IMMUNOPATHOLOGY AND INFECTIOUS DISEASES

The Antiviral Cytokines IFN- α and IFN- β Modulate Parietal Epithelial Cells and Promote Podocyte Loss

Implications for IFN Toxicity, Viral Glomerulonephritis, and Glomerular Regeneration

Adriana Migliorini,^{*} Maria L. Angelotti,[†] Shrikant R. Mulay,^{*} Onkar O. Kulkarni,^{*} Jana Demleitner,[‡] Alexander Dietrich,[‡] Costanza Sagrinati,[†] Lara Ballerini,[†] Anna Peired,[†] Stuart J. Shankland,[§] Helen Liapis,[¶] Paola Romagnani,[†] and Hans-Joachim Anders^{*}

From the Nephrological Center,^{*} Medizinische Klinik und Poliklinik IV, Klinikum der Universität München-Ludwig Maximilian University, Campus Innenstadt, Munich, Germany; the Excellence Centre for Research,[†] Transfer and High Education for the Development of De Novo Therapies, University of Florence, Florence, Italy; the Walther-Straub Institute for Pharmacology and Toxicology,[‡] School of Medicine, Ludwig Maximilian University, Munich, Germany; the Division of Nephrology,[§] University of Washington, Seattle, Washington; and the Department of Pathology and Immunology,[¶] Washington University, School of Medicine, St. Louis, Missouri

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Address correspondence to
Hans-Joachim Anders, M.D.,
Nephrologisches Zentrum der
LMU, Medizinische Klinik und
Poliklinik IV, Ziemsenstr. 1,
80336 München, Germany.
E-mail: hjanders@med.uni-muenchen.de.

Interferon (IFN)- α and IFN- β are the central regulators of antiviral immunity but little is known about their roles in viral glomerulonephritis (eg, HIV nephropathy). We hypothesized that IFN- α and IFN- β would trigger local inflammation and podocyte loss. We found that both IFNs consistently activated human and mouse podocytes and parietal epithelial cells to express numerous IFN-stimulated genes. However, only IFN- β significantly induced podocyte death and increased the permeability of podocyte monolayers. In contrast, only IFN- α caused cell-cycle arrest and inhibited the migration of parietal epithelial cells. Both IFNs suppressed renal progenitor differentiation into mature podocytes. In Adriamycin nephropathy, injections with either IFN- α or IFN- β aggravated proteinuria, macrophage influx, and glomerulosclerosis. A detailed analysis showed that only IFN- β induced podocyte mitosis. This did not, however, lead to proliferation, but was associated with podocyte loss via podocyte detachment and/or mitotic podocyte death (mitotic catastrophe). We did not detect TUNEL-positive podocytes. Thus, IFN- α and IFN- β have both common and differential effects on podocytes and parietal epithelial cells, which together promote glomerulosclerosis by enhancing podocyte loss while suppressing podocyte regeneration from local progenitors. (*Am J Pathol* 2013, 183: 431–440; <http://dx.doi.org/10.1016/j.ajpath.2013.04.017>)

Systemic viral infections frequently are associated with immune complex glomerulonephritis, which deposits viral nucleic acids inside the glomerulus.¹ Once viral nucleic acids reach the intracellular cytosol of mesangial and glomerular endothelial cells they are recognized by distinct recognition receptors to induce massive secretion of interferon (IFN)- α and

IFN- β , a process that aggravates immune complex glomerulonephritis and proteinuria.^{2–6} Generally, the induction of IFN- α and IFN- β is a central element of the host's antiviral

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response because these interact with IFN receptors on adjacent cells in a paracrine manner.⁷ This induces STAT1/2 signaling and subsequent induction of more than 200 IFN-stimulated genes (ISG), which display a wide range of nonspecific proinflammatory and specific antiviral activities.⁸

Some viruses cause glomerulopathies also by directly infecting glomerular epithelial cells. For example, HIV and parvoviruses can infect glomerular epithelial cells [ie, podocytes and parietal epithelial cells (PECs)].^{9–13} It is tempting to speculate that viral replication inside podocytes and PECs triggers IFN-dependent antiviral responses, and that this process contributes to HIV-associated nephropathy and to other viral glomerulopathies. Proteinuria, collapsing nephropathy, and pseudocrescents are hallmarks of viral glomerulopathies and implicate podocyte- and PEC-related pathology.^{1,9} However, the molecular mechanisms of viral infection, viral recognition, antiviral IFNs, and their primary and secondary effects on podocytes and PECs are largely unknown.¹⁴

Assuming that viral glomerulopathies will expose podocytes and PECs to IFN- α and IFN- β , we sought to determine their functional impact on both epithelial cell types. We hypothesized that IFN- α and IFN- β would induce ISGs in both podocytes and PECs. Furthermore, we speculated that IFN- α and IFN- β would affect the cell cycle and favor cell death of these glomerular cell types, a mechanism that should aggravate proteinuria and glomerular pathology *in vivo*.

Materials and Methods

In Vitro Studies

Renal progenitor cells with PEC characteristics were isolated from human kidneys and cultured as previously described.¹⁵ For their differentiation into mature podocytes they were cultured for 48 hours with vitamin D3, retinoic acid, and dexamethasone in Dulbecco's modified Eagle's medium/F12 medium as described.^{16,17} Mouse PECs were extracted from preparations of mouse glomeruli as described¹⁸ and cultured at 33°C on 10-cm plates (BD Biosciences, Heidelberg, Germany) coated with type I collagen in RPMI 1640 containing 5% fetal bovine serum and 50 U/mL IFN- γ as described.¹⁸ To induce their differentiation, the cells were cultured under growth-restrictive conditions (at 37°C, in the absence of IFN- γ) for 14 days, which lead to acquisition of a polygonal shape and replication arrest. Cells showing these features were used to perform the experiments. Mouse podocyte and mouse PECs were isolated and cultured as previously described.¹⁹ For all of the experiments, cells were cultured under nonpermissive conditions (at 37°C, in the absence of IFN- γ) to induce differentiation into mature arborized podocytes expressing synaptodin.

Proliferation Assay, Flow Cytometry, and Migration Assay

The cells were seeded in 96- or 6-well plates and stimulated with 5000 U/mL mouse IFN- α and IFN- β (mouse IFN- α 4:

molecular weight, 19.5 kDa; mouse IFN- β : molecular weight, 19.6 kDa), or 5000 U/mL human IFN- α and IFN- β (human IFN- α 4: molecular weight, 19.4 kDa; human IFN- β : molecular weight, 23 kDa; all from PBL Laboratories, Piscataway, NJ), respectively, for 24 hours. Cell viability was determined using an MTT assay according to the manufacturer's instructions (Promega, Mannheim, Germany). Cell death was evaluated by flow cytometry using a FACScalibur machine (BD Biosciences). Cells were stimulated for 24 hours with 5000 U/mL mouse IFN- α and IFN- β or 5000 U/mL human IFN- α and IFN- β . Cell suspension together with supernatant was prepared and stained using propidium iodide (PI) and the Annexin V kit (BD Biosciences) following the manufacturer's instructions. Cell migration was determined using a culture insert (Ibidi, Munich, Germany). The inserts were placed on 12-well plates and the cells were seeded at 100% confluence. After 24 hours the insert was removed and the cells were stimulated with medium or 5000 U/mL mouse IFN- α and IFN- β or 5000 U/mL human IFN- α and IFN- β . To evaluate the cell migration rate, images were captured at 0 hours and every 12 hours on a digital phase-contrast microscope, and then analyzed by digital image analysis software (Photoshop Extended CS4 Software; Adobe Software, San Jose, CA). Two laboratory members independently performed digital image analysis to minimize acquisition bias.

ECIS

IFN- α - and IFN- β -induced changes in podocyte resistance were analyzed using an electric cell impedance sensing assay (ECIS) device (Applied Biophysics, Troy, NY). Murine podocytes were cultured as described.²⁰ Human renal progenitor cells were differentiated into mature podocytes as described.^{16,17} Cells were seeded on ECIS cultureware (80BW10E+; Applied Biophysics), which was preincubated with podocyte cell medium overnight and connected to the ECIS device. Confluent podocyte cultures were incubated overnight at 37°C in 5% CO₂ before the medium was changed to HBSS $\pm$ IFN- α and IFN- β or solvent. Resistance (Ω) was analyzed for 30 minutes or 12 hours, for murine and human podocytes respectively.

Protein Isolation and Western Blotting

Protein was extracted from renal cells using RIPA buffer (Sigma, Munich, Germany) containing protease inhibitors (Roche, Mannheim, Germany) and processed for Western blotting as described.²¹ The membranes were incubated overnight at 4°C with goat antibody against mouse p21 (clone F-5; Santa Cruz, Heidelberg, Germany). Secondary antibodies were peroxidase-conjugated donkey anti-goat IgG (Dianova, Hamburg, Germany) and anti-rabbit IgG (Cell Signaling, Frankfurt am Main, Germany). To quantify p21 expression, Western blots were analyzed with digital image analysis software (Photoshop Photoshop Extended

Table 1 Murine Oligonucleotide Primers Used for SYBR-Green Real-Time qPCR

Gene	Sequence
<i>18s</i>	Forward: 5'-GCAATTATTCCCCATGAACG-3' Reverse: 5'-CCTACCAAATCACTCCGGA-3'
<i>mIFIT1</i>	Forward: 5'-CAAGGCAGGTTTCTGAGGAG-3' Reverse: 5'-TACGACTACCACTGGTCCAG-3'
<i>mIFIT3</i>	Forward: 5'-TTCACGACGACACAGAAAC-3' Reverse: 5'-ACGGTAAAGTGGACCTTAAA-3'
<i>mMX1</i>	Forward: 5'-TCTGAGGAGAGCCAGACGAT-3' Reverse: 5'-CTGGAGTAGCTGTGGGACTC-3'
<i>mCXCL10</i>	Forward: 5'-GGCTGGTCACCTTTCAGAAG-3' Reverse: 5'-CGAGAGACGACAGGTAGGAT-3'
<i>mPUMA</i>	Forward: 5'-CACCTAGTTGGGCTCCATTT-3' Reverse: 5'-GCATGACGCGCAACTCCA-3'
<i>mBAK</i>	Forward: 5'-AGACCTCCTCTGTCTCCTGG-3' Reverse: 5'-GGAACAGGTCTACGGTAAAA-3'
<i>mBID</i>	Forward: 5'-GTGTAGCTCCAAGCACTGCC-3' Reverse: 5'-CCGATTCCGTTTCCAAACG-3'
<i>mBAX</i>	Forward: 5'-GATCAGCTCGGGCACTTTAG-3' Reverse: 5'-CAACTTCAACGGTAGTCGTT-3'
<i>mTNFα</i>	Forward: 5'-AGGGTCTGGGCCATAGAACT-3' Reverse: 5'-CATCTGTCTTCTCGCACCACC-3'
<i>mIL6</i>	Forward: 5'-TGATGCACTTGACAGAAAACA-3' Reverse: 5'-CGGATAACTTTTAAAGGAGACCA-3'
<i>mCXCL9</i>	Forward: 5'-CCTAGTGATAAGGAATGCACGATG-3' Reverse: 5'-CTTGCCCTCTAGTTTGGACGGATC-3'
<i>mCXCL11</i>	Forward: 5'-CCGAGTAACGGCTGCGACAAAG-3' Reverse: 5'-GTTCGAGCGGAGTATTACGTCC-3'
<i>mIFNR1</i>	Forward: 5'-CCAAGGCAAGAGCTATGTCTTG-3' Reverse: 5'-CGTTTACAGGTCTGATGCGTGAC-3'
<i>mIFNR2</i>	Forward: 5'-GAGCCTAGAGACTATCACACCG-3' Reverse: 5'-GGCGATTGATGTGGGAGACCAT-3'

CS4 Software; Adobe Software). Data were expressed relative to β -actin expression.

Real-Time RT-qPCR

Cells were seeded and stimulated for 12 hours with 5000 U/mL IFN- α and IFN- β or 5000 U/mL human IFN- α and IFN- β . Total RNA was isolated from cells or kidneys using an RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. cDNA was generated using reverse transcriptase (Superscript II; Invitrogen, Carlsbad, CA). For ISG detection, the SYBR Green Dye detection system was used for real-time quantitative PCR (qPCR) on a Light Cycler 480 (Roche) using 18S rRNA as a house-keeping gene. Three hundred nanomolar gene-specific primers (Metabion, Munich, Germany) were used as listed in Tables 1 and 2. Controls consisting of double-distilled water were negative for target and housekeeping genes. High-confidence algorithms were used to reduce the risk of false-positive crossing point. Melting curve profiles were analyzed for every sample to detect eventual unspecific products or primer dimers. For cell differentiation experiments TaqMan Reverse Transcription-PCR was performed

as described.²² Nephrin quantification was performed using an assay-on-demand kit (Applied Biosystems, Warrington, Cheshire, UK).

Animal Studies

Adriamycin nephropathy was induced in 6-week-old female severe combined immunodeficient (SCID) mice obtained from Harlan (Udine, Italy), by a single i.v. injection of Adriamycin (6.5 mg/kg in PBS; Sigma, Munich, Germany) on day 0 followed by daily subcutaneous injections of either saline (vehicle), 1000 U IFN- α 6, or 1000 U IFN- β . Group size was $n = 6$ in each experiment in triplicate. All mice were sacrificed on day 7 by cervical dislocation. Urinary albumin and creatinine excretion were evaluated at days 0 and 7 using the Albuwell M kit (Exocell, Philadelphia, PA) and a creatinine assay kit (Diasys, Holzheim, Germany) as described. All experimental procedures were performed according to the Italian animal care and ethics legislation and were approved by the local government authorities.

Immunofluorescence and Confocal Microscopy

All immunofluorescent studies were performed on frozen sections (5 μ m thick) and were analyzed with a LSM510 META laser confocal microscope (Carl Zeiss, Jena, Germany). The following primary antibodies were used: anti-CD68 monoclonal antibody (clone FA-11; Abcam, Cambridge, UK), anti-claudin-1 monoclonal antibody (clone 2H10D10; Invitrogen), antinephrin (clone C17; Santa Cruz Biotechnologies, Santa Cruz, CA), anti-wild-type-1 (clone

Table 2 Human Oligonucleotide Primers Used for SYBR-Green Real-Time qPCR

Gene	Sequence
<i>18s</i>	Forward: 5'-GCAATTATTCCCCATGAACG-3' Reverse: 5'-CCTACCAAATCACTCCGGA-3'
<i>hIFIT1</i>	Forward: 5'-ATCCAGGCGATAGGCAGAGATC-3' Reverse: 5'-AAGGAGGTGTGAAGTCGTTCCG-3'
<i>hIFIT3</i>	Forward: 5'-CCTGGAATGCTTACGGCAAGCT-3' Reverse: 5'-AACCCGTCTGAGAGTCTACGAG-3'
<i>hMX1</i>	Forward: 5'-GGCTGTTTACCAGACTCCGACA-3' Reverse: 5'-ATCTCTCGACGGTCCGAAACAC-3'
<i>hCXCL10</i>	Forward: 5'-GGTGAGAAGAGATGTCTGAATCC-3' Reverse: 5'-ACGTACGAAGGTTCCCTACCTG-3'
<i>hPUMA</i>	Forward: 5'-ACGACCTCAACGCACAGTACGA-3' Reverse: 5'-GGGCTCTACCTCGGGTTAATCC-3'
<i>hBAK</i>	Forward: 5'-TTACCGCCATCAGCAGGAACAG-3' Reverse: 5'-GCTGCGATACTGAGTCTCAAGG-3'
<i>hBID</i>	Forward: 5'-TGGGACACTGTGAACCAAGGAGT-3' Reverse: 5'-GGATGACCACAAACCGAAGGAG-3'
<i>hBAX</i>	Forward: 5'-TCAGGATGCGTCCACCAAGAAG-3' Reverse: 5'-CTACTAACGGCGGCACCTGTGT-3'
<i>hIFNR1</i>	Forward: 5'-CGCCTGTGATCCAGGATTATCC-3' Reverse: 5'-CACTTTCGGTCTCGTGTGTGGT-3'
<i>hIFNR2</i>	Forward: 5'-ACCGTCTTAGAAGGATTACGCG-3' Reverse: 5'-GGTGGTCTCAACTCTAACAAC-3'

F-6; Santa Cruz Biotechnologies), anti-H3-Ser10 (anti-phospho-histone H3; Abcam), and antipodocin (anti-NPHS2; Abcam). Double immunolabeling was performed using the following secondary antibody: Alexa Fluor 488 goat anti-rat IgG (Invitrogen, Life Technologies, GmbH, Darmstadt, Germany), Alexa Fluor 546 goat anti-rabbit IgG (Invitrogen), Alexa Fluor 488 rabbit and goat IgG (Invitrogen), Alexa Fluor 546 goat anti-mouse IgG1 (Invitrogen), and Alexa Fluor 488 goat anti-mouse IgG1 (Invitrogen). Double-positive cells were quantified in 15 glomeruli of at least six sections for each mouse by two independent and blinded observers (A.M. and M.L.A.).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software version 4 (GraphPad Software, La Jolla, CA) for analysis of variance followed by the Student-Newman-Keuls test. We used the Student's *t*-test for direct comparisons between two groups. We considered a *P* value of <0.05 as statistically significant. Data were expressed as means $\pm$ SEM or SD.

Results

IFN- α and IFN- β Trigger Expression of Multiple ISGs in Glomerular Epithelial Cells

We first tested the ability of type I IFNs to activate glomerular epithelial cells. Expression of both IFN-receptor chains (IFNAR1 and IFNAR2) was confirmed by real-time RT-qPCR in podocytes as well as in PECs from human and murine origin (Supplemental Figure S1). IFN- α and IFN- β significantly induced multiple ISGs, such as MX1, IFIT1, IFIT3, and CXCL10, in podocytes and parietal epithelial cells of both species (Supplemental Figure S2). Thus, the antiviral cytokines IFN- α and IFN- β activate both podocytes and PECs.

Podocyte Culture: Only IFN- β Increases Monolayer Permeability and Induces Expression of Pro-Apoptotic Genes and Podocyte Death

Based on the consistent activation of podocytes by IFN- α and IFN- β , we next questioned their effect on podocyte permeability. We measured changes in podocyte resistance on IFN stimulation to quantitate alterations of barrier function.^{20,23} Cellular resistance was decreased significantly in response to IFN- β , with as little as 5 minutes of stimulation (Figure 1A) in murine podocytes. In human podocytes, IFN- β affected cellular resistance in a similar manner but only after 2 hours of stimulation (Figure 1A). In contrast, IFN- α stimulation had no effect on podocyte monolayer resistance. Changes in podocyte activation can be transient or persistent, and eventually may lead to podocyte detachment and/or death. Flow cytometry analysis of human podocyte culture supernatant showed an

Figure 1 IFN- β increases podocyte monolayer permeability by causing podocyte detachment and death. **A:** Human and mouse podocytes were seeded into the ECIS cultureware and grown to confluency. Monolayer resistance was analyzed for 30 minutes or 12 hours, respectively. Data are expressed as normalized resistance $\pm$ SEM for three independent experiments. **B:** PI and annexin V flow cytometry of human and mouse podocytes quantifies their viability after 24 hours of exposure to 5000 U/mL IFN- β .

increased number of PI-positive cells on IFN- β stimulation compared with control (31.6% versus 16.4% of detached cells) (Supplemental Figure S3). Therefore, we questioned, how IFN- α and IFN- β affected podocyte adhesion and podocyte viability. Human and mouse podocytes were exposed to IFN- β for 24 hours and were analyzed by flow cytometry for PI and annexin V. IFN- β exposure significantly increased the numbers of PI-positive podocytes from both species whereas the effect on annexin V staining was stronger in mouse podocytes (Figure 1B). IFN- α exposure did not affect podocyte viability (not shown). In addition, only IFN- β significantly increased the mRNA expression levels of the pro-apoptotic genes PUMA, BAK, BAX, and BID (Supplemental Figure S4). Thus, although IFN- α and IFN- β both activate ISG expression in podocytes, only IFN- β rapidly alters podocyte permeability, followed by podocyte loss via detachment or death.

PECs: IFN- α Induces p21 to Block Proliferation and Migration But Both IFNs Suppress Progenitor Differentiation into Podocytes

In contrast to differentiated podocytes, PECs readily proliferate in culture and *in vivo*. For example, when glomerular basement membranes rupture they are exposed to serum, which drives PEC overgrowth in Bowman's space (ie, glomerular crescent formation).²⁴ Human CD133/CD24-positive renal progenitor cells within the PEC layer along the inner aspect of Bowman's capsule have the capacity to differentiate into podocytes in culture and may restore podocyte loss *in vivo*.^{15,17} This concept of progenitor PEC-derived podocytes is supported by lineage tracing experiments in transgenic mice during renal development and growth.²⁵ Persistent proteinuria and glomerular scarring may imply that the activation of quiescent PEC progenitors, their coordinated proliferation and migration to the glomerular tuft, and their subsequent differentiation into mature podocytes is impaired significantly.²⁶ Because the regulators of these events are not well defined, we questioned whether IFN- α and IFN- β affects any of these steps of podocyte regeneration from their PEC progenitors.

Effects on Proliferation and Viability

We assessed PEC viability and growth on IFN- α and IFN- β stimulation using the MTT assay. IFN- α , but not IFN- β , significantly reduced the viability of both human and murine PECs (Figure 2, A and D). This was associated with an induction of the cyclin-dependent kinase inhibitor-1 p21, which stops cell-cycle progression in the G1 phase (Figure 2, B and E, and Supplemental Figure S5). PI/annexin V flow cytometry showed that the reduced viability of PECs on IFN- α stimulation did not relate to the death of PECs (Figure 2, C and F). Thus, IFN- α inhibits PEC proliferation (eg, by p21-mediated cell-cycle arrest).

Effects on Migration

Next, we studied the impact of IFN- α and IFN- β on renal progenitor/PEC migration. We used wound healing assays on human renal progenitor monolayers and murine PECs, which involve the activation and migration of surviving cells to close the wound, consistent with its negative regulation of migration. Exposure to IFN- α , but not to IFN- β , significantly reduced the capacity to close the wound (Figure 3, A and B).

Effects on Differentiation

Finally, we tested the potential of renal progenitors to differentiate into podocytes. This was achieved by exposing the renal progenitors to the appropriate medium and by quantifying nephrin mRNA expression after 48 hours of differentiation. IFN- α and IFN- β both significantly reduced the capacity of podocyte progenitors to induce nephrin mRNA expression (Figure 3C). The effect was stronger for IFN- β because IFN- β also destroyed mature podocytes, as

Figure 2 IFN- α arrests PEC growth by affecting the cell cycle. **A** and **D**: Human and murine renal PECs were cultured with 5000 U/mL human IFN- α and IFN- β for 24 hours and cell proliferation was assessed. Data are means $\pm$ SEM of three experiments, each analyzed in quadruplicate. * $P < 0.05$ versus control. **B** and **E**: In a similar experiment, cellular lysate was analyzed by Western blot for the expression of p21. β -Actin staining is shown as loading control. One representative blot of several blots from three independent experiments is displayed. **C** and **F**: Human and murine PECs stimulated for 24 hours with 5000 U/mL IFN- α and IFN- β were stained with annexin V and PI and analyzed by flow cytometry. Increase of PEC death was not detected on IFN- α or IFN- β stimulation.

shown earlier. These data show two critical effects of the IFN subtypes on glomerular epithelial cells. First, IFN- α specifically suppresses PEC proliferation and migration; second, both IFN- α and IFN- β inhibit renal progenitor differentiation into mature podocytes. The latter augment podocyte loss after IFN- β -induced podocyte death.

IFN- α and IFN- β Induce Renal ISG Expression and Aggravate Glomerulosclerosis in SCID Mice

These *in vitro* data suggest that IFN- α and IFN- β compromise the function and structure of glomerular epithelial cells in terms of maintaining the glomerular filtration barrier. To test the functional significance of these observations *in vivo*, we injected either recombinant IFN- α or IFN- β into mice with Adriamycin-induced focal segmental glomerulosclerosis.²⁷

Figure 3 IFN- α and IFN- β modulate PEC migration and podocyte progenitor differentiation. Cell migration assay $\pm$ 5000 U/mL IFN- α and IFN- β was performed with human (A) and murine (B) PECs using culture inserts as described in *Materials and Methods*. Data shown represent the mean wound size at the end of the experiment. Data are expressed as means $\pm$ SEM of three independent experiments. ** P < 0.01, *** P < 0.001 versus control. C: Human renal progenitors were cultured in the vitamin D3, retinoic acid, and dexamethasone in Dulbecco's modified Eagle's medium/F12 (VRADD) medium $\pm$ 5000 U/mL human IFN- α and IFN- β for 48 hours, and nephrin mRNA expression was quantified by TaqMan real-time qPCR as described in *Materials and Methods*. As control, renal progenitors were cultured for 48 hours in normal growth medium as described in *Materials and Methods*. Data are expressed relative to their respective 18S rRNA expression and are means $\pm$ SEM of three independent experiments. ** P < 0.01, *** P < 0.001 versus control.

This model involves a significant component of extrarenal adaptive immunity, which certainly would be modulated by systemic IFN- α and IFN- β injections.²⁷ Because the focus of this work was the intrarenal effects of the IFNs, we performed all experiments in SCID mice lacking adaptive immunity. Injections with either recombinant IFN- α or IFN- β significantly aggravated proteinuria by day 7 of mice with Adriamycin nephropathy. However, the effect of IFN- β was much more prominent than that of IFN- α (Figure 4A), with increased numbers of glomerular CD68-positive macrophages for both IFNs (Figure 4B) and with increased intrarenal mRNA expression of the ISGs (MX1, IFIT1, IFIT3, and CXCL10), proinflammatory cytokines (IL-6, tumor necrosis factor- α), and chemokines (CXCL9, CXCL11) (Figure 5, A and B). Healthy SCID mice injected with either recombinant IFN- α or IFN- β did not show any histopathologic alterations or proteinuria (data not shown). Thus, we conclude that IFN- α and IFN- β both have the potential to induce ISG in the kidney *in vivo* and to aggravate proteinuria and glomerular pathology in SCID mice with Adriamycin nephropathy.

IFN- α and IFN- β Have Distinct Effects on Podocytes and PECs *in Vivo*

Because the *in vitro* studies showed that IFN- α and IFN- β elicit differential effects on podocytes and PECs, we extended these studies *in vivo*. Consistent with our *in vitro* data, IFN- β injections reduced podocyte numbers in SCID mice with Adriamycin nephropathy as compared with the vehicle control group (Figure 6, A and B). The impact of IFN- α injections on podocyte counts was less potent. Next, we performed double immunostaining for the podocyte marker podocin and for histone H3 phosphorylated at serine 10 (H3-P), which marks the prophase of nuclear division of cells that are about to complete mitosis. This process frequently leads to podocyte detachment or death because podocytes can

neither complete cytokinesis nor maintain their function as aneuploidy cells.^{16,28–32} Podocyte H3-P staining was absent in healthy mouse kidneys (not shown). Control SCID mice with Adriamycin nephropathy had a reduction in the number of H3-P–positive podocytes. However, H3-P staining was increased significantly in mice that had received IFN- β injections (Figure 6, C and D). IFN- α injections had no effect on podocyte mitosis. The results of our *in vitro* studies instead implied that IFN- α predominately affects PECs (eg, by inducing PEC senescence), which we validated by double immunostaining for the PEC marker claudin-1 and for H3-P. Claudin-1/H3-P positivity was absent in healthy mouse kidneys (not shown). Control SCID mice with Adriamycin nephropathy displayed H3-P–positive PECs but their numbers were decreased significantly on IFN- α injections, whereas IFN- β injections had no such effect (Figure 7, A and

Figure 4 IFN- α and IFN- β aggravate Adriamycin-induced nephropathy in SCID mice. A: Urine albumin/creatinine ratios were measured in mice injected with Adriamycin alone in comparison with Adriamycin + 1000 U IFN- α – and Adriamycin + 1000 U IFN- β –injected mice after 7 days. B: CD68-positive macrophages were quantified per glomerulus after 7 days of injury. Data are expressed as means $\pm$ SEM and are representative of three independent experiments (n = 6 mice for each experiment and for each treatment group). * P < 0.05, ** P < 0.01, and *** P < 0.001 versus Adriamycin.

Figure 5 Both IFN- α and IFN- β induce intrarenal expression of ISGs and inflammatory markers. mRNA expression of the ISGs (MX1, IFIT1, IFIT3, and CXCL10) (A), proinflammatory cytokines (IL-6, tumor necrosis factor- α), and chemokines (CXCL9, CXCL11) (B). Total kidney mRNA was extracted from mice injected with Adriamycin or with Adriamycin + 1000 U IFN- α and IFN- β after 7 days of treatment. Real-time qPCR was performed for the targeted ISG and proinflammatory markers as described in *Materials and Methods*. Data are expressed relative to their respective 18S rRNA expression and are means $\pm$ SEM of three experiments ($n = 6$ mice for each experiment and for each treatment group). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$ versus Adriamycin.

B). Thus, although IFN- α and IFN- β both aggravate Adriamycin-induced proteinuria and focal segmental glomerulosclerosis, both have different effects on podocytes and PECs, which are consistent with our *in vitro* results.

Discussion

IFN- α and IFN- β coordinate antiviral immunity, which involves the activation of innate and adaptive immunity in addition to numerous other biological effects that support antiviral host defense.^{8,33} Although viral nephropathies represent an important entity in clinical nephropathy, there is

little known about the biological effects of these enigmatic cytokines inside the kidney. Satchell et al³⁴ reported anti-proteinuric effects of IFN- β in glomerular diseases of immunocompetent rodents and Schwarting et al³⁵ reported protective effects of IFN- β on lupus nephritis of MRL-lpr mice. However, IFNAR deficiency protects mice from glomerulonephritis.^{36–38} To dissect these discrepant results we studied how the type I IFNs affect glomerular epithelial cells *in vitro* and *in vivo* in SCID mice to exclude adaptive immunity as a confounding factor. Here, we show that IFN- α and IFN- β both increase glomerular inflammation and proteinuria. As a novel finding, we show that their biological actions are direct and have distinct effects on podocytes and PECs. Our results have implications for the pathophysiology and pathology of viral glomerulonephritis, regarding podocyte loss in general, and regarding renal side effects of IFN therapy.

IFN- β ameliorates glomerular injury and proteinuria in immunocompetent rodents^{34,35} but aggravates Adriamycin nephropathy in SCID mice lacking adaptive immunity. This finding implies that the type I IFNs have complex immunoregulatory effects on innate and adaptive immunity and distinct direct effects on glomerular epithelial cells. Injections with recombinant IFN- α and IFN- β both increased the intrarenal expression of ISG including the chemokine CXCL10. CXCL10 (also known as IP-10) attracts proinflammatory macrophages (and cytotoxic T cells) via the chemokine receptor CXCR3, which explains the increased numbers of intrarenal CD68-positive macrophages of SCID mice with both IFNs.³⁹ This in itself may be sufficient to explain the associated increase in proteinuria,⁴⁰ but IFN- β also affected the glomerular filtration barrier via direct effects on podocytes. Within a few minutes of IFN- β exposure, cultured podocytes changed their shape, potentially rounded up by retracting cytoplasmic extensions, which altered ECIS bioimpedance measurements (ie, functional parameters of podocyte monolayer permeability and cellular shape).²⁰ Satchell et al³⁴ used the same bioimpedance device and reported opposite results on IFN- β stimulation. Their studies were performed with immature and proliferating glomerular epithelial cells of human origin,³⁴ which does not allow predictions on the filtration barrier generated by terminally differentiated podocytes in our study. This immediate effect on podocyte permeability was accompanied by podocyte detachment and by reduced podocyte viability as assessed after 24 hours of IFN- β stimulation by PI/annexin V flow cytometry. Our *in vivo* results supported this *in vitro* finding because IFN- β —treated SCID mice suffered from increased podocyte loss and more proteinuria during Adriamycin nephropathy compared with IFN- α or vehicle treatment.

We detected an increased podocyte mitotic activity in IFN- β —treated SCID mice, which is similar to the typical features of viral glomerulonephritides that display multinucleated podocytes in association with podocyte loss and glomerulosclerosis.^{13,41} This is consistent with previous

Figure 6 IFN- β aggravates podocyte loss in Adriamycin nephropathy. **A**: Quantification of number of podocytes (nephlin-positive positive) Wilms tumor 1/ glomerulus after 7 days of treatment. Data are expressed as means $\pm$ SEM and are representative of three independent experiments ($n = 6$ mice for each experiment and for each treatment group). **B**: Representative images of double-label immunofluorescence for WT-1 (red) and nephrin (green). **C**: Quantification of number of mitotic podocyte (podocin-positive P-H3-positive)/glomerulus after 7 days of treatment. Data are expressed as means $\pm$ SEM and are representative of three independent experiments ($n = 6$ mice for each experiment and for each treatment group). **D**: Representative image of double-label immunofluorescence for podocin (red) and P-H3 (green). * $P < 0.05$, ** $P < 0.01$ versus Adriamycin.

reports that showed how podocytes enter the cell cycle to undergo hypertrophy while the M phase of nuclear division is inhibited by cyclin kinase inhibitors at the G1 and G2/M restriction points.^{30,42} When podocytes are forced to pass the M phase (eg, by sublytic complement activation, Notch, or mouse double minute 2 homolog signaling), podocytes may undergo aberrant nuclear division, resulting in multinucleated cells, often with an asymmetric chromosome segregation during the anaphase of the cell cycle.⁴³ Also, differentiated podocytes lack proteins such as aurora B, which is necessary to complete cytokinesis. Histomorphologically, such aneuploid podocytes are characterized by two, three, or even more (micro-)nuclei that altogether indicate a severely damaged cell, rather than podocyte proliferation to compensate podocyte loss.^{16,31,41,44} Instead, this process is an underlying mechanism of podocyte loss.⁴³ This is because podocytes are terminally differentiated cells with a complex

and sophisticated cytoskeletal structure, which links them to neighboring cells via the slit membrane. Forming the mitotic spindle requires extensive cytoskeletal rearrangements, which are not compatible with maintaining the filtration barrier and their structural integration.⁴² Therefore, stimuli that force podocytes to pass the M phase of nuclear division trigger podocyte detachment or death within or shortly after mitosis.^{16,29–31,42,44} This variant of cell death was called mitotic catastrophe.^{28,32,45}

IFN- α had an effect similar to IFN- β on proteinuria, glomerular inflammation, and scarring, which should relate to their consistent induction of ISG and chemokines. IFN- α and IFN- β also shared the inhibitory effect on renal progenitor differentiation to podocytes, which favors glomerular scarring instead of regeneration.²⁶ However, IFN- α did not cause immediate changes in cell shape that affected the resistance and capacity of podocyte monolayers and did not affect

Figure 7 IFN- α affects PEC proliferation in Adriamycin nephropathy. **A**: Quantification of the number of proliferating PECs (P-H3-positive claudin-1-positive)/glomerulus after 7 days of treatment. Data are expressed as means $\pm$ SEM and are representative of three independent experiments ($n = 6$ mice for each experiment and for each treatment group). ** $P < 0.01$ versus Adriamycin. **B**: Representative images of double-label immunofluorescence for claudin-1 (red) and P-H3 (green).

podocyte viability within 24 hours. IFN- α -induced cell-cycle arrest in PECs *in vitro* and *in vivo* further impairs podocyte repair. This antimitotic effect of IFN- α should relate to p21 induction, a cell-cycle inhibitor that protects podocytes from completing mitosis.⁴⁶

IFN- α and IFN- β elicit unique biological effects, however, they both bind to the same IFN receptor. The IFNAR consists of two transmembranous receptor chains with different binding affinities for the 16 type I IFN variants including IFN- α and IFN- β , setting off different signaling events that can explain their shared and distinct biological activities.⁴⁷

Our findings have several clinical implications. Type I IFN are important mediators of viral infection, thus their local effects on podocytes and PECs may contribute to podocyte loss, focal segmental glomerulosclerosis, and collapsing glomerulopathy (eg, in viral nephropathies).^{9,11,12,48} HIV replicates in podocytes and PECs and viral nucleic acids induce IFN production and ISG expression in glomerular cells.^{2,4–6,9} Type I IFNs also contribute to other forms of glomerulonephritis as evidenced by reduced disease activity in IFNAR-deficient mice.^{36–38} Type I IFNs are also essential mediators of systemic lupus and lupus nephritis including local effects on podocytes as evidenced by the IFN- α - and IFN- β -specific reticular inclusions in podocytes.^{49,50} Finally, recombinant type I IFNs frequently are used for the treatment of a number of autoimmune diseases and chronic viral hepatitis.^{51,52} The aggravation of proteinuria has been reported as a side effect, which may relate to the pathomechanisms described in this study.^{53–55}

In summary, IFN- α and IFN- β both aggravate Adriamycin nephropathy in SCID mice by aggravating intrarenal inflammation. IFN- β specifically promotes podocyte loss by inducing mitotic catastrophe of podocytes. IFN- α affects proliferation and migration of PECs. Both IFNs also impair the differentiation of renal progenitors into mature podocytes, a mechanism that favors focal scarring over glomerular repair. All together, these data provide a mechanistic model on how antiviral responses triggered by IFN- α and IFN- β drive podocyte loss, proteinuria, and glomerulosclerosis.

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Supplemental Data

Supplemental material for this article can be found at <http://dx.doi.org/j.ajpath.2013.04.017>.

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