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Effects of Bone Marrow-Derived Mesenchymal Stromal Cells on Gene Expression in Human Alveolar Type II Cells Exposed to TNF-α, IL-1β, and IFN-γ

Matthew Schwede[1]; Erin Wilfong[2]; Rachel L. Zemans[3,4]; Patty J. Lee[5]; Claudia dos Santos, MD[6,7,8]; Xiaohui Fang[9]; Michael A. Matthay[1,9,10]

Affiliations:

1. Department of Medicine, University of California, San Francisco, San Francisco, California, USA
2. Department of Medicine, Division of Allergy, Pulmonary and Critical Care Medicine, Vanderbilt University Medical Center, Nashville, Tennessee, USA
3. Division of Pulmonary and Critical Care Medicine and
4. Cellular and Molecular Biology Program, University of Michigan Medical School, Ann Arbor, Michigan, USA
5. Yale University School of Medicine, Section of Pulmonary, Critical Care, & Sleep Medicine, New Haven, Connecticut, USA
6. Interdepartmental Division of Critical Care Medicine and

7. Division of Respirology, Department of Medicine, St. Michael's Hospital, Toronto, Ontario, Canada
8. Li Ka Shing Knowledge Institute, Toronto, Ontario, Canada
9. Cardiovascular Research Institute, University of California, San Francisco, San Francisco, California, USA
10. Department of Anesthesia, University of California, San Francisco, San Francisco, California, USA

Corresponding author:
Matthew Schwede, MD
505 Parnassus Avenue
San Francisco, CA 94143
Phone: 415-476-1528
Fax: 415-353-1990
Email: Matthew.Schwede@ucsf.edu

Authors contributions:

Matthew Schwede: Conception and designed research, analyzed data, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript, approved final version of manuscript.

Erin Wilfong: Conceived and designed research, interpreted results of experiments, edited and revised manuscript, approved final version of manuscript.

Rachel L. Zemans: Conceived and designed research, interpreted results of experiments, edited and revised manuscript, approved final version of manuscript.

Patty J. Lee: Edited and revised manuscript, approved final version of manuscript.

Claudia dos Santos: Edited and revised manuscript, approved final version of manuscript.

Xiaohui Fang: Conceived and designed research, performed experiments, interpreted results of experiments, prepared figures, drafted manuscript, edited and revised manuscript, approved final version of manuscript.

Michael A. Matthay: Conceived and designed research, interpreted results of experiments, edited and revised manuscript, approved final version of manuscript.

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Abstract

The acute respiratory distress syndrome (ARDS) is common in critically ill patients and has a high mortality rate. Mesenchymal stromal cells (MSCs) have demonstrated therapeutic potential in animal models of ARDS, and their benefits occur in part through interactions with alveolar type II (ATII) cells. However, the effects that MSCs have on human ATII cells have not been well studied. Using previously published microarray data, we performed genome-wide differential gene expression analyses of human ATII cells that were 1) unstimulated, 2) exposed to proinflammatory cytokines (CytoMix), or 3) exposed to proinflammatory cytokines plus MSCs. Findings were validated by qPCR. Alveolar type II cells differentially expressed hundreds of genes when exposed either to

proinflammatory cytokines or proinflammatory cytokines plus MSCs. Stimulation with proinflammatory cytokines increased expression of inflammatory genes and down-regulated genes related to surfactant function and alveolar fluid clearance. Some of these changes, including expression of some cytokines and genes related to surfactant, were reversed by exposure to MSCs. In addition, MSCs induced up-regulation of other potentially beneficial genes, such as those related to extracellular matrix remodeling. We confirmed several of these gene expression changes by qPCR. Thus, ATII cells down-regulate genes associated with surfactant and alveolar fluid clearance when exposed to inflammatory cytokines, and mesenchymal stromal cells partially reverse many of these gene expression changes.

Introduction

The acute respiratory distress syndrome (ARDS) is common, affecting approximately 10% of adult intensive care unit patients worldwide (3). With an in-hospital mortality of almost 40% for ARDS (44) and a lack of disease-modifying therapies (27), developing new treatments is important (52).

Pathologically, ARDS is characterized by injury to the lung parenchyma, which is due in part to an immune response (27). Mesenchymal stromal cells (MSCs) have significant therapeutic potential in ARDS through their anti-inflammatory effects and barrier enhancing effects (27, 38), and they are currently the focus of clinical trials for ARDS , sepsis , and bronchopulmonary dysplasia . These self-renewing multipotent stem cells have shown benefits in several pre-clinical studies (27). MSCs reduce pulmonary edema and the inflammatory responses to endotoxin in mouse models of ARDS (17). They improve animal survival in part through the release of pro-resolving lipids (15) and anti-inflammatory proteins, such as the IL-1 receptor antagonist (40). MSCs are known to interact with several types of immune cells (32, 48, 59). Recently, they were also shown to interact with alveolar type II (ATII) cells (14, 15), attenuating the increased protein permeability and cytoskeletal changes that proinflammatory cytokines induce in cultured ATII cell monolayers (15). These effects were mediated by

MSC-dependent angiopoietin-1 secretion, suggesting a vascular-mediated mechanism by which they could reduce lung injury and pulmonary edema.

ATII cells make up approximately 2-5% of the alveolar surface area (24) and have several important functions. They produce surfactant, serve as progenitor cells for the alveolar epithelium (2) thus helping restore epithelial barriers (24), and play a key role in vectorial sodium-dependent transport of fluid to maintain dry airspaces (36, 37). Progenitor function is regulated by EGFR-KRAS (9), Wnt/β-catenin (39, 60), keratinocyte growth factor (KGF), and hepatocyte growth factor (HGF) (55) signaling. ATII cells also play a role in host defense through cytokine and chemokine expression (53) and by producing surfactant proteins A and D, which are collectins that opsonize microorganisms (18). However, ATII cell responses both to inflammatory environments and to MSCs are incompletely understood, and their response to MSCs has not been studied on a genome-wide level.

The objective of this study was to elucidate how MSCs affect epithelial cell biology in an inflammatory environment. Using previously published microarrays (14), we performed an unbiased, genome-wide exploratory analysis of human ATII cell gene expression in response to stimulation with proinflammatory cytokines in the presence or absence of MSCs. We first examined the gene expression of ATII cells exposed to CytoMix (a mixture of TNF-α, IL-1β, and IFN-γ), which models the proinflammatory edema fluid of ARDS (30). We also studied the gene expression of ATII cells co-cultured with human bone marrow-derived MSCs and stimulated with CytoMix. We confirmed many of the gene expression changes with qPCR.

In response to CytoMix, ATII cells up-regulated cytokines and down-regulated genes that code for proteins related to surfactant and alveolar fluid clearance. Exposure to MSCs plus CytoMix partially reversed some of the gene expression changes induced by CytoMix.

Materials and Methods

Cell cultures and Transwell system

The microarray data used in this study were from a previously described experiment of type II alveolar cells (ATII) and mesenchymal stromal cells (MSCs) (14).

The ATII cells for the microarray were isolated from cadaver human lung tissues of five adult males with no lung disease using previously published methods (15), and the ATII cells used for quantitative PCR (qPCR) validation were harvested from a different donor using the same methods. The lungs were harvested from brain-dead subjects, maintained at 4°C, and transported to the University of California, San Francisco within 6 hours. Within 24 hours of arrival in our laboratory, the ATII cells were isolated from the right middle lobe, if there was no evidence of injury or consolidation to that lobe. Allogeneic human MSCs were obtained from the Tulane Center for Gene Therapy (New Orleans, LA) for microarray studies and from the Institute for Regenerative Medicine at the Texas A&M Health Science Center for qPCR studies. The ATII cells were plated at a density of 1×10^6 cells/well in the upper compartment of Transwell systems (0.4-mm pore size and collagen I coated; Costar, Corning). The Transwell system was used to study the paracrine effects of MSCs on ATII cells (20). As previously described (15), the cells were cultured in a 37°C and 5% CO_2 incubator in mixed media of DMEM high glucose 50% and F-12 50% containing 10% FBS and antibiotics (gentamicin, penicillin, streptomycin, and amphotericin). Where indicated, CytoMix (IL-1β, TNF-α, and IFN-γ, 50 ng/ml each; R&D Systems) was added to the culture medium, and MSCs were plated in the bottom compartment of the Transwell at a density of 250,000 cells/well with no direct contact with ATII cells, as described previously (14). ATII cells, MSCs, and CytoMix were added simultaneously, and conditions were maintained for 24 hours (14). Three conditions were examined: 1) ATII cells alone (control), 2) ATII cells plus CytoMix, and 3) ATII cells plus MSCs and CytoMix.

Microarray data processing

The gene expression data used for this study were previously published (14) and made publicly available as GSE68610 on the Gene Expression Omnibus (GEO) (11). These data were generated using Affymetrix Human Genome U133A 2.0 Arrays. We downloaded the raw data from GEO and processed them using the Robust Multiarray Average, or RMA (22). Only four arrays were available for ATII cells exposed to CytoMix but not MSCs, but five arrays were available for the other conditions. No batch correction was performed because all array experiments were done in a single batch.

During data processing, we applied a custom cell description file (CDF) as provided by the Microarray Lab at the University of Michigan (7). Custom CDFs were used to ensure that probe set annotations were updated. This method excludes probes that do not match a gene sequence perfectly and uniquely, which may reduce the number of genes used for comparisons (7). We used custom CDF version 21 and specifically a CDF that summarizes gene expression data to Entrez gene identifiers. We did not exclude any gene based on its level of expression in the dataset. For quality control, we also checked interarray Pearson correlation within each of the three conditions, using a cut-off of below 0.90 to consider an array for exclusion. Replicate arrays demonstrated within-condition correlation of at least 0.94.

Quantitative PCR

Using qPCR, we validated differential expression for genes that had statistically significant expression changes and were of interest because of their biological functions. Total RNA from ATII cells was isolated with the RNeasy Mini kit (Qiagen) and reverse transcribed to cDNA using the "High Capacity RNA-to-cDNA Kit" (Applied Biosystems). The assay IDs for TaqMan specific gene primers (Applied Biosystems) were: SFTPB (Hs00167036_m1), SFTPD (Hs01108490_m1), IL23A (Hs00372324_m1), CCL2 (Hs00234140_m1), AQP1 (Hs01028916_m1), AQP3 (Hs00185020_m1), AQP5 (Hs00387048_m1), CXCL10 (Hs00171042_m1), CXCL11 (Hs00171138_m1), POSTN (Hs01566750_m1), LOX (Hs00942480_m1), CASP8 (Hs01018151_m1), SCNN1A (Hs00168906_m1), SCNN1B (Hs01548617_m1), GAPDH (Hs02786624_g1), and EIF2B2 (Hs00204540_m1). qPCR was performed with TaqMan gene primers and TaqMan Fast Advanced Master Mix (Applied Biosystems) using the StepOnePlus System (Applied Biosystems). The average threshold count (Ct) value of 3 technical replicates was used in all calculations. GAPDH and EIF2B2 were used as a housekeeping genes because they displayed the lowest standard deviation amongst groups compared to other housekeeping genes tested. Data analysis was performed using the $2^{-\Delta Ct}$ method (46). Relative mRNA data are expressed as mean ± standard deviation.

Statistical analyses

All statistical analyses were performed using R version 3.3.2. We performed principal components analysis using the "prcomp" function in R and hierarchical clustering using tools from the Bioconductor package made4 (6). Gene symbols and descriptions for each gene were generated using the "getBM" function from the biomaRt package in R, and orthologous gene lists between species were generated using the "getLDS" package in biomaRt (10). We excluded arrays that appeared to be outliers based on hierarchical clustering and principal components analysis. We performed differential gene expression analysis using the Bioconductor package limma (43), and we focused on two comparisons: 1) ATII cell controls vs. ATII cells exposed to CytoMix, and 2) ATII cells exposed to CytoMix vs. ATII cells exposed to both CytoMix and MSCs. Genes were considered differentially expressed if they were at least two-fold differentially expressed between conditions and if the p-value was less than 0.05 after adjusting for multiple testing using the Benjamini-Hochberg method (4).

For gene set enrichment analysis of up- and down-regulated genes, we used gene sets that were downloaded from MSigDB (51) to look for functional enrichment. P-values for enrichment were generated in R using Fisher's exact test, where the number of genes in our processed microarray dataset was the background number. We adjusted Fisher's exact test p-values for multiple testing using the Benjamini-Hochberg method (4). We used the "Hallmark" gene sets (33) from MSigDB in the initial exploratory analyses. "Hallmark" gene sets are derived from published gene sets and describe specific biological states. They were developed in order to reduce heterogeneity and redundancy among descriptive gene sets (33). We also downloaded the "Canonical pathway" gene sets from MSigDB, which are curated from pathway databases, such as KEGG (26) or Reactome (5).

Hierarchical clustering dendrograms and heatmaps were created using the "heatplot" function in the Bioconductor pathway made4 (6). Hierarchical clustering was performed with distance function 1-Pearson correlation, as previously done (12). We visualized KEGG pathways using the pathview package in Bioconductor (34).

Results

Mesenchymal stromal cells and CytoMix both modulate ATII cell gene expression

We analyzed a total of 12,264 genes in alveolar type II (ATII) cells derived from human lungs of five individuals. Principle component analysis revealed that most of the gene expression variation (61%) in this dataset could be explained by two principal components (Figure 1A). The first component (50% of variation) separated ATII cells that were exposed or unexposed to MSCs, and the second (11% of variation) separated ATII cells exposed to only CytoMix from control ATII cells and ATII cells exposed to both CytoMix and MSCs. This suggests that CytoMix and MSCs had independent effects on ATII cell gene expression. For future analyses, we removed one outlier array based on the principal components analysis and hierarchical clustering analysis (Figure 1). This ATII replicate exposed to only CytoMix clustered with control ATII cells, suggesting that the CytoMix was ineffective in this sample.

CytoMix induces alveolar type II cells to up-regulate proinflammatory genes and down-regulate genes required for surfactant production and alveolar fluid clearance

A total of 409 and 454 genes were up- and down- regulated, respectively, in ATII cells in response to CytoMix (Supplementary table 1). Up-regulated genes were enriched for several MSigDB Hallmark gene sets and pathways related to inflammation (Figure 2, Supplementary table 2). These include gene sets describing response to TNF-α via NF-κB signaling (enrichment adjusted p-value 8×10^{-63}) and response to interferon gamma ($p = 1 \times 10^{-73}$). For example, the most strongly up-regulated genes included those encoding chemokines CXCL10 and CXCL11 (Figure 3), both of which are up-regulated over 100-fold and are known to be induced by interferon gamma (25). Genes coding CCL5, CCL7, and CCL8 were also upregulated, as was IL1β. Of the canonical pathways, the Reactome cytokines pathway is the most statistically significantly up-regulated (p-value 2×10^{-20}, Supplementary table 1). Using the "pathview" function in Bioconductor (34), we also visualized up- and down-regulated genes within the KEGG TNF pathway. Many elements of the TNF pathway were up-regulated, as were numerous cytokines and chemokines (Figure 4).

Gene sets down-regulated in ATII cells following exposure to CytoMix were related to lipid metabolism and alveolar fluid transport. The most enriched MSigDB Hallmark gene set was adipogenesis ($p = 4 \times 10^{-6}$, Supplementary table 3), which includes genes coding for proteins important for pulmonary surfactant function, such as long chain Acyl-CoA dehydrogenase (16). Surfactant proteins B and D were downregulated 23- and 10-fold, respectively (Figure 3). Down-regulated genes also included those involved in ion and water channel function (Figure 3, Supplementary table 1). The alpha and beta subunits of epithelial sodium channel (ENaC) were not differentially expressed in the initial genome-wide analysis adjusted for multiple testing. However, given the importance of the ENaC channel to alveolar fluid clearance (36), we carried out focused, hypothesis-driven statistical testing without multiple testing adjustment. We found that genes encoding both ENaC alpha and beta subunits were down-regulated ($p = 6 \times 10^{-5}$ and 0.03, respectively).

Mesenchymal stromal cells alter multiple aspects of ATII gene expression

Exposure of ATII cells to CytoMix plus MSCs resulted in the up-regulation of 215 and down-regulation of 938 genes compared to CytoMix alone (Supplementary table 4).

Functional enrichment analysis identified various up-regulated genes involved in epithelial-mesenchymal transition ($p = 2 \times 10^{-11}$, Figure 2, Supplementary table 5). For example, we identified up-regulation of SNAIL (adjusted p = 0.005), and down-regulation of E-cadherin (adjusted p = 0.001). However, several EMT genes (28) were not differentially expressed, such as genes coding Twist, Vimentin, MMP2, MMP9, N-cadherin, ZEB1, or ZEB2.

Among the genes in this Hallmark gene set, two of the most strongly up-regulated were genes coding for periostin and lysyl oxidase (Supplementary table 4), proteins involved in collagen cross-linking (35) (Figure 3), which suggests extracellular matrix modification. Consistently, the canonical pathways that were most enriched in these up-regulated genes included several pathways related to the extracellular matrix (Supplementary table 5). Genes coding for fibronectin and matrix metalloproteinases were also strongly up-regulated, consistent with wound healing and repair.

To further analyze the ATII cells' reparative potential, we examined differential expression of other factors, such as EGFR (9) and genes related to Wnt signaling (39, 60). Although several of these genes did not meet our original cut-off of fold-change greater than 2, EGFR, WNT2, WNT8B, WNT10B, WNT11, and WNT16 were all significantly up-regulated ($p < 0.05$, after multiple testing adjustment) with MSC exposure. Some genes related to proliferation, such as KI67, E2F1, E2F2, and E2F4, were similarly up-regulated, although other genes related to proliferation, such as MYC signaling (Supplementary table 7), were down-regulated. Lastly, we noted increased expression of angiopoeitin-1, which helps restore more normal type II alveolar cell paracellular permeability to protein (15), and nearly significantly increased HGF expression ($p = 0.052$), which also helps with repair (55).

Although MSC exposure was associated with up-regulation of some genes related to TNF-α signaling (Figure 2, Supplementary table 5), the specific pathway genes were different from those up-regulated with CytoMix exposure (Supplementary table 6). Figure 4 shows that several genes from the KEGG TNF pathway were down-regulated with MSC exposure, particularly chemokines, suggesting that MSCs have an anti-inflammatory effect. Genes associated with apoptosis such those coding for FADD and caspase proteins and the Reactome apoptosis pathway (Supplementary table 7) were also down-regulated, suggesting an anti-apoptotic effect of MSCs on ATII cells. Similarly, genes coding for other anti-apoptotic proteins, such as SODD or CHOP, were up-regulated, with *DDIT3*, the gene that codes for CHOP, being eight-fold increased and the fifth most strongly up-regulated gene. The TNF-α pathway genes up-regulated by MSCs but not by CytoMix also included several coding for transcription factors such as HES1, FOS, JUNB, and KLF2, the last of which is essential for type 1 pneumocyte differentiation during development in mice (41), a process also important for lung injury repair (24).

MSCs attenuate inflammatory changes induced by CytoMix

In genome wide analyses adjusted for multiple testing, genes up-regulated by CytoMix were significantly down-regulated by MSCs (Fisher's test $p = 7 \times 10^{-11}$), and those down-regulated by CytoMix were also significantly up-regulated by MSCs ($p = 6 \times$

10^{-9}). Genes with transcriptional changes induced by CytoMix and reversed by exposure to MSCs included those coding surfactant protein B, IL-23, and CCL2, which is a chemokine involved in ARDS pathogenesis (57) (Supplementary table 8). In hypothesis-driven tests for genes that we found were down-regulated by CytoMix, we also found that the alpha and beta subunits of ENaC were up-regulated with MSC exposure ($p = 0.01$ and 4×10^{-5}, respectively).

Notably, not all genes related to inflammation were reversed following 24 hours of MSC exposure. For example, ATII cells are known to express pattern recognition receptors (PRR) (13); several of these were up-regulated by CytoMix but their expression did not change in response to MSCs. For example, NOD2, which is a PRR expressed by epithelial cells (54), was up-regulated over 3-fold in response to CytoMix and remained up-regulated in cells exposed to CytoMix plus MSCs. A similar pattern was observed for *DDX58*, the gene coding for the PRR RIG-I. The only Toll-like receptor that was down-regulated with MSC exposure in our initial analysis was TLR3 (Supplementary table 4).

Comparison of MSC effects to those from a mouse model

In order to validate these differentially expressed genes, we examined comparable differentially expressed genes from a mouse model (45). In dos Santos et al., mice underwent cecal ligation with or without exposure to MSCs, and differential gene expression analysis of several murine tissues, including lung, was performed. We found that 33% of the genes that were differentially expressed in ATII cells exposed to MSCs (Supplementary table 4) overlapped with those from dos Santos et al. (Fisher's test $p = 0.0004$), suggesting that the MSCs affected similar pathways in both models. The down-regulated genes had particularly strong overlap with those down-regulated in dos Santos et al. (19%, $p = 4 \times 10^{-6}$), with similar down-regulation of caspase 3 and chemokine ligands CXCL1, CXCL2, CXCL3, and CCL4. The up-regulated genes were under-enriched in those up-regulated in dos Santos et al. (4%, $p = 0.005$).

Validation with quantitative PCR

For the genes tested, all gene expression changes induced by CytoMix were confirmed with qPCR (Figure 5). CytoMix induced up-regulation of genes coding for several cytokines, including CXCL10, CXCL11, CCL2, and IL-23, and it induced down-regulation of genes coding for surfactant protein B, surfactant protein D, and the ENaC subunits alpha and beta.

The effects of MSCs did not all validate, but the gene expression trends were overall similar to those of the microarray. Exposure to MSCs resulted in down-regulation of CXCL10, CXCL11, and caspase 8, but IL-23 was not substantially down-regulated, and CCL2 was actually up-regulated with MSC exposure by qPCR. Additionally, surfactant protein D and ENaC beta expression increased with MSC exposure, but surfactant protein B and ENaC alpha expression remained unchanged. MSCs caused increased gene expression of lysyl oxidase and periostin, similar to the microarray results. Interestingly, in the microarray analysis, surfactant protein D, CXCL10, and CXCL11 were not differentially expressed with MSC plus CytoMix compared to CytoMix alone ($p > 0.10$ for those comparisons).

Discussion

The primary findings of this study can be summarized as follows. Several gene expression pathways in ATII cells were modified in response to a mixture of TNF-α, IL-1β, and IFN-γ, which models the proinflammatory edema fluid of ARDS (30). MSCs reversed some, but not all of the effects of CytoMix on ATII cells, including up-regulation of inflammatory genes and down-regulation of genes related to key ATII cell functions, such as surfactant production and alveolar fluid clearance.

ATII cells exposed to CytoMix up-regulated genes coding several cytokines and pro-apoptotic proteins. It has been previously shown that ATII cells can express cytokines in infection or inflammatory environments (49). In response to CytoMix, ATII cells also down-regulated genes important for normal ATII cell function, such as genes related to surfactant and vectorial alveolar fluid transport. Consistently, previous studies have shown that the inflammatory environment induced by endotoxin reduced surfactant production in mice (23) and alveolar fluid clearance in human lung (31). In

addition, several genes related to lipid production and genes encoding surfactant B, which is important for maintaining alveolar tension (56), and surfactant D, which is thought to support host defense in the alveolus (19), were down-regulated. Genes important for fluid transport were also down-regulated, such as those coding for the alpha and beta subunits of ENaC. CytoMix was previously shown to induce down-regulation of ENaC subunits alpha and beta in ATII cells (30).

In contrast, MSCs induced ATII cells to reverse some of the gene expression changes induced by CytoMix. With MSCs, ATII cells up-regulated the genes coding surfactant protein D and ENaC subunit beta while they down-regulated gene expression for genes associated with apoptosis, such as caspase 8, and cytokines, such as CXCL10, which has been linked to ARDS pathogenesis (21).

Our research group has previously reported that MSCs partially restore fluid clearance in injured lung through ENaC-mediated sodium transport (31). While the effect of MSCs on ENaC alpha did not validate with qPCR, ENaC activity in alveolar cells may also change independent of gene or protein expression (42). The same is true for caspases, where post-translational modification alters their catalytic activity (58).

MSCs also induced ATII cells to up-regulate several genes, including those in pathways related to injury repair and the epithelial-mesenchymal transition. The significance of epithelial-mesenchymal transition genes is unclear because many other genes associated with epithelial-mesenchymal transition were not differentially expressed. However, the most strongly up-regulated of these genes were related to extracellular matrix modification. For example, ATII cells exposed to MSCs up-regulated the gene coding for periostin, which has been shown to enhance wound repair in alveolar epithelial cells (1). Periostin also causes airway fibroblasts to over-express collagen and helps stiffen the collagen matrix (47), making it potentially beneficial for epithelial repair. Consistently, several genes related to repair, such as EGFR, angiopoietin-1, and HGF were also statistically significantly up-regulated.

MSCs did not reverse all genes associated with CytoMix exposure, including genes that could be relevant for host defense, such as pattern recognition receptors (PRR). Toll-like receptor 3 (TLR3) was down-regulated with MSC exposure, but this

may be advantageous since TLR3-deficient mice with influenza have a survival advantage, potentially because of an attenuated inflammatory response (29).

Most of our findings validated with qPCR, which is expected using arrays and data processing methods similar to our own (8). In our experiment, a few gene expression changes in the microarray analyses were not validated by qPCR, particularly those related to MSC exposure. For example, with MSC exposure, chemokine CCL2 gene expression increased in the qPCR analysis but decreased in the microarray analysis. Several factors may have contributed to discrepancies between microarray and qPCR, including biological variability. The qPCR was performed using a new ATII biological replicate, and genes used for qPCR were in part chosen because of biological interest, not always because of magnitude of differential expression. The qPCR studies were also performed using MSCs derived from an individual different from those in the original microarray. Regardless, most of the trends related to MSC exposure were confirmed with qPCR, such as reduced ATII cytokine gene expression and increased gene expression associated with alveolar fluid clearance and surfactant. It is also possible that different MSC experiments alter similar transcriptional programs but potentially different genes. This is further supported by the significant overlap of these genes differentially expressed in ATII cells and genes differentially expressed in a mouse model of sepsis, in which mice were also exposed to MSCs (45).

Our study has several advantages. Genome-wide analysis of human alveolar type II cells exposed to human mesenchymal stromal cells in an inflammatory milieu is novel and allowed us to identify previously unexplored changes in ATII cells. Our study offers the added benefit of enriching for the low abundance ATII cells, compared to whole-tissue expression analyses (50). We also studied human rather than animal cells. The volume of data offered by our genome-wide approach revealed several ATII cell adaptations to both inflammation and MSCs, which sets the stage for mechanistic experiments.

The limitations to these studies include the *in vitro* approach, the modest sample size, the lack of an MSC-only group, and the reliance on gene expression analyses. The *in vitro* Transwell model has been useful in studies of alveolar cell protein permeability (15), alveolar fluid clearance (30), and interactions with MSCs (14, 15). In addition, the

pro-inflammatory cytokines in this study reproduce many of the effects of pulmonary edema fluid from ARDS patients (30), and *in vivo* experiments have generated complementary results to Transwell experiments (14). However, the environment encountered by ATII cells *in vivo* is more complex, with inflammatory cells, endothelial cells, and lymphatics, which were not accounted for in this study. In the future, the genes identified here as upregulated by ATII cells in response to MSCs could be inhibited in *in vivo* models of lung injury to confirm that these genes indeed mediate the protective effect of MSCs *in vivo*. Although the sample size is limited, we were able to discover statistically significantly differentially expressed genes that had at least two-fold change across biological replicates of ATII cells. Because we did not have a group of MSC-only condition, we could not distinguish the effects of MSCs on ATII cells from the interaction of MSCs and CytoMix on ATII cells. However, the comparison of MSCs plus an inflammatory environment versus the inflammatory environment alone is the most clinical relevant comparison and has been previously used (45). Lastly, our study only explored mRNA expression changes, and future studies could include protein or micro RNA studies to further validate and explore these changes induced by MSCs.

This study shows that exposure to an acute inflammatory environment induces ATII cells to up-regulate genes that code cytokines and down-regulate those associated with alveolar fluid clearance and surfactant. Exposure to MSCs partially reversed several of these changes.

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Figure Legends

Figure 1: A) Principle components analysis of all arrays in the type II alveolar cell microarray dataset. B) Hierarchical clustering of all arrays in the dataset using all genes on the arrays.

Figure 2: Gene set enrichment of differentially expressed genes. A) MSigDB Hallmark gene sets up-regulated in alveolar type II cells exposed to CytoMix. B) MSigDB Hallmark gene sets up-regulated in alveolar type II cells exposed to mesenchymal stromal cells and CytoMix compared to CytoMix alone.

Figure 3: Heatmap of genes differentially expressed by type II alveolar cells. We included genes differentially expressed when ATII cells were exposed to either CytoMix or to CytoMix plus mesenchymal stromal cells in a Transwell system. The top ten most up- and down-regulated genes in each comparison are displayed. Red and blue indicate increased and decreased expression, respectively, normalized to a z-score for each gene.

Figure 4: Differential expression of TNF pathway genes. Figures were generated using the "pathview" package in Bioconductor (34). Red means up-regulated and green means down-regulated. A) Genes from the KEGG (26) TNF pathway that are differentially expressed in ATII cells exposed to CytoMix compared to control. B) Genes from the KEGG TNF pathway that are differentially expressed in ATII cells exposed to mesenchymal stromal cells and CytoMix compared to CytoMix alone.

Figure 5: Quantitative PCR validation. Gene expression changes in ATII cells for several genes across three conditions: control, exposure to CytoMix, and exposure to both CytoMix and MSCs. Gene expression values are from qPCR, normalized to reference gene GAPDH. The genes featured are those coding for A) ENaC subunit alpha, B) ENaC subunit beta, C) Caspase 8, D) Periostin, E) Lysyl oxidase, F) IL-23, G) CXCL10, H) CXCL11, I) CCL2, J) Aquaporin 1, K) Aquaporin 3, L) Aquaporin 5, M) Surfactant protein B, N) Surfactant protein D.

Supplementary table 1: Genes differentially expressed by ATII cells that were exposed to CytoMix alone vs. control. P-values were generated using the Bioconductor package limma, and they were adjusted for multiple testing using the Benjamini-Hochberg method.

Supplementary table 2: MSigDB Hallmark gene sets and canonical pathway gene sets up-regulated in ATII cells that were exposed to CytoMix alone vs. control. Enrichment p-values in each analysis were adjusted using the Benjamini-Hochberg method.

Supplementary table 3: MSigDB Hallmark gene sets and canonical pathway gene sets down-regulated in ATII cells that were exposed to CytoMix alone vs. control. Enrichment p-values in each analysis were adjusted using the Benjamini-Hochberg method.

Supplementary table 4: Genes differentially expressed by ATII cells that were exposed to MSCs plus CytoMix vs. CytoMix alone. P-values were generated using the Bioconductor package limma, and they were adjusted for multiple testing using the Benjamini-Hochberg method.

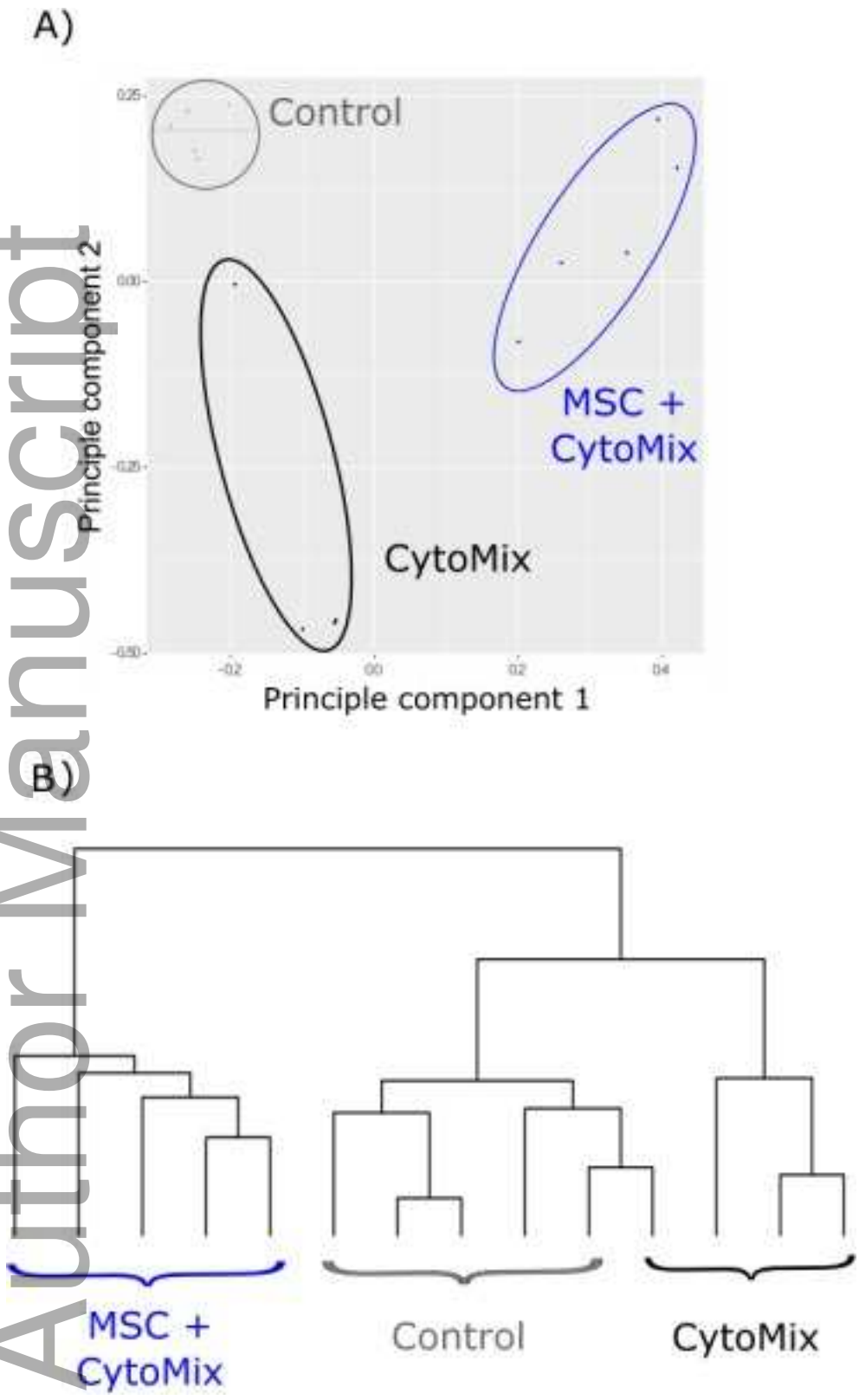
Supplementary table 5: MSigDB Hallmark gene sets and canonical pathway gene sets up-regulated in ATII cells that were exposed to MSCs plus CytoMix vs.

CytoMix alone. Enrichment p-values in each analysis were adjusted using the Benjamini-Hochberg method.

Supplementary table 6: TNF-α signaling genes that are up-regulated with CytoMix or MSC exposure. This table lists genes that are up-regulated in ATII cells exposed to either CytoMix alone or CytoMix plus MSCs, and which are in the Hallmark gene set describing TNF-α signaling through NF-κB. We also show the overlap between the TNF-α genes that are up with CytoMix and those that are up with both CytoMix and MSCs.

Supplementary table 7: MSigDB Hallmark gene sets and canonical pathway gene sets down-regulated in ATII cells that were exposed to MSCs and CytoMix compared to CytoMix alone. Enrichment p-values were adjusted for multiple testing using the Benjamini-Hochberg method.

Supplementary table 8: Genes whose expression pattern reverses with MSC exposure in the microarray. We examined genes that had differential expression in both comparisons (CytoMix vs. control and MSCs + CytoMix vs. CytoMix alone) but whose direction of differential expression (up- vs. down-regulated) was reversed by MSC exposure. This table includes genes from Supplementary table 1 and Supplementary table 4 but not genes that were up- or down-regulated in hypothesis-driven tests for specific genes, such as for the ENaC subunits.



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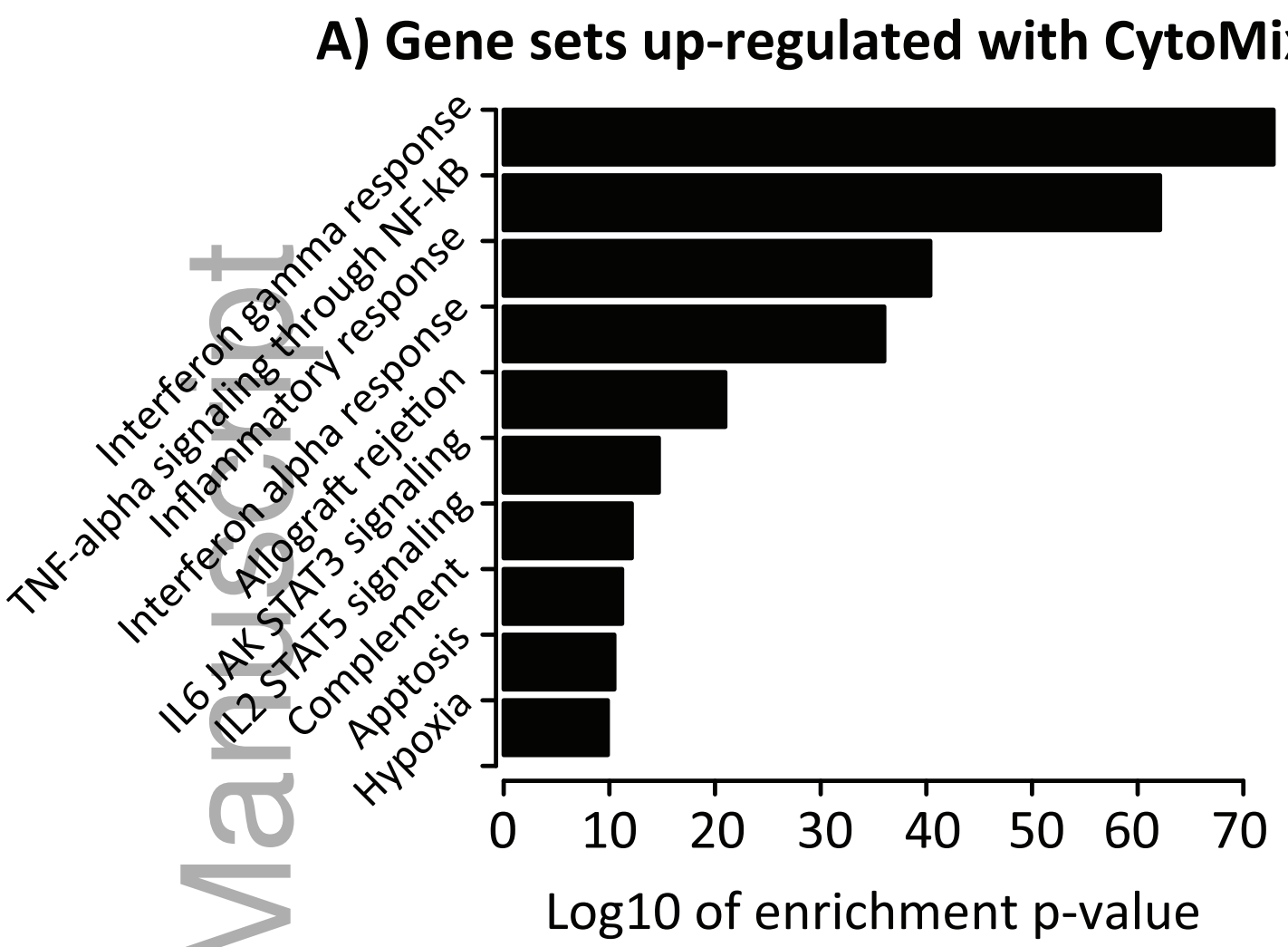

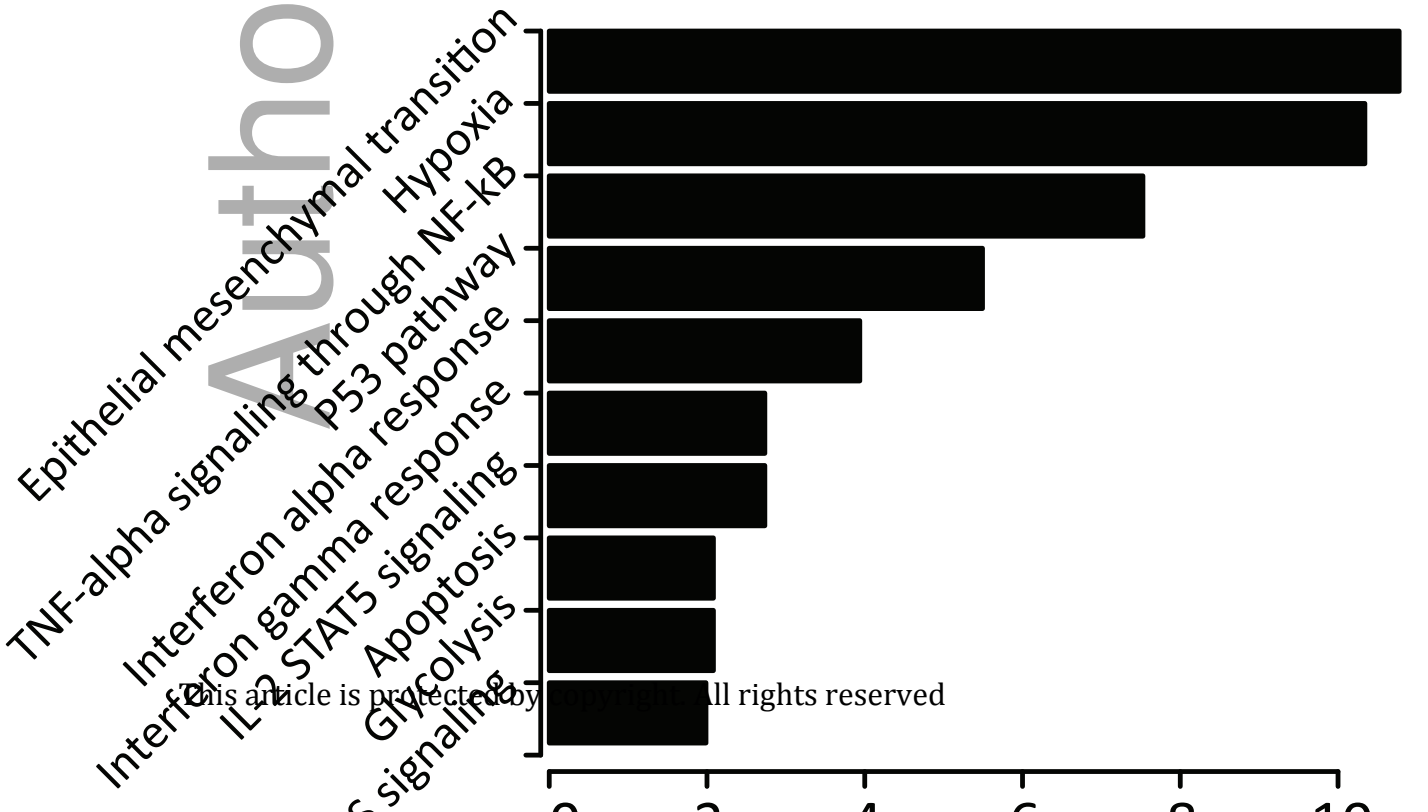

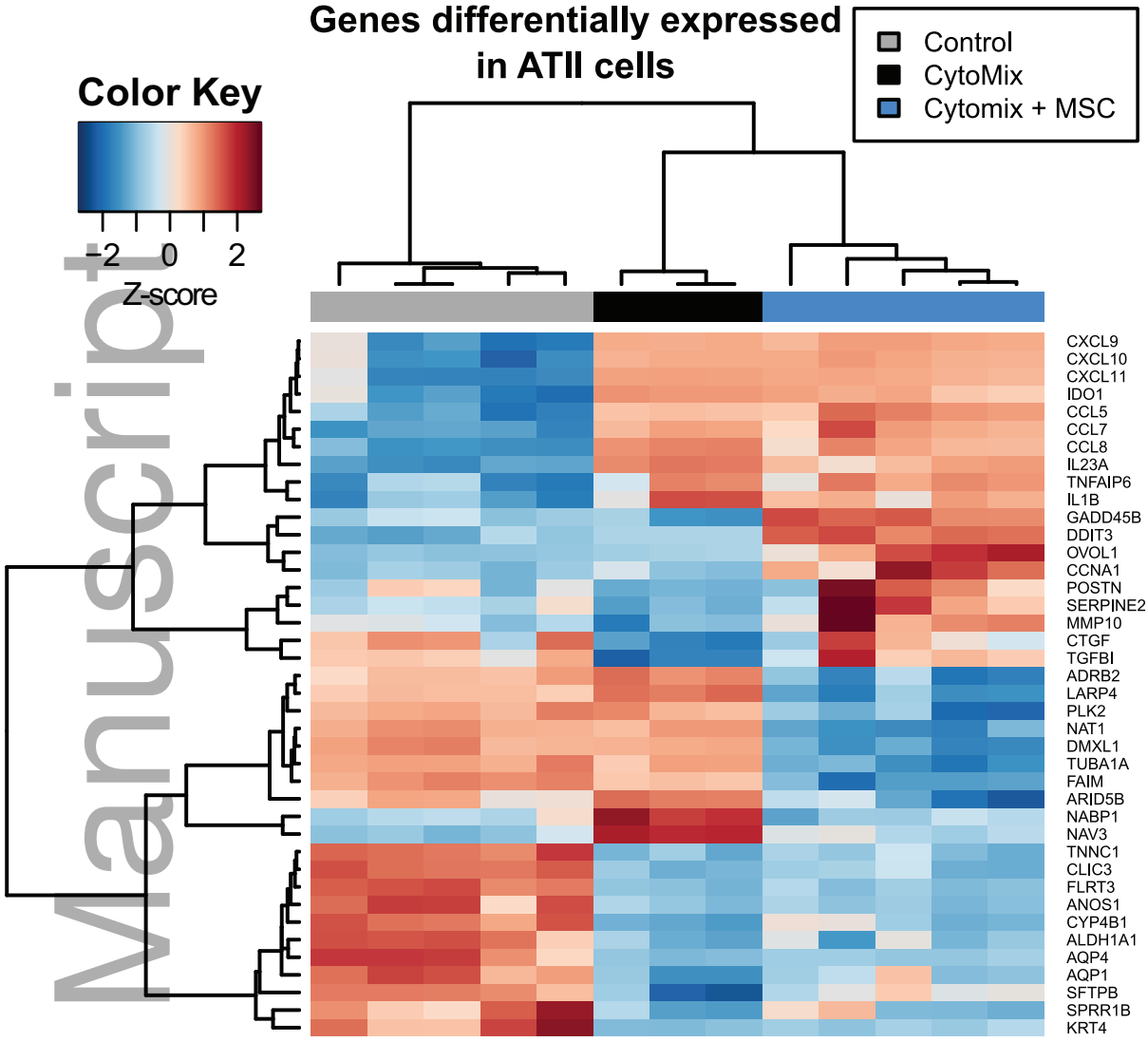

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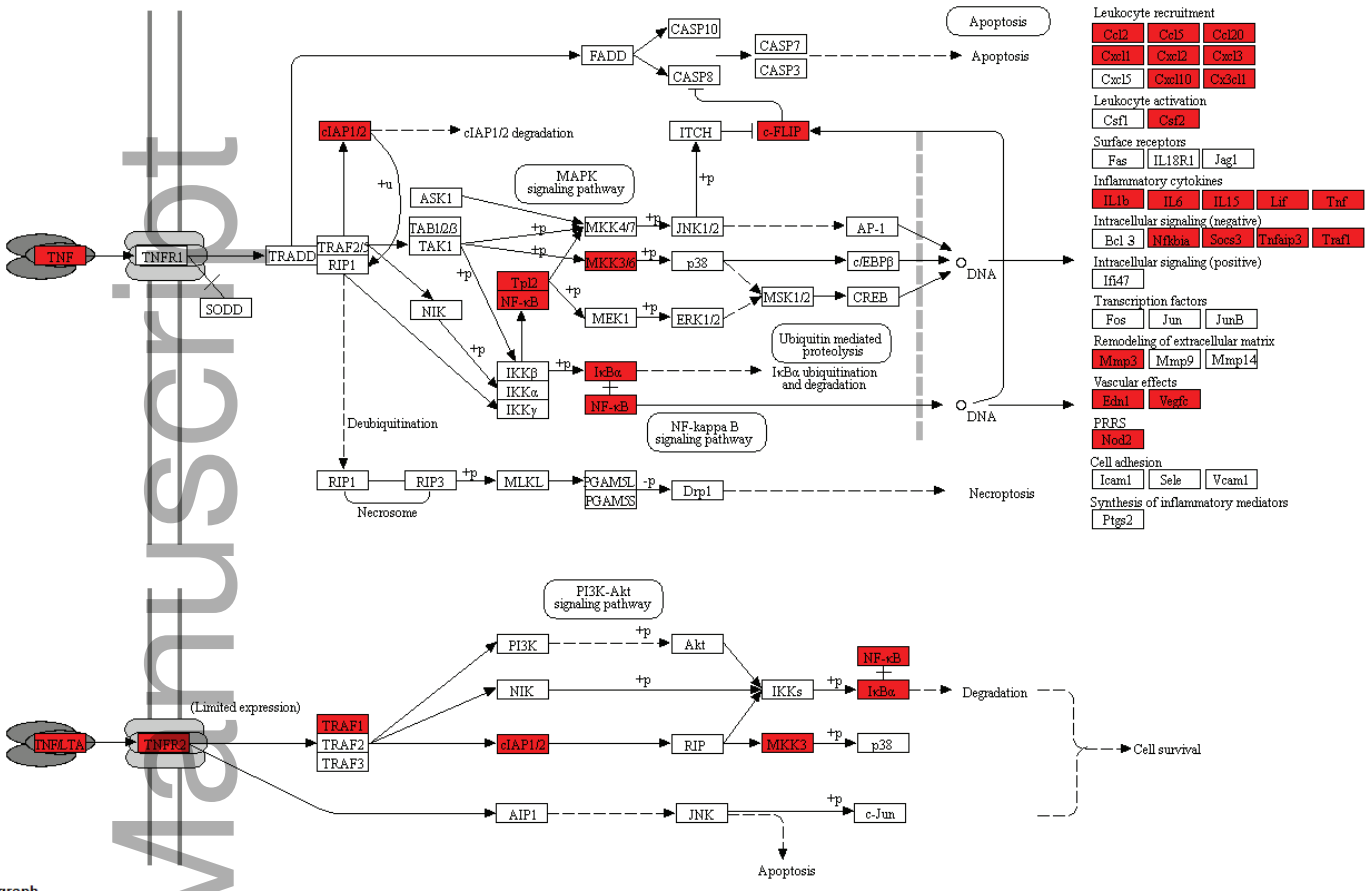
A) TNF pathway genes altered with CytoMix



Data on KEGG graph
Rendered by Pathview

B) TNF pathway genes altered with MSCs