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The lipopolysaccharide-triggered mesangial transcriptome: Evaluating the role of interferon regulatory factor-1

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Background. Presently, we do not have a clear picture of how the mesangial transcriptome evolves following stimulation. The present study was designed to address this, using an innate trigger to stimulate murine mesangial cells.

Methods. Three independent mesangial cell lines derived from C57BL/6 mice were stimulated with lipopolysaccharide (LPS). The mesangial cell transcriptomes were defined 1, 6, 24, and 60 hours poststimulation with LPS, using a 17,000 gene oligonucleotide array.

Results. Interferon regulatory factor-1 (*IRF-1*), *ScyA2/MCP1*, *ScyA20/MIP3α* (*ScyB1/Gro1*, and *ScyB2/MIP2α/Gro2* were the earliest genes to be hyperexpressed after LPS stimulation. Later-appearing genes included *ScyA7/MCP3*, *ScyD1/fractalkine*, *GM-CSF/CSF-2*, *PDGF, epiregulin, NfKb*, *C/EBP*, *TIMP-1*, *MMP11*, *MMP13*, *PTGS2/COX2*, *SpI2-1*, *Spp1*, *PAI-1*, *VCAM-1, C3*, and *defensin-β1*, among others. Several of these changes were validated by real-time polymerase chain reaction (PCR) or enzyme-linked immunosorbent assay (ELISA). Rapid *IRF-1* hyperexpression was also noted following stimulation of mesangial cells with peptidoglycan, poly I:poly C, interferon-γ ?(IFN-γ), and heat-aggregated IgG. However, the blocking of *IRF-1* using RNA interference and the use of mesangial cells isolated from *IRF-1*–deficient mice could not substantiate an obligatory role for *IRF-1* in LPS-induced mesangial cell activation. Likewise, IRF-1 deficiency did not impact the development of anti-glomerular basement membrane (GBM)-induced immune nephritis.

Conclusion. Innate stimuli such as LPS appear to trigger successive waves of mesangial cell gene expression. Although *IRF-1* surfaces as an "early-on, early-off" transcription factor following several different triggers, it does not appear to be an essential molecule for mesangial cell activation by innate triggers or for anti-GBM disease.

The mesangial cell constitutes a key glomerular cell type that plays a potentially important role in the pathogenesis of several renal diseases [1–3]. In particular, it has been recognized to produce a rich array of mediators, including cytokines and chemokines, reactive oxygen species, nitric oxide, prostaglandins, etc. In addition, these cells possess unique contractile properties that allow them to regulate the local blood flow in the glomerulus. Finally, they also elaborate the glomerular basement membrane (GBM) matrix and contribute to the fibrosclerotic lesions seen later in disease, following a variety of primary triggers.

Although a rich literature exists detailing the up-regulation of several different molecules in mesangial cells upon stimulation, little information is currently available concerning the transcriptome of stimulated mesangial cells. This is an important impetus for the current study. In addition, ongoing research in our laboratory implicates genetically encoded differences in the end organs as being potentially important in facilitating the development of immune nephritis [4, 5]. Clearly, it is imperative to define the "normal" mesangial transcriptome in detail before any potential strain or locus specific genetic aberrations can be examined in the future. This is a second factor that has inspired the present study.

A wide variety of stimuli have been documented to trigger mesangial cells, including innate stimuli triggering different Toll receptors, interferon-γ (IFN-γ), interleukin (IL)-1, and other cytokines, and immune complexes [1–3]. This manuscript focuses on mesangial transcriptomic changes following stimulation of Toll-like receptor 4 (TLR4), using lipopolysaccharide (LPS). As a follow-up, this study also tests the functional importance of one of the earliest transcription factors up-regulated following mesangial cell stimulation, interferon regulatory factor-1 (*IRF-1*).

METHODS

Mesangial cell isolation and stimulation

Three independent mesangial cell lines (labeled B6-M3, B6-M4, and B6-M14) were derived from three 2-month-old C57BL/6 (B6)[3] mice following previously documented protocols [6]. Likewise, mesangial cells were

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also prepared from B6.IRF-1$^{-/-}$ mice, purchased from Jackson Laboratories (Bar Harbor, ME, USA). Essentially, renal cortices from the kidneys were minced and then pressed through a series of sieves of decreasing pore size (250 μm mesh, 150 μm, and 75 μm). The glomeruli were collected on the finest sieve (>95% purity, as assessed microscopically), washed with sterile phosphate-buffered saline (PBS), and cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with nonessential amino acids, 2-mercaptoethanol (2-ME), antibiotics, and 10% horse serum. Importantly, this medium contained D-valine, instead of L-valine, to suppress fibroblast outgrowth. Although these cultures initially possessed other glomerulocyte cell types, notably podocytes, mesangial cells outgrew other cell types after 2 weeks of culture. Flow cytometric analyses of the cultured cells revealed them to be large (based on forward scatter), complex (as ascertained by side scatter) and myosin positive, but negative for keratin, von Willebrand factor, and CD45. All mesangial cells were used between the 10th and 20th passage of cultivation. Cells were plated in serum-free medium and stimulated with LPS at 10 ng/mL. At the indicated time points (0, 1, 6, 24, and 60 hours after LPS stimulation), aliquots of cells were processed for RNA isolation. In other experiments, mesangial cells were stimulated with peptidoglycan (100 ug/mL), poly I:C (100 ug/mL), cytosine phosphate guanosine (CpG) oligonucleotides (10 nmol/L), IL-1β (1 ng/mL), INF-γ (5 ng/mL), or total rabbit IgG (10 ug/mL), purchased from Sigma Chemical Co. (St. Louis, MO, USA). These concentrations were selected based on preliminary dose-response experiments with each individual trigger.

Microarray studies

All protocols used for the microarray studies are detailed at the following Web site: *http://microarraycore.swmed.edu/tech_support.html*. Total RNA was prepared using RNAeasy Mini Kit (Qiagen, Valencia, CA, USA), using manufacturer-suggested protocols. Isolated RNA was amplified once using MessageAmp aRNA kit (Ambion, Woodward, TX, USA), following manufacturer-suggested protocols. We have previously established the relative utility and reliability of using amplified RNA (aRNA) versus unamplified RNA (*http://microarraycore.swmed.edu/*). The aRNA prepared was then labeled with cyanine-3 (Cy3) or cyanine-5 (Cy5), using the ASAP aRNA Labeling Kit (Perkin-Elmer, Wellesley, MA, USA). Both sets of labeled probes were mixed and hybridized to a mouse 17K-oligonucleotide array, consisting of oligonucleotides drawn from the Unigene cluster. The gene content of this array is detailed at the University of Texas Southwestern Medical Center Microarray Core's Web site (*http://microarraycore.swmed.edu/*). Following a series of washes with standard sodium citrate (SSC) and sodium dodecyl sulfate (SDS)-containing buffers, the slides were spin-dried, and scanned using Genepix 4000B. Recorded pixel intensities in the Cy3 and Cy5 fluorescent channels were digitally stored, and analyzed as described below.

Microarray data analysis

The chips were first examined for hybridization quality; isolated subgrids where the hybridization was not satisfactory were flagged and genes from those regions were excluded from further analysis. Although there were 17,000 gene spots on the arrays, about 44% of the spots were flagged away for being unsatisfactory, so that each array yielded only about 9500 data points, on the average. Among the genes that displayed differential expression, the chip-to-chip consistency was good, with the interchip correlation coefficients ranging from 0.35 to 0.78. Fluorescence intensities on all arrays were next subgrid normalized. To remove noise, the mean pixel intensity of each gene was then compared to the local background intensity. Genes that exhibited intensities that were <2 SD above the mean background intensity in >75% of component pixels were excluded from analysis. In addition, genes whose mean fluorescence intensities fell below 1% of the array-wide maximal fluorescence intensity were also excluded.

Of the remaining array spots, those that differed in expression between the control (i.e., unstimulated) and experimental (i.e., LPS-stimulated) by > twofold were examined further. Hierarchic clustering was performed using Gene Traffic (Iobion, La Jolla, CA, USA), based the Pearson correlation distance metric and the "average" clustering algorithm. Statistical significance of any observed expression differences between the unstimulated and stimulated samples were determined using the Student *t* test (Sigmastat) (Jandel Scientific, San Rafael, CA, USA). Since the expression differences failed to maintain significance after multiple testing correction (because of the small number of arrays studied, and the large number of genes being analyzed), the validity of the observed expression differences were confirmed by one of two orthogonal methods, real-time reverse transcription-polymerase chain reaction (RT-PCR) or enzyme-linked immunosorbent assay (ELISA) measurement of corresponding protein levels, for selected genes showing maximal expression differences at the different time points studied. All microarray data detailed in this communication are freely available from the corresponding author.

Potential transcription factor binding sites were identified as follows. The sequence of each differentially expressed gene was accessed on the Ensembl database (*www.ensembl.org*). One thousand base pairs upstream