix (Bio-Rad 1725271) in a CFX Connect Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA). The primer pair sequences are shown in **Supplementary Table S2.2** with glucuronidase β (*GUSB*) serving as a housekeeping gene ²¹. Standard curves and primer efficiencies were previously determined ²². Data are expressed as fold changes of control using the comparative cycle threshold ($\Delta\Delta C_t$) method where ΔC_t = C_t of the target gene (e.g., *IL6*) – C_t of the housekeeping gene (*GUSB*); $\Delta\Delta C_t$ = ΔC_t of treated samples (e.g., “HLF + EOS (IL-3 IgG)”) – ΔC_t of control samples (“HLF (IL-3 IgG)”); and fold change = $2^{-\Delta\Delta C_t}$ ²⁰.

2.3.7 Multiplex immunoassays

At the end of experiments, all culture supernatants were harvested from each device (about 95 μ L). Cells were removed by centrifugation at 1,500 rpm (about 1,000 g) for 10 min at 4 °C, and 75 μ L of each supernatant were collected and stored at -80 °C until use. Soluble factor analyses were performed using a custom multiplex immunoassay panel for IL-6, IL-8/CXCL8,

and GM-CSF (MILLIPLEX HCYTA-60K, MilliporeSigma). Samples were centrifuged again before use according to the manufacturer's protocol. Assays were read on a Luminex 200 instrument (Luminex Corp.) with xPONENT software. Standard curves were obtained using Belysa® Immunoassay Curve Fitting Software (MilliporeSigma). Data below the limit of quantification were plotted as zero on graphs.

2.3.8 Statistical analyses

Data are expressed as the mean of culture (technical) replicates $\pm$ the standard error of the mean (SEM) (n=3-4); each plotted point represents a culture replicate within the same device chip. Data were analyzed and visualized using Graphpad Prism 9 software (San Diego, CA). Differences among culture conditions were analyzed using ordinary one-way analysis of variance tests (one-way ANOVA), followed by Holm-Šidák's multiple comparisons test to compare the means of preselected pairs of conditions. $p < 0.05$ was considered statistically significant.

2.4 Conclusion

In summary, we demonstrated the use of a novel *ex vivo* microfluidic coculture model of degranulating eosinophils and HLFs to investigate the mechanisms of airway inflammation. Our open microfluidic coculture devices present a unique platform for interrogating these cell interactions, as a method of modeling airway inflammation and fibroblast activation *ex vivo*. In this model system, IL-3-activated eosinophils strongly degranulate on HA-IgG and induce HLFs into a proinflammatory phenotype via coculture. Here, we validated the findings from our previous eosinophil-conditioned media studies; with the signaling molecules we characterized in this study (*i.e.*, IL-6, IL-8), we found similar trends to our previous unidirectional culture system.

In addition, we presented the results of a new analyte not reported before (*i.e.*, GM-CSF), the upregulation of which could provide us with further directions in bidirectional signaling effects, especially from HLFs to eosinophils. In the future, we will continue discovering additional signaling molecules that may only be captured in our coculture (bidirectional signaling) system. Further, we plan to reengineer the device to enable triculture with additional relevant immune cells.

Our coculture model suggests an important interplay between the immune (*e.g.*, eosinophils) and mesenchymal (*e.g.*, HLFs) compartments leading to a proinflammatory fibroblast phenotype that may in turn amplify the overall inflammatory response. More importantly, although the present study uses eosinophils derived from people with allergy and asthma, in the future, we envision that this coculture model system could be more broadly applied to other organs (*e.g.*, liver, breast, prostate, gastrointestinal tract) with immune-mesenchymal cell interactions in both normal and diseased states.

2.5 References

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Chapter 3 | Injury-induced Paracrine Effects on the Podocyte's Transcriptome

Reproduced in part from Y. Zeng, R. A. Schweickart, J. C. Maggiore, C. J. Loretz, N. A. Hukriede, J. W. Pippin, O. Wessely§, A. B. Theberge§, S. J. Shankland§, "Injury-Induced Paracrine Effects on the Podocyte's Transcriptome," Submitted. (§co-corresponding)

YZ, ABT, and SJS conceptualized the research. OW, ABT, and SJS supervised the research. YZ, RAS, JWP, OW, and SJS planned the experiments. YZ, JCM, and CJL conducted experiments and collected data. YZ and RAS analyzed the data. YZ, JWP, OW, and SJS interpreted the results of the experiments. YZ, RAS, JCM, and SJS drafted the manuscript. YZ prepared the figures. YZ, NAH, JWP, OW, ABT, and SJS edited the manuscript. All authors reviewed and approved the manuscript.

Abstract: Although injury in glomerular disease might only damage a subset of podocytes in any given glomerulus, the response of the healthy neighboring podocytes to the injured podocytes oftentimes determines the course of the disease. To investigate this relationship, we designed a dual chamber open microfluidic co-culture device to specifically examine paracrine signaling from podocytes undergoing targeted injury by either Adriamycin, Puromycin Aminonucleoside or a cytopathic anti-podocyte antibody to healthy podocytes. Global transcriptomic analysis measured by RNA-sequencing revealed shared and unique pathways between the three forms of targeted injury, with temporal differences in the transcriptomic responses to each form of injury. Transcriptional changes also showed that paracrine-induced injury to neighboring podocytes was similar to the targeted primary injured podocytes and was specific for each podocyte injury model. Ligand-receptor analysis of ligands secreted by the insult targeted podocytes and receptors expressed by the responsive, paracrine injured counterparts identified 19 candidate mediator pairs. These findings critically define a new concept for future studies to understand the pathways involved in animal models and ultimately human studies.

3.1 Introduction

In primary glomerular diseases such as minimal change disease, focal segmental glomerulosclerosis (FSGS) and membranous nephropathy ^{1,2} podocytes are the initial glomerular cell type that becomes injured. They are also injured in other common diseases such as diabetic kidney disease and IgA nephropathy which impairs critical podocyte functions. As podocytes are terminally differentiated epithelial cells, they cannot self-renew when lost in disease ^{3,4}. Instead, other glomerular cells, parietal epithelial cells and cells of the renin lineage, provide a limited reservoir of podocyte progenitors by means of trans-differentiation ³. In addition, podocytes are critical in coordinating glomerular development and homeostasis. A physiological paracrine-mediated crosstalk between podocytes and neighboring glomerular cells has been shown. For example, podocytes produce vascular endothelial growth factor alpha which in turn is required for the survival of glomerular endothelial cells ⁵.

These paracrine signaling networks become modified in glomerular diseases. For example, injured podocytes release factors that cause secondary injury to mesangial and parietal epithelial cells ^{6,7}, resulting in proliferative, fibrotic and migratory phenotypes that cause glomerular scarring ⁸. Recent studies have also shown paracrine effects of injured podocytes on neighboring healthy podocytes, which further augments glomerular disease progression ⁹⁻¹¹. Studies in a mosaic mouse line demonstrated that selective injury of tdTomato-positive podocytes by the immunotoxin LMB2, caused damage to neighboring EGFP-positive podocytes not-targeted by LMB2 in the same glomerulus ^{12,13}. However, underlying molecular mechanisms of paracrine podocyte-to-podocyte signaling networks have not been identified. Moreover, it is unknown whether different forms of podocyte injury trigger the same or different paracrine signaling to neighboring healthy podocytes.

To address these questions, we established an *in vitro* microfluidic co-culture model that allows us to directly assess paracrine signaling events. Using an open microfluidic co-culture platform, we were able to determine what mediators from injured podocytes cause paracrine damage to neighboring podocytes and how the podocyte responses differed between different forms of experimental injury models.

3.2 Results

3.2.1 Open microfluidic co-culture model to assess paracrine signaling during podocyte injury

To investigate the paracrine effects of human podocyte injuries on neighboring podocytes *in vitro* we adapted our previously engineered dual chamber open microfluidic co-culture device,^{14, 15} composed of an inner and an outer chamber, separated by a solid half wall, so that cells seeded in each chamber are cultured independently (**Figure 3.1a,b**). To determine the mass transport efficiency across the chambers we embedded 40 kDa FITC-labeled dextran in a thin layer of Collagen I at the bottom of the outer chamber to address secretion/diffusion from the outer towards the inner chamber (**Figure 3.1c**). Samples from the inner chamber were assessed at 3 minutes (min), 3, 7, 15, and 24 hours (h), as well as 10 days (d). Fluorescence signal increases as the co-culture time progresses and starts to plateau after 7 h (i.e., reaches a similar level as the 10-d samples) (**Figure 3.1d**). We thus concluded that in a co-culture experiment, 72 h would be a suitable time point for both cell populations to release, receive, and respond to paracrine signals. This conclusion was also bolstered by our prior work using this device to observe paracrine signaling in two different cell model systems.^{14, 15}

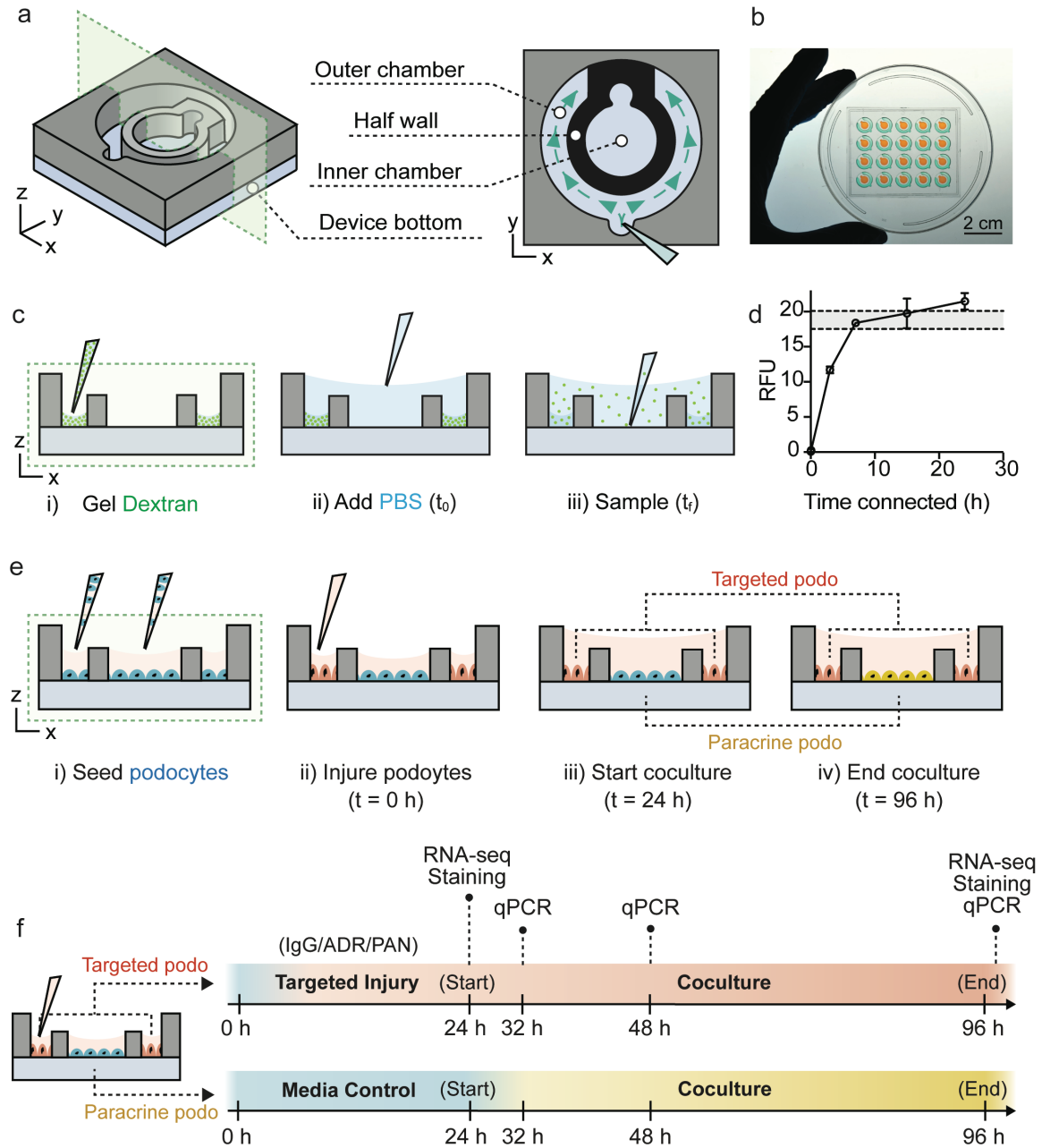


Figure 3.1. Open microfluidic co-culture model. **(a)** Schematics of an isometric (left) and top (right) view of a single co-culture device. Fluid can be delivered to the entire chamber via a single pipetting motion at the notch. **(b)** The engineered device chip contains 20 co-culture devices in a 4 x 5 array fitted in a 10-cm Petri dish as the primary container. The inner and outer chambers are loaded with orange and green dyes respectively for visualization. **(c)** A cross-sectional view of the device in the model mass transport experiment. (i) A thin layer of collagen I with 40 kDa FITC-Dextran is gelled at the bottom of outer chambers; (ii) PBS is added at t_0 to connect both chambers; (iii) samples are taken at the bottom of the inner chamber center at specific times (t_r) and measured for fluorescent intensity. **(d)** Fluorescence intensities of samples taken over time. Data are expressed as mean $\pm$ SEM ($n=4$). The grey shaded region reflects the

95% confidence interval of the signal at $t = 10$ d. RFU: relative fluorescence unit. **(e)** Simplified workflow of the podocyte-podocyte co-culture experiment. (i) Primary human podocytes are seeded and cultured in both chambers of the device until confluent; (ii) upon confluence, cytopathic sheep antibodies (IgG), Adriamycin (ADR), puromycin (PAN), or media control is added to the podocytes in the outer chambers at t_0 to start targeted injuries for 24 h; (iii) at $t = 24$ h, the outer and inner chambers are connected with fresh culture media to initiate the co-culture of targeted podocytes and paracrine podocytes, respectively; (iv) at $t = 96$ h, paracrine podocytes are assessed for paracrine injuries after 72 h in co-culture. **(f)** Schematic showing timelines for the targeted and paracrine injured podocytes. RNA-seq and immunofluorescence staining was performed on samples collected at the beginning ($t = 24$ h) and the end (96 h) of the co-culture; qPCR was performed on samples throughout the co-culture ($t = 32$ h, 48 h, and 96 h).

Next, we used the microfluidics system to co-culture podocytes and assess paracrine signaling following three podocyte injury models (**Figure 3.1e**). Primary human podocytes were seeded in both the inner and outer chambers. While the chambers were still separated, the podocytes in the outer chambers were injured separately by 3 well-described forms of injury: (i) a cytopathic anti-podocyte antibody (IgG)¹⁶⁻²², (ii) Adriamycin/doxorubicin (ADR)^{23, 24}, and (iii) puromycin aminonucleoside (PAN)⁹. These cells are herein referred to as insult-targeted podocytes. After 24 h, the injury inducers were washed off and fresh media was added to connect both chambers, allowing the targeted podocytes from the outer chamber to communicate with the healthy uninjured podocytes in the inner chamber. The latter are herein referred to as paracrine injured podocytes, as they were not exposed to the injury-inducing agents, but to paracrine signaling from the injured podocytes of the outer chamber. Cells from both chambers were processed for mRNA-sequencing (mRNA-seq), qRT-PCR, and immunofluorescence at multiple time points during the process (**Figure 3.1f**).

3.2.2 Podocyte injuries characterized by changes to mitochondria dynamics

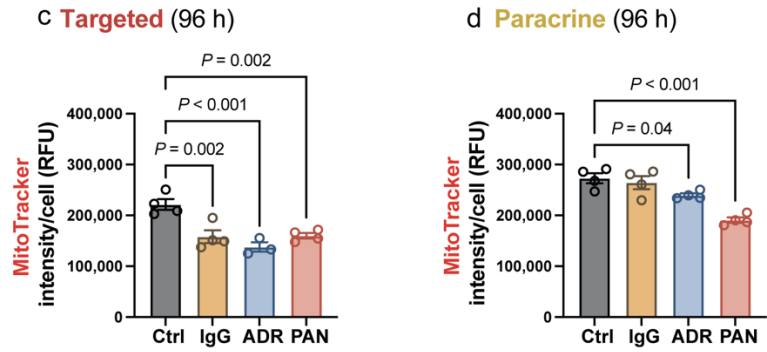
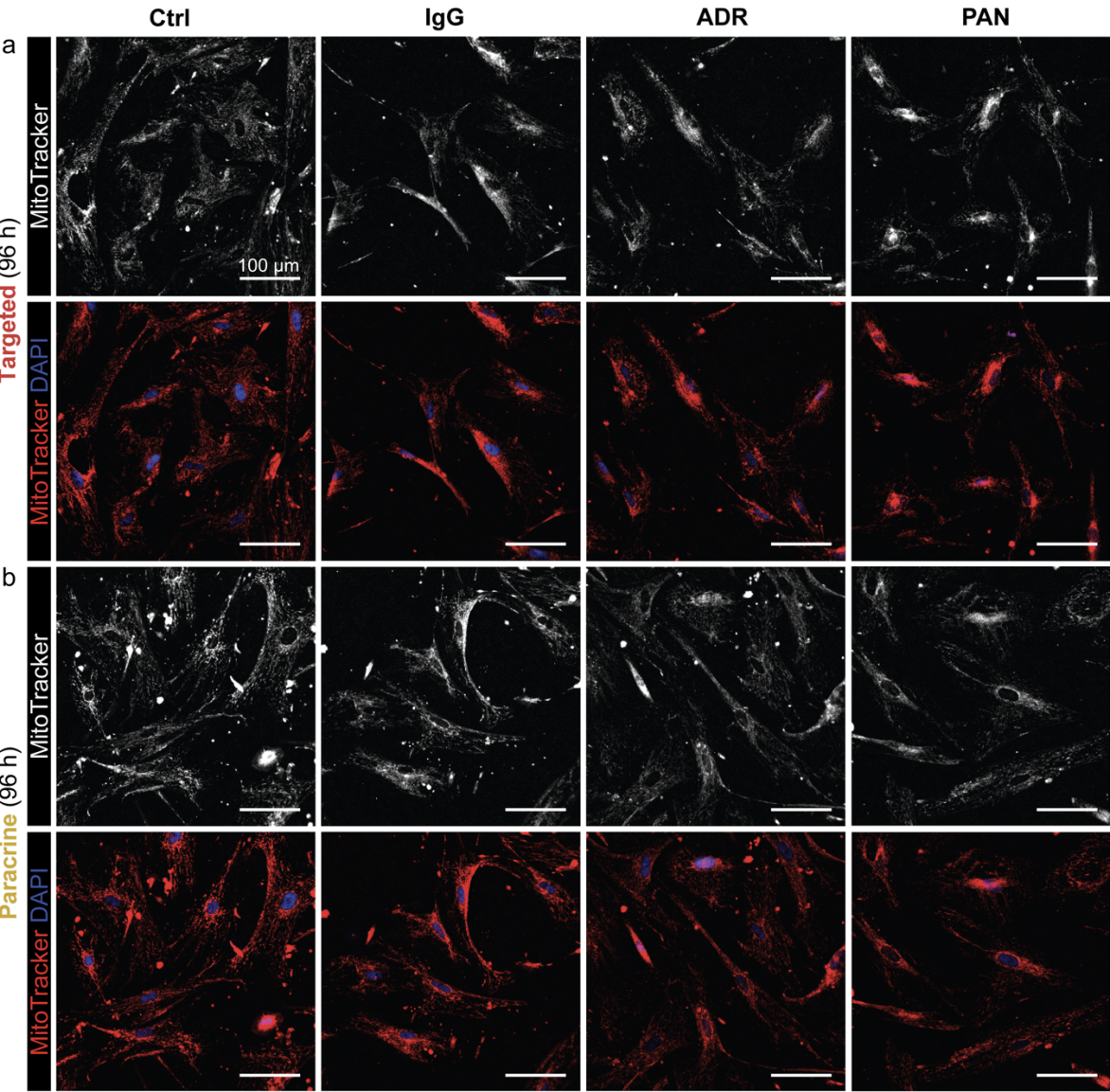


Figure 3.2. Mitochondrial redistribution and fragmentation following injury. **(a, b)** Representative images of MitoTracker staining of targeted (a) and paracrine (b) podocytes. The top panels show single-channel images of MitoTracker in greyscale; the bottom panels show merged channel images of MitoTracker and DAPI with pseudo colors (red: MitoTracker, blue: DAPI). **(c, d)** MitoTracker fluorescence decreased in all forms of the targeted (c) and paracrine (d) injury. Data are expressed as mean $\pm$ SEM (n = 4); each point is one culture replicate.

To assess injury, we assessed mitochondrial changes that are impacted by podocyte injury²⁶ at t = 96 h by MitoTracker staining²⁷. Mitochondria in healthy control podocytes exhibited a filamentous network of elongated mitochondria dispersed throughout the entire cytoplasm (**Figure 3.2a**). In all three forms of targeted podocyte injury (IgG, ADR, or PAN) in the outer chamber, two major mitochondrial changes were detected. First, insult targeted cells displayed a fragmented network of mitochondria with redistribution to the perinuclear region. Second, the mitochondria were significantly less active compared to untreated control cells (**Figure 3.2c**).

Similarly the mitochondrial network of the paracrine injured podocytes in the inner chambers also exhibited fragmentation and redistribution, albeit lower than in the podocytes with targeted injury (**Figure 3.2b,d**). The IgG-injury driven paracrine signaling caused perinuclearly mitochondrial clustering in the paracrine podocytes but did not change mitochondrial activity; on the other hand, ADR and PAN caused highly fragmented mitochondrial networks and significantly decreased mitochondrial activity in the paracrine podocytes. Together, these data indicate that targeted podocyte injury in the outer chamber causes a paracrine effect on the mitochondria of the podocytes in inner chamber of the co-culture device that were not targeted by IgG, ADR, or PAN injury.

3.2.3 Targeted and paracrine podocyte injury causes cytoskeleton remodeling

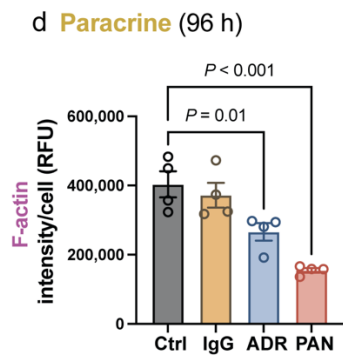
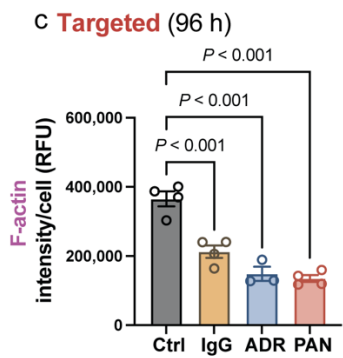
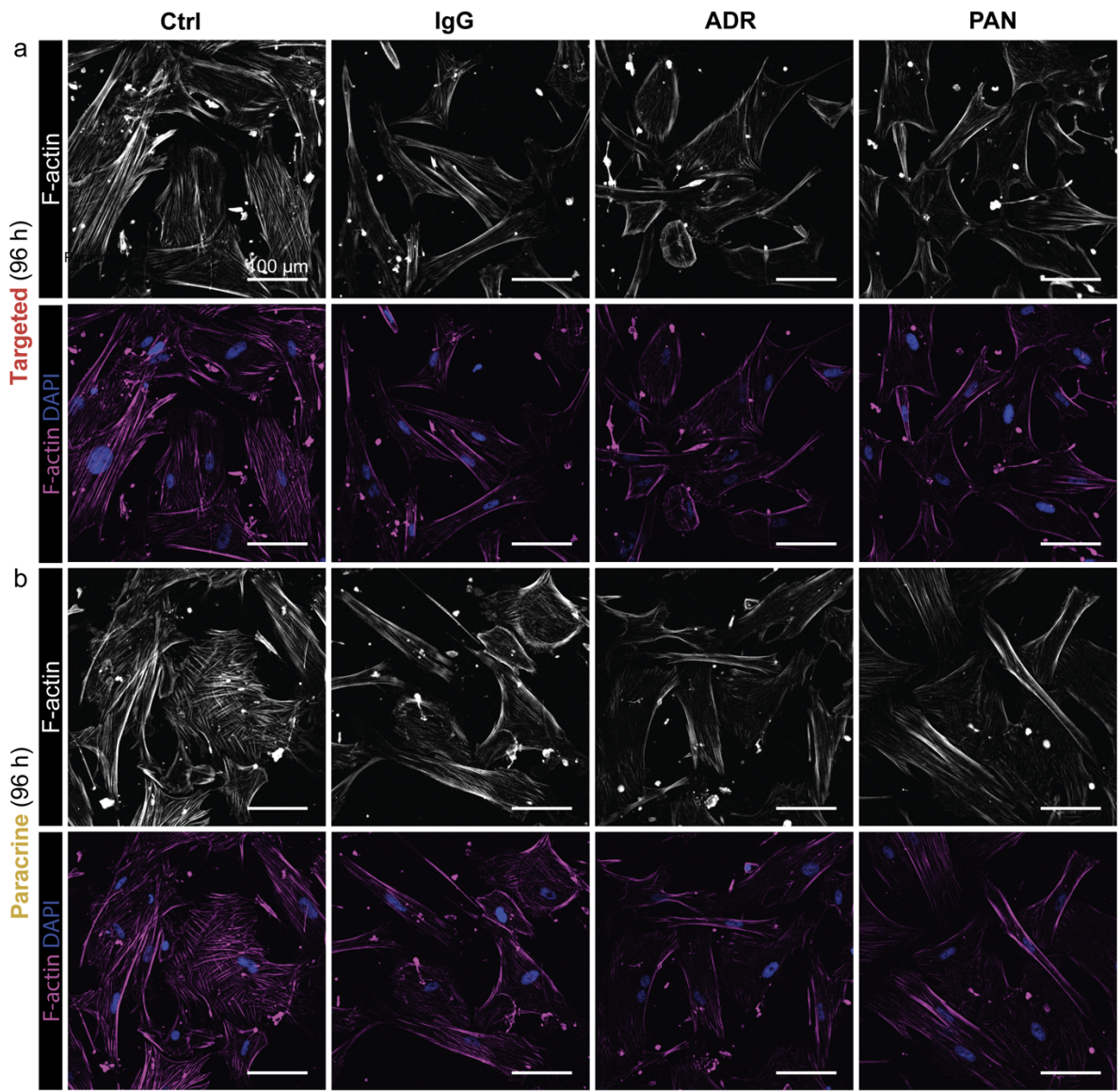


Figure 3.3. Targeted and paracrine injured podocytes demonstrate actin cytoskeletal remodeling. **(a, b)** Representative images of F-actin staining are shown for targeted (a) and paracrine (b) injured podocytes. The top panels show single-channel images of F-actin in greyscale; the bottom panels show merged channel images of F-actin and DAPI with pseudo colors (cyan: F-actin, blue: DAPI). **(c, d)** F-actin fluorescence signals per cell decreased in targeted (c) and paracrine (d) injured podocytes. Data are expressed as mean $\pm$ SEM ($n = 4$); each point is one culture replicate.

Next, we investigated cytoskeletal remodeling of injured podocytes at 96 h by staining the actin cytoskeleton for F-actin. Healthy control podocytes exhibited long, thick actin bundles (i.e. stress fibers) extending in parallel throughout the cell body and with both ends connected to focal adhesions (**Figure 3.3a** and **Supplementary Figure S3.1**). The podocytes with targeted injury had thinner and fewer actin fibers in the central cell body with cortical actin predominant along the cell border. This was further supported by measuring F-actin staining intensity per cell, which was significantly decreased following targeted injury by IgG, ADR, or PAN (**Figure 3.3c**). The paracrine injured podocytes demonstrated a less pronounced, yet similar phenotype, with a loss of central actin fibers in the cell body compared to control podocytes (**Figure 3.3b, d**). Again, the paracrine effects of IgG were not as severe as that of ADR and PAN, showing mainly a redistribution of actin fibers near the cell periphery, but no significant decrease in the overall F-actin intensity per cell (**Figure 3.3c**).

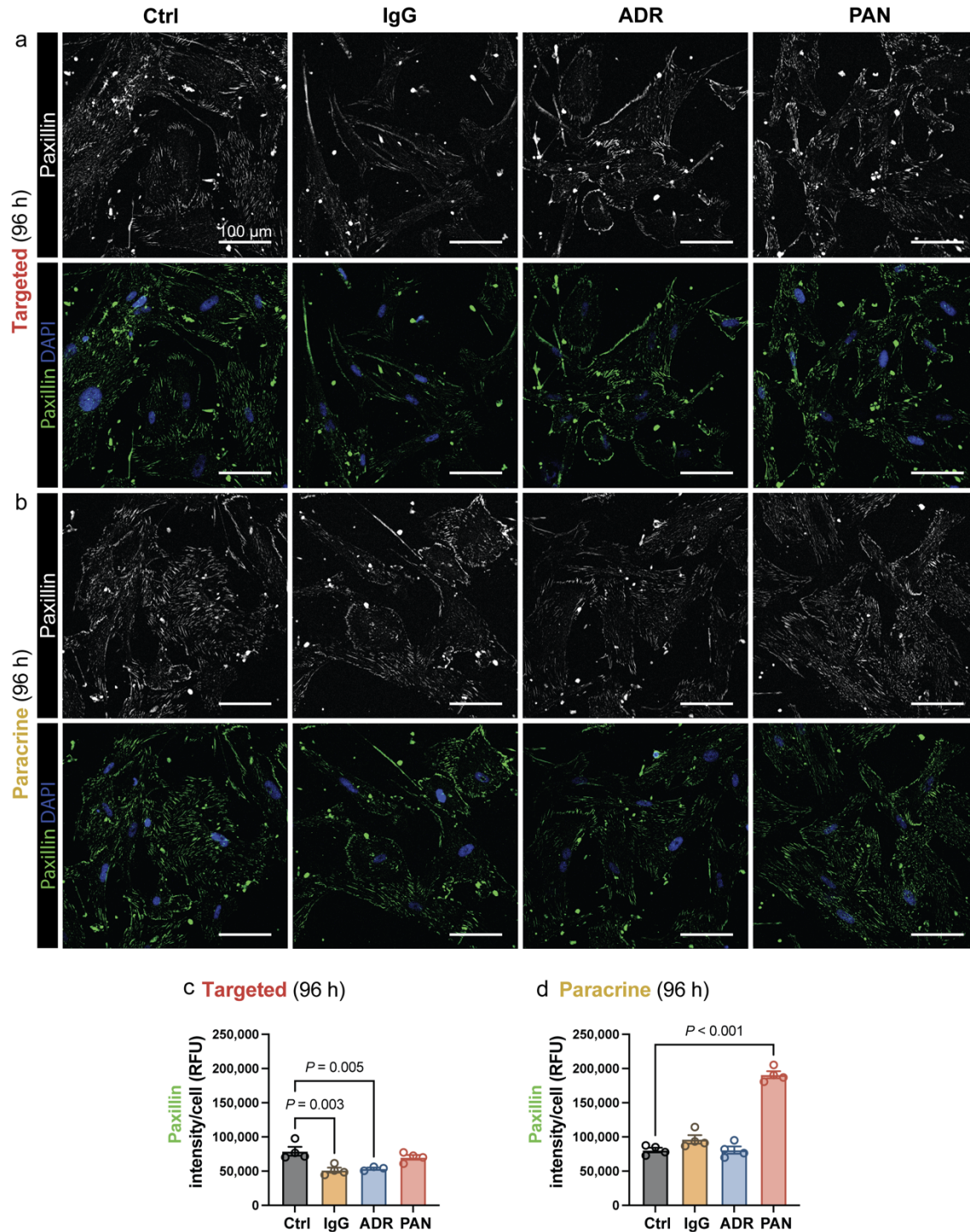


Figure 3.4. The size and number of focal adhesions are reduced in injured podocytes. **(a, b)** Representative greyscale (upper panels) and merged channel images of Paxillin and DAPI with pseudo colors (green: Paxillin, blue: DAPI, lower panels) images of Paxillin staining of targeted (a) and paracrine (b) injured podocytes. Paxillin fluorescence signals per cell was lower in IgG- and ADR-induced targeted injury (c) targeted and was higher in PAN-paracrine injury (d). Data are expressed as mean $\pm$ SEM (n = 4); each point is one culture replicate.

Finally, we investigated the morphology and distribution of focal adhesions using Paxillin staining. Healthy control podocytes had elongated mature focal adhesions near the cell periphery and throughout the cell body that were connected to actin stress fibers (**Figure 3.4a** and **Supplementary Figure S3.1**). Upon targeted injury with IgG and ADR, the size and number of focal adhesions decreased, and most focal adhesions were localized near the cell edges and absent from the central area of the cell body (**Figure 3.4a**). Targeted injury with PAN maintained strong focal adhesions along the cell edges and had many focal adhesions within the cell bodies, although not necessarily linked to actin stress fibers (**Supplementary Figure S3.1**). This was confirmed by quantifying the intensity of Paxillin staining (**Figure 3.4c**).

The paracrine injured podocytes by IgG and ADR showed similar changes with fewer focal adhesions in the main cell body, and most localized to the cell borders (**Figure 3.4b**). Yet, this was not accompanied by changes in Paxillin intensity (**Figure 3.4c**). Again, podocytes under paracrine effects of PAN injury maintained strong focal adhesions along the cell edges and within the cell bodies but were not linked to actin stress fibers (**Figure 3.4** and **Supplementary Figure S3.1**). This was further confirmed by the quantification of the Paxillin intensity, which increased following PAN injury (**Figure 3.4d**). Together these results show that both targeted and paracrine injury results in multiple changes to the podocyte cytoskeleton.

3.2.4 Global transcriptomic changes of podocytes from bulk RNA-sequencing

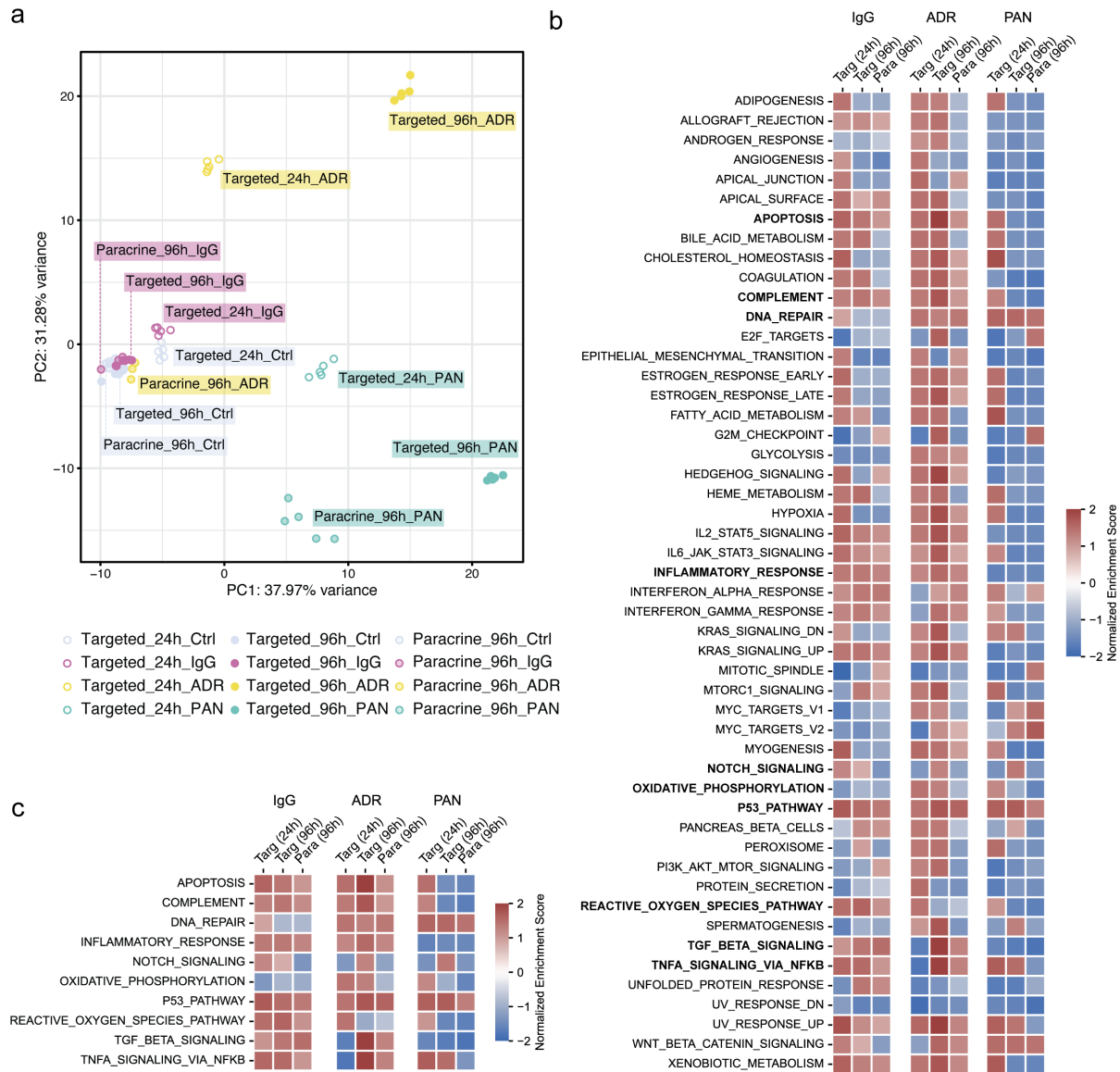


Figure 3.5. Global transcriptomic changes based on bulk RNA-sequencing data post targeted and paracrine podocyte injury. **(a)** Principal Component Analysis reveals the clustering and separation of targeted and paracrine podocytes in response to IgG (pink), ADR (yellow), PAN (green), or control (blue) treatments. **(b)** A heatmap of all 50 human hallmark gene sets from Gene Set Enrichment Analysis for all three forms of targeted injury (Targ) at 24 h and 96 h and paracrine injury (Para) at 96 h. Changes in the Normalized Enrichment Scores (NES) were determined in comparison to their corresponding control samples. **(c)** A heatmap of 10 curated hallmark gene sets from (b) that are critical to podocyte functions.

To obtain an unbiased assessment of the changes caused by the three different experimental models of podocyte injury and the targeted and paracrine consequences, we performed bulk mRNA sequencing of podocytes at t=24 and t=96 h of injury (**Figure 3.1f**). Principal component analysis (PCA) showed that samples from the same injury conditions clustered together with clear separation between the samples from each of the three different forms of injury (IgG, ADR, PAN) and between the two time points (24 h or 96 h) with 37.97% (PC1) and 31.28% (PC2) variances, respectively (**Figure 3.5a**). Moreover, healthy control podocytes for targeted or paracrine cells at the different time points (“Targeted/Paracrine_24h/96h_Ctrl”) clustered together. This indicates each of the three forms of injury generated different podocyte injury response profiles.

Yet, the most revealing was the temporal resolution. The different models of targeted injured podocytes at 24 h (“Targeted_24h_IgG”, “Targeted_24h_ADR”, “Targeted_24h_PAN”) were separated from each other and from the healthy control podocytes. At 96 h, the targeted ADR and PAN-injured podocytes (“Targeted_96h_ADR” and “Targeted_96h_PAN”) were still separated, but the IgG-injured cells (“Targeted_96h_IgG”) were inseparable from the controls. With respect to the paracrine injured podocytes, only the cells receiving paracrine signals from the PAN injured podocytes (“Paracrine_96h_PAN”) were separated from the others, while the cells under the paracrine effects of IgG and ADR clustered with the control cells.

Figure 3.5b shows gene set enrichment analysis (GSEA) using the human 50 hallmark gene set collection and **Supplementary Figure S3.2** lists the top 15 positively or negatively enriched gene sets for each condition at each time point. In IgG, ADR, or PAN targeted injured podocytes, the immediately downregulated pathways at 24 h were mostly cell proliferation related, such as E2F targets, cell cycle G2/M checkpoint, mitotic spindle assembly, and MYC

targets variant 1 and 2. However, comparing 24 h and 96 h timepoints provided injury-specific insights.

In targeted IgG injury the strongest response was at 24 h, with 34/50 upregulated and 16/50 downregulated gene sets. Of these 22 of the 34 upregulated and 12 of the 16 downregulated pathways remained enriched at 96 h. Moreover, 16 of the 22 upregulated pathways were also observed in the paracrine injured podocytes at 96 h, such as apoptosis, complement, inflammatory response, interferon alpha response, p53 pathway, reactive oxygen species (ROS) pathway, TGF β signaling, and TNF α signaling via NF κ B.

Similarly, ADR-induced injury resulted in a broad gene set enrichment response at 24 h (35/50 upregulated gene sets), but the strongest response was at 96 h (41/50 upregulated gene sets) and 28/50 were upregulated in the paracrine injured podocytes at 96 h. Among the top upregulated pathways shared between targeted and paracrine injured podocytes were the p53 pathway, DNA repair, TNF α signaling via NF κ B, KRAS signaling, IL2 STAT5 signaling, TGF β signaling, and interferon alpha response. Moreover, although many upregulated pathways were shared with IgG-induced injury, several pathways were unique to ADR-induced injury such as cholesterol homeostasis, coagulation, and DNA repair.

Finally, in contrast to IgG and ADR, PAN-induced targeted injury resulted in mostly downregulated gene sets (25/50 and 39/50 at 24 h and 96 h respectively), while 41/50 gene sets were downregulated in paracrine podocytes at 96 h. Yet, a few common upregulated gene sets were also seen in PAN-induced targeted and paracrine injuries, including DNA repair, p53 pathway, and Wnt/ β -catenin signaling.

To further highlight the common and unique hallmark pathways enriched in IgG, ADR, and PAN-induced podocyte injuries, we curated a list of 10 gene sets reported in individual

studies to be involved in podocyte injury^{1, 2, 28} and that were enriched in more than one of the targeted injuries used in this study (**Figure 3.5c**). The results show that all three targeted podocyte injury models increased pathways for apoptosis, complement, DNA repair, p53 pathway, TNF α signaling, and ROS. The upregulation of the ROS pathways was further supported by the MitoTracker staining results shown in **Figure 3.2**. In contrast, there were several examples of differences between the three injuries: TGF β signaling, and the inflammatory response were increased in IgG- and ADR-, but not in PAN-induced injury; oxidative phosphorylation increased in ADR- and PAN-, but not IgG-induced injury. It is also noteworthy that the targeted and paracrine injured podocytes showed similar enrichments at 96 h for each mode of injury.

3.2.5 Differentially expressed genes (DEG) in targeted and paracrine injured podocytes

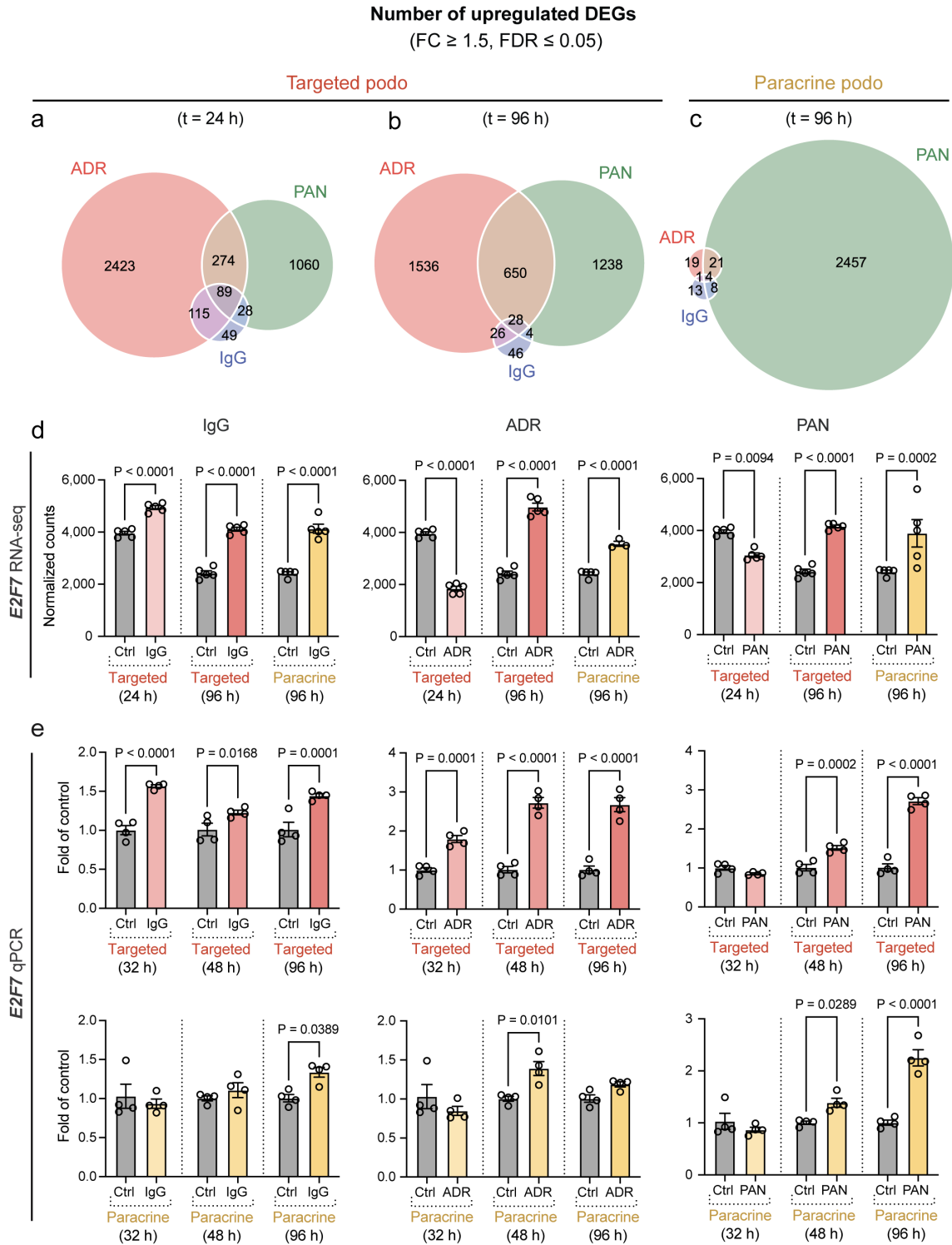


Figure 3.6. Differentially expressed genes (DEGs) in targeted and paracrine injured podocytes. **(a-c)** Venn diagrams showing the number of upregulated DEGs in targeted injury at 24h (a) and 96h (b) and in paracrine podocytes at 96h (c) after ADR (pink), IgG (blue) and PAN (green) treatment versus controls, and the overlap between the different forms of injury. The upregulated DEGs are defined as a fold change (FC) ≥ 1.5 and a false discovery rate (FDR) ≤ 0.05 . **(d, e)** Bulk RNAseq gene expression (d) of *E2F7* in targeted podocytes (t = 24 h and 96 h) and paracrine podocytes (t = 96 h) after IgG, ADR, or PAN treatments that was validated by RT-PCR at 32 h, 48 h, and 96 h (e).

To compare individual genes changed in podocytes targeted injured by IgG, ADR, and PAN as well as impacted by paracrine signaling we performed a DEG analysis using DESeq2 on the mRNA-seq data using a fold change (FC) ≥ 1.5 , and a false discovery rate (FDR) ≤ 0.05 as cutoffs (**Figure 3.6a**). In targeted injured human podocytes at 24 h, 49 were changed by IgG, 2423 by ADR, and 1060 by PAN, while 46, 1536, and 1238 were changed at 96 h, respectively. Moreover, between the three forms of targeted podocyte injury 89 were shared at 24 h and 28 at 96 h. In the paracrine injured paracrine situation at 96 h: 13, 19, and 2457 DEGs were changed by either IgG, ADR, or PAN, respectively (**Figure 3.6a**). In this set only 4 upregulated DEGs were shared between all three different injury models. The normalized gene counts, p-values, FDR, and FC of the 89, 28, and 4 overlapped DEGs in targeted and paracrine injured podocytes are listed in **Supplementary Table S3.1**. By reducing the FC threshold to ≥ 1 we identified additional upregulated and overlapping DEGs (i.e. 200, 86, and 11) (**Supplementary Figure S3.3, Supplementary Table S3.2**). Of those we focused on the 11 DEGs upregulated in paracrine injured podocytes and validated the 5 genes with the highest gene counts and/or fold changes (i.e., *DUSP4*, *E2F7*, *FBXO32*, *PDK4*, and *SIPRI*) by qRT-PCR. Moreover, we included additional injury time points (t = 32 h and 48 h) to follow their changes in a more detailed fashion (**Figure 3.6b,c** and **Supplemental Figure S3.4-S3.7**).

3.2.6 Paracrine injury is accompanied by changes to ligand-receptor pairs

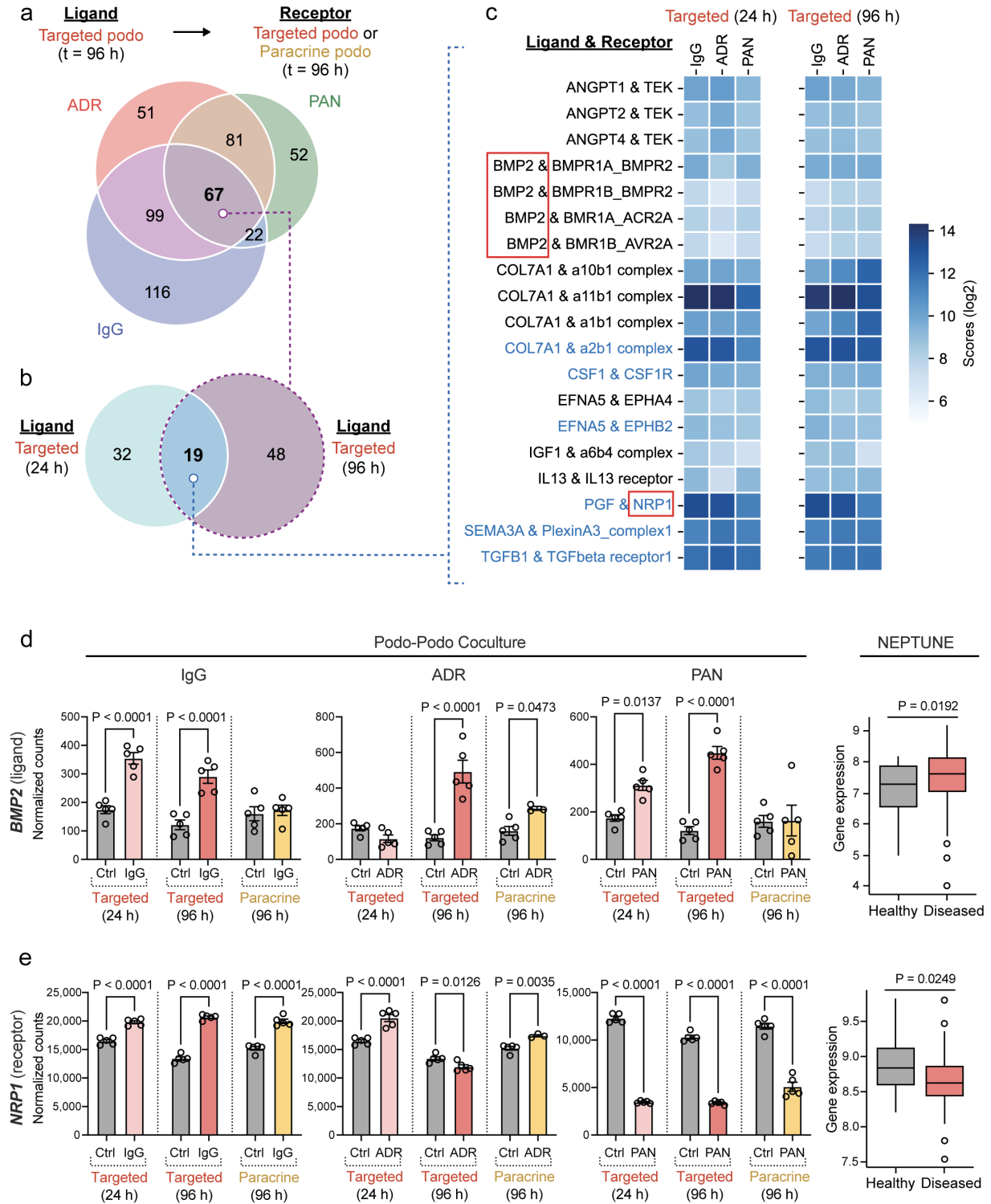


Figure 3.7. Ligand-receptor analysis in targeted-to-paracrine podocyte signaling. **(a)** A Venn diagram summarizing the number of enriched ligand-receptor pairs in IgG, ADR, and PAN-induced injuries, defined as the ligand gene in the targeted podocytes at 96 h or the receptor gene in the paracrine podocytes at 96 h is significantly upregulated compared to their healthy controls. **(b)** A Venn diagram showing the number of enriched ligand-receptor pairs based on ligands at $t = 24$ h and ligands at $t = 96$ h. **(c)** A heatmap showing a curated list of ligand (left)-receptor (right) pairs that overlap among the three forms of injury and between $t = 24$ h and 96 h. *BMP2* and *NRP1* are marked by the red box. **(d, e)** Graphs showing the gene expression of the candidate ligand *BMP2* (d) and the receptor *NRP1* (e) measured in RNA-seq (mean $\pm$ SEM, $n = 3-5$). The graphs on the right show the expression levels for *BMP2* and *NRP1* from the Neptune dataset. p-values were obtained in NEPTUNE from pairwise t-tests.

To address the paracrine crosstalk between injured and healthy podocytes, we next performed a ligand-receptor analysis using the bulk RNA-seq data. We compared the targeted and paracrine injured podocytes at the early (24 hours) and the late timepoint (96 hours) for all three injury models. A candidate ligand-receptor pair was defined by a ligand gene significantly upregulated ($FC \geq 1$, $p\text{-value} \geq 0.05$) in the podocytes with targeted injury and its receptor significantly upregulated in the paracrine injured podocytes. **Figure 3.7a** summarizes the number of unique and overlapping ligand-receptor pairs in all three injury forms at 96 h, where we identified 116, 51, and 52 unique enriched ligand-receptor pairs for IgG, ADR, and PAN, respectively. Amongst all three injury models, there were 67 overlapping enriched ligand-receptor pairs (**Supplementary Table S3.3**). To identify those pathways that were persistent throughout the 72 h co-culture period, we next compared the ligands expressed in the podocytes with targeted injury at $t = 24$ h with receptor genes expressed in the paracrine injured cells at $t = 96$ h. This identified 51 overlapping enriched ligand-receptor pairs among IgG, ADR, and PAN-induced injuries (**Supplementary Figure S3.8** and **Supplementary Table S3.4**). Finally, by comparing the two lists of ligand-receptor pairs we identified 19 that were consistently upregulated (**Figure 3.7b** and **Supplementary Table S3.5**). Expression of these were visualized via heatmap using log2 transformed expression scores calculated based on the normalized counts

of the ligand-receptor gene at 24h and 96 h (**Figure 3.7c**). The signaling molecules and their ligand-receptor genes are summarized in **Supplementary Table S3.6**.

3.2.7 Overlap of podocyte paracrine signaling genes with human NEPTUNE data

To determine if the results from the ligand-receptor analysis obtained from the cultured cell models were also changed in human disease, we cross-referenced our results with the glomerular RNAseq from the NEPTUNE database ²⁵ focusing on patients with histological evidence of glomerular epithelial cell injury (i.e., podocytes or parietal epithelial cells). The genes for the 19 ligand-receptor pairs identified in **Figure 3.7c** were extracted from the NEPTUNE database and plotted (**Supplementary Figures S3.9-S3.10**). From those, we selected the ligand *Bone Morphogenetic Protein 2 (BMP2)*, a member of the TGF- β superfamily, and the *Neuropilin1 (NRPI)* receptor (**Figure 3.7d,e**).

In all three forms of podocyte injury, *BMP2* was upregulated in the targeted podocytes especially by 96 h. *BMP2* was also upregulated in the paracrine injured podocytes by ADR, but not by IgG and PAN (**Figure 3.7d, middle panel**). *NRPI* was downregulated in patients with glomerular epithelial injury in NEPTUNE (**Figure 3.7e, right panel**). In the podocyte-podocyte co-culture, *NRPI* had differential expression dynamics for each of the three injuries (**Figure 3.7e, left panel**). The IgG-induced injury led to upregulated *NRPI* in targeted podocytes at 24 h and 96 h, as well as in paracrine podocytes at 96 h. The ADR-induced injury led to upregulation of *NRPI* at 24 h and then downregulation at 96 h in targeted podocytes; *NRPI* was also upregulated in paracrine podocytes. The PAN-induced injury led to the opposite response as the IgG and *NRPI* was downregulated in targeted podocytes at 24 h and 96 h, and at 96 h in the paracrine podocytes.

3.3 Discussion

Podocytes are vulnerable to many forms of injury resulting in proteinuria and oftentimes glomerulosclerosis²⁹. Although injury might only damage a subset of podocytes in any given glomerulus, the response of the healthy neighboring podocytes oftentimes determines the course of the disease¹³. In this respect, paracrine effects of injured podocytes on healthy podocytes (and the neighboring parietal epithelial cells, endothelial cells, mesangial cells) are likely critical for disease progression. Unfortunately, paracrine signaling has not been well defined in large part due to a lack of suitable experimental models. This study is the first to use a microfluidic co-culture model to investigate how injured podocytes communicate with healthy podocytes via paracrine signaling in a controlled microenvironment. This model adds a new layer of complexity to classical podocyte cell culture approaches that have successfully elucidated mechanisms that are challenging to study *in vivo* experimentally and clinically.

We developed a scalable open microfluidic two-chamber co-culture device to study podocyte-podocyte communication. In separate experiments, podocytes were targeted in one chamber with each of three forms of experimental podocyte injury (IgG, ADR, PAN). Subsequently, the system was switched to co-culture by allowing the injured podocytes to communicate with healthy podocytes in the second chamber. It is important to note that following the initial form of injury, the supernatant was removed, and the cells were thoroughly washed before initiating the co-culture conditions. As such, the paracrine injured podocytes were prevented from ever coming into contact with either IgG, ADR, or PAN and the only injury trigger was derived only from the podocytes with targeted injuries. This model provides multiple advantages over traditional cell culture models to study paracrine signaling: (1) Compared to macroscale culture systems such as petri dishes or multi-well plates, microfluidic devices operate

with less volume. This allows to minimize process-critical resources such as primary human samples or growth factors. (2) Compared to the use of conditioned media, i.e., the collection of media from one culture plate and the manual transfer to another, the microfluidic co-culture system allows for real-time two-way communication between two types of cells for the entire exposure time. (3) Compared to closed microfluidic systems, our open microfluidic platform allows direct access to the cell chambers using pipettes and as such is more translatable to standard biological laboratories practices. (4) Compared to transwell cell culture inserts, our microfluidic co-culture device has both chambers on the same focal plane, thus allowing easy daily monitoring and post-fixation imaging.

The current study, for the first time to our knowledge, defines the transcriptional responses of cultured primary human podocytes to three different experimental forms of targeted podocyte injuries, which are commonly used both in cell culture models and *in vivo*, ADR, PAN, and a cytotoxic anti-podocyte antibody (IgG). Global transcriptomic analysis revealed three major themes. First, there are common pathways such as the p53 signaling that were increased in all three forms of injury and that were sustained over the course of the study (i.e. 96 h). Interestingly, the transcription factor *E2F7* that contributes to p53-dependent cell cycle arrest in response to DNA damage,³⁰ expression was significantly upregulated immediately post targeted IgG injury and remained elevated. Following ADR and PAN injuries, *E2F7* levels were initially downregulated but then increased progressively with the highest level at t = 96 h. Paracrine injured podocytes followed a similar trend.

Second, certain pathways are unique to specific forms of injury. For example, the inflammatory response was increased by IgG and ADR but was decreased in the PAN injury model. Third, there are temporal differences in the transcriptomic responses to the three injury

models. For example, ROS signaling was upregulated at 24 h in all three forms of injury but was down-regulated by 96 h in the ADR and PAN-treated podocytes. This was expanded upon by a more granular analysis of the DEGs for each of the three forms of injury. This analysis showed (1) each injury has its own unique set of DEGs, (2) there were overlaps between any two forms of injuries and (3) that 2% (n=129) DEGs overlap between all three forms at 24 h and 1% (n=61) at 96 h. These distinct and shared transcriptomic responses to the three different forms of experimental podocyte injury open many possibilities for future investigations on individual components or signaling pathways identified here. It also highlights the importance of carefully considering which injury model to choose to test a specific hypothesis and underscores the importance of examining multiple injury models.

One of the most intriguing findings of the mRNA-seq data analysis was that the paracrine injury of neighboring podocytes was similar to the targeted primary injury and was specific for the chosen podocyte injury model. Pathways enriched in the paracrine injured podocytes were very similar to their targeted counterparts for each form of injury. These results suggest that following targeted injury there are common pathways that are responsible for both the targeted and paracrine injury response. But the differences between the different injury models also indicate that the paracrine signaling response is not universal, and that the paracrine mechanisms are dependent on the defects occurring in the initially injured podocytes.

Finally, ligand-receptor analysis of the ligands secreted by the targeted podocytes and receptors expressed by the paracrine injured counterparts allowed us to identify candidate paracrine mediators. We focused on the top 23 ligand-receptor pairs that overlapped among the three forms of injury that were consistently upregulated at 24 h and 96 h. These included several pathways that have been implicated in podocyte biology previously. *BMP2*, a member of the

TGF- β superfamily, increases ROS production in differentiated podocytes via the overexpression of inhibitor of DNA binding 1 (*ID1*)³¹. NRP1, which forms a receptor complex with Plexin A3 (PLXNA3), is also a coreceptor for VEGFA which competes with semaphorin 3A (SEMA3A) for NRP1 binding³². In fact, SEMA3A signaling has been shown to be secreted by podocytes and can induce F-actin cytoskeleton collapse in the podocytes if present in excess³³. Moreover, as these candidate ligand-receptor pairs are shared between our three models of experimental podocyte injury, they may constitute a common signaling network mediating paracrine induced injury regardless of the specifics of the injury.

We recognize the limitations of the current study, many of which are inherent to cell culture models. First, podocytes wrap around the capillaries *in vivo*, where the topography, pressure, and extracellular matrix components play vital roles in maintaining healthy podocyte function,³⁴ none of which are well represented in 2D culture *in vitro*.³⁵ Nevertheless, our study presents - in our opinion - a large step forward. Using a novel open microfluidic co-culture model allows efficient studying of paracrine signaling between healthy and injured podocytes *in vitro*. It emphasizes (1) that podocytes have unique and common transcriptomic responses to different forms of injury and (2) that the paracrine induced injury responses are similar to the targeted injury responses. Second, while we focused on probing the signaling between injured and healthy podocytes, the two-chamber co-culture device is directly amendable to the other glomerular cell types such as endothelial cells and PECs, that have been shown to contribute to repair and/or exacerbation of podocyte injuries³⁶.

Finally, these findings will directly impact future studies to determine the criticality of those pathways in animal models and ultimately human studies. Moreover, they also stress the importance of a personalized medicine approach in nephrology, enabled by the reduced culture

volumes of the microfluidic devices that allow working with limited numbers of cells from patients. Treating patients with glomerular diseases will not be a one-drug-fits-all approach, but will require careful molecular phenotyping of each glomerular disease to determine which drug(s) are most effective for an individual patient.

3.4 Methods

See Supplementary Methods for detailed description.

3.4.1 Open microfluidic co-culture model description

The dual chamber open microfluidic co-culture device comprises two polystyrene pieces that are milled using a computer numerical control mill (Datron Neo) and then solvent bonded together as we previously reported ^{14, 15}. A detailed description of the mass transport characterization experiment is provided in the Supplementary Methods.

3.4.2 Primary human podocyte isolation

A detailed description of podocyte isolation and characterization is provided in the Supplementary Methods.

3.4.3 Injuring podocytes in the microfluidic device

At $t = 0$ h, while the chambers were still separated, primary human podocytes in the outer chambers were injured with (i) a cytopathic anti-podocyte antibody (IgG; 10 mg/ml), ¹⁶⁻²² (ii) Adriamycin/doxorubicin (ADR; 2 ug/ml; Sigma-Aldrich D1515) ^{23, 24}, and (iii) puromycin aminonucleoside (PAN; 50 ug/ml; Sigma-Aldrich SBR00017) ⁹. A detailed description of the targeted and paracrine podocyte injury is provided in the Supplementary Methods.

3.4.4 Staining and fluorescence microscopy

A detailed description of Mitochondrial function assessment and focal adhesion and actin cytoskeleton measurements are provided in the Supplementary Methods.

3.4.5 Bulk mRNA-sequencing, qRT-PCR and bioinformatic methodologies

A detailed description of the RNA-seq data analysis is provided in the Supplementary Methods. The sequences data are deposited into the Gene Expression Omnibus (GEO) database (GSE255482) and are available online.

3.4.6 Interrogation of human kidney injury data

A detailed description of how the NEPTUNE tranSMART ²⁵ was utilized to align selected data from the co-culture studies with transcriptomic data of patients with glomerular disease is provided in the Supplementary Methods.

3.4.7 Statistics

See Supplementary Methods.

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Chapter 4 | A Flexible and Responsive Approach to Assess the Effects of Wildfire Smoke

Exposure with homeRNA

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AJH, EB and ABT contributed to the conception and design of the study. KNA, AJH, DSK, EB and ABT designed and executed the human subjects study. AJH, DSK, FYL, EB, and ABT developed homeRNA and the protocols implemented in home sampling in this chapter. AJH, DSK, KNA, JS, GWH and MGT conducted/contributed to the human subjects research and collection of samples. YZ and LGB performed the RNA extractions on samples and collected the RIN and yield data. AJH, YZ, LGB, JWM, TKB, JS, EB, DC, and ABT analyzed/interpreted the data. AJH, YZ, LGB, JS, IHJ and ABT prepared the figures and wrote the chapter.

Abstract: We developed a flexible decentralized study design to investigate the blood transcriptomic responses to disasters such as wildfire smoke exposure, which require a responsive approach to changing conditions. We use our previously developed homeRNA kit, which allows for at-home blood collection and RNA stabilization. Fifty-eight participants across 10 U.S. states collected their own samples throughout June 2021 to April 2022. To determine sampling time, we monitored average daily PM_{2.5} levels from the nearest EPA sensor. We received 635 homeRNA-stabilized blood samples with concurrent surveys on exposure-related symptoms and behavior before, during, and after wildfire smoke exposure to assess the immediate and longitudinal effects on blood transcriptomics. We demonstrate that homeRNA is a suitable tool for investigating the regional effects of wildfire smoke exposure—enabling a targeted, flexible, and responsive longitudinal sampling approach.

4.1 Introduction

The frequency, size, and intensity of wildfire events have increased in the past decades, particularly across the western United States.¹⁻⁴ Since 2016, wildfire smoke has led to steady increases in daily and annual average concentrations of fine particulate matter (PM_{2.5}) in the Western US.⁵⁻⁷ Okanogan County in Washington state, for example, experienced one of its worst summers for wildfire smoke in 2021 as smoke from nearby wildfires blanketed the region, causing several days of “hazardous” air quality index (AQI) category (hazardous air quality is a PM_{2.5} ≥ 225.5 $\mu\text{g}/\text{m}^3$; for reference, good air quality is PM_{2.5} ≤ 9.0 $\mu\text{g}/\text{m}^3$). It was an unprecedented level of smoke exposure for an area that is already a historically wildfire smoke impacted region.

Wildfires not only have a severe impact on the environmental landscape but also are a large contributor to adverse human health outcomes.⁸⁻¹¹ Wildfire smoke and resulting PM_{2.5} exposures can lead to acute respiratory symptoms and worsen chronic health conditions, including chronic obstructive pulmonary disease (COPD)¹²⁻¹⁵, asthma^{16,17}, and cardiovascular disease¹⁸⁻²¹. In addition to investigating the effects of wildfire smoke on overall health, there has also been a push to understand the impacts of PM_{2.5} exposure from a mechanistic perspective, particularly on the respiratory tract and systemic inflammation.²²⁻²⁵ Understanding the biological mechanisms involved in wildfire smoke exposure could enable biomarker discovery, targeted therapy development, and development of risk assessment tools for smoke exposure prevention and response. Previous studies have monitored the inflammatory response in wildland firefighters in the Eastern US and Western Canada before and after responding to fires using whole blood samples and dried blood spots; these studies reported elevated levels of cytokines and chemokines, such as interleukin-6 (IL-6), interleukin-8 (IL-8), and C-reactive protein (CRP),

correlated with increased wildland fire PM_{2.5} exposure.^{18,22,25,26} These results implicate acute inflammatory activation in response to occupational smoke exposure, but there is still a need to investigate health effects in general populations who experience routine wildfire smoke exposure by the nature of where they live. Towards this goal, Aguilera et al. collected blood samples from 15 participants in the San Francisco Bay area before (AQI category of good) and during continuous 11-day average wildfire smoke exposure (AQI category of moderate) in 2020; this study found increased levels of various immune cells and their proteins, including interleukin-16 (IL-16) and granzymes via proteomic and immune cell phenotyping analysis. While this study was the first of its kind to immunophenotype the same group of participants before and during a wildfire, there is still a need to systematically profile blood transcriptome response to wildfire smoke exposure before, during, and after wildfire smoke exposure.

Typically, clinic-based blood draws are used to study longitudinal immune responses in blood transcriptomes. However, this study design for investigating the effects of wildfire smoke exposure is logistically challenging due to the unpredictable nature of wildfires and the low accessibility to healthcare and research centers in rural areas, where many fires occur. Running a clinic-based blood draw study on the general population experiencing wildfire smoke exposure would require either (1) relying on historical data to choose a location that has the infrastructure to support frequent clinic-based blood draws (usually in an urban or suburban environment) and a high prevalence of wildfire smoke (usually in rural areas), (2) setting up a multi-site clinic-based study to increase the chances that at least one site has a wildfire smoke event, or (3) relying on retrospective studies or capturing smoke exposure incidentally on a study investigating a different question where longitudinal blood samples are collected.^{27,28} These

factors make designing studies where blood is collected at multiple time points in response to wildfire smoke exposure both expensive and logistically challenging.

To address this challenge, we employed a remote and flexible study design using homeRNA: a self-sampling kit made of a commercially available blood collection device, Tasso SST, and a custom engineered RNA stabilization tube we previously developed.²⁹ The homeRNA kit allows study participants to self-collect and stabilize blood by themselves in their own homes for shipment back to a centralized lab for downstream analysis. Our primary objectives were to (1) evaluate the feasibility of a flexible study design in response to disaster and (2) assess the immediate and longitudinal effects of wildfire smoke exposure on blood transcriptomics.

By self-sampling at home, homeRNA allows for a study design that includes a wider reach of participants living in remote locations who are traditionally inaccessible by mobile phlebotomists or clinic-based blood draws. Study kits can be mailed to participants within a day of a disaster warning or occurrence (such as a wildfire event), thus reducing the need to predict where the disaster would occur ahead of the study (as would be required for a clinic-based blood draw study design). Furthermore, multiple kits can be sent in one shipment that can be used by participants over several time points upon receiving, allowing for repeated sampling across an event, enabling targeted regional response to disasters. Here, we implemented homeRNA into a wildfire smoke exposure study to collect blood from 58 participants across the Western US before, during, and after wildfire smoke exposure. With a subset of these samples, we also analyzed the blood RNA transcriptome from 12 participants located in Okanogan County, WA during a major wildfire in 2021 (AQI category of hazardous) with 15 other concurrently sampled participants located in the Western US (AQI categories range between good and unhealthy).

4.2 Results

4.2.1 homeRNA allows for investigating the effects of wildfire smoke exposure across the Western US

homeRNA makes use of a commercially available lancet-based blood sampling device, the Tasso device, which collects up to 0.5 mL of capillary blood from the upper arm. The RNA stabilizing effect is achieved by a custom-built stabilizer tube engineered by our group, which contains a commercially available stabilizer, *RNAlater*[™] and is designed to interface with the Tasso blood collection compartment (**Figure 4.1A**). Once blood is collected by the Tasso device, the user assembles the Tasso SST tube with the stabilizer tube and shakes vigorously, allowing the *RNAlater*[™] to mix thoroughly with the blood and therefore stabilize the blood RNA (**Figure 4.1A**). In our first pilot study, we demonstrated the feasibility of homeRNA for remote blood sampling and RNA stabilization, receiving blood samples with RNA yields and quality suitable for standard downstream analysis such as RNA sequencing.²⁹ We further showed the robustness of homeRNA in high ambient temperature settings (>30°C), where we were able to extract suitable RNA quality and yield from blood samples that experienced high external temperatures in the Western US and Doha, Qatar.³⁰

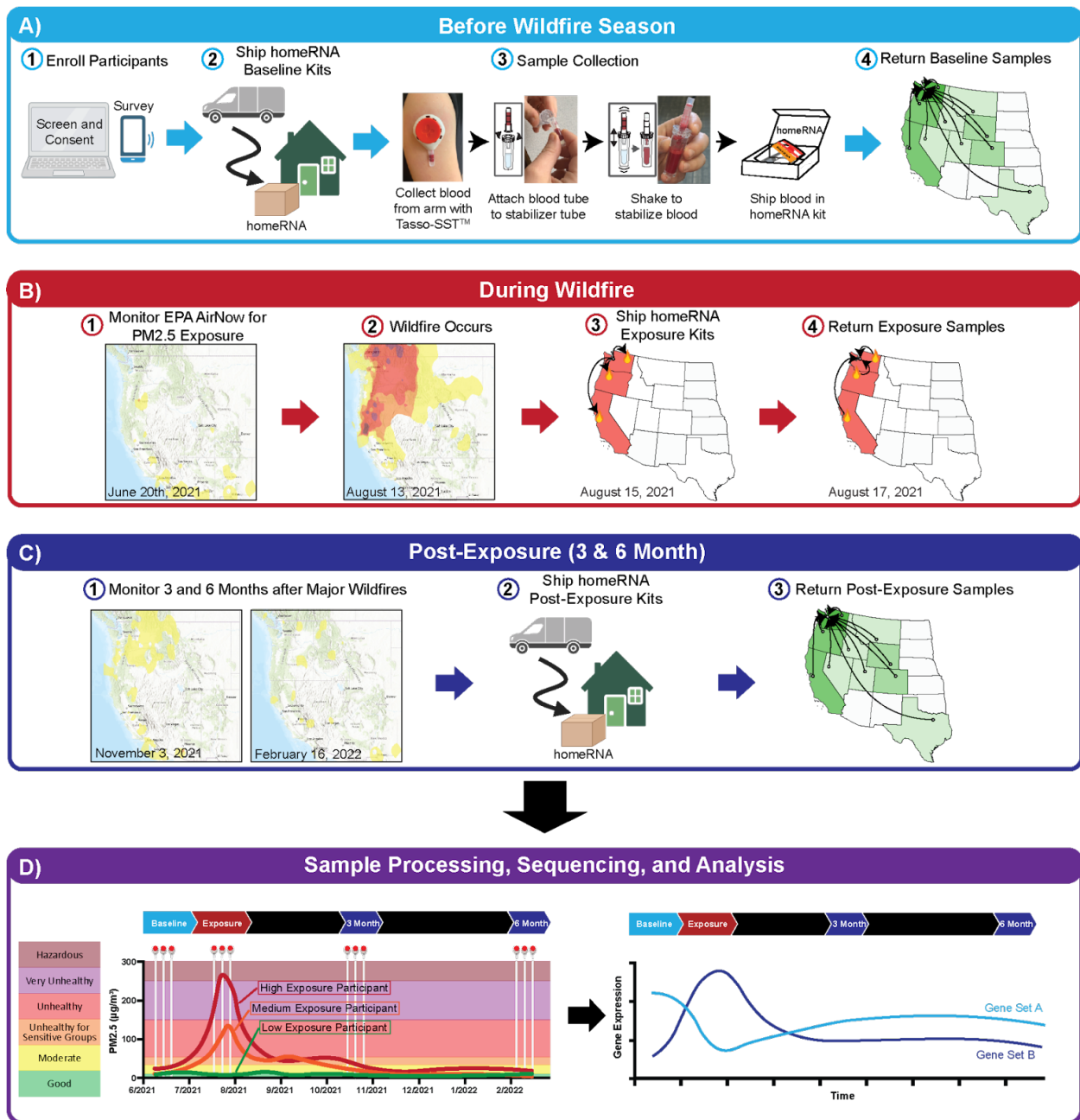


Figure 4.1. Study design for using homeRNA to investigate the effects of wildfire smoke exposure. **A)** Participants were enrolled across the Western United States and sent three homeRNA kits to collect baseline blood samples, which were then returned to the study team (Seattle, Washington). **B)** Throughout the 2021 wildfire season (June-September), wildfire occurrence and smoke exposure (PM_{2.5}) were monitored using the EPA AirNow site. When a wildfire occurred, three homeRNA kits were sent to participants experiencing wildfire smoke exposure to collect exposure samples. Eighteen participants were located in the Methow Valley in Washington, where three severe wildfires occurred. A subset of participants located outside of the Methow Valley were also sent three homeRNA kits and sampled at similar time to serve as low exposure controls. **C)** All participants were sent three homeRNA kits between October-early

November (~3 months after wildfire event in Okanogan County in Washington), then again in February-April (~6 months after the wildfire event in Okanogan County in Washington) to collect post-exposure samples. **D** i) Representative timeline of participant's PM_{2.5} exposure as collected from the nearest outdoor EPA sensor. White vertical lines indicate blood sampling timepoints. Participants were categorized as High Exposure, Medium Exposure, or Low Exposure (see Methods). ii) A subset of blood samples from participants in each exposure level were processed and sequenced using 3' mRNA sequencing and analyzed using a time-course gene-set analysis model.

In this work, we designed a longitudinal remote sampling study using homeRNA for blood sampling of participants across the Western United States in response to wildfires. This study design consists of three stages of sampling: 1) collecting baseline blood samples before wildfire season, 2) collecting exposure samples during wildfire season, and 3) collecting post-exposure samples three and six months after wildfire smoke exposure (**Figure 4.1**). In the first stage of sampling, after a participant was enrolled into the study, they were immediately sent a package containing three homeRNA kits to collect three samples typically three to four days apart (**Figure 4.1A**). These samples then serve as baseline samples prior to wildfire season. After each sample collection, the participant then returned their stabilized blood sample back to the study team location (Seattle, Washington).

In the second stage of sampling, the study team monitored the daily average PM_{2.5} level and wildfire occurrence in each participant's location using the EPA AirNow website. Once wildfire smoke occurrence and increased PM_{2.5} levels were detected, participants were immediately shipped a package containing three homeRNA kits, typically two to three days after a wildfire event (**Figure 4.1B**). Because participants were asked to use a set of three homeRNA kits 3 to 4 days apart, each sampling window lasted for about 1.5 weeks, thereby capturing multiple time points throughout a wildfire event (e.g., before, during, and after the wildfire-specific PM_{2.5} spike). These samples then serve as wildfire smoke exposure samples. Lastly, in

the third stage of sampling, post-exposure samples were collected from all participants to assess the potential long-term effects of wildfire smoke exposure (**Figure 4.1C**). In this case, participants were sent a first set of three homeRNA kits three months after the wildfire events and a second set of three homeRNA kits six months after the wildfire events. After completion of the study, each participant's relative PM_{2.5} exposure from wildfire smoke can be examined to then elucidate the changes in blood transcriptome via sequencing (**Figure 4.1D**).

In our specific study implementation, to reduce the risk of failing to capture wildfire smoke exposure, we took a dual approach of enrolling participants broadly throughout the Western US, while also utilizing a more targeted enrollment strategy in Okanogan County in Washington state, a region with historically impactful wildfire seasons and active community efforts related to wildfires. This recruitment geography not only reduces the risk of a wildfire not occurring in a single study location, but also makes it possible to collect samples from participants who are getting exposed to wildfire smoke and those who are not, allowing for a more comprehensive understanding of the effects of wildfire smoke.

In the present study, a total of 119 individuals were screened beginning in June 2021 (**Figure 4.2**); of these individuals screened, 10 were not eligible and 17 did not finish the screening process. Of the 92 eligible individuals, 63 participants were enrolled into the study for homeRNA sampling, with preference given to those located in Okanogan County, WA, as well as individuals who add to geographic, demographic, and exposure diversity. Of the 63 enrolled participants, 5 did not complete the study because no sample was returned. In the end, 58 participants across 10 Western US States completed the study and we received a total of 635 homeRNA-stabilized samples before, during, and after the wildfire season. Of the 58 sampled participants, the median age was 41 (range 20-76), 71% participants (n=41) reported female sex

at birth, 50% participants (n=29) were from Washington, and 31% participants (n=18) were from Okanogan County (**Figure 4.3B**).

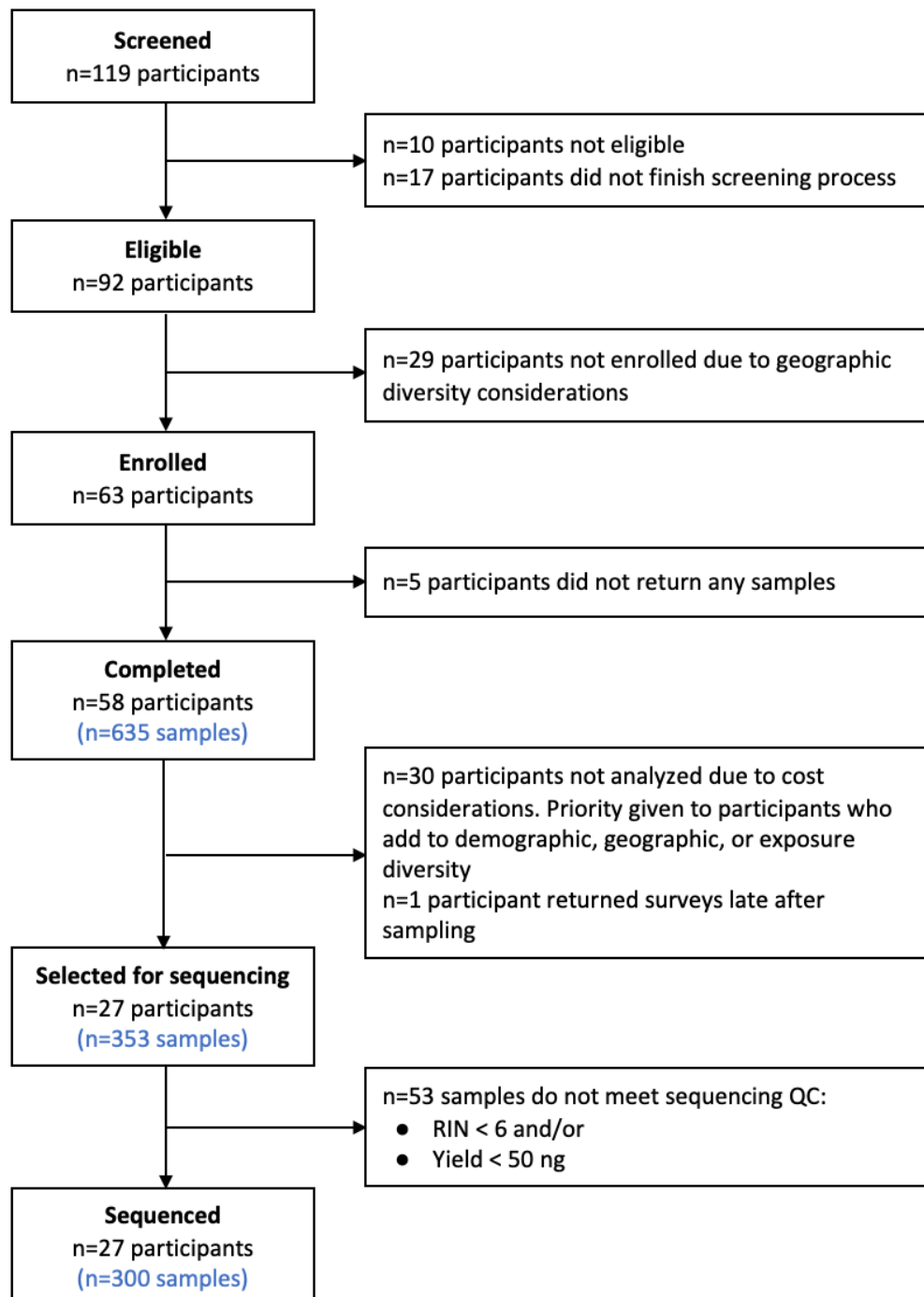


Figure 4.2. Study participant and sample flow chart. Participants were selected with a preference given to geographic diversity and those in highly wildfire-prone areas. RNA extracted from homeRNA collected blood samples was sequenced using 3'mRNA-sequencing.

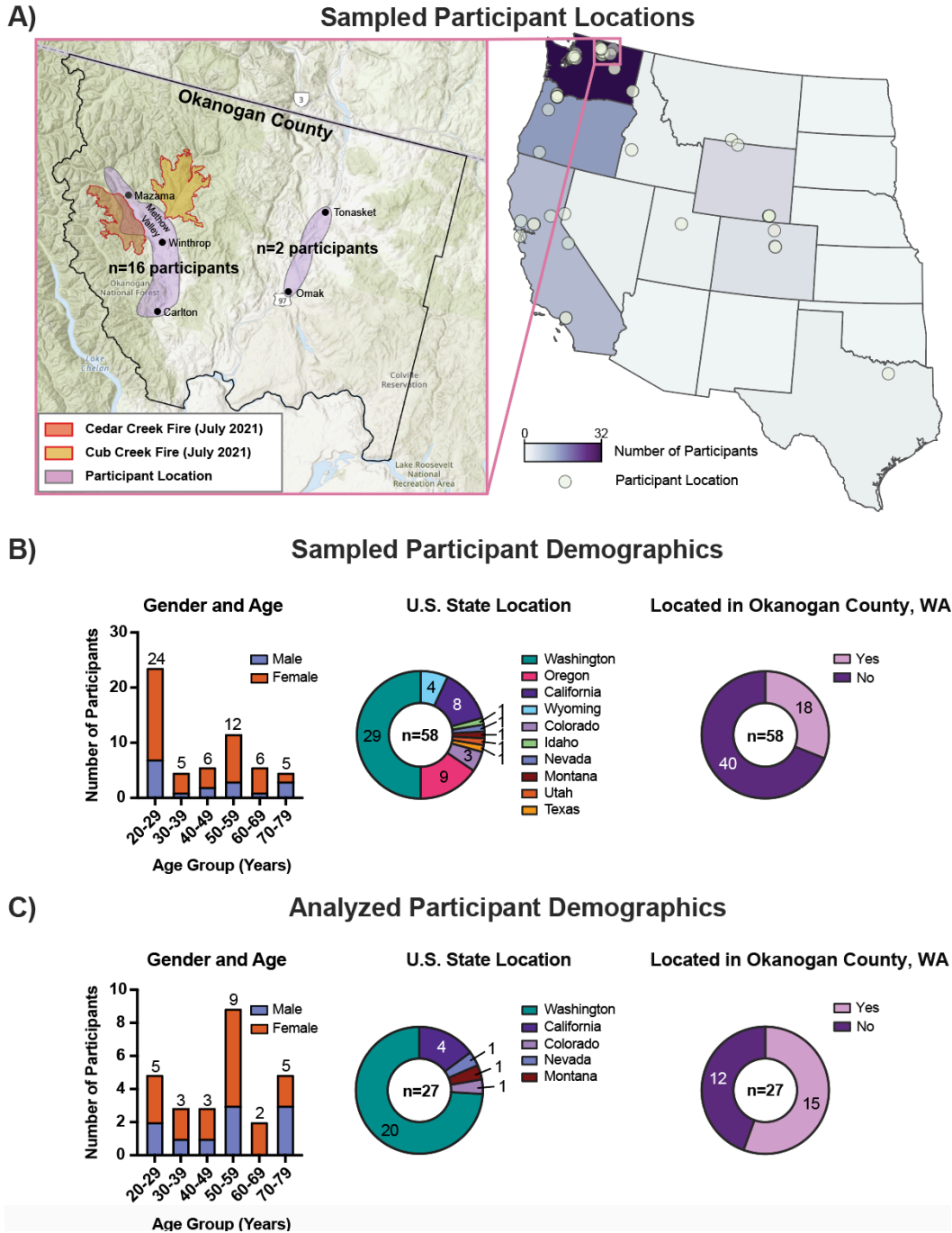


Figure 4.3. Location and demographics of study participants. A) Location of study participants in the Western United States. Each participant is represented by a circle and each state is colored based on the number of participants located within the state. Inset depicts Okanogan County in Washington state, where 18 total participants are located, with the burned area of two major wildfires, the Cedar Creek and Cub Creek fire, that started in July 2021. Inset map was generated with ArcGIS software, where each participant's reported zip code was used to generate coordinates (arcgis.com). Demographics of B) all sampled participants who completed the study (n=58 participants) and C) participants whose samples were analyzed with 3'mRNA-sequencing (n=27 participants).

Our study captured two major wildfires, both of which occurred in Okanogan County, WA in July 2021: the Cedar Creek Fire and the Cub Creek Fire (**Figure 4.3A**).³¹ Therefore, we will discuss the 58 sampled participants in the context of two groups: 18 participants located in Okanogan County who were exposed to wildfire smoke and 40 participants located in all other sampled counties who were not exposed to wildfire smoke (**Figure 4.3B**). Since the major wildfires took place in Okanogan County, all participants located within this county were shipped a set of three homeRNA kits to collect exposure samples. Participants located outside of Okanogan County were also sent a set of three homeRNA kits at similar times to collect low-exposure control samples. After the wildfire season, post-exposure samples were collected from all participants, who were sent a set of three homeRNA kits in October-early November 2021 (approximately 3 months after the major wildfires in Okanogan County), and a second set of three homeRNA kits in February-April 2022 (approximately 6 months after the major wildfires in Okanogan County).

From the 58 participants who completed the study, , 27 participants (353 samples) were selected for total RNA extraction and sequencing. Of the 353 samples, 85% samples (n=300) met our internal QC criteria ($RIN \geq 6$, $yield \geq 50$ ng) for 3'mRNA-sequencing. We enrolled more participants (n=58) than what we planned to analyze (n=27) by design to increase the likelihood of capturing high quality exposure samples. For the 27 analyzed participants, we selected 15 out of 18 participants in Okanogan County who were exposed to wildfire smoke, and 12 out of 40 participants in other counties who were not exposed to wildfire smoke. Of the 27 analyzed participants, the median age was 54 (range 21-76), 63% participants (n=17) reported female sex at birth, 74% participants (n=20) were from Washington, and 56% participants (n=15) were from Okanogan County (**Figure 4.3C**).

For sequencing data analysis, participants were categorized into high, medium, low exposure groups based on their local daily PM_{2.5} levels. Of the 15 participants located in Okanogan County, 14 experienced high and 1 experienced medium levels of smoke exposure in 2021. Of the 12 participants located in all other analyzed counties, one experienced high smoke exposure, two experienced medium smoke exposure, and nine experienced low smoke exposure in 2021. Their transcriptomic profiles were analyzed by examining genes differentially expressed between different exposure groups as well as different time points of a single participant.

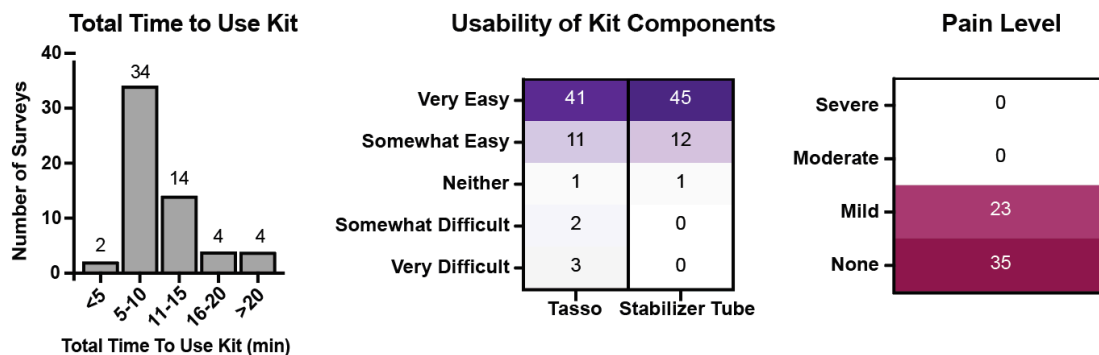
4.2.2 homeRNA is an easy-to-use kit that allows high retention and flexibility across a 10-month-long study

To address the challenges of setting up a long-term, multi-location, clinic-based blood draw study, we used homeRNA for a remote study design with the advantages of flexible study logistics, broad participant recruitment, and at-home self-sampling. Our longitudinal study to assess the short and long-term effects of wildfire smoke exposure spanned 10 months (June 2021-April 2022) where each participant collected up to 15 blood samples with the homeRNA kit before, during, and after the wildfire season. Despite this prolonged study design and frequent sampling needs, there was a completion rate of 92% (n=58/63 participants originally enrolled) where participants returned at least one sample. In the closing survey, 93% of participants (n=50/54 participants who completed the closing survey) reported that they would like to be contacted again to participate in a second year of the study for the 2022 wildfire season. In fact, a total of 13/63 participants enrolled in this first study (2021 wildfire season) enrolled again in the second year of the study (manuscript in preparation).

To understand the usability of homeRNA, we sent a survey accompanying each sample collection to the participants. After collecting their first blood sample for the study, 59% of

participants (n=34/58) reported that it took them a total of 5-10 minutes to use the homeRNA kit (**Figure 4.4A**), which includes collecting blood with the Tasso-SST device, interfacing the Tasso collection tube with our custom-engineered stabilizer tube, shaking to mix the contents between the tubes, and sealing the sample for return shipment. Further, 90% (n=52/58) and 98% (n=57/58) of participants reported that the Tasso blood collection device and stabilizer tube, respectively, were “very easy” or “somewhat easy” to use; 9% of participants (n=5/58) reported that the Tasso device was “somewhat difficult” or “very difficult” to use and no participants reported that the stabilizer tube was difficult to use (**Figure 4.4A**). Lastly, all participants reported no or mild pain (n=35/58 reported no pain and n=23/58 reported mild pain) associated with the blood collection using homeRNA for the first time. These initial responses suggest that the homeRNA kit was easy to use even at the first time.

A) First Collected Sample (n=58 surveys)



B) All Collected Samples (n=636 surveys)

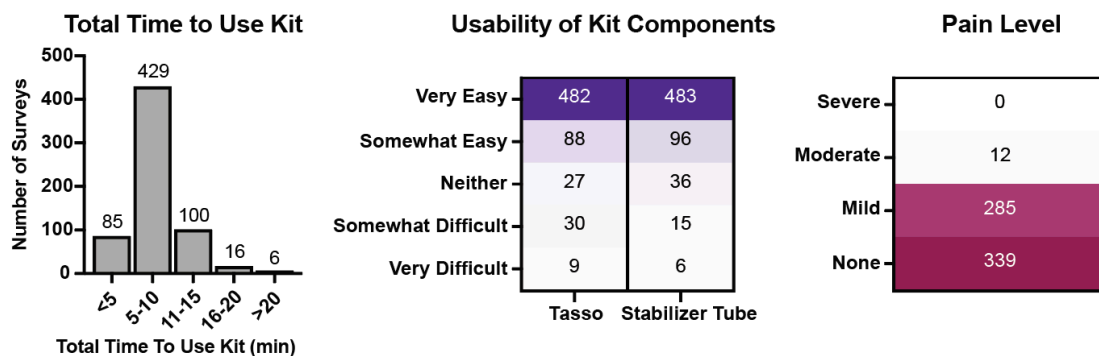


Figure 4.4. Usability survey responses for using the homeRNA kit to self-collect and stabilize blood throughout the study. Participant survey responses on the total time to use the homeRNA kit (left), the usability of the Tasso-SST blood collection device and the stabilizer tube (middle), and the pain level associated with blood collection (right) from **A**) first collected sample (n=58 surveys) and **B**) all collected samples (n=636 surveys) from 58 participants.

Throughout the study, we collected a total of 636 sampling surveys from the 58 participants that showed similar responses about their homeRNA user experience to their first survey response (**Figure 4.4B**). 67% of surveys (n=429/636; increased from 59% for the first time) reported 5-10 minutes for homeRNA kit use; 90% (n=570/636) and 91% (n=579/636) of surveys indicated that the commercial Tasso device and custom stabilizer tube, respectively, were “very easy” or “somewhat easy” to use; 98% of surveys reported no or mild pain (n=339/636 reported no pain and n=285/636 reported mild pain) associated with blood collection. The similar survey responses between the first sample collection and all sample collections demonstrate that homeRNA was easy to use in the beginning and throughout the study. Overall, the survey responses regarding the usability of homeRNA suggest the effectiveness of deploying homeRNA in a remote study design.

Finally, we sent a follow-up survey in November 2023, 18 months after the completion of the study on the 2021 wildfire season, to assess participants' preference for the remote study design over a clinic-based blood draw study design. In this survey (Supplementary Table SX), 93% of the respondents (n=28/30) considered participating in a remote blood sampling study (the current study) to be “significantly easier” or “somewhat easier” than an in-person blood sampling study (e.g., clinic-based blood draw). When asked what reasons would prevent the participant from participating in an in-person blood sampling study, 77% of respondents (n=23/30) answered the length of commute to the nearest clinic or blood draw facility and/or the difficulty of fitting these visits into their work schedule. Further, 77% of participants (n=23/30)

agreed or strongly agreed that the flexibility in when they could take a blood sample was an important factor in their ability to participate in this study. Lastly, 97% of the respondents (n=29/30) indicated that they would be willing to participate in this or a similar remote blood sampling study again and 70% of the respondents (n=21/30) indicated that they would be willing to participate in this or a similar remote blood sampling study for 5 years (with this option being the longest period we provided in the survey).

4.2.3 Flexible study design captures a wide range of PM_{2.5} exposure throughout wildfire season

The current standard practice to quantify wildfire smoke exposure is by measuring the concentration of ambient PM_{2.5}. PM_{2.5} is particulate matter with an aerodynamic diameter of 2.5 µm or smaller, also known as fine particulate matter. PM_{2.5} can be inhaled into the deepest accesses of the lungs and absorbed by the bloodstream, making it a great health concern.³² Specifically, wildfire-specific PM_{2.5} was shown to increase respiratory hospitalizations than PM_{2.5} from other sources (e.g., ports, industrial plants, and traffic emissions).³³

We estimated the daily average PM_{2.5} exposure for each participant using data from their nearest available EPA sensor for outdoor PM_{2.5}, and if available, data from PurpleAir indoor air monitors for indoor PM_{2.5}. Further, we categorized the 27 analyzed participants' wildfire smoke exposure into high (n=15), medium (n=3), and low (n=9) levels based on the standard deviation of their outdoor PM_{2.5} data across the study period (see Methods). The daily average PM_{2.5} data (indoor and outdoor) of an example participant from each exposure group across the study timeline (June 2021 - March 2022) was plotted against their homeRNA sampling dates (**Figure 4.5A-C**); these graphs are further color coded based on the EPA's AQI categories based on

PM_{2.5} exposures (Table S1). Similar graphs on all analyzed participants can be found in **Supplementary Figure S4.3**.

A subset of participants were selected to receive Indoor PurpleAir sensors to track indoor PM_{2.5} concentrations. In some participants, we observed higher levels of indoor PM_{2.5} concentrations than outdoor PM_{2.5} concentrations. For example, participants in Okanogan County, WA with the same outdoor PM_{2.5} data (P4, P5, and P7) experienced drastically different indoor PM_{2.5} in different households (**Supplementary Figure S4.3A-C**). Additionally, when outdoor PM_{2.5} was low after wildfire seasons, some participants (P4, P13, and P17) still experienced periodic spikes of high indoor PM_{2.5} (**Supplementary Figure S4.3A,D,E**). This discrepancy in indoor and outdoor PM_{2.5} levels on certain days may be due to lack of home ventilation or indoor behavioral activities (e.g., smoke from cooking, burning candles, etc.).

Figure 4.5. Participant PM_{2.5} exposures across the study period. **A-C)** Data from example participants exposed to high (A), medium (B), or low (C) levels of wildfire smoke, plotted against the homeRNA sampling timepoints (white vertical lines with Tasso symbols on top), outdoor EPA sensor (black lines with data points), indoor PurpleAir sensor (grey lines with data points), and AQI categories (colored background). **D)** Hierarchically clustered heatmap summarizing the outdoor PM_{2.5} levels during the wildfire season (June 2021-September 2021) for the 27 participants analyzed with 3'mRNA-sequencing. Participants are color-coded based on their geographic region (magenta: Okanogan County, teal: non-Okanogan County) and exposure levels (red: high exposure, orange: medium exposure, green: low exposure).

To have a close-up look at the PM_{2.5} exposure for all participants throughout the wildfire season, we summarized the outdoor PM_{2.5} data of the 27 analyzed participants during June-September 2021 in a hierarchically clustered heatmap (**Figure 4.5D**). The 15 participants (P1-P15) from Okanogan County, WA, and 12 participants (P16-P27) from other counties were placed in distinct clusters, reaffirming our decision to compare Okanogan County versus non-Okanogan counties for subsequent data analysis. Participants 1-10 lived closest to the two major wildfires in Okanogan County, WA. Participants 12-15 lived further away from the major wildfires but still experienced high levels of smoke exposure. Participant 11 lived the furthest away from the two major wildfires in Okanogan County and experienced a medium level of wildfire smoke exposure. Participant 16 lived in Washoe County, Nevada and experienced high levels of wildfire smoke exposure in late August 2021 in response to the Caldor fire that started August 14th in El Dorado County. Participants 17-18 lived in Lincoln County, WA, adjacent to Okanogan County, and experienced a medium level of wildfire smoke exposure in August 2021 due to winds drifting smoke into nearby counties. Participants 19-27 lived in other counties in WA or other Western US states, and experienced low levels of wildfire smoke exposure.

4.2.4 Symptoms and mitigation strategies in relation to PM_{2.5}

With each sampling survey we asked participants to report any symptoms they were experiencing which could be related to wildfire smoke exposure.³⁴ The reported symptoms are plotted against the AQI categories determined based on the maximum PM_{2.5} average in the previous 7 days (**Figure 4.6**). To exclude the confounding factors for the symptoms besides smoke exposure, survey responses that answered “Yes” to the question “Are you currently feeling sick (e.g. cold or flu)?” were not included in the symptom results.

When the AQI category was Good or Moderate, most participants did report no physical symptoms in their surveys. When the AQI category was Unhealthy or Very Unhealthy, the percentages of surveys indicating wildfire related symptoms (e.g., Coughing, Stinging eyes, A scratchy throat, Headaches, Tireness) were increased compared to the Good or Moderate group. Notably, when the AQI category was Hazardous, the percentages of surveys reporting symptoms were decreased compared to the Unhealthy or Very Unhealth group.

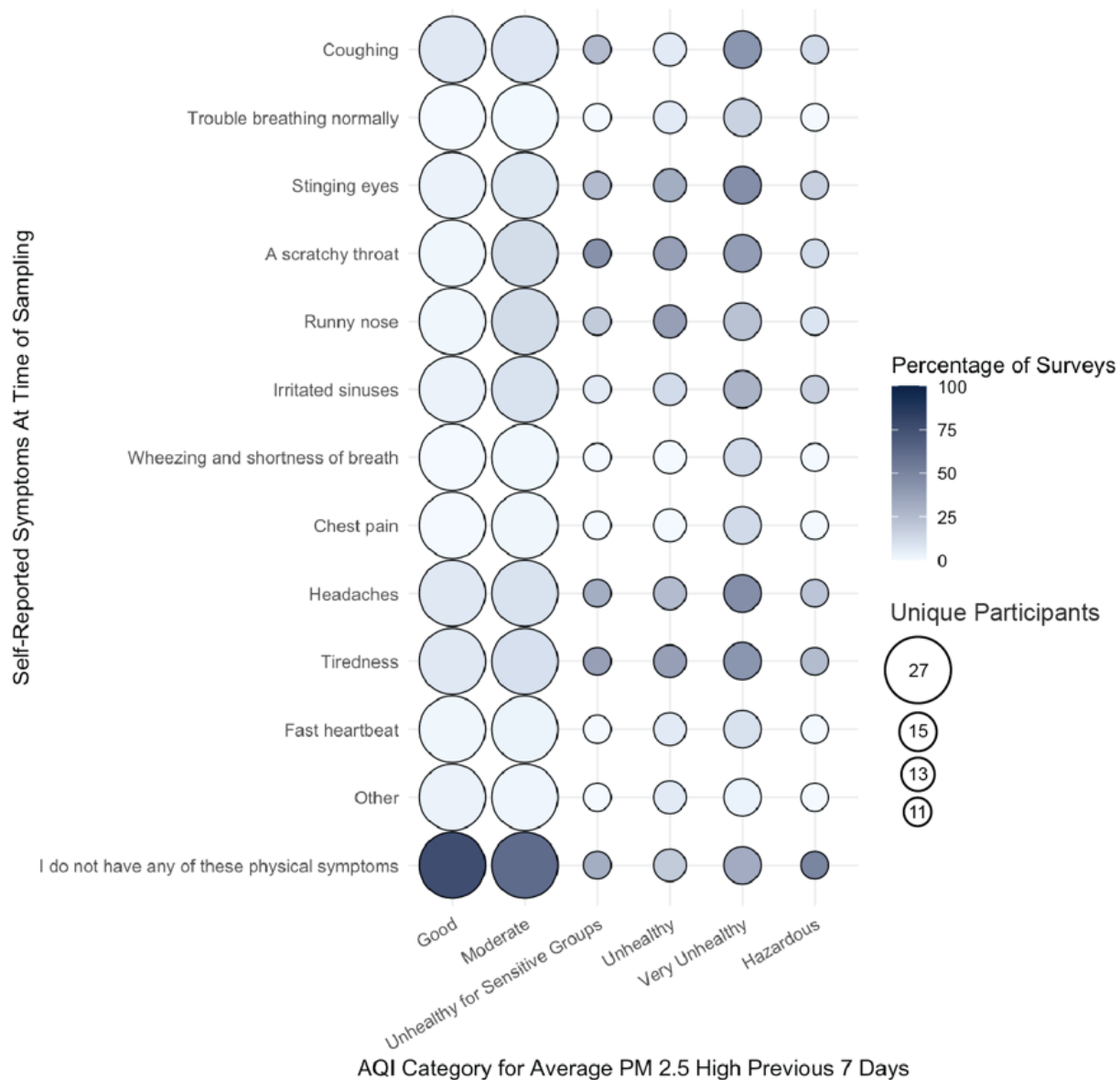


Figure 4.6. Dot plot depicting self-reported symptoms in sample collection surveys and corresponding AQI categories for their highest daily average PM_{2.5} in the previous 7 days. A total of 363 samples with associated surveys were collected from 27 participants. Excluded from symptom data were surveys that answered “Yes” to the question “Are you currently feeling sick?”, which decreases the total to 353 surveys. Dot size represents the number of unique participants and color gradient represents the percentage of surveys reporting a symptom (or not having any physical symptoms) within a given AQI category. “Asthma attack” was not plotted as no one reported this symptom at any time point.

4.2.5 Quality and quantity of RNA extracted from homeRNA are suitable for RNA sequencing

At the end of the study, a total of 635 homeRNA samples were returned from 58 participants exposed to varying levels of wildfire smoke before and during the wildfire season (June-September 2021), as well as 3 and 6 months after to assess the short and long effects of wildfire smoke on blood transcriptome. The average blood volume collected using the Tasso SST device was 250-300 μ L over a span of 3-5 min (**Supplementary Figure S4.1**). Total blood RNA was extracted from 431 samples for RNA yield and quality assessment. Of the extracted samples, the average RNA yield was 500 ng with 97% samples (n=420/431) above 50 ng (cutoff for 3'mRNA seq by Lexogen); the average RNA Integrity Number (RIN) was 7.5 with 94% samples (n=398/424) above 6 (cutoff for 3'mRNA seq by Lexogen) (**Supplementary Figure S4.2**). Samples from 27 participants (n=353 samples) were selected for 3'mRNA sequencing where 300 samples that passed our internal QC standards were sent to Lexogen, Inc for sequencing. Sequencing results and corresponding time-course genet set analysis³⁵ are pending.

4.3 Discussion

Blood transcriptomic response to wildfire smoke exposure is difficult to capture, largely due to the unpredictable nature of wildfires. Smoke exposure can occur in remote areas and can quickly move from one region to another depending on many factors. Setting up a large-scale study to study wildfire smoke exposure in the general population using traditional clinical-based blood draw is difficult as it either limits participant enrollment to a specific region where an academic or clinic laboratory is located that can provide phlebotomy blood draws or requires complicated logistics to set up multiple or remote phlebotomy blood draw locations. Moreover, a clinic-based study requires study participants to travel physically to a blood draw location, which

can be challenging or even impossible during a natural disaster. homeRNA kits provide flexibility to participants, making it easier for them to collect samples even during a stressful event such as a wildfire occurring nearby. Notably, at one point in the study, some participants needed to evacuate due to the locations of their homes with regard to a wildfire. Some of these participants chose to take their homeRNA kits with them and continued sampling. Such anecdotes exemplified the flexible nature of homeRNA and remote study design and the potential utility of such tools for the study of natural disasters broadly. Utilizing homeRNA as we have done in this study allows for facile remote study logistics and broad recruitment, while allowing for participants to sample in their own homes easily throughout a yearlong study.

Furthermore, to understand the effect of wildfire smoke on immune activation and inflammatory responses, it is important to have a baseline measurement of the inflammatory transcriptome prior to wildfire smoke exposure, given recent studies showing that immune gene regulation can persist as long as three months after exposure.³⁶ However, if a study is located only in one location, collecting baseline samples before any smoke exposure has occurred could be logistically risky from a study design perspective, as there is always a chance that no smoke exposure will occur in that region in a given wildfire season. Previous studies utilize several strategies to allow for the collection of a baseline while de-risking the possibility that a wildfire smoke event will not occur. Apart from utilizing a firefighter population who will more than likely be exposed to smoke,^{22,25,26,37} another strategy is using controlled burns as a proxy for wildfire smoke exposure^{25,36,38} as these can be more readily predicted, but many studies have found varying responses to controlled burns versus a true wildfire event.³⁶ As such, many existing literature on wildfire smoke exposure where blood samples are collected either miss a baseline measurement as they only begin sampling after smoke exposure has occurred,²⁷ take

place over multiple sites over multiple years,^{38,39} or take place in a region prone to wildfire smoke.^{17,36,40} Other studies that do contain a pre-smoke exposure sample, are collected incidentally; study participants were enrolled in a separate study in which longitudinal samples were being collected, while a subset of participants happens to have experienced wildfire smoke exposure during the study period.^{24,27,28}

homeRNA kits provide an additional strategy by enabling fully remote, flexible, and responsive sampling throughout a wildfire season. To de-risk the possibility of smoke exposure not occurring, we took a dual approach of enrolling participants broadly throughout the Western US, while also utilizing a more targeted enrollment strategy in Okanogan County in Washington state, a region that historically has had high wildfire smoke exposures yearly.^{41–44} We sampled 58 participants from 10 states throughout the West and Southwest US, including Washington, Oregon, Colorado, Idaho, Montana, Nevada, Utah, Wyoming, and Texas. While enrolling broadly, the plurality of study participants were located in Okanogan County, WA (n=18). This recruitment geography not only reduces the risk of a wildfire not occurring in a single study location, but also makes it possible to collect samples from participants who are getting exposed to wildfire smoke and those who are not, allowing for a more comprehensive understanding of the effects of wildfire smoke.

To determine when kits should be sent to capture wildfire smoke exposure, we monitored the EPA AirNow site to track daily PM_{2.5} averages. Using the nearest EPA sensor to each study participant, we estimate the average daily PM_{2.5} exposure for each participant. This creates a standardized way of estimating exposure (barring sensor-to-sensor differences) by utilizing an EPA sensor which has some standardization on how the PM_{2.5} measurement is made.

However, the methodology of using a single nearest EPA sensor has many limitations. First, the distance between each participant's location and their nearest EPA sensor varies amongst the study participants. Many participants, particularly those who were located in Okanogan County, WA live closest to the same sensor, and therefore have the same outdoor PM_{2.5} data. However, because they vary in distance from the sensor, it is more likely that the PM_{2.5} exposure differs amongst the participants and the accuracy likely follows how close they live to the sensor. Second, variations in terrain, wind, and other weather patterns may further complicate the actual outdoor exposure estimation, beyond simply correcting for the relative distance to the nearest EPA sensor. Finally, individual behavior may also change their effective PM_{2.5} exposure, depending on their time spent indoors versus outdoors as well as protective measures (e.g., wearing an N95 mask or installing HEPA filters at home).

Other limitations of the study include potential confounding factors that may affect blood transcriptome besides wildfire smoke exposure, including seasonal flu, allergies, and psychological stress from wildfire. However, we were able to collect samples from people who did not experience smoke exposure throughout the year which may help correct for this confounding factor. Another concern was that participants would not remain enrolled throughout the study due to the prolonged year-long nature (June 2021-April 2022) and the requirement to collect up to 15 blood samples with the homeRNA kit. Nonetheless, the study participants reported that the homeRNA kit was easy to use, and most participants remained in the study (n=58/62 originally enrolled) until the end and returned an average of 11 samples per participant (635 samples from 58 participants). Furthermore, 93% of participants indicated in their survey responses that they would like to be contacted for enrollment for an additional year.

4.4 Conclusion

We demonstrated that homeRNA facilitated an effective and flexible approach to studying the effects of wildfire smoke on human health. The homeRNA kits enabled participants to collect and stabilize blood samples in their own homes, allowing for a fully decentralized study. By enabling fully remote, flexible, and responsive sampling throughout a wildfire season, homeRNA kits provide an opportunity to capture multiple time points at the presence or absence of a particular wildfire event, allowing for comparisons across exposure groups. The study also retained over 95% of participants, with some participants even choosing to continue using homeRNA during a wildfire evacuation, demonstrating the feasibility of using homeRNA for longitudinal studies on wildfires and other disasters. Most RNA extracted from homeRNA-collected blood samples had suitable quality and quantity for downstream analysis such as RNA sequencing, indicating that this study design can help elucidate blood transcriptomic response to wildfire smoke in the general population. Further studies utilizing homeRNA kits in a larger study population could provide more information on the health effects of wildfire smoke as well as their socio-economic, occupational, and household-level determinants.

4.5 Methods

4.5.1 homeRNA kit components

homeRNA is a self-sampling kit for the collection and stabilization of blood RNA. The kit uses a commercially available Tasso-SST™ blood collection device for the self-collection of peripheral blood and a specially engineered stabilizer tube containing RNA*later*™ for immediate stabilization of cellular RNA. The Tasso-SST™ blood collection device was purchased from Tasso, Inc (Seattle, WA). The stabilizer tube was injection molded out of polycarbonate (PC:

Makrolon 2407) by Protolabs, Inc (Maple Plain, MN) and was designed to hold an RNA stabilization solution and connect to the Tasso-SST™ blood collection tube. The stabilizer tube was filled with 1.3 mL RNAlater™ (Thermo Fisher) stabilization solution and capped. The stabilizer tube insert was injection molded out of polycarbonate (PC: Makrolon 2407) by Protolabs, Inc (Maple Plain, MN) and was designed to hold the stabilizer tube containing blood in a 50 mL conical tube during shipping. All components were cleaned via sonication in 70% ethanol (v/v) for 30 min and air dried prior to assembly. Finally, all kit components were placed in a rigid custom-designed mailer box (The BoxMaker, Inc.). Detailed design files and the workflow of assembling and using the homeRNA kit can be found in an initial pilot study in which we characterized the feasibility of using homeRNA for remote blood collection and RNA stabilization.²⁹

4.5.2 homeRNA kit use workflow

The workflow of the homeRNA kit is described in detail in Haack, Lim, et al. ³⁷. Briefly, to use the homeRNA kit, participants first collected capillary blood from their upper arm using the Tasso-SST™ device. Next, participants were asked to estimate the volume of the blood collected based on a reference photo of the Tasso-SST™ tube in the sample collection survey (**Supplementary Figure S1C**). The participants then stabilized the blood in RNAlater™ by interfacing the RNA stabilizer tube with the Tasso-SST™ blood tube and shaking vigorously to mix the blood with the stabilizing solution. Finally, after mixing, the stabilized blood sample was placed inside a 50 mL conical tube containing an insert to hold the sample tube in place, which was then packaged and mailed back to the lab for downstream analysis.

4.5.3 Study design of using homeRNA to monitor wildfire smoke exposure across the Western US

This is a prospective longitudinal study aimed at profiling detailed kinetics of blood transcriptome in participants before, during, and 3 months and 6 months after exposure to wildfire smoke between June 2021 – April 2022 using a self-sampling kit, homeRNA. We compared participants who were exposed to high, medium, or low levels of wildfire smoke based on the PM_{2.5} levels collected by their nearest outdoor EPA sensor.⁴⁵ Self-reported symptoms and behavior were assessed via survey with each sampling. The study was approved by the University of Washington (UW) Institutional Review Board (IRB) under protocol STUDY00012463. All study procedures were performed after informed consent was obtained. All samples were collected remotely by study participants and online surveys were administered through REDCap.

4.5.4 Participant recruitment and enrollment

Participants were recruited via social media and community outreach between June - August 2021. After completing a screening survey online,, individuals were invited to complete the informed consent and enrollment by the study team. To be eligible for the study, participants had to be: (1) 18 years old or older; (2) living in area prone to wildfire including the states of Alaska, Arizona, California, Colorado, Florida, Idaho, Montana, Nevada, New Mexico, Oklahoma, Oregon, Texas, Utah, Washington, and Wyoming; (3) not pregnant (upon enrollment); (4) not residing in a correctional facility; and (5) not a friend or family member of researchers conducting study.

Participants were enrolled with preference given to (1) those located in Okanogan County, Washington (WA) (a historically wildfire-prone area); (2) Clean Air Methow⁴⁶ (a community air quality program) participants with an indoor Purple Air monitor; and (3) those with diverse geographic locations outside of Okanogan County.

4.5.5 Sample collection timeline

Throughout the study, participants were sent a package containing three homeRNA kits at each sampling window (before, during, and 3 months and 6 months after wildfire season)e. Each participant was also given a schedule for when to use each kit. Typically, each of the three samples was taken 3 to 4 days apart, and returned immediately via overnight shipping, if available.

Before wildfire season: Immediately upon enrollment, participants were sent a set of three homeRNA kits to collect baseline samples before wildfire season (June 2021).

During wildfire season: The location of wildfires, smoke plumes, and PM_{2.5} values were monitored throughout the wildfire season (July-September 2021). If smoke was present in a participant's zip code, participants were immediately sent a box with three kits and asked to sample every 3-4 days. During the wildfire smoke events in Okanogan County, WA, participants outside of this region who were not experiencing smoke exposure were also asked to sample at similar times to match participants located in Okanogan County.

After wildfire season: All participants were sent an additional set of three homeRNA kits between October - November (3 months after the wildfire events in Okanogan County), then again in February - April (approximately 6 months after the wildfire events in Okanogan County).

4.5.6 PM_{2.5} data collection

The average daily concentration of PM_{2.5} that each participant was exposed to during the study period was taken from each participant's nearest outdoor EPA sensor. A subset of participants were also provided an indoor PurpleAir monitor to collect indoor PM_{2.5} data.

Outdoor PM_{2.5}: Daily average PM_{2.5} data for each day of the study period collected by the closest EPA sensor to each participant was downloaded from the EPA website.⁴⁵ The closest sensor was determined by mapping the latitude and longitude of each participant's home address with the reported latitude and longitude of EPA sensors. If no average PM_{2.5} data was available on a certain day with a participant's nearest sensor, then PM_{2.5} data was taken from the second nearest EPA sensor. If the second nearest EPA sensor did not have available data, then it was taken from the third sensor.

Indoor PM_{2.5}: A subset of participants (n=15) was provided with indoor PurpleAir monitors and the study team was provided with the sensor ID. Indoor PM_{2.5} data collected by participant's PurpleAir sensors was downloaded from the PurpleAir website⁴⁷, and the daily average PM_{2.5} was calculated for each day of the study period.

Exposure level categorization: Participant exposure levels were categorized as high, medium, and low based on the standard deviation of their outdoor PM_{2.5} values throughout the study period.

4.5.7 Survey data collection

Participants were surveyed throughout the study period through REDCap. Specific surveys are described below:

Enrollment Survey: This survey was given immediately after informed consent was acquired. It collects information about participant demographics, home addresses (for sending samples and tracking PM_{2.5} data), as well as underlying health conditions, recent vaccinations, and medications that may affect immune activation.

Baseline Survey: This survey was given with the first blood sample collected, whether it was before the first wildfire smoke exposure or not. The baseline survey collects information on exposures a participant may have experienced in the past year, the participant's occupation (outdoor work or not), and the participant's typical daily activities (and to list general activities they engage in as a means of reducing psychological stress). Questions on daily activities and stress were adapted from standardized surveys on stress assessment available on PhenX.⁴⁸ This survey also includes all the questions in the Sample Collection survey (below), and if the participant was experiencing smoke exposure for the first sample, they were asked additional questions that are specific to smoke exposure listed in the Smoke Exposure Survey (below).

Sample Collection Surveys: This survey was given with every blood sample collected. It includes questions about the date and time of the sample collection, sample collection metrics (e.g., blood volume estimation, time used for the collection), usability questions (e.g., ease of use with the kit, pain level using the Tasso device), as well as any symptoms (the list of symptoms was adopted from a CDC website on health effects of wildfire smoke³⁴), stress levels and daily activities unrelated to wildfire smoke exposure. If a participant received an indoor PurpleAir monitor, they were asked to report the PM_{2.5} value on the air monitor at the time of collection.

Smoke Exposure Surveys: This survey was given if a study participant was using the homeRNA kit during wildfire smoke exposure. It contains all the questions in the Sample Collection Survey (above) with additional questions specifically about behaviors in response to wildfire smoke

exposure. These questions included how much time the participants spent outdoors, in a car or bus, and indoors and if they utilized any mitigation strategies, such as using an air filter, wearing masks, avoiding outside activities, or keeping windows closed.

Closing Survey: This survey was given at the end of the study period (April 2022). It included demographic and behavior questions, including if a participant had a major change in behavior, health status, or location (e.g., on vacation) during the study.

Followup Survey: This survey was given 18 months after the study was concluded in November 2023. It asked for the participant's motivation to enroll in the study, comparisons between remote blood sampling and in-person blood draw, feedback on using the homeRNA kit, and willingness to participate again.

4.5.8 Sample processing

Upon arrival at the lab, the packaged homeRNA kit was immediately stored at -20°C by direct delivery to a -20°C freezer on campus. Within a week, the 50 mL conical tube containing the stabilized blood sample was removed from the kit, and transferred to -80°C until ready for further processing.

4.5.9 Participant selection for analysis

Participants from Okanogan County were selected for sequencing analysis if they had (1) at least 4 high-quality samples collected with associated surveys and (2) at least 1 sample collected with an associated survey during the wildfire smoke exposure. Samples were considered high quality with visual inspection upon return if they: (1) had no visible clotting in the tube; (2) appeared sufficiently mixed; (3) had enough blood collected; and (4) did not leak. Participants from non-Okanogan regions were selected for analysis if they (1) had at least 4 high-

quality samples collected with associated surveys and (2) were matched to the Okanogan participants with combinations of age, gender, and location.

4.5.10 RNA isolation

Total cellular RNA was isolated from all selected participant's samples using the Ribopure™ - Blood RNA Isolation Kit (Thermo Fisher) according to the manufacturer's protocol and eluted in two 50 µL aliquots. Isolated RNA was stored at -80°C until ready for further analysis.

4.5.11 Assessment of total cellular RNA integrity and yield

The RNA Integrity Number (RIN) scores of the first 50 µL elution were obtained on a Bioanalyzer 2100 (Agilent) using the Agilent RNA 6000 Pico Kit (Agilent 5067-1513). All RNA samples were diluted 1:20 in nuclease-free water before RIN measurement to ensure the RNA concentration of the loaded sample was in the qualitative range for the Agilent RNA 6000 Pico Kit. The RNA concentrations of the first 50 µL elution were measured using the Qubit Flex Fluorometer (Thermo Fisher) using the Qubit RNA High Sensitivity Assay Kit (Invitrogen Q32855).

4.5.12 RNA sample selection and processing

After isolation of all participant samples chosen for extraction, samples were selected for 3'mRNA-sequencing analysis based on if they met our internal quality control standards (RIN $\geq$ 6 and yield $\geq$ 50ng). We note that some samples (n=107 samples from 9 participants) were previously run as a pilot gene expression analysis using the nanoString nCounter Autoimmune Profiling Panel. Additional RNA processing was performed on a subset of these previously run

samples to meet nanoString's sample submission requirements ($RIN \geq 7$ and RNA input ≥ 100 ng). These RNA samples were treated with DNase I (RNase-free) (New England Biolabs, M0303S) to remove contaminating genomic DNA and/or Monarch® RNA Cleanup Kit (New England Biolabs, T2030L) to purify and concentrate the RNA according to the manufacturer's protocols.

4.5.13 3'mRNA sequencing and analysis

After isolation and processing, 100 ng of total RNA from 300 selected samples were sequenced by the Lexogen facility (Vienna, Austria) using 3'mRNA-sequencing technology (Lexogen QuantSeq 3' mRNA-Seq V2 Library Prep Kit FWD with UDI) on an Illumina NextSeq 2000 instrument according to the manufacturers' instructions. Prior to library preparation, all samples were treated with Ambion DNase I (Invitrogen) for 10 minutes at 37°C followed by heat inactivation with EDTA at 75°C for 10 minutes. Libraries were prepared using 17 ng of RNA as input. Libraries were amplified for 18 cycles and were quality controlled on a Fragment Analyzer device using the DNF-474 HS NGS Fragment kit (1-6000 bp) (Agilent). The samples were sequenced to a read depth of 5 million reads.

4.6 References

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Chapter 5 | Conclusion and Outlook

This dissertation demonstrated the significant potential of open microfluidic technologies in advancing biomedical research and elucidating human disease mechanisms. The innovative design and versatile application of open microfluidic platforms provide unique opportunities to investigate complex biological mechanisms and disease pathways in ways that were previously unobtainable with traditional approaches. The key findings detailed in previous chapters showcased the diverse applications and profound impact of open microfluidic technologies as a bioanalytical chemistry tool to facilitate mechanistic studies for medicine and the environment, leading to potential new therapeutic targets and strategies.

Chapter 1 laid the groundwork by introducing open microfluidic systems and highlighting their advantages in bioanalytical research. Chapter 2 presented a novel open microfluidic coculture device for investigating cell-cell signaling with an application in airway inflammation. The ability to coculture primary human fibroblasts and eosinophils in an open system facilitated the discovery of new signaling factors, advancing our understanding of fibroblast-eosinophil crosstalk in allergy and asthma. Chapter 3 extended the application of the microfluidic coculture device to glomerular disease, allowing for direct measurement of paracrine signals from injured podocytes on neighboring healthy podocytes. This provoked new insights into the pathophysiology of podocyte injury and its paracrine effects on other cell types in the glomerulus (e.g., endothelial cells, glomerular epithelial cells). Chapter 4 showcased homeRNA as a versatile tool for remote blood sampling to assess the effects of wildfire smoke exposure. This chapter demonstrated the logistical advantages of a flexible study design with homeRNA which can be used in other disaster research or decentralized study.

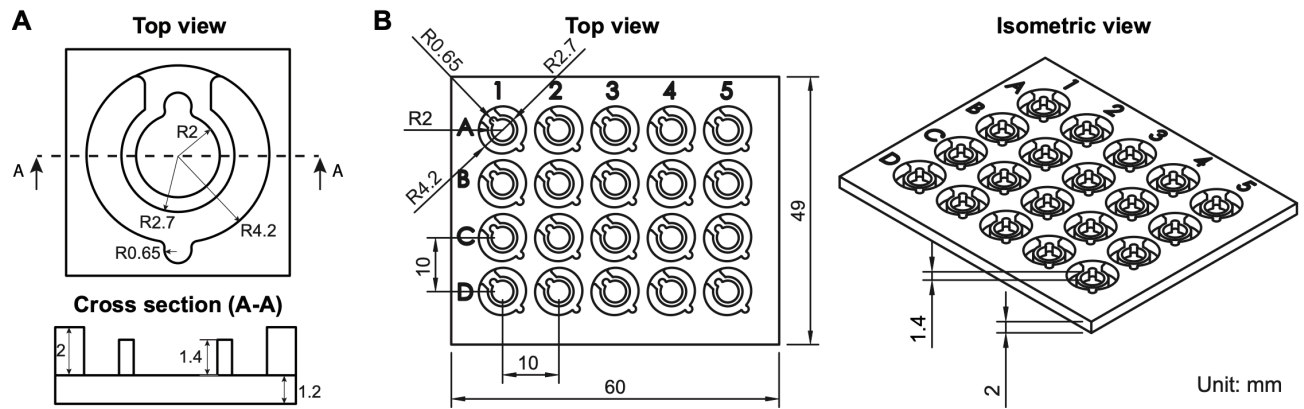
The future of open microfluidic technologies in biomedical research is promising, with several avenues for further development and application. Integrating open microfluidic platforms with advanced analytical methods such as mass spectrometry, single-cell RNA sequencing, and high-resolution imaging will enhance their capability to dissect complex biological systems in great detail. Additionally, the versatility and small volume of open microfluidic systems have immense potential in personalized medicine, as they can be customized with primary samples to create patient-specific models, enabling personalized disease modeling and drug screening. Moreover, the simplicity and portability of open microfluidic platforms make them ideal candidates for point-of-care and digital health applications, particularly in remote and resource-limited settings. Furthermore, the accessibility and user-friendly design democratize the application of open microfluidic technologies, enabling broader and faster adoption among researchers and fostering cross-functional collaborations.

With unique capabilities and promising applications, open microfluidic technologies will continue to evolve and contribute significantly to the field of biomedical research, offering novel solutions and insights into complex human disease mechanisms. The advancements made in this dissertation will serve as a foundation for future explorations, unlocking the transformative potential of open microfluidic technologies.

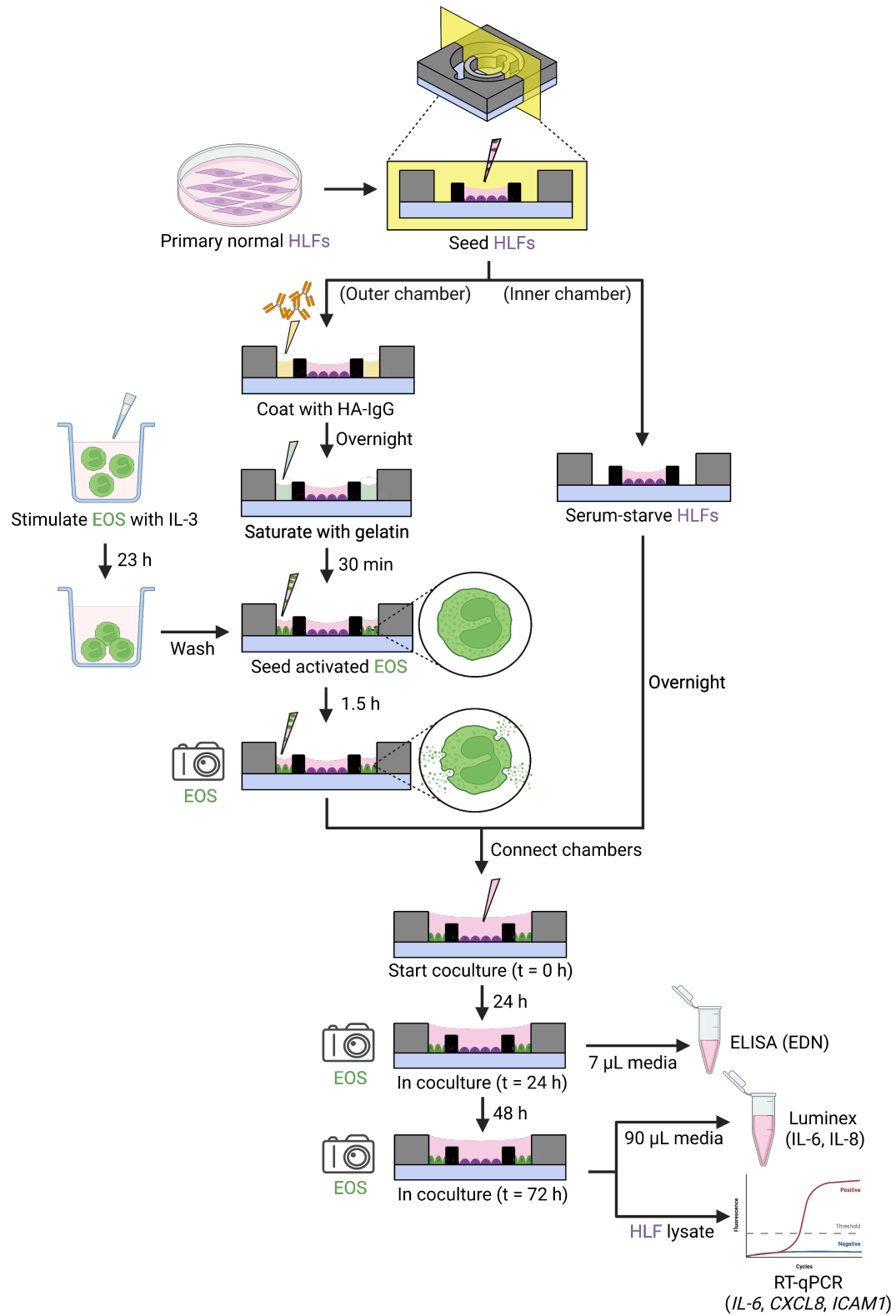
Appendix

A. Supplementary information for Chapter 2

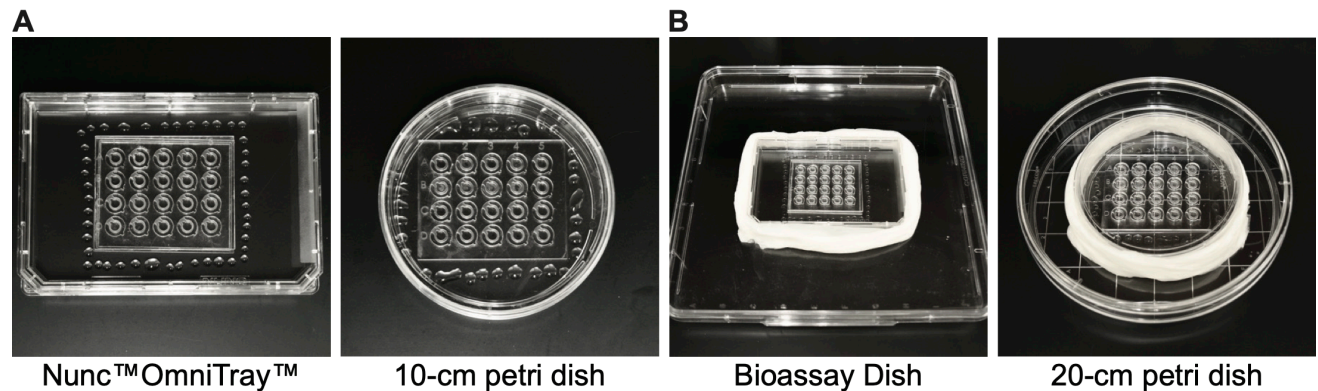
1. Supplementary Figures



Supplementary Figure S2.1. Detailed dimensions of the open microfluidic coculture device. (A) Dimensions of a single coculture device. Reproduced from Zhang et al., (2020) as allowed per journal policy. Computer aided design file can be found in the Supplementary Material of Zhang et al. (B) Dimensions of a milled polystyrene top piece with the coculture devices in a 4×5 array.

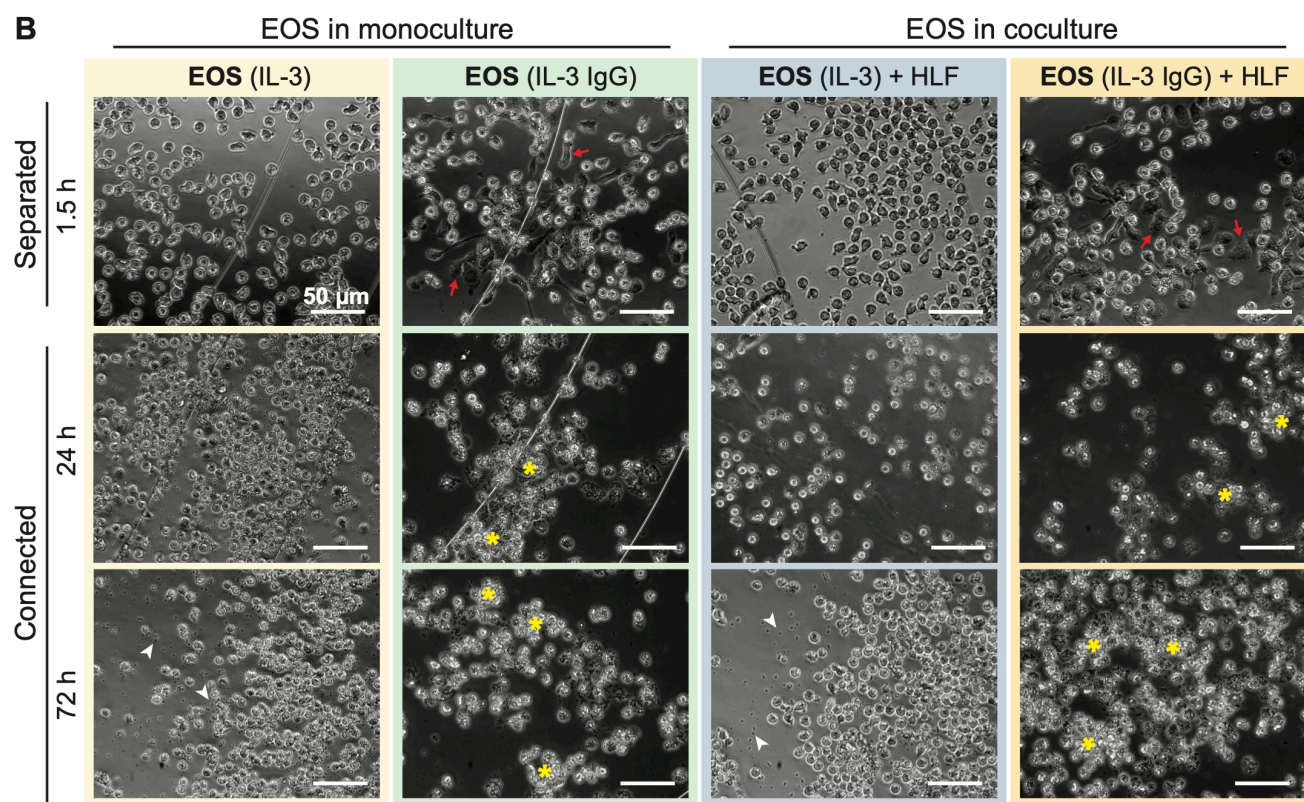
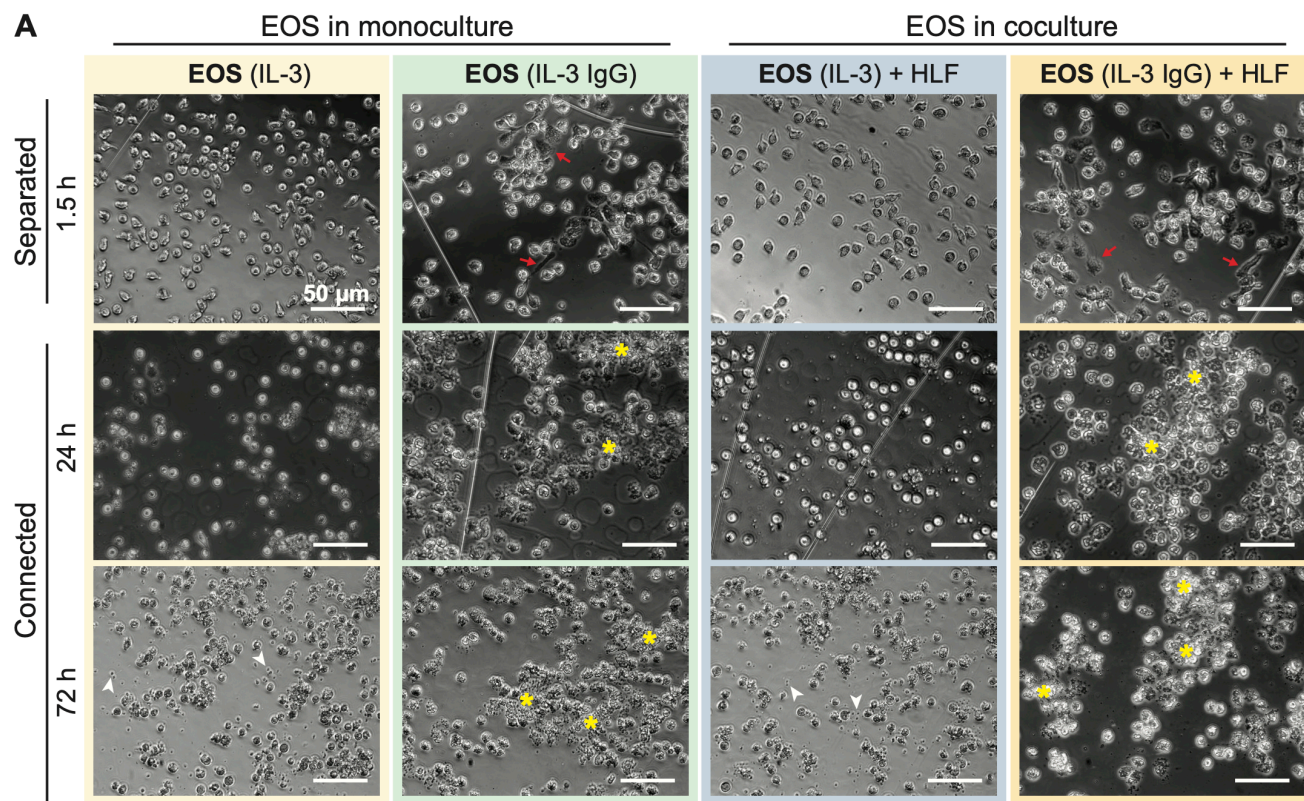


Supplementary Figure S2.2. Schematic workflow showing cell culture procedures and readouts of the eosinophil (EOS)-human lung fibroblast (HLF) coculture in the open microfluidic coculture device. Created with BioRender.com. HA-IgG: heat-aggregated immunoglobulin G. ELISA: enzyme-linked immunosorbent assay. EDN: eosinophil-derived neurotoxin. IL-6: interleukin-6. IL-8: interleukin-8.

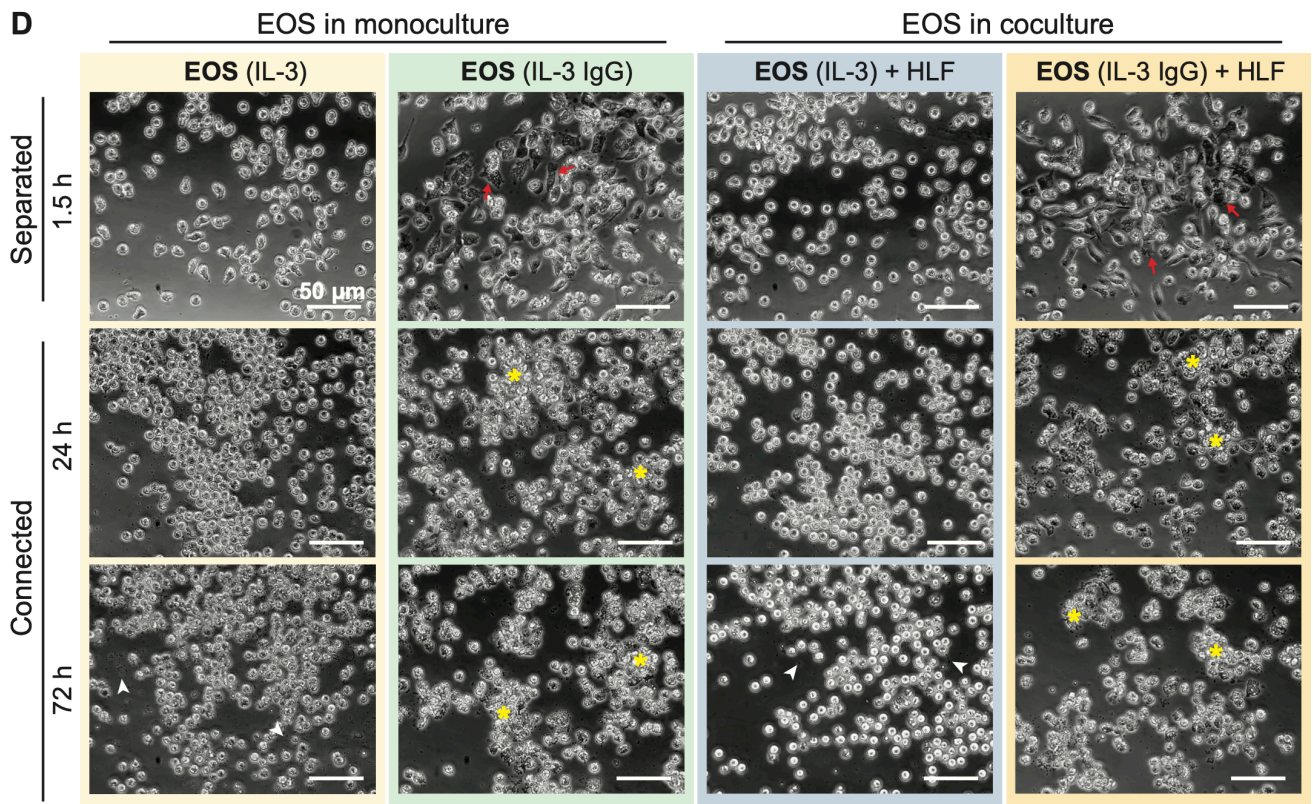
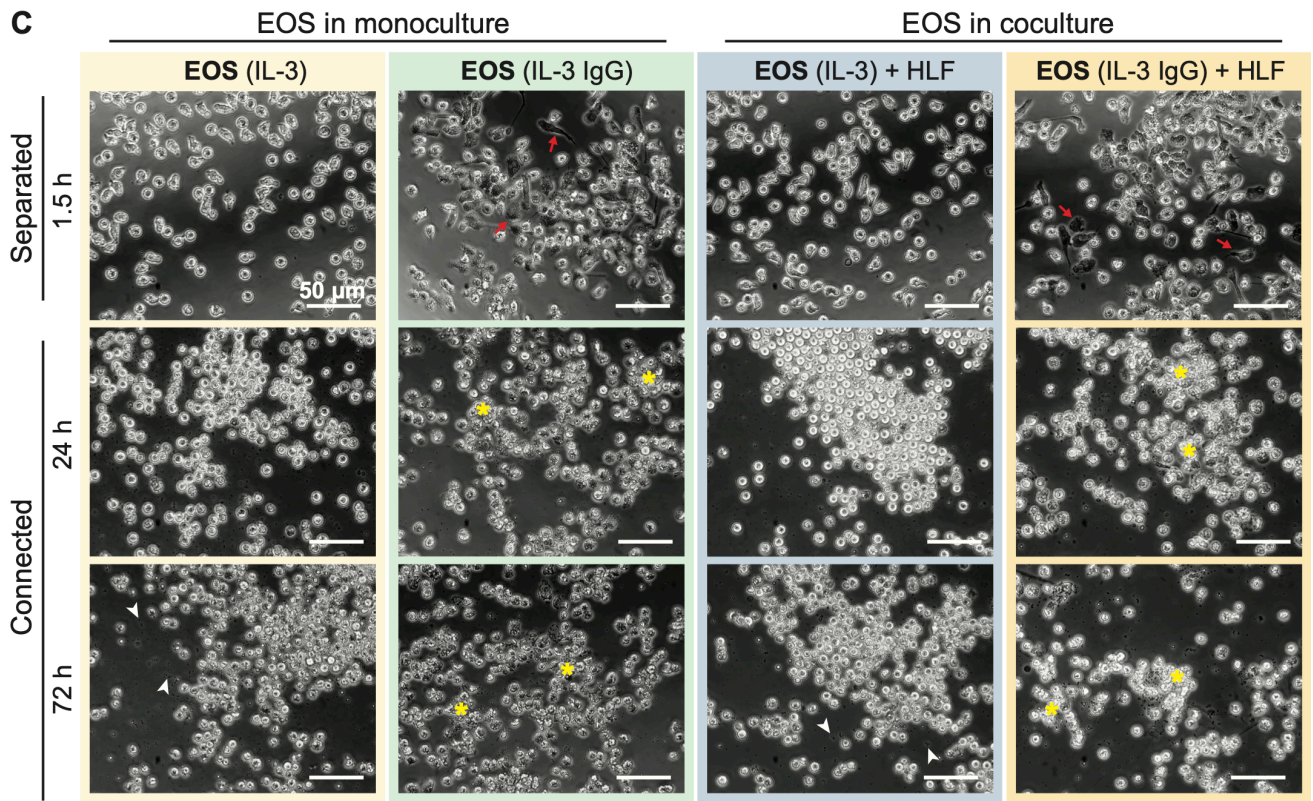


Supplementary Figure S2.3. Examples of the evaporation control setup for the open microfluidic device. **(A)** Approximately 1 mL of sterile sacrificial water was placed as droplets evenly around the perimeter of the device chip in the primary container (e.g., OmniTray™, 10-cm petri dish). **(B)** The primary container was placed into a secondary container (e.g., 30×30 cm bioassay dish or 20-cm petri dish). The perimeter of the primary container was wrapped tightly with Kimwipes soaked with sterile water. In this study, we used OmniTray™ and Bioassay Dish as the primary and secondary container, respectively.

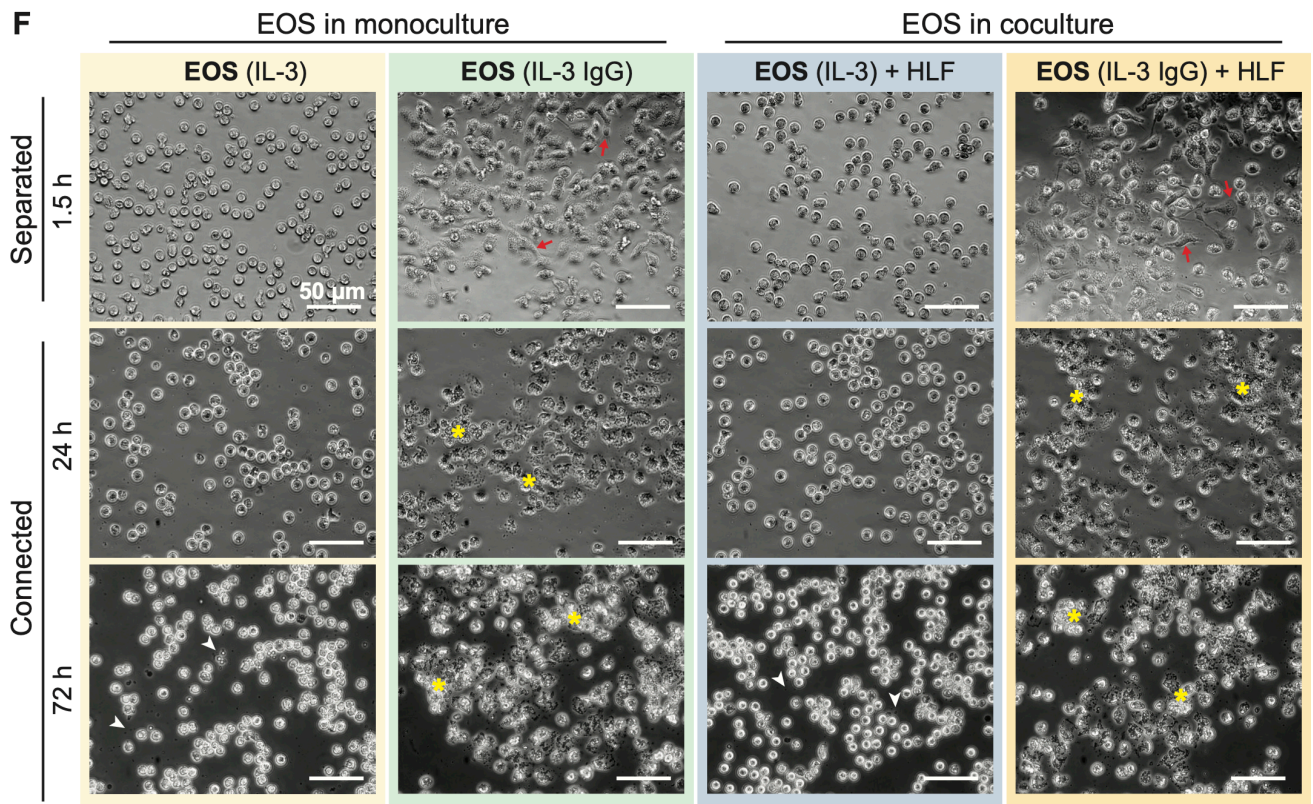
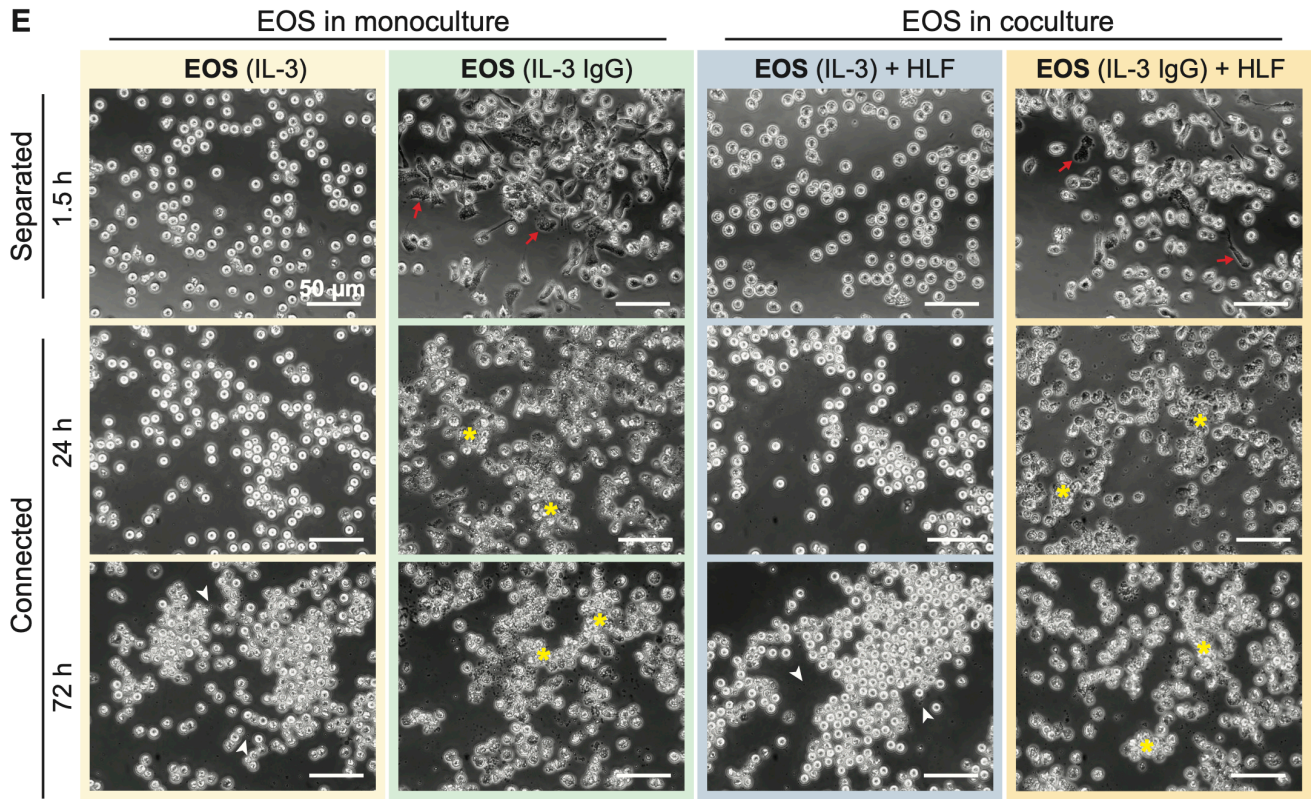
In the next pages, we show a complete collection of results from all donor pairs (including the 2 pairs shown in the main manuscript). Figure captions might be on a different page due to large figures.



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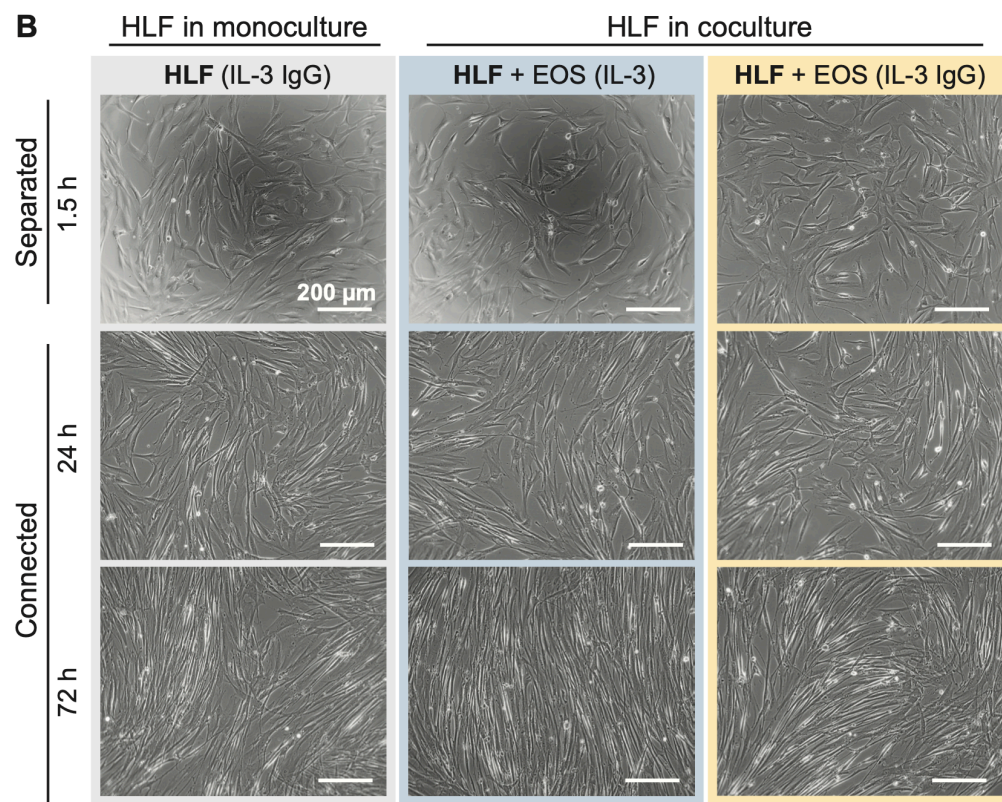
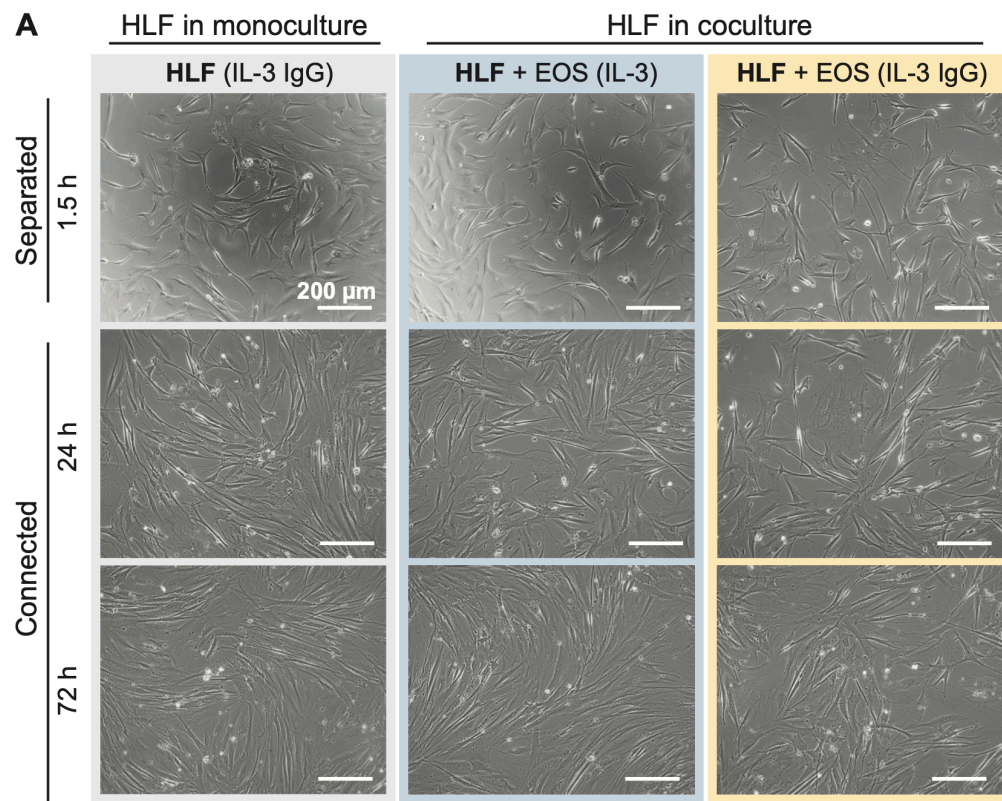


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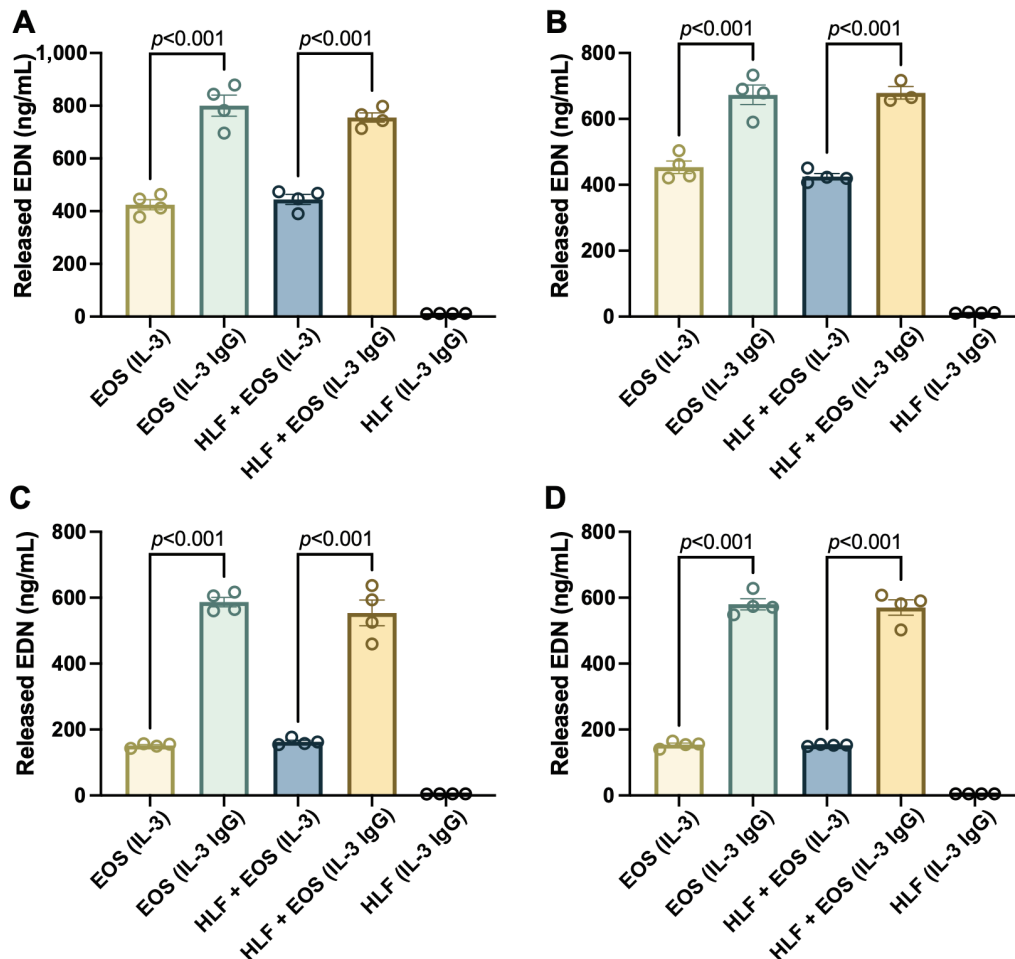
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Supplementary Figure S2.4. Phase contrast images of eosinophils activated with IL-3 (“IL-3”) and in some conditions also seeded onto HA-IgG (“IL-3 IgG”) in coculture devices at the indicated times. Representative images from 6 donor pairs are shown: **(A)** eosinophil donor 1 and HLF donor 1, **(B)** eosinophil donor 1 and HLF donor 2, **(C)** eosinophil donor 2 and HLF donor 1, **(D)** eosinophil donor 2 and HLF donor 2, **(E)** eosinophil donor 3 and HLF donor 1, **(F)** eosinophil donor 3 and HLF donor 2. The chambers remained separated for 1.5 h and then were connected for up to 72 h. Red arrows show eosinophils spreading on HA-IgG. Yellow asterisks show aggregates of eosinophil cell debris. White arrowheads show eosinophil cell-free granules. Images are representative of 4 culture replicates.

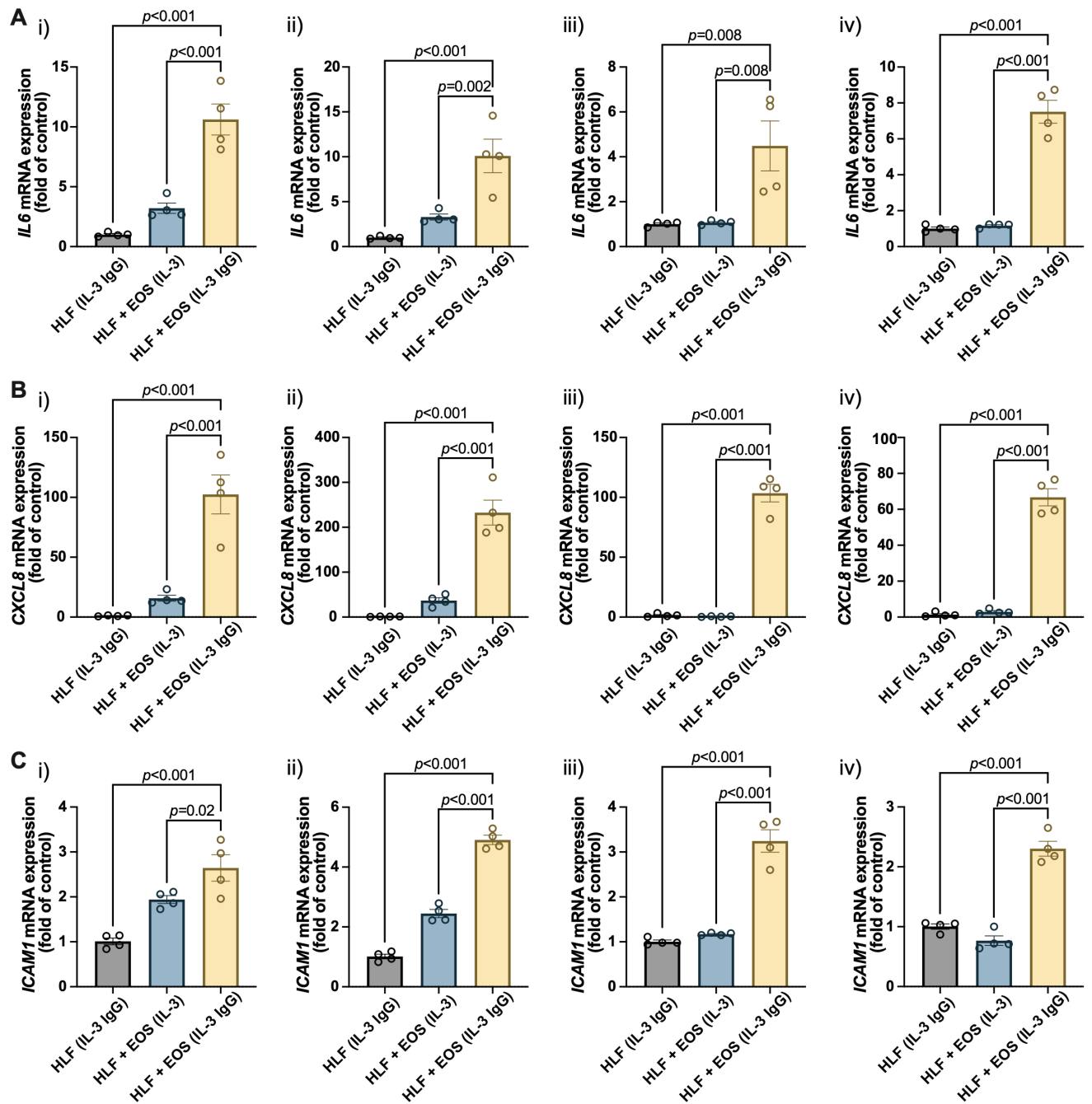


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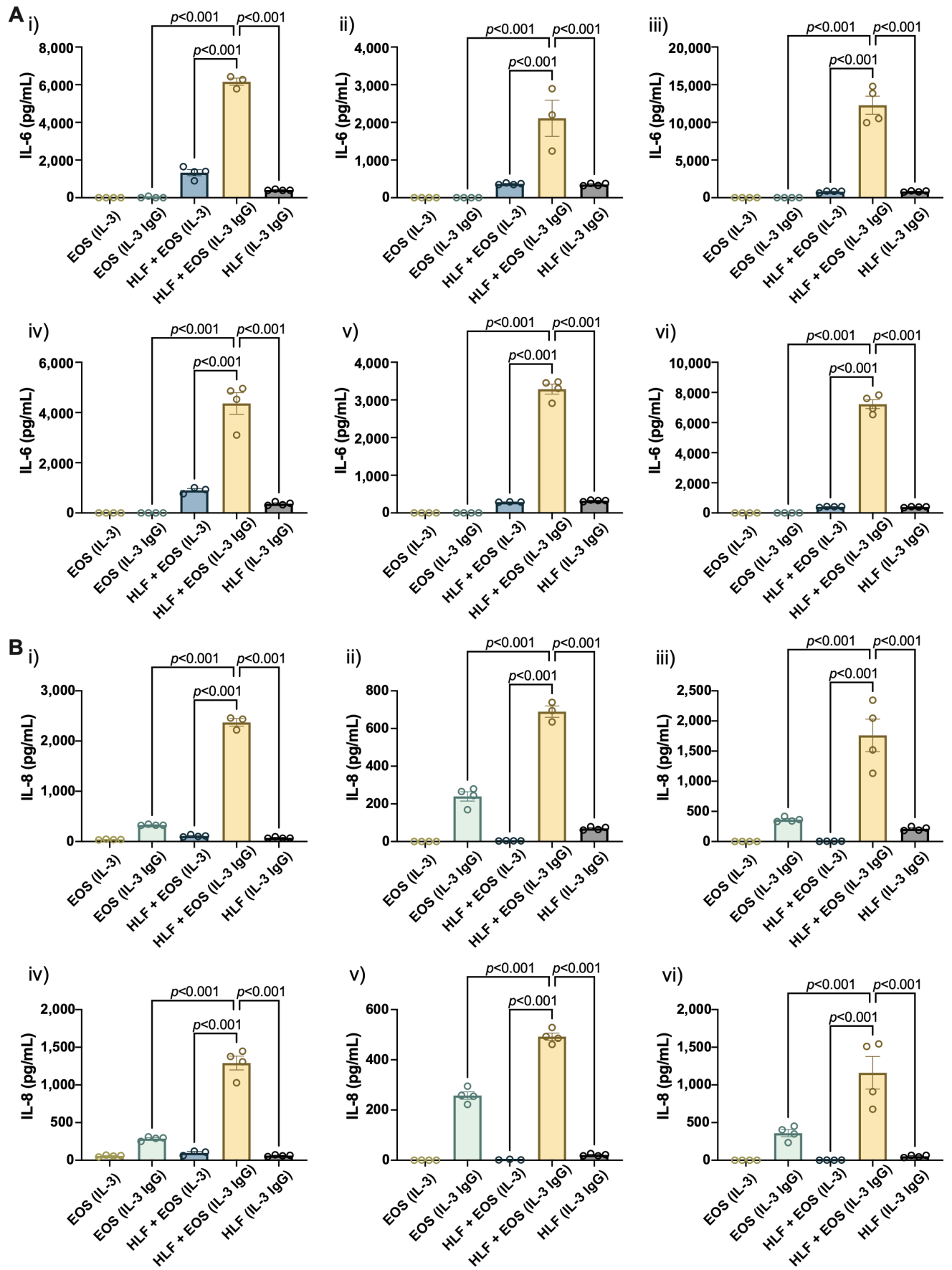
Supplementary Figure S2.5. Phase contrast images of HLFs in coculture devices at the indicated times. Representative images from 2 donor pairs are shown: **(A)** eosinophil donor 2 and HLF donor 1, **(B)** eosinophil donor 2 and HLF donor 2. The chambers remained separated for 1.5 h and then were connected for up to 72 h. Images are representative of 4 culture replicates, as well as 3 eosinophil donors and 2 HLF donors.



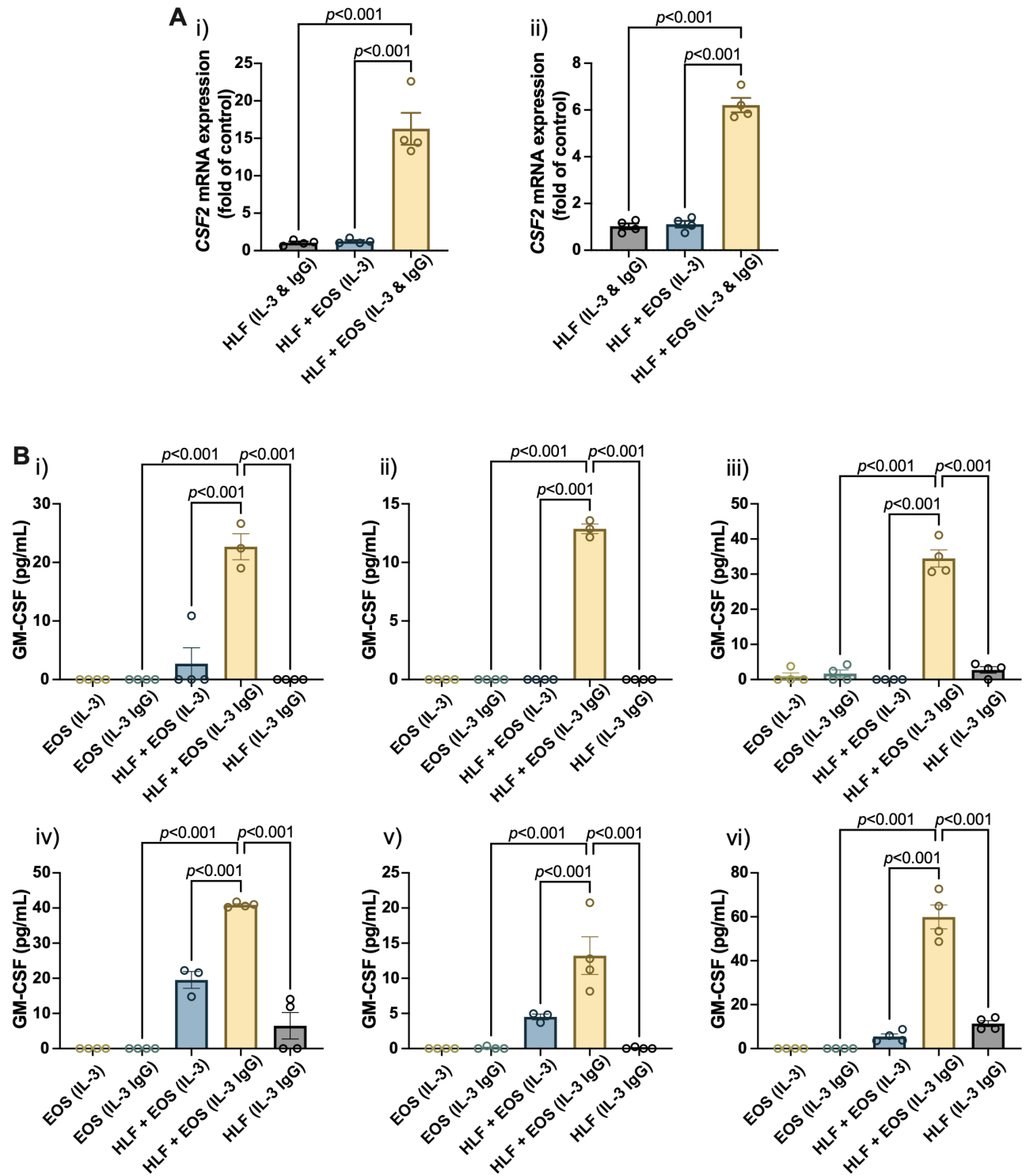
Supplementary Figure 6. Levels of released eosinophil-derived neurotoxin (EDN) in culture media after 24 h measured by ELISA. Eosinophils release the highest level of EDN under IL-3 IgG conditions regardless of coculture or monoculture. Results from 4 donor pairs are shown: **(A)** eosinophil donor 1 and HLF donor 1, **(B)** eosinophil donor 1 and HLF donor 2, **(C)** eosinophil donor 3 and HLF donor 1, **(D)** eosinophil donor 3 and HLF donor 2. The 2 donor pairs with eosinophil donor 2 were excluded due to protocol differences in ELISA. Data are expressed as mean $\pm$ SEM (n=4); each point is one culture replicate. Data were analyzed using one-way ANOVA, followed by Holm-Sídák's multiple comparisons test.



Supplementary Figure S2.7. Proinflammatory genes are upregulated in HLFs cocultured with degranulating eosinophils. HLFs in coculture with degranulating eosinophils (“HLF + EOS (IL-3 IgG)”) for 72 h showed the highest mRNA level upregulation of (A) IL6, (B) CXCL8, and (C) ICAM1. Results from 4 donor pairs are shown: (i) eosinophil donor 1 and HLF donor 1, (ii) eosinophil donor 1 and HLF donor 2, (iii) eosinophil donor 2 and HLF donor 1, (iv) eosinophil donor 2 and HLF donor 2. Data from the 2 donor pairs with eosinophil donor 3 were not included because the samples were used to develop a separate readout. Data are expressed as mean $\pm$ SEM ($n=4$); each point is one culture replicate. Data were analyzed using one-way ANOVA, followed by Holm-Šidák's multiple comparisons test.



Supplementary Figure S2.8. The coculture of HLFs with degranulating eosinophils releases the highest levels of proinflammatory cytokines compared to HLF and eosinophil monocultures and other control conditions. Levels of soluble factors, **(A)** IL-6 and **(B)** IL-8, were measured in the conditioned media after 72 h in culture. Results from 6 donor pairs are shown: **(i)** eosinophil donor 1 and HLF donor 1, **(ii)** eosinophil donor 2 and HLF donor 1, **(iii)** eosinophil donor 3 and HLF donor 1, **(iv)** eosinophil donor 1 and HLF donor 2, **(v)** eosinophil donor 2 and HLF donor 2, **(vi)** eosinophil donor 3 and HLF donor 2. Data are expressed as mean $\pm$ SEM (n=3-4); each point is one culture replicate. Data were analyzed using one-way ANOVA, followed by Holm-Šídák's multiple comparisons test.



Supplementary Figure S2.9. The coculture of HLFs with degranulating eosinophils induces the highest levels of expression and secretion of *CSF2*/GM-CSF than other control conditions. **(A)** mRNA levels of *CSF2* in HLFs after 72 h in culture. Results from 2 donor pairs are shown: **(i)** eosinophil donor 2 and HLF donor 1, **(ii)** eosinophil donor 2 and HLF donor 2. Data from the 4 donor pairs with eosinophil donors 1 and 3 were not included, because the samples were limited and used up to develop a separate readout. **(B)** Levels of GM-CSF measured in the conditioned

media after 72 h in culture. Results from 6 donor pairs are shown: **(i)** eosinophil donor 1 and HLF donor 1, **(ii)** eosinophil donor 2 and HLF donor 1, **(iii)** eosinophil donor 3 and HLF donor 1, **(iv)** eosinophil donor 1 and HLF donor 2, **(v)** eosinophil donor 2 and HLF donor 2, **(vi)** eosinophil donor 3 and HLF donor 2. Data are expressed as mean $\pm$ SEM (n=3-4); each point is one culture replicate. Data were analyzed using one-way ANOVA, followed by Holm-Šidák's multiple comparisons test.

2. Supplementary Tables

Supplementary Table S2.1. Calculation of eosinophil seeding densities in 96-well plates (Esnault et al., 2017) or outer chambers of the microfluidic coculture device. The surface area seeding density was kept consistent in both cultureware.

	96-well plate	Device outer chamber
Media volume	100 μ L	36 μ L
Seeding area	32 mm ²	29.3 mm ²
Volume density	1×10^6 cells/mL	2.6×10^6 cells/mL
Area density	3125 cells/mm ²	
Total cell number per well	1×10^5 cells	9.2×10^4 cells

Supplementary Table S2.2. Forward and reverse sequences of primers used in reverse transcription quantitative polymerase chain reaction (RT-qPCR). *GUSB*: glucuronidase β . *IL6*: interleukin 6. *CXCL8*: C-X-C motif chemokine ligand 8. *ICAM1*: intercellular adhesion molecule 1. *CSF2*: colony stimulating factor 2.

Primer	Forward	Reverse
<i>GUSB</i>	CAGGACCTGCGCACAAGAG	AGCGTGTCGACCCCATTC
<i>IL6</i>	TGCAGATGAGTACAAAAGTCCTGAT	GTGGTTATTGCATCTAGATTCTTTGC
<i>CXCL8</i>	CTTGCGAGCCTTCCTGATTT	TTCTTTAGCACTCCTTGGCAAAA
<i>ICAM1</i>	GCCAGGAGACACTGCAGACA	TGGCTTCGTCAGAATCACGTT
<i>CSF2</i>	TGAATGAAACAGTAGAAGTCATCTCAGAAAT	GCTCCAGGCGGGTCTGTA

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B. Supplementary information for Chapter 3

1. Supplementary Methods

Open microfluidic co-culture model description

The dual chamber open microfluidic co-culture device comprises two polystyrene pieces that are milled using a computer numerical control (CNC) mill (Datron Neo) and then solvent bonded together (**Figure 3.1a**). The fabrication, surface treatment, and sterilization procedures prior to cell culture were performed as we previously reported ^{1,2}. The geometry of the chambers allows them to be loaded with a standard pipette; by anchoring the pipette tip at the notch of each chamber, liquid can fill the entire chamber with a single dispensing step due to the spontaneous capillary flow ³. A chip containing an array of chambers is designed to fit in a standard 10-cm Petri dish (**Figure 3.1b**), which is used as a primary container for culture sterility and evaporation control during incubation and live-cell imaging.

Mass transport efficiency across the chambers was tested by embedding 40 kDa FITC-labeled dextran in a thin layer of collagen I (3 mg/ml) to achieve a concentration of 25 mg/ml (**Figure 3.1c**). Twenty microliters of dextran-collagen mixture was gelled at the bottom of the outer chamber for 30 min at 37°C to mimic the molecules secreted from a monolayer of cells cultured in the outer chamber (source chamber). At t_0 , the inner and outer chambers were connected by adding 80 ml of PBS which flooded the half wall and initiated simulated co-culture conditions. The co-culture was kept in an incubator at 37°C for the desired lengths of time, during which the dextran molecules began to diffuse out of the collagen layer, into the aqueous solution, and across the half wall. At $t_f = 3$ minutes (min), 3, 7, 15, and 24 hours (h), as well as 10 days (d) 5 μ l samples of the aqueous solution were carefully taken from the center bottom of the inner chamber in each device as a representation of the level of soluble factors that receiving

cells cultured in the inner chamber could be exposed to. The samples were stored at -80°C until fluorescence intensities were measured using a SpectraMax M5 plate reader (Molecular Devices) with the Ex/Em wavelengths of fluorescein (494 nm/521 nm).

Primary human podocyte isolation

Deidentified human kidney tissue was obtained from Novabiosis or as previously described ⁴, in accordance with a protocol approved by the University of Washington Human Subjects Institutional Review Board (IRB STUDY00001297). Glomeruli were isolated through differential sieving. Specifically, minced kidney cortex was pressed and washed with sterile Dubelcco's Phosphate Buffered Saline (Corning, Corning, NY) through a 50 mesh (300µm opening) stainless steel sieve to dissociate the tissue and break glomeruli away from tubules. Glomeruli were captured on an 80 mesh (180µm opening) stainless steel sieve. Captured glomeruli were collected, pelleted by centrifugation at 200G and re-suspended in RPMI 1640 medium (w/o L-glutamine and phenol red) supplemented with 1mM sodium pyruvate (ThermoFisher Scientific, Waltham, MA), 9% Nu-Serum™ IV Growth Medium Supplement (Corning Incorporated - Life Sciences, Durham, NC) and 100U/ml Penicillin-Streptomycin (ThermoFisher Scientific, Waltham, MA). Glomeruli were plated onto bovine collagen type 1 (10 µg/cm²) (Santa Cruz Biotechnology, Dallas, TX) coated Primaria™ Petri dishes (Corning, Corning, NY) and placed at 37°C with 5%CO₂. Following 14 days of culturing, podocyte outgrowths were removed from plates with Accutase (ThermoFisher Scientific, Waltham, MA), and filtered through a 100 µm cell strainer (Sigma-Aldrich, St. Louis, MO) to remove remaining glomeruli. Podocytes were re-plated for expansion and subsequently cryopreserved in liquid nitrogen until use (Isolation A1B11406).

Injuring podocytes in the microfluidic device

Primary podocyte cultures were maintained in the conditions described above and used from passages 3–6. In the microfluidics devices, primary human podocytes were seeded (150 cells/mm²) in both the inner and outer chambers coated with bovine collagen I (10 µg/cm²) and allowed to grow to confluence. At t = 0 h, while the chambers were still separated, the podocytes in the outer chambers were injured with (i) a cytopathic anti-podocyte antibody (IgG; 10 mg/ml),⁵⁻¹¹ (ii) Adriamycin/doxorubicin (ADR; 2 µg/ml; Sigma-Aldrich D1515)^{12, 13}, and (iii) puromycin aminonucleoside (PAN; 50 µg/ml; Sigma-Aldrich SBR00017)¹⁴. During this time, podocytes in the inner chamber were incubated with podocyte media alone.

After 24 hours of injury, the three injury inducers were removed, fresh media was added, and both chambers were connected. Co-culture was maintained via the shared media for up to 72 hours without further media changes. The devices were kept in a primary container (i.e. a 10-cm petri dish), where 1 ml of sterile sacrificial liquid (water: PBS = 1:1) was added around the device chip to mitigate evaporation. The primary container was placed in a secondary container (i.e., 100 × 20 mm Petri Dish), in which the peripheral of the primary container was surrounded with sterile cotton balls soaked with water. The secondary container was then placed at the back of the bottom shelf of an incubator at 37°C and 5% CO₂.

Staining and fluorescence microscopy

Mitochondrial function assessment: Mitochondrial dysfunction¹⁵ and loss of focal adhesions¹⁶ are typical responses to podocyte injury. For mitochondria labeling, at the end of co-culture (t = 96 h), targeted and paracrine injured podocytes were washed with fresh media and then

incubated with MitoTracker[®] Red CMXRos (200 nM; Invitrogen, M7512) prepared in podocyte growth medium for 40 min at 37°C. After incubation, the cells were washed again with fresh media, fixed with 2% PFA + 4% sucrose solution for 20 min, and permeabilized with 0.1% Triton X-100 for 10 min at room temperature.

Focal adhesion and actin cytoskeleton measurements: For focal adhesion staining, the permeabilized cells were blocked with Background Buster (Innovex Biosciences, NB306) for 30 min and Rabbit IgG Fab Blocking (1:50 dilution in 2% BSA; Jackson ImmunoResearch, 011-000-007) for 30 min at room temperature. The cells were then incubated with the rabbit anti-paxillin antibody (1:200 dilution; abcam, ab32084) overnight at 4°C and the AF488 goat anti-rabbit antibody (1:200 dilution; Jackson, 111-545-144) for 60 min at room temperature. For F-actin and nuclei counterstaining, the cells were incubated with AF647 phalloidin (1:400 dilution; Invitrogen, A22287) for 30 min and DAPI (1:500 dilution; Sigma, 1023676001) for 10 min at room temperature. For imaging, the cells were mounted in SlowFase Diamond Antifade Mountant (Invitrogen, S36963), and the device chip was sealed with a 48 × 60 mm No. 1 glass coverslip (Gold Seal[®] Cover Glasses, thickness 0.13–0.17 mm, Thermo Scientific, 48 × 60-1-002G). Fluorescence images were acquired using an EVOS FL Auto Imaging System (Invitrogen AMAFD1000).

Bulk mRNA-sequencing and qRT-PCR

Total mRNA was harvested from podocytes in both chambers at different time points (**Figure 3.1f**) using the miRNeasy Micro kit (Qiagen, 217084) with on-column DNase I digestion according to the manufacturer's protocols. The yield and quality of the purified total RNA were measured using a Qubit High Sensitivity RNA kit (Invitrogen, Q32855) and a 2100

Bioanalyzer (Agilent Technologies), respectively. Bulk mRNA-sequencing (mRNA-seq) was performed by Psomagen on samples from $t = 24$ h (i.e., 0 h in co-culture) and 96 h (i.e., 72 h in co-culture). Sequencing libraries were prepared using the SMARTer Stranded Total RNA-Seq Kit v2 - Pico Input Mammalian (Takara Bio Inc.) and sequencing was performed using the NovaSeq6000 S4 (2x150bp) system (Illumina Inc.) The sequence data are deposited into the Gene Expression Omnibus (GEO) database (GSE255482) and are available online.

Validation of select genes from the RNAseq data was performed by qRT-PCR using samples from $t = 32$ h (i.e., 8 h of co-culture), 48 h (24 h in co-culture), and 96 h (72 h in co-culture). The reverse transcription reaction was performed using the High-Capacity RNA-to-cDNA™ Kit (Applied Biosystems, 4387406) per the manufacturer's instructions. Gene expression levels were determined by qPCR using iTaq Universal SYBR Green Supermix (Bio-Rad, 1725124) in a QuantStudio 6 Flex Real-Time PCR System (Applied Biosystems, 4485691). QuantiTect Primer Assays (Qiagen, 249900) were utilized for *BMP2*, *DUSP4*, *FBXO32*, *E2F7*, *NRP1*, *PDK4*, and *18S* genes. Relative mRNA expression levels were determined using the 18S as internal control.

Bioinformatic methodologies for RNA-sequencing

The fastq files for the bulk mRNA-sequencing data were trimmed using TrimGalore (v0.6.10), aligned to GRCh38 using STAR¹⁷ and gene-level read counts were obtained using featureCounts¹⁸. The data were analyzed with Principal Component Analysis (PCA) using pcaExplorer (v2.26.1)¹⁹. Gene Set Enrichment Analysis (GSEA) was used to identify normalized enrichment scores of 50 human hallmark gene sets²⁰. DESeq2 was used to identify differentially expressed genes (DEGs) and calculate their fold changes (FC), p-values, and false discovery rate (FDR)-adjusted p-values²¹.

To determine putative paracrine signaling loops the DEG-based method CellphoneDB (v3.0.0) ²² was used to analyze the normalized counts for ligand-receptor pairs. The comparisons were performed separately between each condition for targeted injured podocytes at 96 h versus paracrine injured podocytes at 96 h as well as targeted injured podocytes at 24 h versus paracrine injured podocytes at 96 h. The DEG list was based on the output of the DESeq2 analysis with a cutoff of a p-value of ≤ 0.05 , $FC \geq 1$, and counts ≥ 10 . The CellphoneDB results were uploaded to InterCellar (v2.6.0) ²³ for data interpretation and visualization. InterCellar generated a table of all the ligand-receptor interactions that was then filtered to include only curated data and signaling from the targeted injured podocytes focusing on paracrine signaling events.

Interrogation of human kidney injury data

The Nephrotic Syndrome Study Network (NEPTUNE) tranSMART ²⁴ was utilized to align selected data from the co-culture studies with transcriptomic data of patients with glomerular disease. Gene expression data from all available patients across disease states was utilized and further subset to include only glomerular RNA-seq. Using the advanced workflow within tranSMART, box plots with ANOVA were generated for select genes with dependent variables of ‘Any glomerular epithelial injury’ on histology. As described on tranSMART, the ‘diseased/healthy’ dependent variable includes presence of ‘halo detachment from original GBM’, ‘hyaline protein droplets in epithelial cells’, ‘epithelial cell hypertrophy’, or ‘epithelial cell hyperplasia’.

Statistics

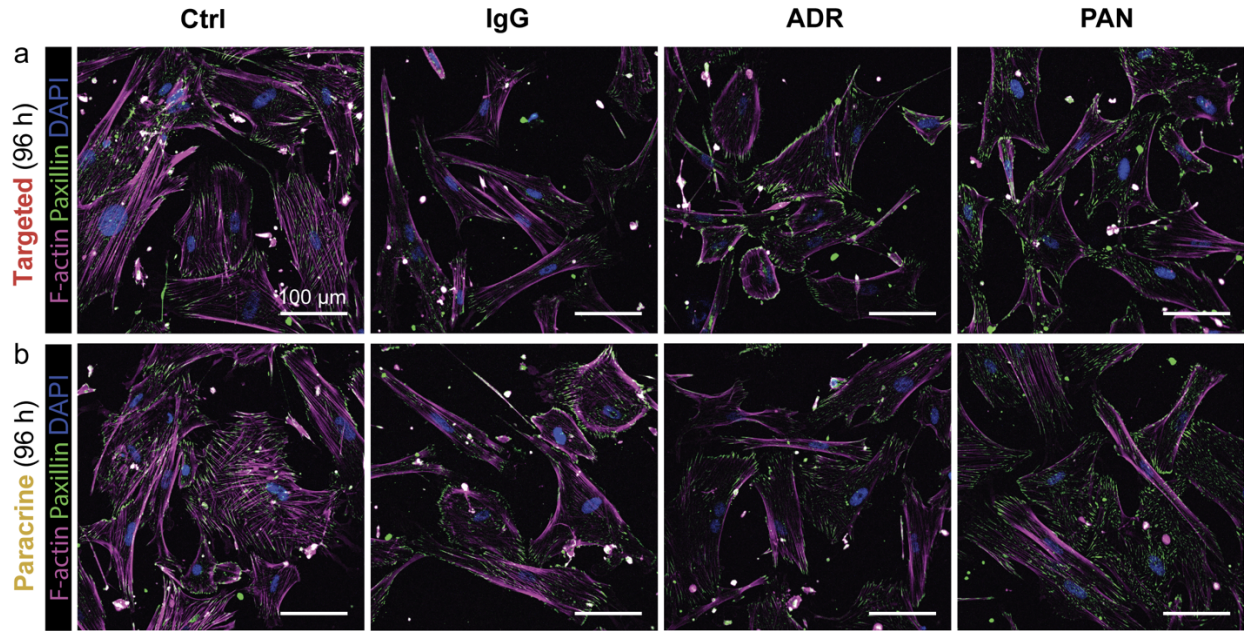
Data in bar graphs are expressed as the mean of the technical (i.e., culture) replicates $\pm$ the standard error of the mean (SEM) (n =3–5); each plotted point represents a culture replicate within the same device chip. Data were analyzed and visualized using GraphPad Prism 10 software (San Diego, CA). Differences among culture conditions were analyzed using ordinary one-way (or two-way) analysis of variance tests (one-way ANOVA or two-way ANOVA), followed by Holm-Šidák's multiple comparisons test to compare the means of pre-selected pairs of conditions. $P < 0.05$ was considered statistically significant.

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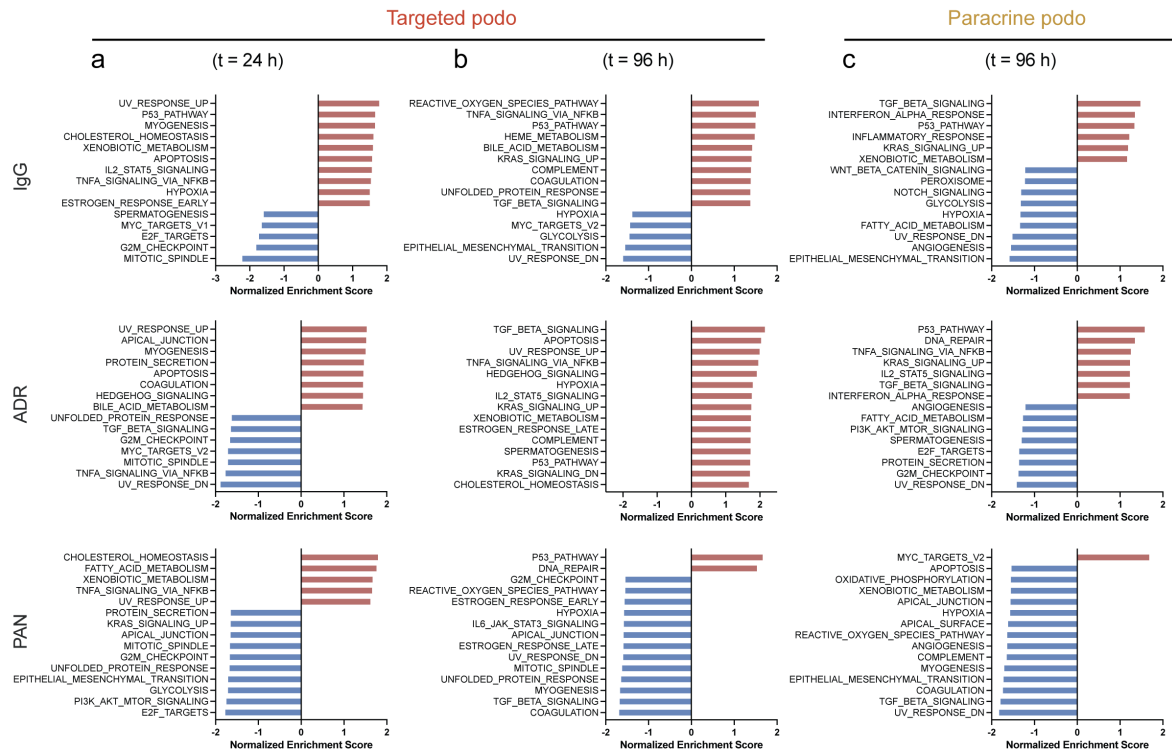
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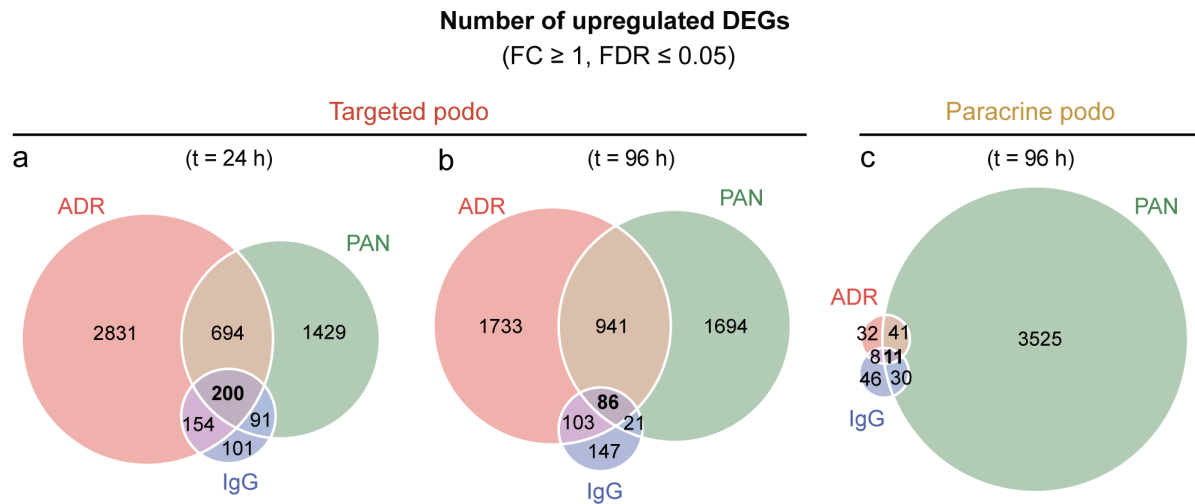
2. Supplementary Figures



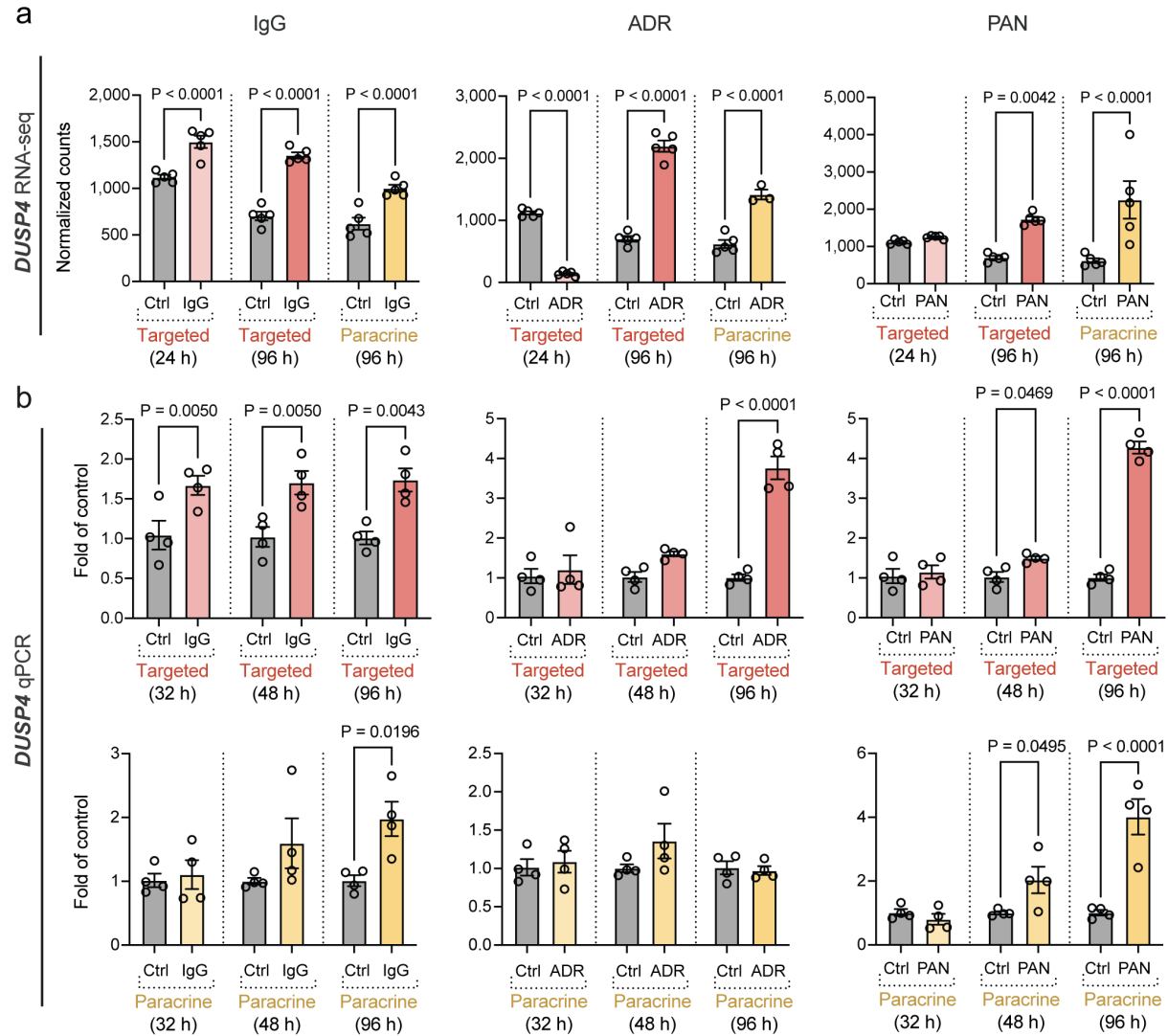
Supplementary Figure S3.1. Targeted and paracrine injured podocytes demonstrate actin cytoskeletal and focal adhesion remodeling. Representative merged channel images of F-actin, Paxillin, and DAPI staining with pseudo colors (cyan: F-actin, green: Paxillin, blue: DAPI) for targeted (a) and paracrine (b) injured podocytes.



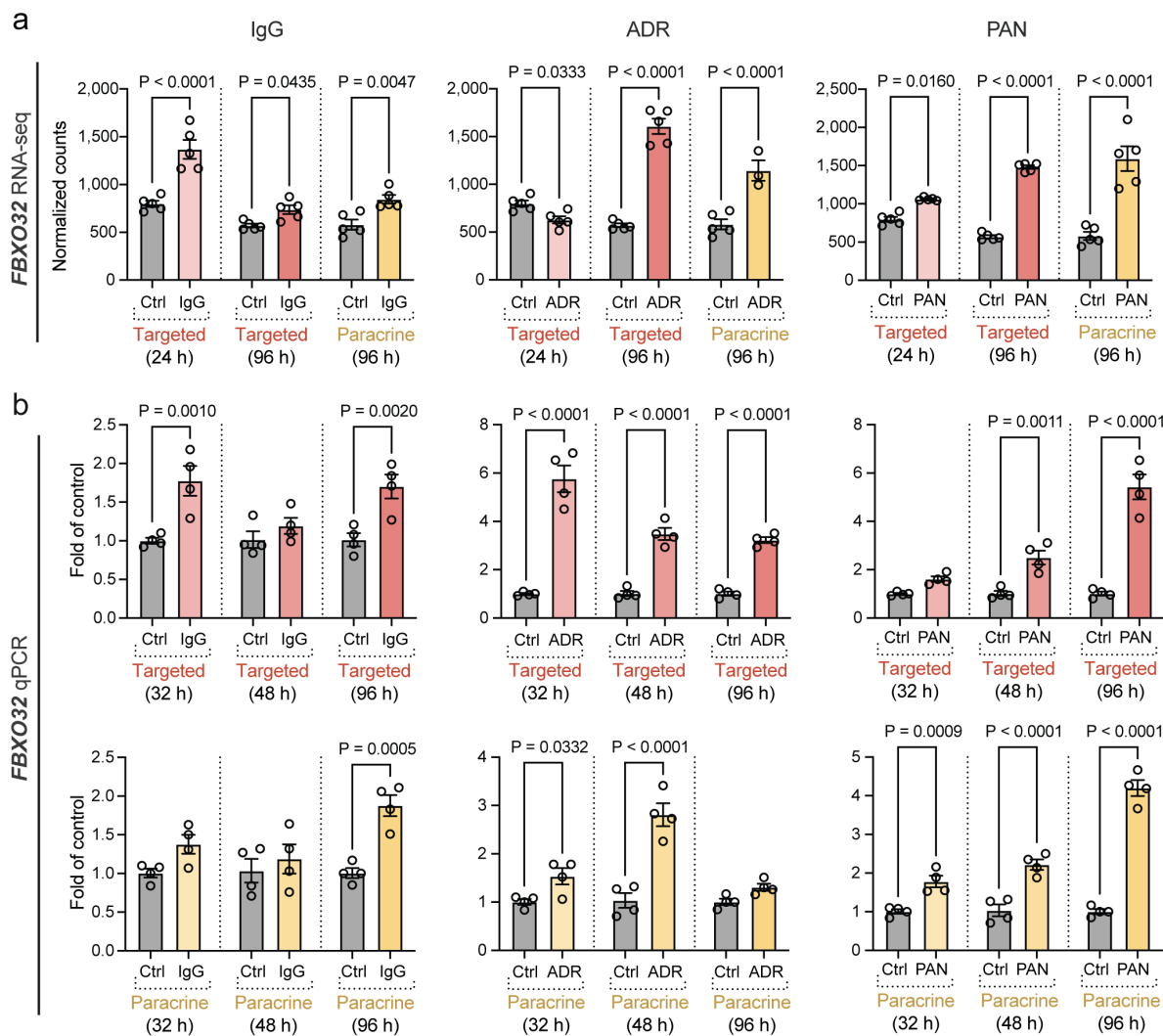
Supplementary Figure S3.2. *Top 15 enriched gene sets in podocytes with targeted and paracrine injuries by IgG, ADR, or PAN. Top 15 positively (red) or negatively (blue) enriched gene sets from Gene Set Enrichment Analysis in podocytes with targeted injuries at 24h (a) and 96h (b) and podocytes with paracrine injuries at 96h (c) after IgG (top), ADR (middle), or PAN (bottom) treatment versus controls.*



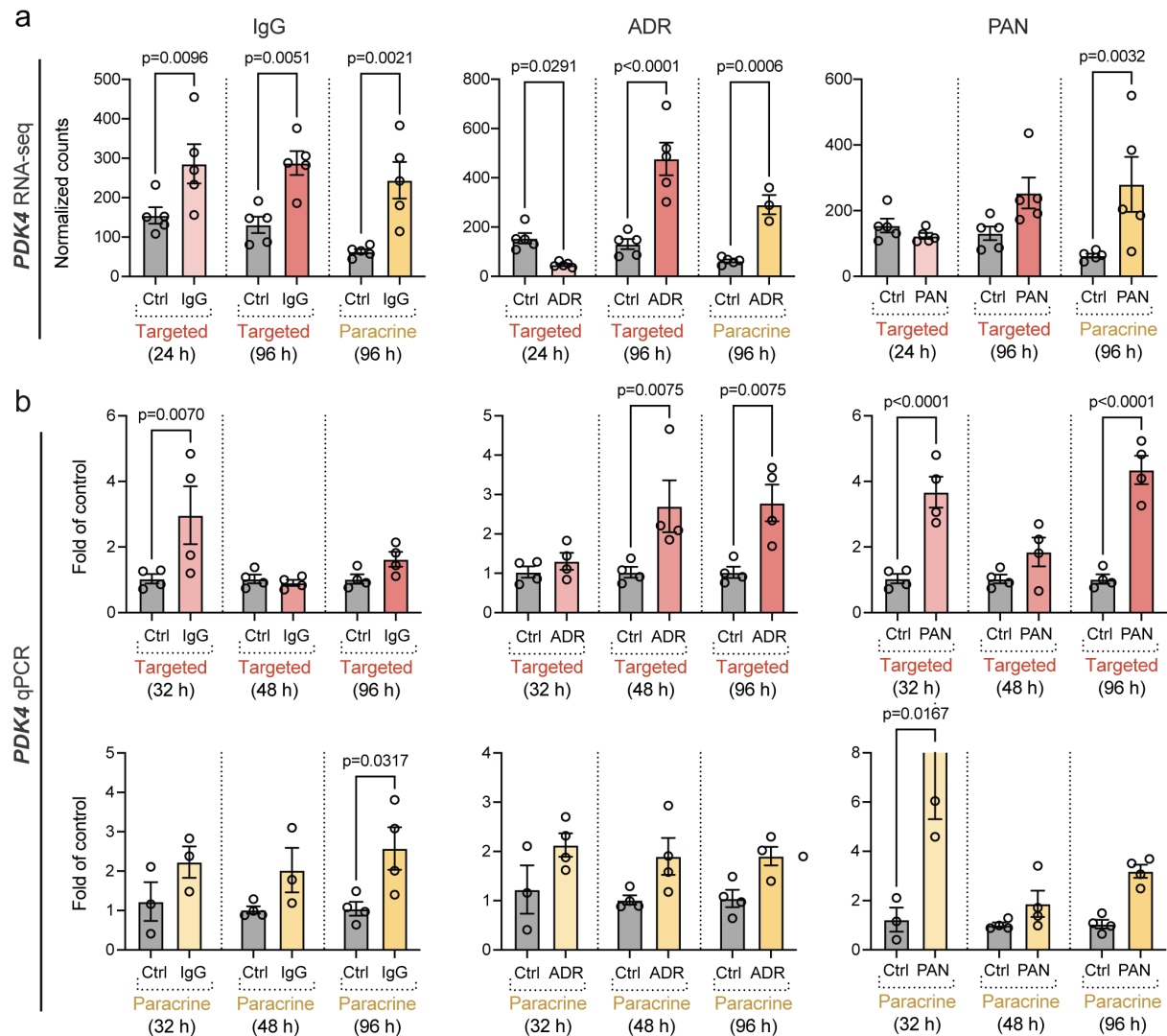
Supplementary Figure S3.3. *Differentially expressed genes (DEGs) in targeted and paracrine injured podocytes. Venn diagrams showing the number of upregulated DEGs in targeted injury at 24h (a) and 96h (b) and in paracrine podocytes at 96h (c) after ADR (pink), IgG (blue) and PAN (green) treatment versus controls, and the overlap between the different forms of injury. The upregulated DEGs are defined as a fold change (FC) ≥ 1 and a false discovery rate (FDR) ≤ 0.05 .*



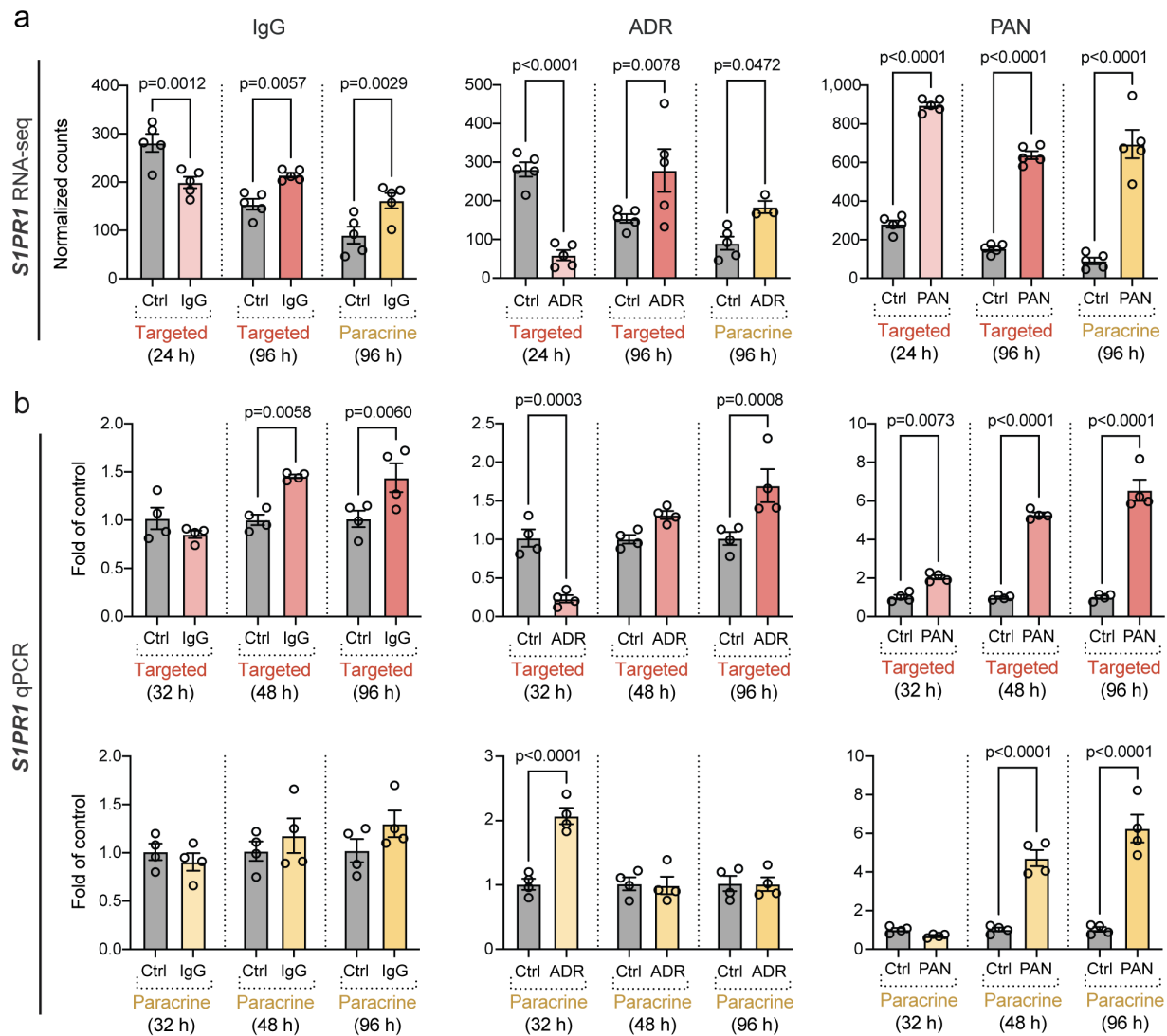
Supplementary Figure S3.4. RNA-seq expression of *DUSP4* was validated by RT-PCR. **(a)** Bulk RNAseq gene expression of *DUSP4* in targeted podocytes (t = 24 h and 96 h) and paracrine podocytes (t = 96 h) after IgG (left), ADR (middle), or PAN (right) treatments. **(b)** RT-PCR expression of *DUSP4* in targeted and paracrine injured podocytes at 32 h, 48 h, and 96 h.



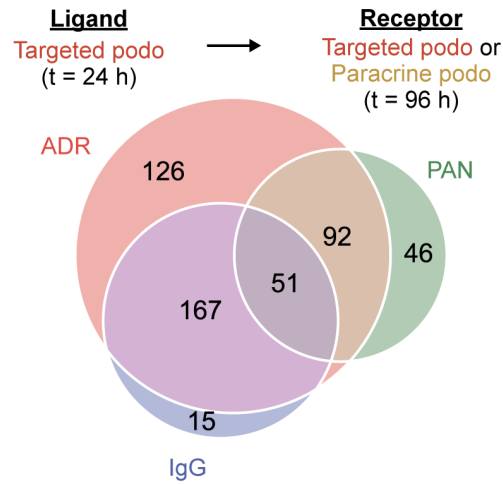
Supplementary Figure S3.5. RNA-seq expression of *FBXO32* was validated by RT-PCR. **(a)** Bulk RNAseq gene expression of *FBXO32* in targeted podocytes (t = 24 h and 96 h) and paracrine podocytes (t = 96 h) after IgG (left), ADR (middle), or PAN (right) treatments. **(b)** RT-PCR expression of *FBXO32* in targeted and paracrine injured podocytes at 32 h, 48 h, and 96 h.



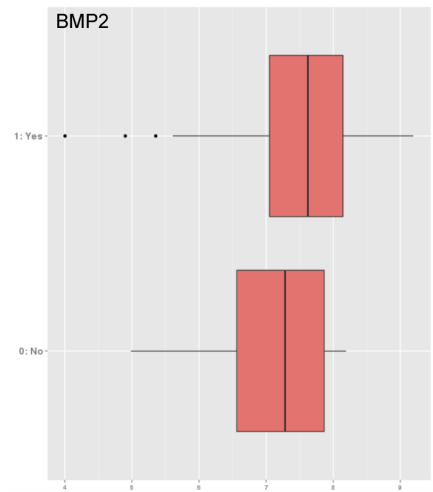
Supplementary Figure S3.6. RNA-seq expression of *PDK4* was validated by RT-PCR. **(a)** Bulk RNAseq gene expression of *PDK4* in targeted podocytes (t = 24 h and 96 h) and paracrine podocytes (t = 96 h) after IgG (left), ADR (middle), or PAN (right) treatments. **(b)** RT-PCR expression of *PDK4* in targeted and paracrine injured podocytes at 32 h, 48 h, and 96 h.



Supplementary Figure S3.7. RNA-seq expression of *S1PR1* was validated by RT-PCR. **(a)** Bulk RNAseq gene expression of *S1PR1* in targeted podocytes (t = 24 h and 96 h) and paracrine podocytes (t = 96 h) after IgG (left), ADR (middle), or PAN (right) treatments. **(b)** RT-PCR expression of *S1PR1* in targeted and paracrine injured podocytes at 32 h, 48 h, and 96 h.



Supplementary Figure S3.8. *Enriched ligand-receptor pairs in podocyte paracrine injury signaling.* A Venn diagram summarizing the number of enriched ligand-receptor pairs in IgG (blue), ADR (pink), and PAN (green)-induced injuries, defined as the ligand gene in the targeted podocytes at 24 h or the receptor gene in the paracrine podocytes at 96 h is significantly upregulated compared to their healthy controls.



ANOVA Result

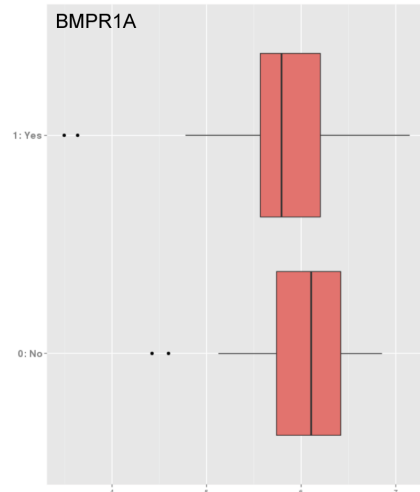
Group	650_at_BMP2
p-value	0.0192
F value	5.66

650_at_BMP2		
Group	Mean	n
0: No	7.07	34
1: Yes	7.53	70

Pairwise t-Test p-Values

650_at_BMP2

	0: No
1: Yes	0.0192



ANOVA Result

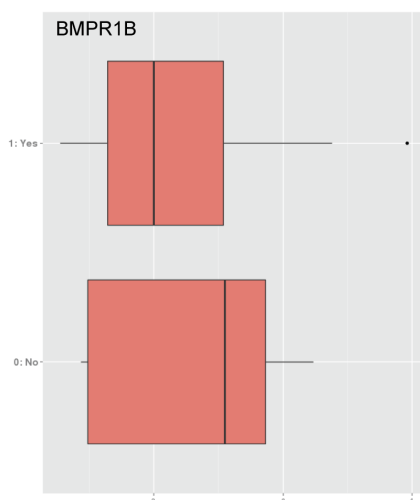
Group	657_at_BMPR1A
p-value	0.11
F value	2.59

657_at_BMPR1A		
Group	Mean	n
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1: Yes	5.8	70

Pairwise t-Test p-Values

657_at_BMPR1A

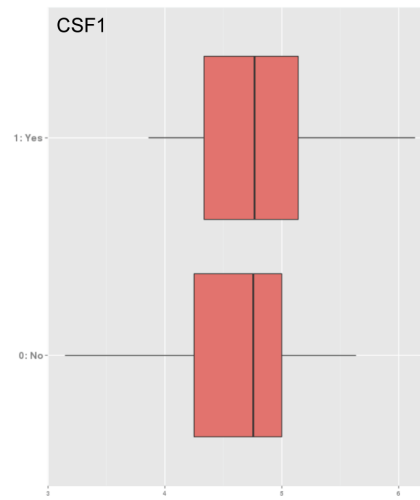
	0: No
1: Yes	0.11



Pairwise t-Test p-Values

658_at_BMPR1B

	0: No
1: Yes	0.647



ANOVA Result

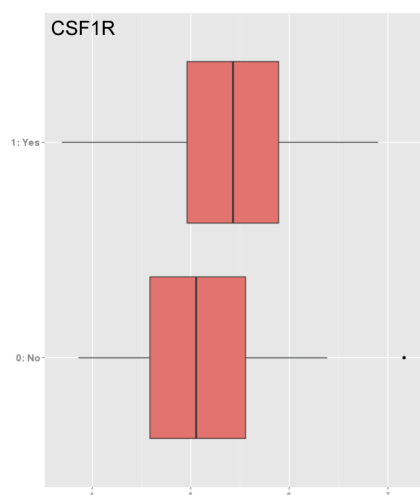
Group	1435_at_CSF1
p-value	0.127
F value	2.37

1435_at_CSF1		
Group	Mean	n
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1: Yes	4.79	70

Pairwise t-Test p-Values

1435_at_CSF1

	0: No
1: Yes	0.127



ANOVA Result

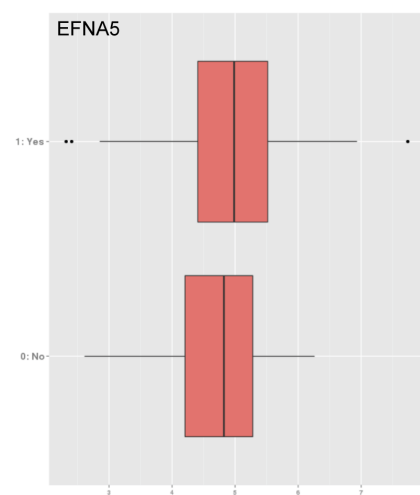
Group	1436_at_CSF1R
p-value	0.0616
F value	3.57

1436_at_CSF1R		
Group	Mean	n
0: No	5.07	34
1: Yes	5.36	70

Pairwise t-Test p-Values

1436_at_CSF1R

	0: No
1: Yes	0.0616



ANOVA Result

Group	1946_at_EFNA5
p-value	0.243
F value	1.38

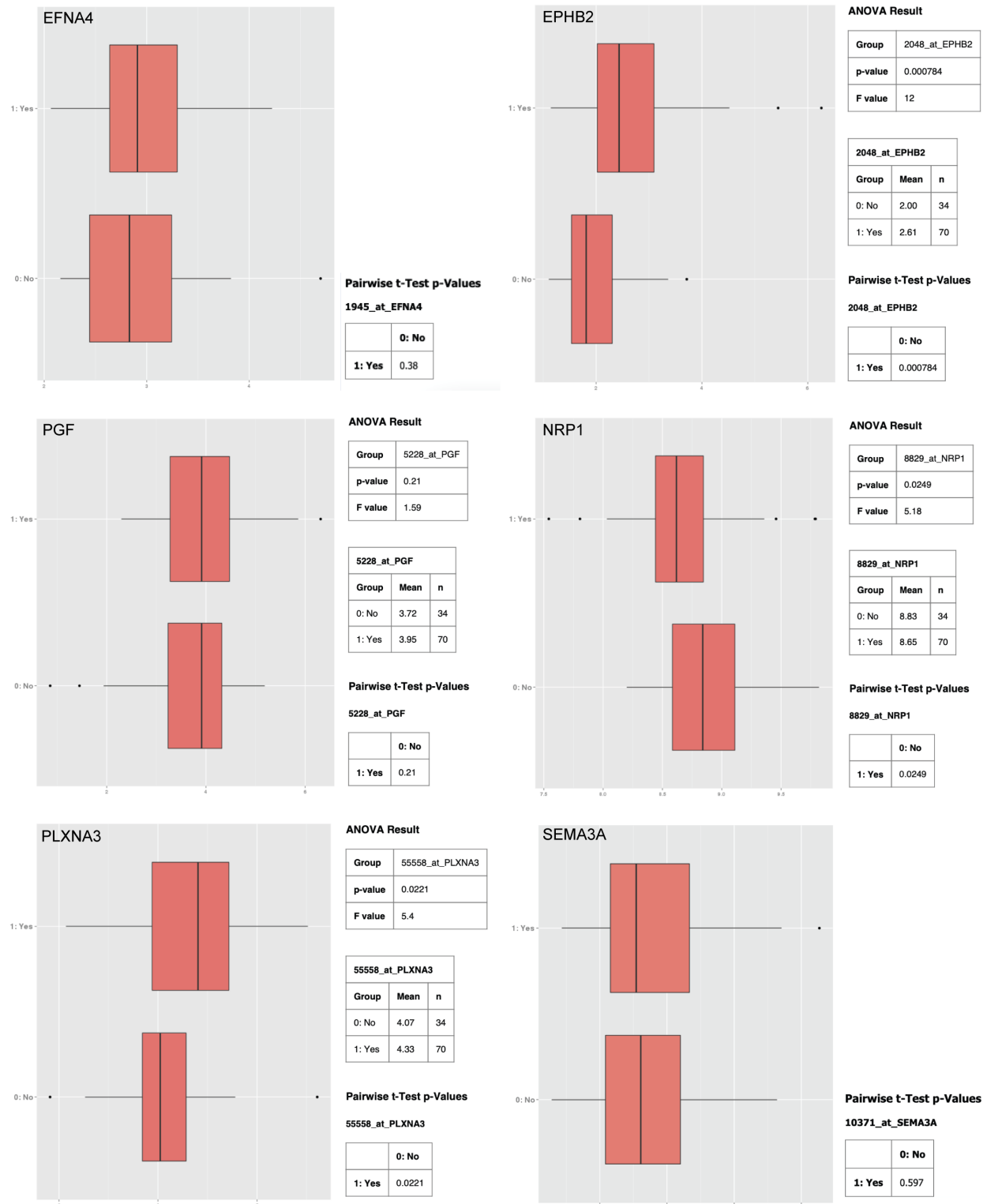
1946_at_EFNA5		
Group	Mean	n
0: No	4.74	34
1: Yes	4.97	70

Pairwise t-Test p-Values

1946_at_EFNA5

	0: No
1: Yes	0.243

Supplementary Figure S3.9. *Gene expression of ligand-receptor pairs from NEPTUNE.* Box plots generated in NEPTUNE tranSMART for the transcriptomic expression of *BMP2*, *BMPRI1A*, *BMPRI1B*, *CSF1*, *CSF1R*, *EFNA5*. The ‘Yes/no’ refers to the presence of ‘Any glomerular epithelial injury’ on histology, including ‘halo detachment from original GBM’, ‘hyaline protein droplets in epithelial cells’, ‘epithelial cell hypertrophy’, or ‘epithelial cell hyperplasia’.



Supplementary Figure S3.10. *Gene expression of ligand-receptor pairs from NEPTUNE (continued).* Box plots generated in NEPTUNE tranSMART for the transcriptomic expression of *EFNA4*, *EPHB2*, *PGF*, *NRP1*, *PLXNA3*, *SEMA3A*. The ‘Yes/no’ refers to the presence of ‘Any glomerular epithelial injury’ on histology, including ‘halo detachment from original GBM’, ‘hyaline protein droplets in epithelial cells’, ‘epithelial cell hypertrophy’, or ‘epithelial cell hyperplasia’.

3. Supplementary Tables

Supplementary Tables S3.1-3.5 are spreadsheets attached separated to this dissertation.

Supplementary Table S3.1. The normalized gene counts, p-values, FDR, and FC of the 89, 28, and 4 overlapped DEGs among IgG, ADR, and PAN treatment in podocytes with targeted (direct) injury at 24h or 96h, and podocytes with paracrine (indirect) injury at 96h. The list of DEGs was based on the output of the DESeq2 analysis with a cutoff of an adjusted p-value (padj) of ≤ 0.05 , fold change (FC) ≥ 1.5 , and counts ≥ 10 .

Supplementary Table S3.2. The normalized gene counts, p-values, FDR, and FC of the 200, 86, and 11 overlapped DEGs among IgG, ADR, or PAN treatment in podocytes with targeted (direct) injury at 24h or 96h, and podocytes with paracrine (indirect) injury at 96h. The list of DEGs was based on the output of the DESeq2 analysis with a cutoff of an adjusted p-value (padj) of ≤ 0.05 , fold change (FC) ≥ 1 , and counts ≥ 10 .

Supplementary Table S3.3. A list of the 67 overlapping enriched ligand-receptor pairs after IgG, ADR, or PAN treatment, filtered based on the output of InterCellar with ligands expressed in the podocytes with targeted injury at t = 96 h and receptors expressed in the targeted or paracrine podocytes at t = 96 h.

Supplementary Table S3.4. A list of the 51 overlapping enriched ligand-receptor pairs after IgG, ADR, or PAN treatment, filtered based on the output of InterCellar with ligands expressed in the podocytes with targeted injury at t = 24 h and receptors expressed in the targeted or paracrine podocytes at t = 96 h.

Supplementary Table S3.5. A list of the 19 overlapping and consistently enriched ligand-receptor pairs after IgG, ADR, or PAN treatment from t = 24 h to t = 96 h.

Supplementary Table S3.6. A summarized list of the consistently upregulated signaling molecules in podocyte paracrine signaling after IgG, ADR, or PAN treatment and their corresponding ligand and receptor genes.

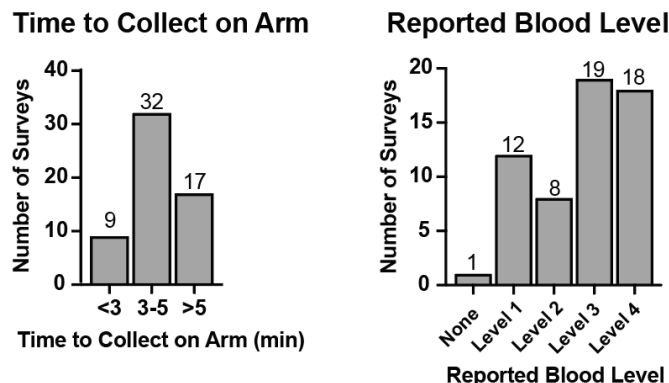
Signaling molecules	Ligand(s)	Receptor(s)
Angiopoietins	ANGPT1, ANGPT2, and ANGPT4	TEK
Bone morphogenetic proteins	BMP2	BMPR1A-BMPR2, BMPR1B-BMPR2, BMPR1A-ACVR2A, and BMPR1B-ACVR2A
Integrins	COL7A1	ITGA1-ITGB1, ITGA2-ITGB1, ITGA10-ITGB1, and ITGA11-ITGB1

Colony stimulating factors	CSF1	CSF1R
Ephrins	EFNA5	EPHA4 and EPHB2
Insulin like growth factors	IGF1	ITGA6-ITGB4
Interleukins	IL13	IL13RA1-IL4R
Placental growth factors	PGF	NRP1
Semaphorins	SEMA3A	NRP1-PLXNA3
Transforming growth factor beta	TGFβ1	TGFβR1-TGFβR2

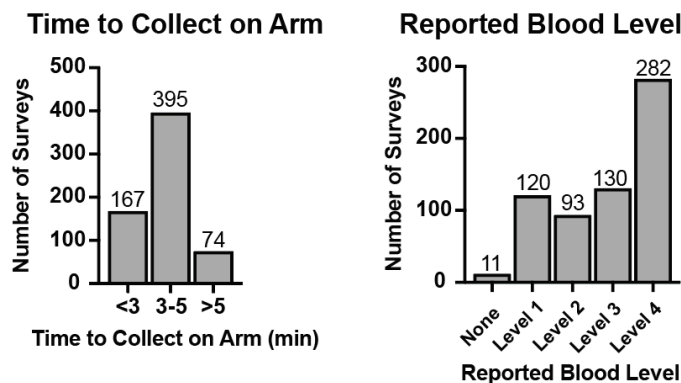
C. Supplementary information for Chapter 4

1. Supplementary Figures

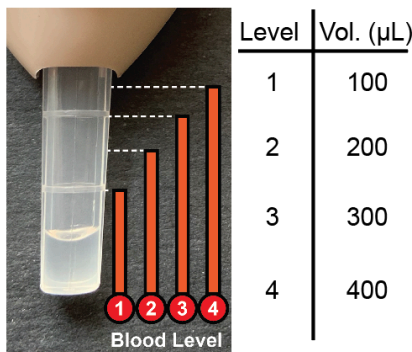
A) First Collected Samples (n=58 surveys)



B) All Collected Samples (n=636 surveys)

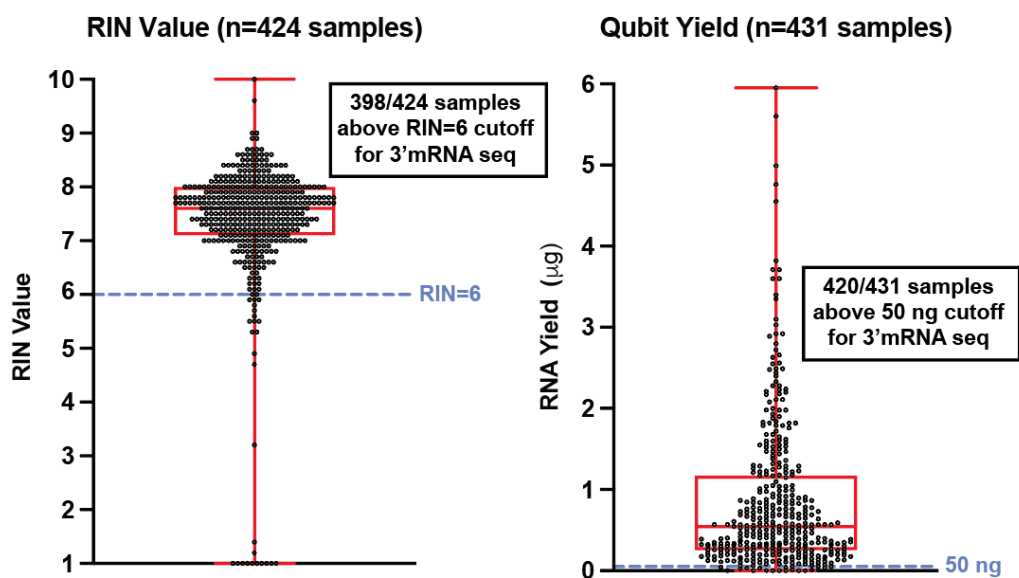


C) Blood Collection Volume

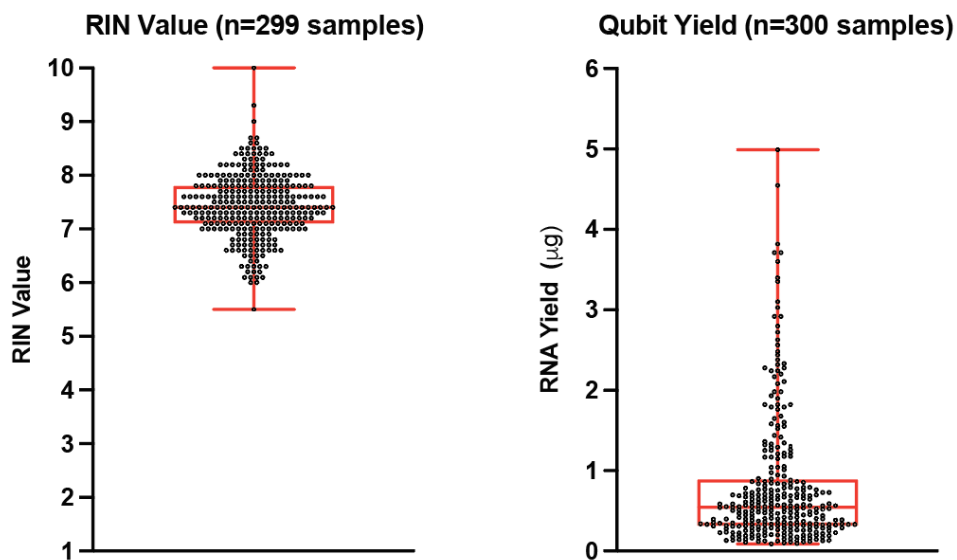


Supplementary Figure S4.1. Survey responses on the blood collection associated with the Tasso-SST device. Participant survey responses on the total time to collect blood on the upper arm with the Tasso-SST device (left) and the reported blood level collected (right) from **A)** first collected sample (n=58 surveys) and **B)** all collected samples (n=636 surveys) from 58 participants. **C)** Image of Tasso-SST blood collection device with approximate volume levels marked. Participants were asked to report blood level by eye after blood collection based on this image.

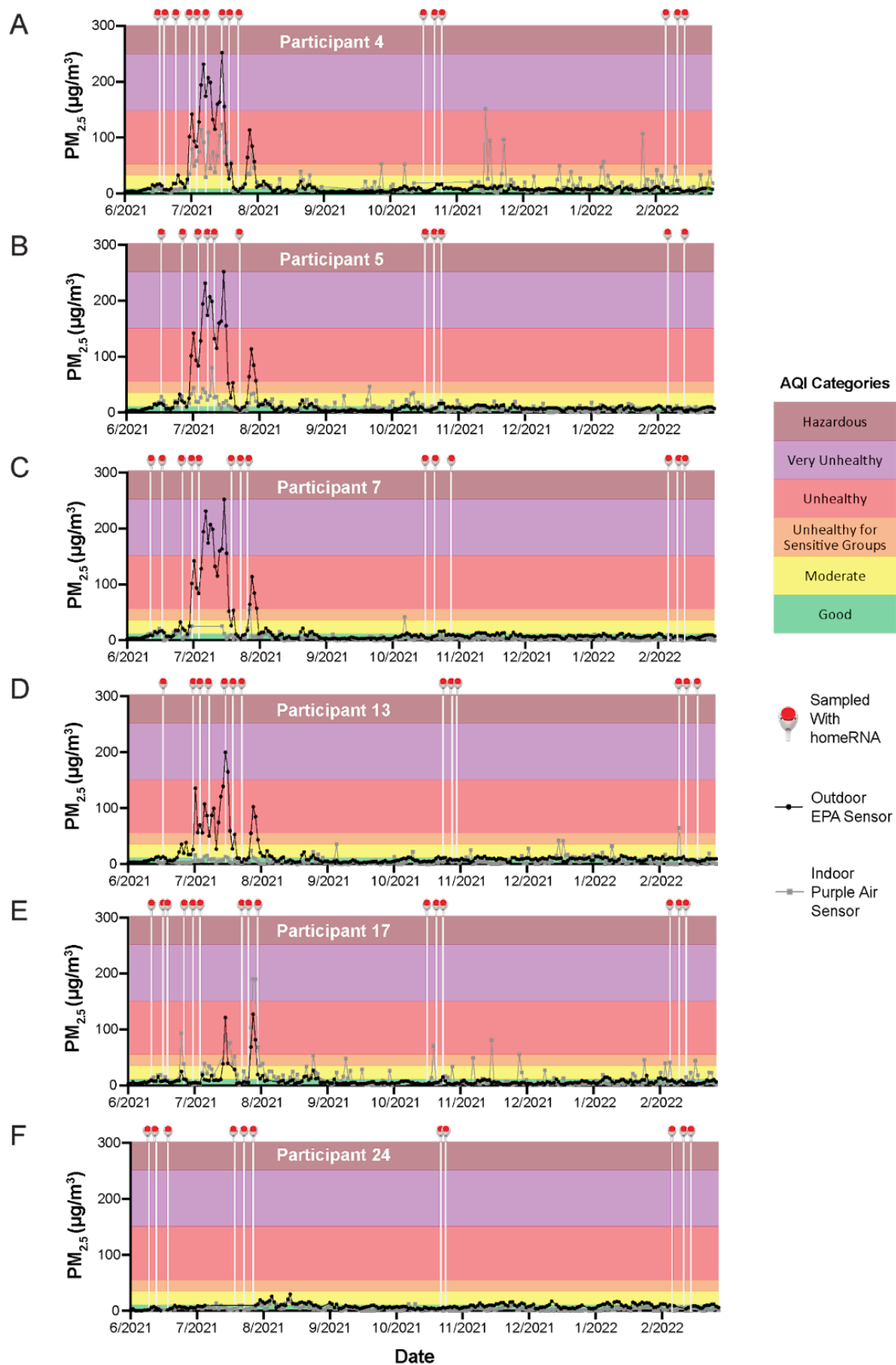
A) RNA Quality and Yield of Extracted Samples



B) RNA Quality and Yield of Analyzed Samples



Supplementary Figure S4.2. RNA quality and yield of homeRNA-stabilized blood samples. **A)** RNA Integrity Number (RIN) values of homeRNA-stabilized blood samples extracted during the study (left) with associated yields of the first elution aliquot (right). These graphs reflect the RINs and yields of the extracted samples without any processing, including cleanup or concentration steps. For analysis with 3' mRNA-sequencing, we set an internal quality control cutoff at RIN=6 with a minimum of 50 ng of RNA input. **B)** RIN values of a subset of extracted samples that were analyzed via 3' mRNA-sequencing (left) with associated yields (right). These graphs reflect any post-processing done to the samples, including DNase treatment and concentration steps. One sample analyzed did not have a RIN value associated with it after concentrating and one sample had a RIN of 5.5 below our internal quality control cutoff after DNase treatment.



Supplementary Figure S4.3. PM_{2.5} data for all analyzed participants across the study period. Data from example participants exposed to high (**A-D**), medium (**E**), or low (**F**) levels of wildfire smoke, plotted against the homeRNA sampling timepoints (white vertical lines with Tasso symbols on top), outdoor EPA sensor (black lines with data points), indoor PurpleAir sensor (grey lines with data points), and AQI categories (colored background).

2. Supplementary Table

Supplementary Table: AQI Categories and PM_{2.5} Cutoffs as defined by the EPA updated in February 2024

AQI Category	PM _{2.5} (ug/m ³) Breakpoints for each AQI Category
Good	0.0 - 9.0
Moderate	9.1 - 35.4
Unhealthy for Sensitive Groups	35.5 - 55.4
Unhealthy	55.5 - 125.4
Very Unhealthy	125.5 - 225.4
Hazardous	≥225.5