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Method Article

A novel immunofluorescence detection method for renal cell-type specific in situ cytokine production by confocal microscopy

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A B S T R A C T

The detection of cytokines production in tissues is subjected to significant limitations: (1) Cytokine protein production frequently does not correlate with mRNA levels. (2) Cytokines are secreted rapidly and dissipate from the cellular source, thus making detection difficult. (3) The synthetic rate of many cytokines are low. (4) Tissue fixation ablates antigenic sites and diminishes detection signals. The identification of the cellular sources of cytokines poses an additional challenge because of the lack of suitable and readily available cellular markers. In our renal cytokine production studies in lupus nephritis, we have established methods to resolve problems associated with the identification of cellular sources of pertinent cytokines in the glomerulus and interstitium. Four-color confocal microscopy was used to colocalize cell-type specific markers with cytokines. The cytokine signal was amplified by the incubation of tissue slices in medium containing pan-specific stimulants plus secretion blockers. Tissue fixation was optimized to provide sharp crisp signals. Commercially available Ab suitable for fluorochrome labeling were used to establish cell-specific markers in the tubules and glomeruli. This combination of optimizations allowed us to define the cellular sources of important glomerular cytokines including TNF-α, IL-6, and IL-1β which appear to form a cytokine circuit in glomerulonephritis pathogenesis.

- Tissue stimulation and secretion blocking for cytokine detection
- Fixation optimization and Ab source identification for direct staining
- Colocalization of cytokines and renal cell-type specific markers

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More specific subject area: cytokines, lupus nephritis	Describe narrower subject area
Method name: Cell-type specific in situ cytokine production	Please specify a name of the method that you customized. The method name should be a word or short phrase to describe the methods used in your paper
Name and reference of original method	If applicable, include full bibliographic details of the main reference(s) describing the original method from which the new method was derived.
Resource availability	If applicable, include links to resources necessary to reproduce the method (e.g. data, software, hardware, reagent)

Method details

Summary

Mice were anesthetized and perfused with RPMI1640 with stimulants and secretion blockers. Kidneys were sliced into 2-3 mm sections and incubated in medium with the same drugs for 6 h. After stimulation, tissue slices were fixed for short periods and equilibrated in sucrose solutions. The OCT blocks from these tissues were sectioned at 5 μm, blocked, and stained with Ab directly conjugated with fluorochromes. Confocal images were captured on a Zeiss LSM700 confocal microscope assembly and analyzed with a Zeiss-provided software.

Procedure

Kidney in situ *cytokine induction*

1. Anesthetize mouse by i.p. injection of 0.225 ml ketamine/xylazine/heparin mix. Heparin will reduce clotting and facilitate kidney perfusion. Wait till animal is unresponsive to toe pinch.
2. Pin mouse on a vinyl dissection flex pad in a dissection pan (Carolina Biol., Burlington, NC; #629005) for perfusion medium collection.
3. Slit open skin along the abdominal midline. Open abdominal cavity. Poke a hole in the diaphragm with scissors to dislodge the lungs against the chest wall. Slit open chest cavity

along the midline and trim off rib cage to expose the heart. Be cautious in avoiding large blood vessels.

4. Use scissors blades to press down the right heart to catch the overhanging segment of the right atrium between the blades. Make certain to excise a large enough piece of the heart wall to allow rapid drainage of perfusate from the vena cavae.
5. Fill a 60 ml plastic syringe with 20 ml of cold stimulation mix containing 20 ng/ml PMA, 1 μM ionomycin, 2 μM monensin, 10 µg/ml brefeldin A (BFA) (PIMB), and 25 mM HEPES, pH 7.4 in RPMI1640 with no serum. Connect to a 21 gauge butterfly vacutainer. The plastic protecting needle sheath is trimmed to allow just enough of the needle point and about 2 mm of the needle stem to be exposed in order to restrict the needle from penetrating too deeply and reemerging from the heart after puncture. After filling the syringe with medium, tap the syringe and catheter line and push the plunger gently to expel air bubbles. Allow the expelled liquid to drain into the thoracic cavity. Immerse the needle point in the liquid after expelling air bubbles to prevent the back suction of air into the needle.
6. Puncture the vacutainer needle into the apex of the left ventricle. Ensure that the needle will not puncture the wall leading into the right ventricle or reemerge from the left ventricle wall to cause leakage. Slowly push through the 20 ml of perfusion solution. If properly done, the liquid will not flow into the lungs and the liver can be seen to be slowly bleaching. The flow of perfusion fluid into the lungs usually indicates that the cut at the right atrium is too small or the perfusion rate is too high. Be mindful of spills and splashes and avoid contact with the perfusate as PMA is known to be a tumor promoter.
7. Excise kidneys and trim off capsule. Press one end of the kidney with index finger and cut with the other hand with a razor blade to 2-3 mm sections. Put into incubation medium (20 ml / 100 mm dish) and rock in a CO_2 incubator for 6 h. Incubation medium contains 10% heat-inactivated (56°C, 30 min) fetal bovine serum in addition to the stimulation reagents (PIMB) used in perfusion. If desired, save 2 kidney slices for unstimulated kidney background cytokine production or for histological staining of formalin fixed tissues.

Tissue fixation

1. Place stimulated tissue slices into histological cassettes and fix in p-formaldehyde/lysine/periodate (PLP) in the cold for 3 h as described [1]. The duration of this fixation is critical. Prolonged fixation obliterates antigenic sites of cytokines and cell-type specific markers recognized by Ab.
2. Rinse samples with PBS and allow formaldehyde to diffuse away from samples in a large volume of 5% sucrose with 50 mM sodium phosphate, pH 7.4 at 4°C overnight. Residual formaldehyde causes high background, especially in the shorter emission wavelength range of 450 nm (violet) to 530 nm (green).
3. Equilibrate in 15% sucrose in 50 mM sodium phosphate buffer, pH 7.4 at 4°C for 2 h.
4. Repeat equilibration in 30% sucrose in 50 mM sodium phosphate buffer, pH 7.4 at 4°C for 3 h.
5. Pass through 4 wells of OCT in 24 well plates to dilute out the sucrose solution on the tissue. Embed in OCT in tissue molds and freeze on dry ice. Store blocks at -70°C. Up to 4 kidney slices can be accommodated in a 15 mm $\times$ 15 mm mold (Fisher; #22863553).

Tissue staining with fluorochrome-conjugated Ab

1. Cut 5 μm tissue sections in a cryostat and place 2 sections per slide on Superfrost plus slides (Fisher Scientific). Circle tissues with a diamond stylus on the underside of the slide. PAP pens can also be used to encircle the samples to contain the staining Ab mixture. However, small sections will be difficult to locate in microscopy without the diamond knife etching.
2. Place slides in a staining rack. Soak slides in PBS in a staining dish for 10 min to dissolve OCT.
3. Extract membrane lipids by immersing in 0.3% Triton X-100 in Dulbecco's MEM (DME) containing 10% horse serum for 30 min in a staining dish.
4. Wash slides twice in PBS, 10 min each.
5. Block with medium containing 2.4G2 rat anti-mouse FcγRII/FcγRIII (CD32/CD16) hybridoma supernatant containing 10% horse serum, and 10% chicken serum for at least 2 h. Purified 2.4G2

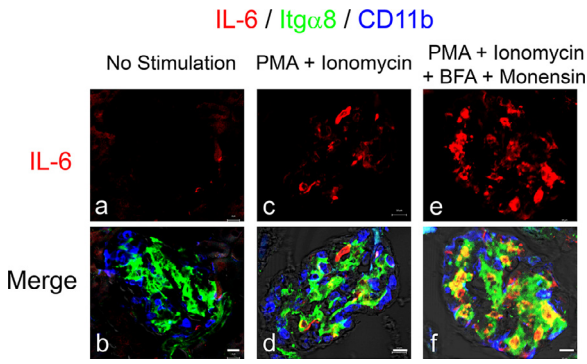

Fig. 1. Stimulation of IL-6 production in kidney glomerulus with pan-specific stimulants and secretion blockers. NZM mice with chronic GN and 3+ proteinuria by dipstick were perfused with cold PBS. Kidney slices were either fixed immediately in p-formaldehyde/lysine/periodate (a,b), or after stimulation for 6 h with either PMA plus ionomycin (c,d), or the combination of PMA, ionomycin, monensin, and brefeldin A. Processed tissues were equilibrated in sucrose, frozen in OCT blocks, and sectioned for staining with A-555-anti-IL-6, A-488-anti-Itgα8, and A-647 anti-CD11b. Images were captured on a LSM700 confocal microscope. Single IL-6 staining (a,c,e) and confocal images (b,d,f) of the respective treatments are shown. Bars equal 10 μm.

mAb (Bio-X-Cell; Lebanon, NH) at 25 μg/ml diluted in serum-containing medium can be used in place of hybridoma supernatant. Blocking can be performed in a staining dish or as a 50 μl bubble on the tissue.

6. Wash off serum with PBS 3 times, 5 min each. Suction off PBS on the slide surface and around the tissue. Do not let tissues go dry.
7. Place slides on a wetted filter paper in a 150 mm culture plate and add 50 μl of Ab mix as a bubble on the tissue. Ab were used at 1 – 2 μg/ml. For 4 color staining, Brilliant Violet (BV) 421-, Alexa fluor (A) 488-, A555-, and A647- conjugated Ab are used. Pacific blue (Pac-Blue)- conjugated Ab or DAPI can be used in place of BV421-conjugated Ab. Ab are diluted with blocking medium containing 2.4G2 and serum. The sections are stained at room temperature for 2 to 4 h, or at 4°C overnight.
8. Dump the Ab mix by tapping the slide vertically on a tissue paper. Place slides in a staining rack. Wash 4 times, 10 min each, in a large volume of PBS in a staining dish.
9. Suction off liquid on the slides and around the tissue slices. Mount slides with Prolong Gold antifade mounting medium (ThermoFisher) with or without DAPI as appropriate.
10. Read slides on a confocal microscope. We use a Zeiss LSM700 assembly with 4 lasers at 405, 488, 543, and 647 nm excitation wavelengths. The images are analyzed by the software ZEN provided by Zeiss as a free download.

Validation of cytokine colocalization with cell-type specific markers in the kidney glomerulus and interstitium

Stimulation of cytokine production

In the lupus-prone NZM2328 (NZM) mouse, various leukocytes and parenchymal cell types cause inflammation by secreting cytokines. However, cytokines from freshly explanted kidneys were difficult to detect. Little staining of highly produced cytokines and chemokines such as IL-6, TNF-α, CXCL-1 and oncostatin M was found in kidneys of NZM mice with severe inflammation and glomerulonephritis (GN). As an example for signal peptide-containing cytokines, the effects of various treatment combinations on the detection of IL-6 is shown in Fig. 1. Little IL-6 staining was detected in unstimulated kidney sections (Fig. 1a, b). To increase cytokine production, PMA and ionomycin which have been used extensively in enhancing hematopoietic cell cytokine production by delivering signals mimicking diacylglycerol and calcium flux stimulation [2,3] were used to stimulate kidney slices *in vitro*. IL-6 production was readily detectable in the glomerulus (Fig. 1c, d). Furthermore, most IL-6

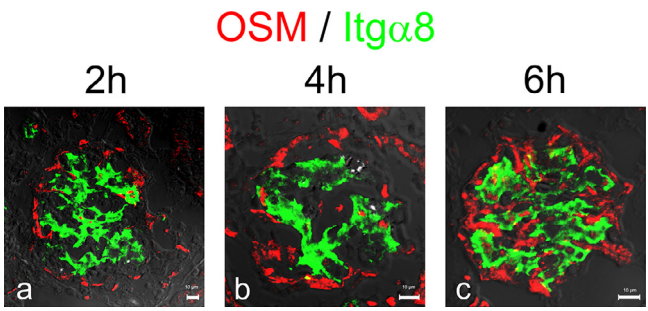

Fig. 2. Time course of *in situ* cytokine production in kidneys. Kidneys slices from NZM mice with chronic GN were incubated in RPMI containing PMA, ionomycin, monensin, and BFA for the indicated time periods and processed for staining. Confocal microscopy showed increasing accumulation of oncostatin M (red) in the glomerulus. Integrin $\alpha 8$ (green) marks the inner core of the glomerulus. Bars are 10 μm.

production was associated with integrin $\alpha 8^+$ mesangial cells. Because cytokines are rapidly secreted, we attempted to further enhance the cytokine signal by adding the secretion blockers monensin and BFA. Because the 2 blockers yield different patterns of cytokine production (see Techniques for Immune Function Analysis; BD Biosciences - Application Handbook, 2003 - research.missouri.edu; reference # [4-6]), a combination of both blockers along with the stimulants was used. Marked increase in IL-6 production was found (Fig. 1e, f). IL-6 production was associated primarily with the mesangial cells and little IL-6 production by the glomerulus-infiltrating CD11b$^+$ macrophages was found. Little increase in IL-6 production over unstimulated cells was found when monensin and BFA were used without the stimulants PMA and ionomycin. Similar enhanced cytokine production patterns were found with TNF-α, oncostatin M (OSM) and CXCL1 in response to the drug combinations.

The effects of incubation time were also examined using OSM production as an example (Fig. 2). Similar to IL-6, little OSM was detected with no stimulation but marked stimulation was found with combined PMA, ionomycin, monensin and BFA treatment. Two hour incubation was marginally effective (Fig. 2a). However, 4 – 6 h incubation boosted the cytokine signal significantly (Fig. 2b, c). Thus an incubation period of 4 – 6 h was used. The incubation did not appear to have caused cell death and the cell morphology was intact. Longer incubation may begin to compromise cellular viabilities as monensin was found to be toxic.

We have also used a combination of TLR ligands PAM_3CSK_4, PolyIC, LPS, R837, and CpG2395 specific for TLR2, TLR3, TLR4, TLR7, and TLR9 stimulation respectively. There was no difference in the cytokine signals induced between PMA plus ionomycin and toll-like receptor ligand stimulation. To further increase the accumulation of the cytosolic cytokines ProIL-1β and Pro-IL-18, we have added the inflammasome or caspase inhibitors MCC950 or parthenolide to the PIMB stimulation mixture but found no difference in Pro-IL-1β levels. Similarly, we added MDL28170 and parthenolide for inhibiting IL-33 secretion and degradation into the PIMB mix during tissue incubation but found no increased IL-33 accumulation.

Fixation

A fixation period of 3 h recommended by Mclean and Nakane [1] was used. Longer fixation periods obliterated the binding epitopes for some Ab and resulted in dim or lost signal. The fixation seemed to have penetrated the tissue slices of about 2 mm as we have found no disintegration of tissue sections due to inadequate fixation during our staining experiments. PLP fixation is far superior to 10% buffered formalin fixation in immunofluorescence staining. Many of our Ab failed to stain paraffin sections even with antigen retrieval and the sections often give high background at the shorter wavelengths.

Ab specificities

We have screened a large panel of Ab against cell specific markers for glomerular and tubular cells. Unlike Ab for hematopoietic cell markers, few mAb against renal intrinsic cells are available and

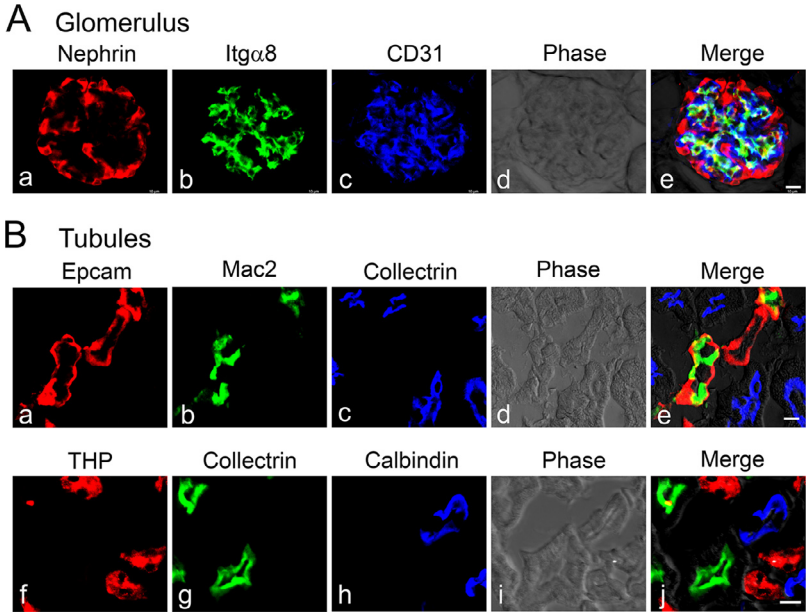

Fig. 3. Markers for renal glomerular cells and tubular cells. NZM mouse kidneys were stained with the indicated Ab for cell type identification. In the glomerulus (A), Ab against nephrin, Itgα8, and CD31 stained podocytes, mesangial cells, and endothelial cells respectively. For the tubular cells (B), anti-collectrin stains proximal tubule cells, anti-THP stains the thick ascending limb, anti-calbindin-1 stains the distal convoluted tubules and connecting tubules, anti-Mac2 stains the principal cells of collectin duct and connecting tubules and anti-Epcam stains the thin ascending limbs, the connecting tubules, and the intercalated cells of the collecting duct. Bars are 10 μm.

fluorochrome-labeled Ab are even more infrequently found. Fortunately many polyclonal Ab raised in goat, sheep, or rabbit of high purity, usually antigen affinity purified, and supplied in powder or liquid form without carriers are available. These Ab are readily labeled with mAb labeling kits for pacific blue or Alexa Fluors (Invitrogen, Grand Island, NY). After extensive testing, we found that for glomerular cells, polyclonal Ab against nephrin, integrin α8 (Itgα8), and CD31 (R & D Systems, Minneapolis, MN) yielded specific and bright staining for podocytes, mesangial cells, and endothelial cells respectively (Fig. 3A; Ref. # [7-9]). In a 4 color analysis, three colors were usually used for cell-type specific marker staining and the 4th color was used for cytokine staining. Many specific polyclonal Ab against cytokines supplied by R & D Systems or Peprotech worked well. Several monoclonal Ab (mAb) for tubular proteins have also worked well in staining sections with short fixation in 2% PLP. Among these mAb are anti-Epcam (G8.8) and anti-Mac2 (galectin-3; M3/38) from Biolegend (San Diego, CA). Mac2 was expressed by connecting tubules and the principal cells of collecting duct (Fig. 3B; [10]). It should be noted that Mac2 has been used as an M2 macrophage marker [11]. Epcam stains the thin ascending limb in the medulla, and collecting duct. We used a rabbit polyclonal Ab generated in our laboratory against calbindin D28K which is localized in the distal convoluted tubules and connecting tubules [12] and Mac2 to validate the localization of Epcam. A polyclonal anti-Tamm Horsfall protein Ab (R & D) was used to identify the thick ascending limb. A rabbit polyclonal anti-claudin-7 (Thermo Fisher) Ab which stains the thin ascending limb, distal convoluted tubule, connecting tubules, and collecting duct was also used (Fig. 3, not shown; [13]).The anti-collectrin Ab (R & D) was used to identify proximal tubules [14].

In the examples shown in Fig. 4, M-CSF production in the glomerulus was found to be produced predominantly by mesangial cells (Fig. 4A, arrows, panels a, b, f, and g), and not by podocytes and endothelial cells. Similarly, stem cell factor (SCF) was produced in the collecting ducts by intercalated cells stained by Epcam (Fig. 4B, arrows, panels a, b, f, g).

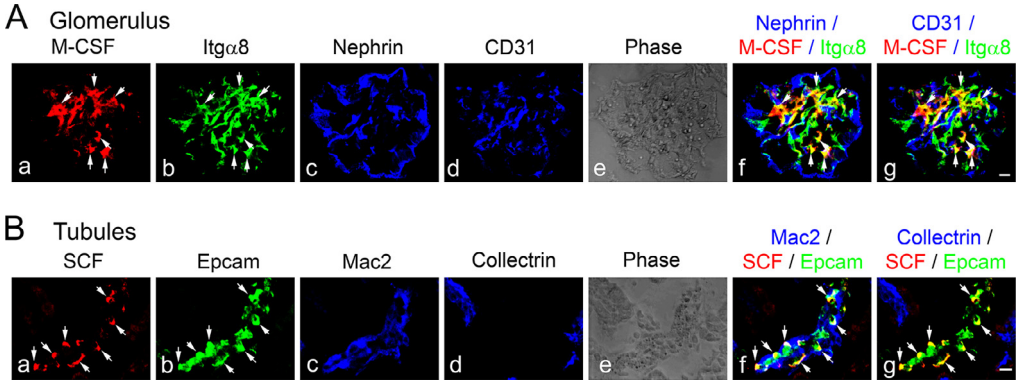

Fig. 4. Cytokine production in the kidney glomerulus and tubules in NZM mice with chronic GN. Kidneys in NZM mice with chronic GN was processed as described in this report and stained with directly conjugated Ab as shown. A. In the glomerulus, M-CSF (a, red) production is colocalized with mesangial cells stained by anti-Itgα8 (green). Colocalization (yellow) is indicated by arrows (panels a, b, f, g). B. SCF (a, red) was produced by the intercalated cells in the collecting duct (b, green) but not by the principal cells in the collecting duct shown by anti-Mac2 staining (c, blue) and not in the proximal tubule (d, blue). Colocalization (yellow) is shown in f and g with arrows in a, b, f, and g. Bars are 10 μm.

We have always used Ab directly conjugated with fluorochromes for kidney staining. This staining usually gives low background readily recognizable as slightly above the autofluorescence background emitted by proximal tubules. Some secondary reagents give very high background that can lead to misinterpretion as positive signals, especially in the outer medulla or inner cortical region of the kidney.

General applicability of this method

This method of *in vitro* tissue stimulation to enhance cytokine signals has general applicability. The stimulant combination PMA and ionomycin has been effective in inducing cellular activation with almost all cell types with few exceptions. Similarly, monensin and BFA blocks signal-peptide mediated secretion of all cell types. In our kidney studies, we have identified the production of TNF-α and IL-6 by macrophages [15], IL-6 and M-CSF production by kidney mesangial cells (Fig. 1,Fig. 4A; ref. # [15]), and OSM and SCF by epithelial cells (Fig. 2,Fig 4). Kidney tubular or glomerular epithelial cells and endothelial cells have also been found to produce IL-6 family cytokines LIF, TAM family ligands Gas6 and Pros1, other chemokines such as CXCL2 and CXCL10, as well as complement components such as C1qa and C3. Thus this method enables the enhanced detection of not only secreted cytokines, but also other secreted proteins such as complement components. The method has been used in cell types including hematopoietic cells, epithelial cells, mesenchymal cells, and endothelial cells. The cytokines that do not strictly follow the accumulation patterns in response to the drug treatments belong to the IL-1 family which comprises IL-1α, IL-1β, IL-18, IL-33, IL-37, IL-38, and IL-1Ra [16,17]. All members except IL-1Ra lack signal peptides and thus are synthesized as cytosolic proteins without transiting through the ER-Golgi secretory pathway. We found that monensin and BFA minimally affect the secretion of these cytokines. However, PMA and ionomycin stimulation is effective in elevating cytokine production as we have observed increased IL-1β signals with PIMB stimulation [15]. Regarding the applicability of this method to tissue types besides the kidney, we have used it to study cytokine production in spleen, lymph nodes, lungs, and liver with good results. The method should be easily adaptable to the studies of cytokine production in other tissues.

Reagents

Anesthesia

Ketamine/xylazine/heparin: Add 1.4 ml of 100 mg/ml ketamine and 0.7 ml of 20 mg/ml xylazine to 9 ml 1,000 U/ml heparin solution.

Fixative

2% p-formaldehyde/lysine/periodate: 50 ml 4% p-formaldehyde in 0.1 M $NaPO_4$, pH 7.4, 20 ml 5X lysine-phosphate, pH 7.4, 30 ml H_2O, and 0.2 g $NaIO_4$. Prepare fresh.

4% p-formaldehyde in 0.1 M $NaPO_4$, pH 7.4: 20 g p-formaldehyde in 100 ml H_2O and 5 drops of 1 N NaOH. Heat to 65°C with stirring until powder is dissolved. Filter through Whatman paper and add 50 ml of 1 M $NaPO_4$, pH 7.4. Add H_2O to make final volume to 500 ml. Store at 4°C.

5X lysine/phosphate, pH 7.4: Titrate 500 ml of 0.75 M lysine HCl with 0.375 M Na_2HPO_4 to pH 7.4. Add 0.375 M $NaPO_4$, pH 7.4 to 1 litre. The final is 0.375 M lysine and 0.1875 M $NaPO_4$, pH 7.4. Store at 4°C.

Chemicals (aliquoted and stored at -70 degrees)

PMA: Sigma; 15,000 X; stock: 300 µg/ml DMSO. Aliquot and store at -70 degrees. For PMA, we buy 1 mg and dissolve in 3.33 ml DMSO. Make 0.5 ml aliquots and 11 μl aliquots. Use 11 μl aliquots and discard after 2 uses.

Ionomycin: Calbiochem; 2000 X, 2mM in DMSO.

Monensin: Sigma; 2500 X; 5 mM in DMSO.

Brefeldin A: LC laboratories; 2,000 X, 20 mg/ml in DMSO.

PAM3CSK4, TLR2 stimulant: Invivogen, 5,000X, 1 mg/ml in H_2O.

PolyIC, TLR3 stimulant: Invivogen, 250 X, 2.5 mg/ml in H_2O.

LPS, TLR4 stimulant: Invivogen, 5,000X, 5 mg/ml in H_2O.

R837, TLR7 stimulant: Tocris, 300 X, 3 mg/ml in DMSO.

CpG2395, phosphorothioate, TLR9 stimulant: IDT synthesis; 500 X, 5 μmole scale; 0.5 mM stock.

MCC 950: Sigma: 2,000 X, make 20 mM in DMSO.

Parthenolide, NLRP1, NLRP3, NFkB, Caspase I inhibitor: MCE, MedChem, 10,000 X, 25 mg/ml in DMSO

MDL28170, Calpain inhibitor: ApexBio, 10 mg/ml in DMSO, 900 X.

Other solutions

1 M HEPES sodium, pH 7.4: Titrate 1 mole of HEPES to pH 7.4 with 10 N NaOH. Make up to 1 L and sterilize with a 0.2 μm filter.

30% sucrose in phosphate buffer, pH 7.4: Dissolve 300 g of sucrose in H_2O in a final volume of 850 ml. Add 50 ml 1 M sodium phosphate, pH 7.4. Warm solution if required. Add 5 mM EDTA, pH 8.0 as preservative. Make up to 1 L.

1 M sodium phosphate pH 7.4: Mix 774 ml of 1 M Na_2HPO_4 with 226 ml of 1 M NaH_2PO_4.

0.5 M EDTA sodium: Weigh 0.5 moles of Na_2EDTA. Add H_2O to 500 ml. Add 10 N NaOH intermittently to allow EDTA to dissolve. Bring pH to 8.0 and make volume up to 1 L.

Additional Information

Cytokines play key roles in inflammation. However, the cellular production of cytokines *in vivo* are difficult to determine because they are rapidly secreted. *In situ* hybridization has been used to determine the cellular source and intracellular levels of cytokine mRNA. Though the method can define the cellular specificity for cytokine production, it does not necessarily provide the rate of cytokine protein production. That cytokine production does not correlate with intracellular cytokine mRNA levels is well documented [18,19]. The most important reason is that most cytokine mRNA contain AU-rich elements (ARE) in the 3'-untranslated region that are bound by ARE-binding proteins. These proteins regulate mRNA stability and translation and their activities are regulated by signaling events involving the MAPK or Jak-Stat pathways [20-22]. An additional problem often associated with the identification of the cellular source of cytokines is the lack of suitable Ab for cell-type specific markers to identify the cytokine-producing cells. To address these problems, and because of the importance of cytokines in lupus nephritis (LN; [23]), we have used an NZM2328 mouse model which manifest a spontaneous female-biased lupus-like disease to examine cytokine production in the glomerulus during LN pathogenesis (Sung and Fu, companion paper to this manuscript;

ref. #[15]). This method of cytokine production will greatly facilitate the understanding of the dynamics of cytokine production and functional interactions of cytokines in diseases.

Animal and Human Rights

All animal experiments have been approved by the University of Virginia Institutional Use and Care of Animals Committee.

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Declaration of Competing Interest

The authors have no conflict of interests.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.mex.2020.100935.

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