**Methods**

*Mice.*  All mice used in this study were on a C57BL/6 genetic background. Female C57BL/6 wild type (WT) mice were purchased from NCI. The *Id3*-/- mice were obtained from Dr. Yuan Zhuang from Duke University backcrossed to C57BL/6 mice for 11 generations and the purity of the line confirmed to be >99% by microsatellite mapping [5]. The mice were bred at the University of Virginia and housed in specific pathogen free conditions. All procedures followed NIH guidelines for humane use of animals and were approved by the Institutional Animal Care and Use Committee. Mice were fed either western diet (TD.88137, 42% calories from fat, 0.2% cholesterol, Harlan-Teklad) or mouse chow (TD 7012, 17% calories from fat, 0% cholesterol) starting at 6-8wks of age. Urinary albumin and creatinine in 24 hour urine samples were measured as previously described [6]. Terminal bleeds from one cohort of mice was submitted for lipid profile analyses to the University of Virginia Clinical Pathology Laboratories.

*Evaluation of renal pathology and inflammatory cells:*  Mice were sacrificed after either 8wks or 15wks on diet and kidneys were studied for renal pathology, fibronectin deposition, and immune cell infiltration [6, 12]. Each glomerulus in one cross section of the kidney (n=42-109/mouse) was evaluated for pathology and scored 0 for normal, and 1 for mild, focal, mesangial involvement, 2 diffuse glomerular involvement of increasing severity and 3 indicating severe mesangial and/or peripheral capillary loop involvement with or without crescents. Presence of chronic changes including glomerulosclerosis / fibrosis was also evaluated. Severity of glomerular pathology is a sum of glomerular severity score multiplied by percent glomeruli affected. Quantitative analysis for fibronectin deposition and immune cell infiltration was carried out by stereology [13, 14] and detailed methods are described in supplemental data.

*Treatment of mesangial cells with oxidized phospholipids.* Primary mesangial cell lines were generated from glomerular isolates using standard methods [15] and used after 5th passage. The cells (5x104cells/well) were cultured in RPMI medium with 0.5% low lipid fetal calf serum for 24 hrs. To stimulate with oxidized phospholipids, Oxidized 1-palmitoyl-2-arachidonoyl-sn-glycero-3-phosphocholine (oxPAPC), an active principle of minimally modified Low Density Lipoproteins (LDL), was reconstituted in RPMI and added to the wells. Control wells received media alone. oxPAPC was a gift from Dr. Norbert Letinger, University of Virginia [16]. After 24 hrs of incubation, supernatants were collected from each well and chemokines and cytokines in the supernatants were measured using the Mouse Grp1 23-plex and Grp2 9-plex Bio-Plex suspension array system (Bio-Rad).These experiments were repeated twice using independently generated mesangial cell lines from Id3-/- and WT mice. In the repeat experiments, CXCL1 measurements were carried out by ELISA (R&D systems).

*Gene expression analyses in mesangial cell lines and isolated glomeruli:* For gene expression analysis, the mesangial cells were harvested in RLT buffer and RNA was isolated using RNA easykit (Qiagen) and followed by cDNA synthesis using QuantiTect Reverse Transcription kit (Qiagen) [17]. CXCL1 gene expression was analyzed by real time PCR Taqman gene expression assays (Applied Biosystems). Data are represented as fold change over untreated B6 mesangial cell cDNA. GAPDH was used as a housekeeping gene control. Glomerular isolation was carried out as previously described [18] and glomerular RNA was extracted using Picopure RNA Isolation kit (Life Technologies). For glomerular gene expression, results are presented as relative expression using GAPDH as a housekeeping control.

*Statistical Analyses:* Statistical analyses were carried out using one way ANOVA with Bonferroni correction for multiple comparisons, two way ANOVA and Mann Whitney test using Graph Pad Prism 3.0 software.

**Supplementary Table 1.** Body weights, serum lipid profiles and blood glucose in WT and *Id3-/-* mice on chow or western diets for 8 wks.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **Strain** | **WT** | **WT** | ***Id3-/-*** | ***Id3-/-*** |
| **Diet** | **Chow** | **Western** | **Chow** | **Western** |
| Body weight (gms) | 19.9+ 0.9 | 25.4+0.7**\*** | 20.8+ 0.3 | 26.2+0.7**\*** |
| (n) | (7) | (14) | (10) | (19) |
| Lipid Profile (mg/dL) |  |  |  |  |
| total cholesterol | 87.4+4.1 | 191.2+5.5**\*** | 82.4+4.8 | 181.5+8.8**\*** |
| HDL | 46.4+1.4 | 85.6+2.2**\*** | 47.6+2.0 | 95.3+4.4**\*** |
| LDL | 26.4+1.8 | 95.8+4.1**\*** | 28.0+2.5 | 78.3+4.4**\*** |
| Triglycerides | 87.8+22.1 | 60.0+8.7 | 41.8+4.2 | 47.3+4.1 |
| (n) | (5) | (5) | (5) | (6) |
| Blood glucose (mg/dL) | 163.8+4.4 | 153.4+11.0 | 144.5+9.3 | 154+13.8 |
| (n) | (5) | (5) | (4) | (5) |

All values are mean+SEM. (n) number of mice studied; **\*** p<0.001 indicating statistical significance compared to chow fed mice using ANOVA for multiple comparisons with Bonferroni post test.

**Supplementary Table 2.** Stereologic analyses of fibronectin and CD68 macrophage positive areas in glomeruli of WT and *Id3-/-* mice fed mouse chow or western diets.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| **STRAIN** | **WT** | **WT** | ***Id3-/-*** | ***Id3-/-*** |
| Diet | Chow | Western | Chow | Western |
| Number of mice | 5 | 5 | 5 | 7 |
| **GLOMERULAR FIBRONECTIN** |  |  |  |
| Glomerular area\* | 184.0 + 18.8 | 200.5 + 16.8 | 179.1 + 12.9 | 203.3 + 27 |
| fibronectin positive area\* | 20.5 + 1.9 | 24.03 + 1.5 | 19.7 + 1.4 | 53.6 + 7.3  |
| **Glomerular fibronectin fraction** | **11.3 + 0.7** | **12.08** + **0.4** | **11.0** + **0.3** | **26.2 + 1.0\*\*** |
| **GLOMERULAR CD68 MACROPHAGES** |  |  |
| Glomerular area\* | 204.8 + 27 | 216.7 + 10.6 | 256.5 + 20.0 | 212.6 + 21 |
| CD68 positive area\* | 2.18 + 0.3 | 1.80 + 0.2 | 2.133 + 0.7 | 4.3 + 0.6 |
| **Glomerular CD68 fraction** | **1.06 + 0.06** | **0.83 + 0.05** | **0.79 + 0.2** | **2.0 + 0.17\*\*** |

AREA\* (x103 sq microns). **\*\*** p<0.001 indicating statistical significance compared to chow fed mice using ANOVA for multiple comparisons with Bonferroni post test.

***Stereology for quantitation of glomerular fibronectin and CD68 macrophages:*** Glomerular area fraction positive for fibronectin or CD68 macrophages was carried out using Stereoinvestigtor software v3.0 (Microbrightfield systems). Briefly, a contour was traced around the renal cortex and a sampling grid of 400mx400m was randomly placed over the contour. Systematic random sampling of the cortex was obtained by positioning a counting frame of 300m x 250m at regular intervals. An average of 75.6+1.9 grids corresponding to 12.1+3 x106 sq microns of each kidney cross section were sampled for stereologic analyses. Using the area fraction fractionator probe, total glomerular areas and glomerular areas positive for fibronectin or CD68 were estimated. Data are presented as fraction of total glomerular area for each mouse and are represented graphically in Figure 1.

****

**Supplementary figure 1:** **Characterization of primary mesangial cell lines** using antibodies to mesangial cells (smooth muscle actin), podocytes (wilm’s tumor 1) and endothelial cells (Von Willebrand factor). Cells were cultured on coverslips and stained by indirect immunofluorescence using standard methods. Cells stained with isotype control antibodies are shown (neg control). The cells were also negative for fibroblast marker, desmin, and pan cytokeratin (data not shown). Immortalized podocyte, fibroblast and epithelial cell lines were used as positive controls for the respective markers. Nuclei (blue) were stained with DAPI. Bottom panel shows purity of the mesangial cell lines studied by flow cytometry. At 4th passage, >90% of the cells express smooth muscle actin and megsin (blue line), markers of mesangial cells. Cells stained with an isotype antibody were used as controls (red line)

****

**Supplementary figure 2: Cytokines and Chemokines produced by mesangial cells in response to oxPAPC .** Primary mesangial cells from Id3+/+ (open bars) and Id3-/- (closed bars) mice were cultured in RPMI supplemented with 0.5% low lipid fetal calf serum for 24hrs followed by incubation with different doses of oxPAPC for an additional 24 hrs. Supernatants were collected and analyzed for levels of cytokine and chemokine using Mouse Grp1 23-plex and Grp2 9-plex Bio-Plex suspension array system (Bio-Rad). Detectable levels of CXCL1, MCSF, PDGFbb, VEGF and MCP1 were found and are shown here. Note that only CXCL1 showed a dose dependent increase in response to increasing amounts of oxPAPC. The following cytokines were included in the array and were not detected in the supernatants of either group: IL1a, IL1b, IL2, IL3, IL4, IL5, IL6, IL9, IL10, IL12p40, IL12p70, IL13, IL17, Eotaxin, GCSF, GMCSF, IFNg, MIP1a, MIP1b, RANTES, TNFa, IL15, IL18, bFGF, LIF, MIP2, MIG.