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Interactions among Glomerulus Infiltrating Macrophages and Intrinsic Cells via Cytokines in Chronic Lupus Glomerulonephritis

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Abstract

Inflammation plays a key role in the pathogenesis of lupus nephritis (LN) and inflammatory cytokines within the glomeruli are critical in this process. However, little information is available for the cell types that are primarily responsible for the production and function of the various cytokines. We have devised a novel method to visualize cytokine signals in the kidney by confocal microscopy and found that cytokine production within the glomerulus is cell type-specific and under translational control. In the lupus-prone NZM2328 mice with chronic glomerulonephritis, IL-6, IL-1 β , and TNF- α in the glomerulus were produced predominantly by mesangial cells, podocytes, and glomerulus-infiltrating blood-derived macrophages, respectively. Microarray and RNASeq analyses showed that these cells expressed the receptors for these cytokines. Together the 3 cell types form a cytokine circuit in amplifying cytokine responses in LN. The intrinsic cells and infiltrating macrophages also produced other cytokines including M-CSF, SCF, and IL-34 that constituted within the enclosed glomerular space the soluble effector milieu which may mediate cellular damage and proliferation, and cytokine transcriptional and translation regulation. IL-10 and IL-1 β were translationally regulated in the glomeruli in the intact kidney in a cell type-specific manner. The production of these 2 cytokines by infiltrating macrophages was undetectable in a visualization system for *in situ* protein accumulation despite high mRNA expression levels. However, these macrophages in isolated glomeruli which are released from Bowman's capsules produced large amounts of IL-10 and IL-1 β . These data reveal the complexity of cytokine regulation, production, and function in the glomerulus and provide a model in which cytokine blocking may be beneficial in LN treatment.

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Keywords

Lupus nephritis; cytokines; mesangial cells; podocytes; macrophages; translational regulation

1. Introduction

Systemic lupus erythematosus (SLE) is an autoimmune disease caused by genetic and environmental factors (Reviewed in [1, 2]). Lupus nephritis (LN) occurs in 40-60% of SLE patients and is a major cause of morbidity and mortality in SLE. Despite improved therapy for the disease, 20% of LN cases progress to end stage renal disease which necessitates dialysis or kidney transplantation. In order to improve therapy, better understanding of the pathogenesis of LN to identify novel therapeutic targets is warranted.

For LN pathogenesis, the deposition of immune complexes at the glomerular basement membrane (GBM) is generally considered the initiating event followed by inflammatory responses involving complement components, cytokines, inflammatory cells, and intrinsic cells. The critical event in LN pathogenesis is the damage and destruction of the selective filtration barrier formed by podocyte foot processes [3]. Podocyte injury is primarily caused by extrinsic factors that induce podocyte activation, migration, detachment, and degeneration. Though the cytokine milieu and cellular interactions within the glomerulus determine podocyte responses, these events are poorly understood. Studies of cytokine protein production *in vivo*, especially by intrinsic glomerular cells are lacking and the available data are largely inferred from cell line studies or mRNA quantification. In normal and pathological human tissues, mRNA expression data have been obtained by microarray analyses of whole isolated glomeruli or glomerular regions by laser capture dissection [4–7]. Murine cell-type specific GFP-expressing podocytes, mesangial cells, and glomerular endothelial cells have been purified and used in microarray or RNASeq for gene expression analyses [8–11]. More recently single cell RNASeq was used to analyze glomerular single cell suspensions [12]. These data provide a general picture of the gene expression of glomerular cells and specifically cytokine mRNA expression in homeostatic and pathological conditions. For cytokine cellular origins, the mRNA expression of several cytokine genes in the glomerulus have been visualized by *in situ* hybridization [13–17]. On the other hand, only a few cytokine proteins in the glomerulus have been visualized by immunohistochemical detection [13–20]. The cellular sources of cytokines in these studies are imprecise because the cytokine-producing cells were identified on the basis of their topographical localization and colocalization with cell type-specific markers were not performed.

Our laboratory has established a murine model of LN with the lupus-susceptible mouse strain NZM2328 (NZM) which develops spontaneous chronic glomerulonephritis (cGN) controlled by the locus *Cgcz1* with tubular and glomerular damage, loss of kidney function, severe proteinuria, and premature mortality; and a milder acute GN (aGN) controlled by *Agnz1* with glomerular inflammation and dilatation but only mild proteinuria without early death and tubular damage [21–23]. With this mouse model, we have defined specific markers for the intrinsic glomerular cells, developed a flow cytometric method for analyzing

glomerular cells, and devised a method to determine the cellular sources of cytokines produced in the glomerulus by intrinsic cells and infiltrating leukocytes. We have shown that cGN correlates with an influx of blood-derived macrophages into the glomeruli [24]. In this report, we have analyzed the glomerular cytokine milieu in NZM mice with cGN by defining cytokine and cytokine receptor mRNA expression by mesangial cells, podocytes, and infiltrating macrophages. We further showed that cytokine protein production in the glomerulus is cell type-specific, with infiltrating macrophages producing TNF- α , mesangial cells producing IL-6, and podocytes producing IL-1 β . Other cytokines and growth factors such as SCF, M-CSF, and IL-34 were also produced. Interestingly, cytokine production of infiltrating macrophages were under translational control as IL-10 and IL-1 β protein production was undetectable in the enclosed glomerular compartment despite high cytokine mRNA levels. We propose that during pathogenesis in LN, a cytokine circuit initiated by immune complexes propagates and amplifies the immune response that leads to glomerular dysregulation and damage and that translational regulation plays an important role in LN pathogenesis.

2. Material and methods

2.1 Materials

DNase I, and Liberase TM were from Roche Diagnostic Corp. (Indianapolis, IN, USA). 7-Amino-actinomycin D (7-AAD) and Dynabead 450 magnetic beads were from Invitrogen (Grand Island, NY). Rat or hamster mAb against the following Ag with or without fluorochrome conjugation were from Biolegend (San Diego, CA, USA): CD11b (M1/70), CD26 (H194-112), CD31 (MEC13.3), CD45 (30-F11), CD73 (Ty/11.8), CD105 (MJ7/18), PDGFR- β (CD140b; APB5), F4/80 (CI:A3-1), I-A/I-E (M5/114.15.2), Ly6G (1A8), Ly6C (HK1.4), Thy1.2 (53.2.1), and TNP (Isotype controls; RTK2071 IgG1; RTK2758 IgG2a; RTK4530 IgG2b). The following affinity-purified polyclonal Ab against recombinant mouse proteins were from the respective companies: R&D (Minneapolis, MN, USA): CD31, IL-1 β , IL-34, integrin α 8 (Itg α 8), N-cadherin, and nephrin; Peprotech (Princeton, NJ): TNF- α , IL-6, SCF, and M-CSF; Sigma Aldrich: claudin 2. Purified goat and rabbit IgG were from Rockland (Limerick, PA) and Pel-Freez (Rogers, AK) respectively. Only Ab directly conjugated with fluorochromes were used in tissue staining because secondary staining reagents gave high non-specific backgrounds. Fluorochrome-labeled isotype control Ab showed no background staining in confocal microscopy.

2.2 Mouse

The lupus-prone NZM mouse strain originally obtained from Jackson Laboratory (Bar Harbor, ME, USA) is maintained in our vivarium. The NZM.Lc1R27 (R27) congenic mouse strain without spontaneous cGN contains an 8 Mb substitution in the *Cgzn1* locus of NZM mice with a C57L/J chromosome segment [21]. NZM mice 5 months or older were monitored weekly for proteinuria by Multistix 10 SG (Bayer Diagnostics Division, Elkhart, IN) and mice with 300 mg/dL urine protein were sacrificed for immunological and histological evaluations. Animal use and manipulation followed protocols approved by the University of Virginia Institutional Animal Use and Care Committee.

2.3. Confocal microscopy

Alexa fluor (A)-, Brilliant Violet (BV)-, and Pacific Blue (PacBlue)- conjugated Ab were obtained either from Biolegend or by labeling with mAb labeling kits (Invitrogen). Kidney sections or magnetic bead-purified glomeruli were fixed in 2% p-formaldehyde-lysine-periodate (PLP) for 3 h, equilibrated in sucrose as described [25, 26], and embedded in OCT. Tissue sections (5 μ m) were stained by fluorochrome-conjugated Ab. Images were captured on a Zeiss LSM-700 confocal microscope at the University of Virginia Advanced Microscopy Center and analyzed by the program ZEN (Zeiss, Thornwood, NY). For analysis, at least 5 NZM or R27 mice with different disease conditions were used and images of more than 15 glomeruli in each mouse were captured.

2.4. Induction of cytokine production

Because cytokines in tissues were difficult to detect by immunofluorescence due to their rapid secretion and dissipation, kidney sections or isolated glomeruli were stimulated *in vitro* in the presence of secretion blockers by a method analogous to *in vitro* single cell cytokine production assays [27, 28]. Mice were anaesthetized with ketamine/xylazine and perfused through the left ventricle with 20 ml of RPMI1640 containing 25 mM HEPES, pH 7.4, 20 ng/ml phorbol myristate acetate (PMA), 1 μ M ionomycin, and secretion blockers brefeldin A (10 μ g/ml) and monensin (2 μ M). The kidneys were sliced into 2-3 mm section and incubated in a CO₂ incubator on a rocker for 6 h with the same stimulation mixture plus 10% heat-inactivated fetal bovine serum (hiFBS). For glomeruli stimulation, mice were perfused with 20 ml cold PBS with magnetic beads and glomeruli isolated [29] before stimulation. Tissue sections or glomeruli were fixed in 2% PLP and processed as described in section 2.3.

2.5. Glomerulus and glomerular cell isolation.

Magnetic bead isolation of glomeruli was performed as described using magnetic beads [24, 29]. The purified glomeruli were 98% pure with yields comparable to published results of 24,000 glomeruli per mouse [29, 30]. A single cell suspension was obtained after Liberase TM digestion with a yield of 3×10^6 cells/mouse.

2.6. Flow cytometry sorting of glomerular intrinsic cells and macrophages

FACS sorting of glomerular cells from single cell suspensions of magnetic-bead isolated glomeruli was performed on a 5-laser, 15-color BD Influx cell sorter at the U. of Virginia Flow Cytometry Core Facility. For glomerular intrinsic cell sorting, single cell suspensions were stained with the following fluorochrome-conjugated Ab: PacBlue-anti-CD73, BV-510-anti-CD11b, A488-anti-Itga8, phycoerythrin (PE)-anti-CD26, Peridinin-Chlorophyll-Protein (PerCP)-Cy5.5-anti-CD31, PE-Cy7-anti-CD105, A647-anti-nephrin, allophycocyanin (APC)-eFluor780-anti-CD45, and A700-anti-I-A plus 7-AAD. Mesangial cells were gated on live singlet CD45⁻CD105⁺Itga8⁺CD73⁺CD31⁻ cells. For myeloid cell analysis, single-cell suspensions from total kidney or glomerular digests were stained with the following fluorochrome-conjugated Ab: BV421-anti-Ly6C, BV510-anti-Ly6G, A488-anti-I-A, PE-anti-CD11c, PE-Cy7-anti-CD11b, A647-anti-CD103, and APC-eFluor 780-anti-CD45. Blood-derived glomerular macrophages were live singlet CD45⁺CD11b⁺Ly6G⁻I-A⁻F4/80^{Lo}

cells. Interstitial conventional CD11b⁺ dendritic cells (DC) were: CD11b⁺CD103⁻CD45⁺CD11c⁺I-A⁺. Interstitial blood-derived macrophages were: CD11b⁺F4/80^{Lo}CD45⁺I-A⁻Ly6G⁻. Flow cytometry data were analyzed by the program FlowJo (Ashland, OR, USA).

2.7. Microarray analysis

Sorted mesangial cells, DC, and macrophages were lysed in Trizol (Invitrogen) for RNA isolation. Affymetrix microarray analysis of mesangial cell, DC, and macrophage gene expression using GeneChip® Mouse Genome 430 2.0 Array (Santa Clara, CA) was performed by Virginia Bioinformatics Institute (Blacksburg, VA). Data analyses by R packages affy and simpleaffy showed that the results were of high quality. The CEL files were further normalized and background subtracted to provide gene expression levels using R packages RMA and LIMMA. Batch effects among different analyses were normalized with the R package COMBAT.

2.8. Real-time PCR

Real-time PCR analysis of DC mRNA—Total RNA from FACS-sorted DC subsets (>98% purity) were reverse transcribed by the Advantage RT-for-PCR kit (BD Clontech). Real-time PCR analysis was performed in a Bio-Rad iCycler Thermal Cycler using Sybr green fluorescence as the readout, and data were analyzed by the iCycler program provided (Bio-Rad). PCR conditions were: 94°C, 22 s; 58–62°C as appropriate, 30 s; and 72°C, 30 s for 39 cycles; 94°C, 22 s; 62°C, 30 s; and 72°C, 5 min for 1 cycle. Melt curves were obtained by increasing the temperature from 65°C to 95°C in 0.5°C increments for 10 s. The primer sequences were generated by the program Primer 3 and the primers synthesized by IDT (Coralville, IA). Primers used for cytokine mRNA have been published [24]. Agarose gel (1.8%) electrophoresis was used to confirm the correct molecular size of the PCR products.

2.9. Statistics

Standard deviation and Student's t test were performed with the program Prism (GraphPad Software, Inc., La Jolla, CA, USA) and by Microsoft Excel (Redmond, WA, USA). R packages affy, simpleaffy, RMA, LIMMA, and COMBAT were used for microarray analysis. Heatmaps were generated with R packages gplots and ggplot2.

3. Results

3.1 Cytokine mRNA expression of intraglomerular bone marrow-derived macrophages

In NZM mice with cGN, CD11b^{hi} bone marrow-derived macrophages are the predominant glomerulus-infiltrating inflammatory cell type with an increase of >8-fold over young non-diseased mice [24]. The role of these CD11b^{hi} macrophages in determining the intraglomerular cytokine environment was first studied by cytokine mRNA quantitation of FACS-purified glomerular CD11b^{hi}I-A⁻F4/80^{Lo}Ly6G^{Lo} macrophages from young mice and mice with 3+–4+ proteinuria by real-time PCR. *Tnf II1b*, and *II10* mRNA levels were high in these macrophages with significant increases on a per cell basis for *II1b* and *II10* mRNA in

sick mice (Fig. 1). Lower levels of *Il6*, *Il18*, and *Il1a* mRNA were detected (Fig. 1) but *Il12a* and *Il12b* mRNA levels were negligible (not shown).

3.2 Intraglomerular TNF- α was produced predominantly by macrophages

Since cytokines are under translational control [24, 31, 32] and are difficult to detect because of their secretion into the intercellular space, we used *in situ* stimulation and secretion blocking to visualize intracellular cytokines and to identify cytokine cellular origins by confocal microscopy. Staining treated tissue sections with anti-TNF- α showed that TNF- α was produced predominantly by CD11b⁺ glomerulus-infiltrating macrophages (Fig. 2A, arrows, panels a, b e, f). Extraglomerular F4/80⁺ resident macrophages in the kidney interstitium produced little TNF- α (Fig. 2A, arrowhead, panels a, c, e). Most CD11b⁺ macrophages produced TNF- α . However, the amounts of TNF- α detected varied from cell to cell. In Fig 2A, those macrophages that produced a large amount of TNF- α are marked by arrows. With careful examination of Fig. 2A, panels a and e, macrophages without obvious colocalization with TNF- α (yellow in panel e) did produce a small amount of TNF- α as shown in panel a. PMN which stain brightly for CD11b (*, Fig. 2A, panels a, b, e, f) and occur in the glomerulus in low numbers in NZM mice with cGN (<1/20 of the number of CD11b⁺ macrophages, ref. # [24]) produced little TNF- α . Little glomerular TNF- α production was detected in kidneys in young NZM mice and no signal was detected with control isotype Ab (not shown).

Glomerular intrinsic cells did not produce TNF- α . TNF- α staining was not associated with mesangial and endothelial cells staining defined earlier by Itga8 and CD31 respectively (Fig. 2B, arrowheads, panels a–c, e; ref #[33]). The topographic localization of TNF- α staining interior of the endothelial cells (Fig. 2B, panel e) further supported the conclusion that podocytes were not the TNF- α producing cells.

3.3 Intraglomerular macrophages produced little IL-6 and IL-1 β

Intraglomerular macrophages in NZM mice with cGN express some IL-6 and high levels IL-1 β mRNA (Fig. 1; ref. #[24]). Staining the kidneys of NZM with cGN showed large amounts of IL-6 and IL-1 β cytokine protein within the glomerulus (Fig. 2C, 2D). However, the vast majority of the IL-6-producing cells did not colocalize with macrophages expressing CD11b (Fig. 2C, arrows, panels a, b, e) while none of the IL-1 β -producing cells stained for CD11b. (Fig. 2C, arrowheads, panels b, f) Thus the IL-6- and IL-1 β -producing cells were largely glomerular intrinsic cells.

3.4 Kidney mesangial cells produce IL-6 in NZM mice with cGN

Though cytokine production has been demonstrated in the glomerulus in diseased kidneys, ambiguities occur regarding their cellular origins because intrinsic cell type-specific markers were not included in the analyses for localization [13, 15, 16, 18]. Thus confocal microscopy was used to identify the glomerular cell type producing IL-6. We have shown previously that podocytes are identified by nephrin and CD26, mesangial cells by Itga8, and endothelial cells by CD31 [33]. IL-6 staining colocalized only with mesangial cell staining (Fig. 3A, arrows, panels a, b, e) and not with endothelial cells (Fig. 3A, arrows, panels c, e) or CD45⁺ cells (Fig. 3A, arrowheads, panels d, f) which comprise mostly of CD11b⁺ macrophages

with a lower number of I-A⁺ dendritic cells (DC), but very few T cells and PMN [24]. IL-6 was not produced by nephrin⁺ podocytes (Fig. 3B, arrowheads, panels a, b, e, f). In agreement with the lack of IL-6 staining in CD45⁺ cells (Fig. 3A, panels d–f), intraglomerular CD11b⁺ macrophages and I-A⁺ DC-like cells produced no IL-6 (Fig. 3B, arrowheads, panels c–f).

Mesangial cell production of IL-6 occurred only in NZM mice with cGN. Young NZM mice with only trace amounts of proteinuria (Fig. 3C), old NZM mice with minimal proteinuria (1+, Fig. 3D), and 6–12 Mo old adult congenic R27 mice which does not exhibit cGN [21] (Fig. 3E), did not produce IL-6. Thus intraglomerular IL-6 was produced predominantly by mesangial cells and detectable only in mice with severe proteinuria and cGN.

3.5 Elevated IL-1 β production by Podocytes but not glomerular macrophages in NZM mice with cGN

In NZM mice with cGN, the high IL-1 β production completely coincided with podocyte staining identified by anti-nephrin (Fig. 4A, panels a, b, d, e; yellow in d, e). No IL-1 β production was associated with mesangial cells and endothelial cells identified by Itga8 and CD31 staining respectively (Fig. 4A, panels c, d, and g–j). Thus IL-1 β production within the glomerulus environment is highly specific to podocytes.

Treatment with stimulant plus secretion blockers clearly enhanced IL-1 β signals. IL-1 β production by podocytes was low in kidney tissues with no treatment (Fig. 4B, panels a, d, e). Podocytes in young NZM mice with little proteinuria and inflammation expressed low but detectable levels of IL-1 β in podocytes (Fig. 4C, panel a, b, d, e). Similarly, podocytes in R27 mice with low proteinuria and kidney inflammation expressed low IL-1 β levels (Fig. 4D, panel a, b, d, e). Thus the inflammatory state of cGN in NZM mice markedly enhanced IL-1 β production by podocytes.

3.6 Intrinsic cell expression of cytokines influence the glomerular cytokine milieu

Among the intrinsic glomerular cells, mesangial cells are considered to resemble macrophages closely in their secretory activities and would significantly shape the intraglomerular cytokine environment [34, 35]. Thus mesangial cell mRNA expression was first assessed by microarray to identify cytokines which may play key roles in the glomerulus. Cytokines of nonhematopoietic origin was further identified by comparing gene expression by mesangial cells, interstitial myeloid-derived CD11b⁺F4/80^{Low} macrophages, and I-A⁺CD11c⁺CD11b⁺ DC from NZM mice with cGN. The results showed the mRNA overexpression by mesangial cells of not only the immune cytokines IL-6, M-CSF, and SCF, but also cytokine of mesenchymal origin including CTGF, BMP6, FGF, PDGF, and VEGFa (Fig. 5A).

SCF and M-SCF encoded by *Kitl* and *Csf1* respectively was readily detected in the glomeruli of NZM mice with cGN (Fig. 6A, 6D). Mesangial cells were the predominant source of SCF within the glomerulus (Fig. 6A, arrows, panels a, b, e, f). Endothelial cells were devoid of SCF staining (Fig. 6A, panels c, e, f), so were the intraglomerular Mac2⁺ M2 macrophages (Fig. 6A, panels h, i). There was extraglomerular SCF staining (*, Fig. 6A,

panels g, l) which did not coincide with proximal (N-cadherin) or distal (claudin 2) convoluted tubule markers (Fig. 6A, panels g–l).

M-CSF was produced by mesangial cells (Fig. 6D, arrows, panels a, b, e, f) but not by podocytes and endothelial cells (Fig. 6E). Glomerular CD11b⁺ and F4/80⁺ macrophages and I-A⁺ DC within the glomerulus did not produce M-CSF (Fig. 6D, panels a, c–f, and g–l).

SCF and M-CSF production were found only during kidney inflammation. Young NZM mice with no kidney inflammation and R27 mice at the age 7–8 Mo and with no proteinuria, did not show glomerular SCF and M-CSF staining (Fig. 6B, 6C, 6F, 6G). Thus SCF and M-CSF were produced in the glomerulus by mesangial cells and only in sick NZM mice when the kidney is inflamed and 3+ proteinuria is observed.

Podocytes may also contribute significantly to the glomerular cytokine milieu. Published RNAseq data (GSE64063; Ref. # [11]) showed that podocytes express high levels of *VEGFa* mRNA and lower levels of *Bmp7*, *Egf*, *Il18*, *Il34* and *Tgfa* mRNA (Fig. 5C). Among these cytokines, IL-34 has been shown to be involved in LN in MRL/lpr mice [36]. IL-34 protein expression in kidneys of young nondiseased mice was low (not shown), but was significantly elevated in mice with cGN. IL-34 protein production was prominent in the glomerulus and podocytes were the only cell type expressing IL-34 (Fig. 6H, panels a–d, arrows). No IL-34 production was detected in mesangial cells and endothelial cells (Fig. 6H, panels a,b,e,f).

3.7 Mesangial cells, podocytes, and macrophages express cytokine receptor mRNA for communication among the glomerular cells

Mesangial cells express a variety of growth factor receptors to respond to paracrine and autocrine factors which are produced within the glomerular space or have diffused across the endothelium (Fig. 5B). High levels of mRNA of receptors for cytokines, IL-6R/GP130 family members, BMP's, TLR's, S1P's, VEGF's, FGF's, and blood components. Significantly, mesangial cells express *Tnfrsf1a*, *Tnfrsf1b*, *Il1r1*, *Il6ra*, and *Flt1* mRNA and their receptor proteins can engage the paracrine factors TNF- α , IL-1 β , and VEGFa produced by macrophages and podocytes or IL-6 as an autocrine during GN pathogenesis (Fig. 5B, arrows).

By the same token, podocytes express mRNA for cytokine receptors, BMP receptors, TLR's, FGFR, and blood component receptors (Fig. 5D, arrows). Of relevance is the podocyte expression of IL-6R α and TNFRI mRNA. Their translation products can respond to stimulation by TNF- α and IL-6 produced by infiltrating macrophages (Fig. 2A) and mesangial cells (Fig. 3A) respectively.

It is noteworthy that interstitial blood-derived CD11b⁺ macrophages and DC express moderate levels of *Tnfrsf1a*, *Tnfrsf1b*, and *Il6ra* but low levels of *Il1r1* mRNA (Fig. 5B, arrows). Since glomerular infiltrating macrophages are blood-derived [24], they are likely to express the same cytokine receptors and respond similarly as the interstitial macrophages.

3.8 In isolated glomeruli without Bowman's capsule i.e decapsulated glomeruli, macrophages produced high levels of IL-10 protein

Because IL-10 is anti-inflammatory and intraglomerular macrophages expressed high levels of IL-10 mRNA (Fig. 1), IL-10 protein production in the kidney was examined (Fig. 7A, 7B). Surprisingly intraglomerular macrophages produced no IL-10 protein when kidney slices were stimulated *in situ* (Fig. 7B, arrows). However, when glomeruli released from the Bowman's capsules were stimulated after isolation, high levels of IL-10 cytokine were produced by the infiltrating macrophages (Fig. 7A, arrows, panels a, b, e, f; IL-10⁺ macrophages in yellow in e, f).

All IL-10 staining was associated with CD11b⁺ macrophages (Fig. 7A, match staining in panels a and b) and not with Itga8⁺ mesangial cells or other intrinsic cells (Fig. 7A, panels c, e). Thus within the enclosed glomerular space as in the case of intact tissue slices, the environmental factors act to suppress IL-10 protein synthesis in macrophages, but when the released factors dissipate into the medium as in isolated glomeruli, robust macrophage IL-10 synthesis occurs.

3.9 Macrophages in isolated decapsulated glomeruli are capable of producing large amounts of IL-1 β

It is intriguing that IL-1 β protein production was enhanced in podocytes (cf. Fig. 4A vs 4B) in cGN whereas no IL-1 β production by macrophages was found in the same glomeruli (Fig. 2D, arrowheads, panels, e, f) and despite the high macrophage *Il1b* mRNA expression (Fig. 1). To explore whether the enclosure of macrophages within the Bowman's capsule in tissue slices causes the regulation IL-1 β production, isolated decapsulated glomeruli were activated and stained for IL-1 β (Fig. 7D). The results are in marked contrast to IL-1 β production detected in glomeruli of tissue slices. Whereas podocytes but not macrophages produced IL-1 β in intact tissue slices (Fig. 5A; Fig. 7C, arrows, macrophages in green), very high IL-1 β protein staining was detected in macrophages in isolated glomeruli (Fig. 7D, arrows, panels a, c, e, f; magenta, IL-1 β and macrophage colocalization). Low but readily detectable IL-1 β production by podocytes was found in isolated glomeruli (Fig. 7D, arrowheads, panels a, b, e, f). Thus within the enclosed glomerular environment which simulates the *in vivo* situation, factors favoring podocyte IL-1 β synthesis and inhibiting macrophage IL-1 β synthesis occur.

The cell type-specific production of IL-6 and TNF- α in isolated glomeruli was similar to that in encapsulated glomeruli within tissue slices, namely TNF- α was produced exclusively by CD11b⁺ macrophages (Fig. 7E, arrows, panels a, b, e, f) and IL-6 was predominantly produced by Itga8⁺ mesangial cells (Fig. 7F, arrows, panels a–c, e, f). For these cytokines, protein production was not affected by the glomerular environment.

4. Discussion

This report showed that major glomerular cytokines TNF- α , IL-6, and IL-1 β in mice with cGN are produced by infiltrating macrophages, mesangial cells, and podocytes respectively in a cell type-specific manner. This specificity occurs independently of mRNA expression

levels, especially in macrophages which exhibit high IL-1 β mRNA expression but produced no cytokine protein when stimulated *in situ* (Figs. 1, 2). Coupled with the expression of TNF-R, IL-6R and IL-1R mRNA by the mesangial cells, podocytes, and macrophages (Fig. 5), the data suggest a mechanism of intensified glomerular inflammation in LN involving cellular interactions and activation cascades through cytokine production. In the pathogenesis of LN, the initial inciting event of immune complex deposition induces mesangial cell and podocyte IL-6 and IL-1 β production respectively through their Fc receptors and receptors for complement components [37–41]. These cytokines will stimulate the upregulation of adhesion molecules and chemokines to induce the glomerular influx of macrophages which are then stimulated by immune complexes, complement components, and intrinsic cell cytokines to produce TNF- α . The combination of TNF- α and IL-1 β have been shown to stimulate mesangial cells efficiently [35, 42, 43] to produce IL-6 and the accumulation of these 3 cytokines in the glomerulus will play a dominant role in regulating other cellular events leading to glomerular damage, cellular proliferation and matrix protein deposition, all hallmarks of GN.

Though we have not directly measured the protein expression of TNFR, IL-1RI, and IL-6R because of their low expression levels, we deem it highly likely that their protein levels will correlate with their mRNA levels as shown by microarray or RNASeq analyses based on the reasoning that follows. A recent study showed that the levels of a surprisingly high percentage (>40%) of cellular proteins correlate with their mRNA expression [44]. Most translational regulation occurs through RNA binding proteins interacting with cis elements such as the AU-rich elements (ARE) in the 3'-untranslated (UT) region and a lower number through the internal stem loops of mRNA [45]. Although it is unclear whether regulation by internal stem loops occurs, we found no ARE (<http://brp.kfshrc.edu.sa/ared>) in these cytokine receptor mRNA [46] and there has no published evidence suggesting that these receptors are under translational control. These reasons suggest the expression of the target cytokine receptors (Fig. 5B, 5D) in intrinsic cells but direct confirmation with improved sensitivity is needed.

Mesangial cells and podocytes are considered mesenchymal- and epithelial- derived cell types [47]. Though these cell type are not considered important contributors of the cytokine milieu, reports of their cytokine production have been amply documented [13–20]. Besides kidney cells, epithelial cells in the lungs are significant producers of IL-25, IL-33, and thymic stromal lymphopoietin [48]. Similarly, mesenchymal derived cell such as smooth muscle cells in kidney, lungs, and intestines produce IL-6 [49–51]. Thus cytokine production by mesangial cells and podocytes are in agreement with the abilities of cells of similar embryonic origins in other tissues in cytokine production.

The lack of detectable IL-10, and IL-1 β protein production despite high mRNA levels in macrophages clearly indicates that cytokine mRNA within the glomerular compartment are under translational regulation (Figs. 1, 7). This regulation is most prominent for IL-10 translation which is almost completely absent in tissue slices following stimulation (Fig. 7B). and is compartment status-specific since large amounts IL-10 protein was produced by macrophages in isolated glomeruli without the Bowman's capsule (Fig. 7A). TNF- α , IL-1 β , IL-6, and most cytokine mRNA contain in their 3'-untranslated region AU-rich elements

(ARE) that regulate the translation, stability, and other properties of the mRNA (Reviewed in [31, 32]).

Translation inhibition occurs when ARE-binding proteins tristetraprolin (TTP), TIAR, or TIA-1 attach to the ARE motifs to sequester the mRNA in translation arrest sites named cytoplasmic P-bodies or stress granules [52–54], and to interact with regulatory proteins such as the general repressor RNA helicase RCK/P54 [55]. TTP also competes for mRNA binding with the ARE-binding and -stabilizing factor human antigen R (HuR) which is required for cytokine translation [56] and phosphorylation of TTP by MK2 in the MAPK/MK2 activation pathway reduces TTP affinity for ARE to allow the translation of ARE-containing mRNA. TTP also recruits the eukaryotic initiation factor 4E2 (eIF4E2) [57] and the TTP bound eIF4E2 competes off eIF4E with its recruited eIF4G, eIF4A, eIF4B proteins and the 43S preinitiation complex from the mRNA cap to arrest translation initiation. Other mRNA-binding proteins such as the ARE/poly(U)-binding/degradation factor (AUF1), fragile-X-mental-retardation-related protein 1, and Argonaute 2 also regulate mRNA translation [58, 59]. In IL-10 translation in THP-1 macrophage cell line, AUF1 is required for LPS-induced IL-10 protein production [60]. For IL-10 translational regulation in the glomerulus, we hypothesize that some glomerular mediator regulates the function of TTP, AUF1, or their associated protein by activating signaling pathway such as p38 or ERK [56, 61] to inhibit translation and these mediators dissipates by diffusion from the immediate macrophage environment in isolated glomeruli, and thus allowing IL-10 protein production. Important systemic lupus erythematosus single nucleotide polymorphism (SNP) risk alleles occur at –1082 (G/A), –819 (C/T), –592 (C/A) upstream of the transcription start site [62, 63]. These SNP most likely control the levels of *Il10* transcription and not translation occurring in intraglomerular macrophages since these SNP's are not present in the *Il10* mRNA.

Translational regulation of IL-1 β is cell type-specific (Fig. 2C, 4A). Macrophages failed to produce cytokine protein in intact tissues despite high IL-1 β mRNA (Fig. 1). In the same environment, high levels of IL-1 β protein was produced by podocytes. An unusual feature of IL-1 β translational regulation is that Tyk2 or IFN-R1 deficient macrophages exhibit enhanced Listeria-induced Pro-IL-1 β and mature IL-1 β production [64] suggesting that Jak-Stat signaling is involved. Based on this report, we hypothesize that Jak-Stat activation in the glomerulus occurs in macrophages but not in podocytes to result in differential IL-1 β production in podocytes and this activation disappears in decapsulated glomeruli, thus allowing IL-1 β protein production in macrophages.

We hypothesize that the translational regulation of IL-10 and IL-1 β is mediated by soluble factors in the glomerulus. With glomerular damage in GN, this regulation may be lost as a result of the disruption of the environmental barrier. However, we have failed to observe this loss of regulation in NZM mice with varying degrees of proteinuria severity. Given that we have not examined the mechanism, the time frame, or the disease state for this translational regulation, it is difficult for us to provide a reasonable explanation for finding exceptions to this regulation in damaged glomeruli.

The glomerulus forms an enclosed environment with complex effector molecules produced by inflammatory and intrinsic cells (Fig. 5). Besides cytokines, growth factors belonging to the IL-6R /GP130, BMP, FGF, and VEGF families (Fig. 5), growth factors, reactive oxygen species, prostaglandins, and other effectors may all play a role in GN progression. It is noteworthy that M-CSF and IL-34 are produced in the glomeruli of NZM mice with cGN (Fig. 7D, 7H). Both growth factors bind to the macrophage CSF-1R mediate macrophage migration and activation, and promote LN in MRL/lpr mice [36]. These 2 cytokines may mediate the glomerular infiltration of blood-derived macrophages in cGN [24], and activate macrophages to produce TNF- α and to participate in the intraglomerular cytokine circuit. Given the wide variety of growth factors produced by the intrinsic cells, a better understanding of this effector milieu will provide more detailed mechanism of LN pathogenesis including podocyte and endothelial cell damage, mesangial cell proliferation, and glomerular matrix deposition. These growth factors may serve as novel targets for improved LN treatment.

It would be remiss not to discuss the role of NLRP3 inflammasome activation in the context of IL-1 β induction in podocytes. Podocyte activation of NLRP3 inflammasomes contributes to the development of proteinuria in lupus nephritis [65], as demonstrated by the activation of NLRP3 inflammasome by IgG from sera from NZM with cGN and anti-dsDNA Abs leading to IL-1 β induction in the stressed podocytes. Recently Guo et al [66] showed that this activation is at least in part through the RIP3-dependent pathway, suggesting the possibility that the TNF- α receptor is involved. In this regard, TNF- α made by the infiltrating macrophages may further fuel NLRP3 inflammasome activation because activation of RIP3 is predominantly through the TNF- α activation pathway [66]. The activation of NLRP3 and inflammasome assembly activates caspase 1 which cleaves pro-IL-1 β and pro-IL-18 to IL-1 β and IL-18 respectively and induces the secretion of the mature cytokines. We have documented that IL-18 is made by the stressed podocytes in cGN. It appears that IL-18 may also play an important role in the pathogenesis of LN (Sung et al, Manuscript in preparation). This aspect of NLRP3 activation will be the topic of another communication.

The cytokine circuit of TNF- α /IL-6/IL-1 β activation most likely plays a significant role in LN and disruption of this circuit may provide an attractive target for therapy. The effects of cytokine blocking on LN have been studied in rodents. Anti-TNFRII has been shown to abrogate IFN- α -accelerated LN in (NZB/W)F1 mice [67], anti-IL-6R mAb preserved kidney filtration functions in MRL/lpr mice [68], and anti-IL-6 mAb lowered glomerulus disease score and inhibited anti-dsDNA production in NZMW mice [69] with spontaneous disease. However, a small clinical trial of 25 LN patients with class III or class IV disease with anti-IL-6 treatment of LN patients failed to demonstrate efficacy and safety [70]. Efficacies in isolated instances nonetheless occur. One LN class IV patient who failed to remit with a combination of full dose steroids, mycophenolate mofetil, and cyclosporine, went into sustained remission with the addition of infliximab infusions [71]. A second patient with severe diffuse proliferative LN was successfully treated with a combination of TNFRII-Ig, intravenous gammaglobulin, and plasmapheresis [72]. These cases suggest that blocking TNF functions may be effective in treating LN, but more definitive clinical trials with adequate power and better design will be needed. IL-1R antagonist IL-1RA-Ig (Anakinra/

Kineret) used in rheumatoid arthritis treatment has not been demonstrated to be successful in treating LN. The modification of treatment protocols may be needed for the successful LN treatment with cytokine antagonists. Manipulating the translational regulation of the anti-inflammatory cytokine IL-10 in macrophages may be effective. However, a better understanding of intraglomerular cytokine circuit will improve the success rate of cytokine intervention in lupus pathogenesis.

In summary, this report shows that TNF- α , IL-6, and IL-1 β are produced in large amount in the glomerulus of kidneys from mice with cGN but the production is cell type-specific with macrophages, mesangial cells, and podocytes each playing a significant role. A cooperative activation by this cytokine circuit formed by these 3 cytokine producers will amplify and propagate inflammatory response in LN to cause GN. This process is under translational control such that cytokine therapies will need to take into account mechanisms that affect cytokine posttranscriptional regulation.

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Highlights

- An *in situ* cytokine detection method was used to visualize cytokines in glomeruli.
- Cell-specific cytokine circuit of TNF- α , IL-6 and IL-1 β production in LN was found.
- Mesangial cells, podocytes and macrophages express receptors for the interactions.
- Mesangial cell and podocyte produced M-CSF and SCF, and IL-34 respectively in cGN.
- The glomerulus microenvironment mediates cytokine translational regulation in LN.

Fig. 1.

Cytokine mRNA expression by glomerulus-infiltrating macrophages. Glomerular CD11b⁺ infiltrating macrophages in 3 Mo old NZM mice with trace proteinuria by dipstick (light gray) or 7 Mo – 12 Mo old NZM mice with 3+ - 4+ proteinuria (dark gray) were purified by cell sorting of single cell suspensions of magnetic bead-isolated glomeruli as described in Materials and Methods. In each sort, glomerular cells from 3-4 mice were pooled. Total RNA were extracted and subjected to reverse transcriptase-real time PCR analysis. The results are pooled from 2 – 4 experiments. *, $p < 0.05$.

Fig. 2.

Glomerulus-infiltrating macrophages in NZM mice with cGN produce TNF- α but not IL-6 and IL-1 β cytokine protein. Mice with 3+ to 4+ proteinuria by dipstick were perfused with 20 ml of medium with PMA, ionomycin, brefeldin A, and monensin and kidney slices were incubated in the same medium with 10% hiFCS for 6 h. Tissue slices were fixed in PLP, passed through a sucrose gradient, and embedded in OCT. Tissue sections (5 μ m) were stained simultaneously with 3 – 4 Ab directly conjugated with fluorochromes of excitation wavelengths of 421, 488, 555, and 647 nm. Photomicrographs were collected on a Zeiss LSM-700 confocal microscope assembly and analyzed by the program ZEN. At least 5 mice were analyzed by the same Ab combinations and >15 glomeruli were recorded for each determination. (A) Arrows show selected CD11b⁺ macrophages producing TNF- α . (B) Arrowheads show that TNF- α staining (panels a, e) is not associated with mesangial cell (panel b, e, f) or endothelial cell (panels c, e, f) staining. (C) CD11b⁺ macrophages did not produce IL-6 (arrows show macrophages, panels a, b, e, f). (D) Arrowheads show CD11b⁺

macrophages with no IL-1 β production (panels b, e, f). In panels f, glomeruli were shown in circles. Bars in f equals 10 μ m.

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Fig. 3.

IL-6 production by mesangial cells in NZM mice with cGN. Kidney slices from NZM or R27 mice with and without severe proteinuria (3+ dipstick) were stimulated in the presence of secretion blockers as described in Fig. 2. Sections were stained with the indicated fluorochrome-conjugated Ab. Photomicrographs were captured by confocal microscopy and analyzed by ZEN software. (A) IL-6 staining colocalized with mesangial cell staining (arrows, panels a, b, e, yellow in e) but not with endothelial cells (arrows, panels c, e) and CD45⁺ leukocytes (arrowheads, panels d, f). (B) IL-6 (arrowheads, panels a, e, f) is not produced by podocytes, CD11b macrophages, and I-A⁺ macrophages/DC (arrowheads,

panels b-f). IL-6 is not detected in the glomeruli of young NZM mice (C) and R27 mice of all ages (E). In old NZM mice without cGN, few mesangial cells (arrows in D, panels a-c) produced IL-6. Bars are 10 μ m.

Fig. 4.

Podocytes produced IL-1 β in kidneys of NZM mice with severe proteinuria and cGN. Tissue slices were processed and stained as described in Fig. 2. (A) IL-1 β staining is associated exclusively with podocytes (panels a, b, d, e; colocalization, yellow in d and e) and not with endothelial cells (panels a, c, d; blue vs. yellow in d and e) and I-A⁺ cells (panel e, blue vs. yellow). No IL-1 β staining (panel f, i, j, red) was associated with mesangial cells (panels g, and i, and j, green). Endothelial cells and I-A⁺ cells are shown in blue in panels i, and j respectively. In A, panels a-e show one glomerulus and f-j show a second glomerulus. (B)

Little IL-1 β was detected in kidneys of NZM mice with severe proteinuria and cGN in the absence of stimulation and secretion blocking (IL-1 β , arrows). (C) Small amounts of IL-1 β was found associated with podocytes in young NZM mice with no proteinuria (IL-1 β , panels a, d, e) and in (D) R27 mice at age 6 Mo to 12 Mo with little proteinuria (IL-1 β , panels a, d, and e). Bars in panels e equals 10 μ m.

Fig. 5.

Cytokine and cytokine receptor mRNA expression by mesangial cells, renal myeloid cells, and podocytes. (A, B) Mesangial cells, and interstitial macrophages and dendritic cells from NZM mice with 3+ - 4+ proteinuria were purified by FACS sorting as described in Materials and Methods and used as the RNA source for microarray analysis. Heat maps of growth factors and growth factor receptors are shown in (A) and (B) respectively. Three independent sorts with pools of 4-5 mice each was used. (C, D) Heat map of podocyte cytokine and

cytokine receptor mRNA expression by RNASeq. The data was obtained from GEO dataset GSE64063 [11].

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Fig. 6.

Mesangial cells produce SCF and M-CSF and podocytes produce IL-34 in NZM mice with cGN. Kidney tissues were prepared as described in Fig. 2 and stained with the indicated fluorochrome-conjugated Ab. (A) SCF staining (arrows, panel a, e, f) colocalizes with mesangial cell staining (panels b, e, f yellow in e, f) but not with Mac2⁺ macrophages (panels h, k, l) or proximal or distal tubular cells (panel i – l). Panels a-f is from 1 glomerulus and g-l is from a second glomerulus. Glomerular cells in young NZM mice (B) and 6-12 Mo old adult R27 mice (C) with no proteinuria did not produce SCF (red). (D) M-

CSF (arrows, panels a-c, e, f, red to yellow in e, f due to cytoplasmic vs. membrane staining) was produced by mesangial cells (arrows, panel b; green in panels e, f) but not by CD11b⁺ macrophages (panel c; blue, panel e) or I-A⁺ macrophage/DC (panel d, blue, panel f). F4/80⁺ macrophages also did not produce M-CSF (panels i, k). (E) Podocytes (panels b, d) and glomerular endothelial cells (panels c, d) in NZM mice with cGN did not produce M-CSF (panels a, d). Young NZM mice (F) and 6-12 Mo old R27 mice (G) without cGN did not produce M-CSF. (H) IL-34 production by podocytes in NZM mice with cGN. Arrows shows colocalization (yellow) of IL-34 (red in a, b) with podocytes (green in a, b). The individual staining of IL-34, nephrin, Itga8, and CD31 are shown in c, d, e, and f respectively. Glomeruli are outlined in white. Bars equal 10 μ m.

Fig. 7.

High levels of IL-10, IL-1 β and TNF- α production by CD11b⁺ macrophages with little IL-6 production in isolated glomeruli. Glomeruli were isolated from NZM mice with cGN by magnetic beads prior to stimulation with PMA and ionomycin, plus secretion blocking with brefeldin A and monensin. The glomeruli were fixed in 2% PLP, equilibrated in sucrose gradient, and frozen in OCT. Sections were stained with fluorochrome-conjugated Ab against cytokines and glomerular intrinsic cell and macrophage markers. (A) In isolated glomeruli, IL-10 was produced by CD11b⁺ macrophages but not mesangial cells (arrows,

IL-10 and macrophages, panels a, b, e, f; yellow for colocalization in e, f). On the other hand, IL-10 was not detected in glomerular macrophages and intrinsic cells when cells were stimulated in tissue slices (B; arrows indicate CD11b⁺ macrophages). (C) Similarly, IL-1 β (panels a, c, red in c) was not produced by CD11b⁺ macrophages in tissues (arrows, macrophages; panel c, green) whereas large amounts of IL-1 β (panels a, c, red in c) was produced by podocytes. In contrast, in isolated decapsulated glomeruli (D), macrophages produced large amounts of IL-1 β (arrows, IL-1 β and macrophages in panels a, c, e; magenta for IL-1 β and CD11b colocalization in e). Lower levels of podocyte IL-1 β production was detected (arrowheads, panels a, b, e, f; yellow for podocyte and IL-1 β colocalization in e, f). (E) TNF- α was produced exclusively by CD11b⁺ macrophages (arrows, TNF- α and macrophages, panels a, b, e, f; yellow for CD11b⁺ TNF- α ⁺ cells in e, f). (F) IL-6 was detectable only in Itg α 8⁺ mesangial cells (arrows, IL-6 and mesangial cells, panels a, b, e, f; yellow for Itg α 8⁺IL-6⁺ cells). Glomeruli are enclosed in white outlines and bars equal 10 μ m. These experiments are repeated 3 times with 2-3 NZM mice with cGN in each experiment.