TLR3-dependent regulation of cytokines in human mesangial cells: a novel role for IP-10 and TNF-α in hepatitis C-associated glomerulonephritis

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Toll-like receptor

IN VIRAL INFECTIONS, DISEASE manifestations often result primarily from immune cells infiltrating target organs on the basis of an ineffectual viral clearance with persistent antigenemia or an inappropriate immune response. Cell types and mediators defining these inflammatory processes are still inadequately understood. In hepatitis C virus-associated glomerulonephritis, analysis of interferon-γ-inducible protein (IP-10) as a chemokine centrally involved in early antiviral response and TNF-α known to balance proinflammatory and immunosuppressive effects in inflammation shows a significant upregulation of both IP-10 and TNF-α mediated specifically by the viral receptor Toll-like receptor 3 expressed on mesangial cells. IP-10 induction is further potentiated by TNF-α signaling, preferentially via the TNF-α receptor subtype 2 selectively increased upon stimulation of viral receptors in the proinflammatory milieu.

Merkle M, Ribeiro A, Wörnle M. TLR3-dependent regulation of cytokines in human mesangial cells: a novel role for IP-10 and TNF-α in hepatitis C-associated glomerulonephritis. Am J Physiol Renal Physiol 301: F57–F69, 2011. First published March 30, 2011; doi:10.1152/ajprenal.00083.2011.—In viral infections, disease manifestations and tissue damage often result primarily from immune cells infiltrating target organs on the basis of an ineffectual viral clearance with persistent antigenemia or an inappropriate immune response. Cell types and mediators defining these inflammatory processes are still inadequately understood. In hepatitis C virus-associated glomerulonephritis, analysis of interferon-γ-inducible protein (IP-10) as a chemokine centrally involved in early antiviral response and TNF-α known to balance proinflammatory and immunosuppressive effects in inflammation shows a significant upregulation of both IP-10 and TNF-α mediated specifically by the viral receptor Toll-like receptor 3 expressed on mesangial cells. IP-10 induction is further potentiated by TNF-α signaling, preferentially via the TNF-α receptor subtype 2 selectively increased upon stimulation of viral receptors in the proinflammatory milieu.

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of viral origin as well as polyriboinosinic:polyribocytidylic acid [poly (I:C)], a synthetic analog of viral dsRNA (1). The helicase retinoic acid-inducible gene I (RIG-I) may also act as a sensor of viral infections by recognition of viral dsRNA (24).

Interferon-γ inducible protein (IP-10) belongs to the family of CXC chemokines and is known also as CXCL10; its receptor CXCR3 is expressed on T-helper (Th1) lymphocytes, which are involved in the restriction of viral spread and viral elimination. In chronic hepatitis C, Th1 inflammatory cells are known to predominate in the liver; correspondingly, a significant increase in Th1-associated chemokines has been shown intrahepatically and in peripheral blood. IP-10 levels correlate with lobular inflammation but not with fibrosis and additionally have prognostic utility as a marker of response to antiviral treatment (30). In patients with chronic HCV infection, significantly higher IP-10 levels have been found in the patient subgroup with mixed cryoglobulinemia and active vasculitis (3). Moreover, an increased urinary concentration of IP-10 has been found in patients with acute kidney injury (22) and is associated with a higher risk of acute rejection in kidney transplant recipients (15, 19). In type 1 diabetes patients with microalbuminuria, urinary concentrations of IP-10 were higher when early progressive renal function decline occurred than with stable renal function (25). As to the ligand of IP-10, CXCR3, it has been shown that the number of infiltrating CXCR3-positive T cells correlates with renal function and proteinuria in glomerular diseases (21). Therefore, IP-10 results to be a marker and mediator of inflammatory processes both in HCV infection and in glomerular diseases of different origin.

TNF-α is a pleiotropic cytokine known to exert multiple effects on the immune system, including the release of IL-1β, IL-6, and IL-8, upregulation of endothelial adhesion molecules and chemokines such as monocyte chemoattractant protein (MCP)-1, macrophage inflammatory protein (MIP)-2, RANTES, and MIP-α, and coordination of the migration of leukocytes to targeted organs. The biological activity of trimeric TNF-α requires binding to one of the TNF-α receptors (TNFR), TNFR1 or TNFR2. The receptors have different affinity for the soluble and the membrane-bound forms of TNF-α; TNFR1 binds both forms equally well, whereas TNFR2 has a higher affinity for the membrane-bound form. A preferential role for TNFR2 during cell-cell interactions has been deduced. In the normal kidney, TNF-α and TNF-R2 are usually not detected; TNFR1 shows a glomerular expression pattern (6). In inflammatory and autoimmune kidney diseases, TNF-α has been implicated in the cascade leading to renal injury, with its expression being related to the injured compartment. An increased expression of TNFR1 has been found in lupus nephritis classes III and IV and in membranous nephropathy. TNFR2
expression has been observed to increase in a pattern similar to TNF-\(\alpha\), suggesting a regulation of TNFR2 by its ligand (2).

To prevent progressive tissue destruction due to persistent inflammation in HCV-associated GN, it is crucial to understand the mechanisms of early virus clearance and the de novo induction of inflammatory processes. We have shown previously that human MC express the viral receptor TLR3 thereby playing an important role in HCV-associated GN (26, 27) and exhibit an increased proliferation and migration in response to TNF-\(\alpha\), suggesting a regulation of TNFR2 by its ligand (2). TNF-\(\alpha\) expression has been observed to increase in a pattern similar to IFN-\(\gamma\) with IP-10 being obviously involved in active HCV-associated GN (21). With IP-10 being obviously involved in active inflammation processes and growing evidence of the pivotal importance of TNF-\(\alpha\) for maintenance of immune homeostasis, we were interested in exploring the effect of TLR3 activation on the expression of IP-10 and TNF-\(\alpha\) in human MC, their mutual regulation, as well as the potential impact of an up-regulation of IP-10 on cytokine and chemokine synthesis.

**METHODS**

Cell culture of human MC. Immortalized human MC were grown as described previously (5) and incubated with culture medium alone or medium containing test substances as indicated. The cytokine concentrations used were 25 ng/ml for TNF-\(\alpha\), 10 ng/ml for IL-1\(\beta\), and 20 ng/ml for IFN-\(\gamma\), with stimulation intervals between 3 and 24 h as indicated. For prestimulation with cytokines, confluent MC were incubated with culture medium containing the cytokines as indicated for 12 h, then washed with PBS, incubated in culture medium containing 10% fetal calf serum for 6 h, and washed again with PBS. Subsequently, MC were incubated with culture medium containing the test substances as indicated. For each stimulation experiment, controls were performed in parallel using culture medium alone. Stimulation with poly (I:C) in cell culture medium was done at concentrations and time intervals as indicated. For analysis of mRNA levels, extraction of total RNA was performed using an RNeasy Mini Kit (Qiagen) with additional DNase digestion.

Quantitative RT-PCR analysis. RT-PCR analysis was done as described (27). Two micrograms of isolated total RNA underwent random primed reverse transcription using a modified Moloney murine leukemia virus reverse transcriptase (Superscript, Life Technologies). In parallel, primed reverse transcription using a modified Moloney murine leukemia virus reverse transcriptase (Superscript, Life Technologies). In parallel, reverse transcription was performed using random hexamer primers. cDNA was used as a template for the quantitative PCR analysis.

Quantitative RT-PCR. Quantitative RT-PCR analysis was performed on a TaqMan ABI 7700 sequence detection system (PE Applied Biosystems). GAPDH served as a reference gene. All water controls were negative for targets and the cytokine reagents or primers and probe, purchased from Applied Biosystems:

- Human TNF-\(\alpha\) (Hs00660834_m1, NM_001065.1, M63197.1 Seq AOD: GCTGCCACTGGTGCTCCTG
- Human IL-1\(\beta\) (Hs00533560_m1, NM_001079821.2, NM_001127462.2 Seq AOD: TGGTGAAGTGAACGTTGATG
- Human IFN-\(\gamma\) (Hs00989291_m1, NM_000619.1, X262468.1, X09H19.2, X026469.1 Seq AOD: GACTAATTTCCGGAATTTCCG
- Human TLR3 (Hs00153550_m1, NM_001066.2, M32315.1 Seq AOD: AGAGAAAGCCAGTGCTACTTCG
- Human IP-10 (Hs00174128_m1, NM_001565.1 Seq AOD: GTGATGGATGCTACTTC
- Human RIG-I (Hs00175457_m1, NM_004833.1, AF024714.1 Seq AOD: CCAAAGTTGTCA
- Human AIM2 (Hs00175454_m1, NM_183395.2, NM_001079821.2, NM_001127462.2 Seq AOD: GCATTCAAGGAGTACCTCTCTC
- Human NALP3 (Hs00175454_m1, NM_001565.2, M63121.1 Seq AOD: GCTGCCACTGGTGCTCCTG
- Human MDA5 (Hs00175454_m1, NM_001565.2, M63121.1 Seq AOD: GCTGCCACTGGTGCT

Statistical analysis. Values are provided as mean \(\pm\) SEM. Statistical analysis was performed by ANOVA, respectively, an unpaired \(t\)-test, which was used only for the proliferation assays including a total of eight experiments. Significant differences in expression levels are indicated for \(P < 0.05\) (*) or 0.01 (**) respectively.

**RESULTS**

Effect of proinflammatory cytokines on expression of TLR3 and RIG-I in cultured human MC. We have previously shown that MC express the viral receptors TLR3 and RIG-I and that expression levels are increased upon stimulation with proinflammatory cytokines. To evaluate the timecourse of induction of TLR3 and RIG-I by cytokines, MC were stimulated with poly (I:C) at a concentration of 10 \(\mu\)g/ml for 24 h; again, culture medium alone served as control.

Preparation of HCV RNA. Exosomes containing viral RNA from human sera of patients with high viral loads were isolated by centrifugation as described before (13). Twenty to fifty milliliters of clotted human whole blood were centrifuged at 1,500 \(g\) for 10 min. The supernatant was collected and centrifuged again at 10,000 \(g\) for 30 min to remove solid remnants, with the resulting supernatant being centrifuged at 70,000 \(g\) for 60 min in a SW28 rotor (Beckman Instruments). The pellet was dissolved in 1 \(ml\) cell culture medium without serum or antibiotics and Lipofectamine was added. Concentration of HCV used for stimulation was 100 \(\times 10^3\) geq/ml confirmed by RT-PCR. For HCV stimulation, confluent human MC in six-well plates were used; once the virus was added, plates were centrifuged at 1,000 \(g\) for 45 min to allow for an efficient virus infection (29). Stimulation was done as indicated.

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combination led to a significant, incremental induction of TLR3 and RIG-I expression from 6 h on (Fig. 1, A and B).

**Effect of poly (I:C) on expression of proinflammatory cytokines.** To test whether activation of viral receptors by poly (I:C) mimicking viral RNA has a mutual effect on the expression of TNF-α, IL-1β, and IFN-γ, MC were stimulated with poly (I:C) (10 μg/ml) for different time intervals (3, 6, 12, 24 h). Both TNF-α and IL-1β showed a barely detectable basal expression. Stimulation with poly (I:C) induced a stable increase in TNF-α expression from 3 to 12 h (Fig. 1C). IL-1β...

Fig. 1. Mutual interactions between proinflammatory cytokines and stimulation of viral receptors on human mesangial cells (MC). MC were cultivated under standard conditions (basal, □) or stimulated with the proinflammatory cytokines TNF-α (○), IL-1β (■), or IFN-γ (○) alone or in combination (comb, ⋆) for different time intervals (3, 6, 9, 12, 24 h). Expression of Toll-like receptor 3 (TLR3; A) and retinoic acid-inducible gene I (RIG-I; B) was analyzed by RT-PCR. MC were cultivated under standard conditions (basal) or stimulated with poly (I:C) (10 μg/ml) for different time intervals (3, 6, 12, 24 h). Expression of TNF-α (C) and IL-1β (D) was analyzed by RT-PCR. E and F: MC were cultivated under standard conditions (squares) or prestimulated with a combination of the cytokines TNF-α, IL-1β, and IFN-γ (diamonds), and then incubated with culture medium alone (open symbols, solid line) or culture medium containing poly (I:C) (10 μg/ml, filled symbols, dotted line) for different time intervals (3, 6, 12, 24 h); expression of TNF-α, IL-1β, and IFN-γ was analyzed by real-time RT-PCR. No expression of IFN-γ was observed (data not shown). Values are means ± SE of 2 experiments done in parallel for each condition, and rRNA served as the reference gene. Comparable results were obtained in 2 series of independent experiments. *P < 0.05. **P < 0.01.
expression was upregulated at all poly(I:C) stimulation time points, with a maximum after 12 h (Fig. 1D). No expression of IFN-γ could be detected (data not shown). Subsequently, MC pretreated with a combination of TNF-α, IL-1β, and IFN-γ were incubated with poly (I:C) (10 μg/ml) for different time intervals (3, 6, 12, 24 h). The maximal expression level of TNF-α was observed after cytokine prestimulation and decreased continuously in both control and poly (I:C)-stimulated cells from 3 h on; the decline in TNF-α expression occurred earlier and was more pronounced in poly (I:C)-treated cells, reaching basal levels at 24 h (Fig. 1E). In contrast, the increase in IL-1β expression observed in prestimulated MC was augmented further in both control and poly (I:C)-stimulated cells until 12 h and was more pronounced in control cells. At 24 h, only the poly (I:C)-treated cells exhibited a decrease in IL-1β expression to initial levels (Fig. 1F). No mesangial expression of IFN-γ was observed, either under baseline conditions or after poly (I:C) stimulation (data not shown).

Effect of proinflammatory cytokines on expression of IP-10. As MC play an important role in virus-associated glomerular disease as well as in antiviral response, we examined IP-10 expression in human MC, simulating a proinflammatory milieu by stimulation with the cytokines TNF-α, IL-1β, or IFN-γ alone or in combination for different time intervals (3, 6, 9, 12, 24 h). No significant increase in the very low basal expression levels of IP-10 was seen upon stimulation with IL-1β or IFN-γ alone. TNF-α led to an increase in IP-10 expression from 6 h on, an induction reaching statistical significance after 24 h. Stimulation with the cytokine combination led to a manifold increase in expression of IP-10 at all the time points, with a maximum after 24 h (Fig. 2A).

Effect of IP-10 on expression of TNF-α and IL-1β. To test for a mutual influence of IP-10 on cytokine expression, MC were stimulated with IP-10 (100 ng/ml) for different time intervals (3, 6, 9, 12, 24 h) and expression of TNF-α and IL-1β was analyzed by RT-PCR; we refrained from analyzing IFN-γ, as we previously did not observe its expression in MC. IP-10 stimulation had no effect on mesangial expression of TNF-α and IL-1β (data not shown).

Effect of incubation with poly (I:C) on mRNA levels of IP-10. Upon activation of viral receptors by stimulation of MC with poly (I:C) (10 μg/ml) for different time intervals (3, 6, 12, 24 h), a significant induction of IP-10 expression is found from 6 h on, with the maximal increase seen at 12 h (Fig. 2B); this time course parallels the one shown for induction of IL-1β expression by poly (I:C) and is consistent with the role of both mediators in early viral clearance in vivo. Incubation of MC with culture medium containing poly (I:C) in different concentrations (0.5, 5, 10 μg/ml) for 12 h resulted in a significant, dose-dependent increase in the expression of IP-10, with a maximum at stimulation with 10 μg/ml poly (I:C) (Fig. 2C). Having shown previously an induction of the viral receptors TLR3 and RIG-I in MC by proinflammatory cytokines, MC were prestimulated with TNF-α, IL-1β, and IFN-γ as described and then incubated in culture medium alone or culture medium containing poly (I:C) (10 μg/ml) for different time intervals (3, 6, 12, 24 h). Again, basal IP-10 expression was barely detectable and did not change over time. Compared with control cells, prestimulated MC showed an earlier and more pronounced IP-10 expression upon incubation with poly (I:C) at 6 h, with IP-10 levels being comparable at 12 and 24 h. At any rate, the highest expression of IP-10 was observed in MC prestimulated with the cytokine combination and then kept in culture medium alone for 12 h (Fig. 2D).

Effect of transfection with siRNA specific for TLR3 and RIG-I on poly (I:C)-induced IP-10 expression. The increase in IP-10 expression induced by stimulation with poly (I:C) (10 μg/ml) for 12 h was significantly reduced by transfection of MC with siRNA specific for TLR3. Transfection with siRNA specific for RIG-I and with negative controls containing unspecific RNA had no effect on poly (I:C) induced expression of IP-10 (Fig. 2E).

Effect of IP-10 stimulation on mesangial expression of viral receptors. To test for a mutual influence of IP-10 on the expression of viral receptors, MC were stimulated with IP-10 (100 ng/ml) for different time intervals (3, 6, 9, 12, 24 h). No effect on expression levels of the viral receptors TLR3 and RIG-I was seen (data not shown).

Effect of proinflammatory cytokines on expression of TNFR1 and TNFR2 in cultured human MC. To allow for analysis of possible signaling pathways of TNF-α in human MC, expression levels of TNFR1 and TNFR2 were measured under basal conditions and after stimulation of MC with the proinflammatory cytokines TNF-α, IL-1β, and IFN-γ alone or in combination for different time intervals (3, 6, 9, 12, 24 h). MC showed a substantially stable basal expression of TNFR1, which was not significantly influenced by any of the cytokines (Fig. 3A), and a comparatively low basal expression of TNFR2. TNFR2 was significantly increased upon stimulation with TNF-α at 9, 12, and 24 h and IL-1β at 6, 12, and 24 h. IFN-γ had no effect on TNFR2 expression. The strongest induction of TNFR2 expression was observed upon stimulation with the combination of cytokines starting at a stimulation time of 3 h (Fig. 3B).

Effect of IP-10 on expression of TNFR1 and TNFR2. Having shown the induction of mesangial IP-10 by TNF-α, we tested whether IP-10 has a reciprocal effect on the expression of TNFR1 and TNFR2. Stimulation of MC with IP-10 (100 ng/ml) for different time intervals (3, 6, 9, 12, 24 h) did not influence basal expression levels of TNFR1 or TNFR2 (data not shown).

Effect of poly (I:C) on expression of TNFR1 and TNFR2. Upon stimulation of viral receptors by incubation of MC with poly (I:C) (10 μg/ml) for different time intervals (3, 6, 12, 24 h), no significant change in the basal expression of TNFR1 was observed (data not shown); expression of TNFR2, however, was significantly upregulated after 12 and 24 h (Fig. 3C). MC prestimulated with the cytokine combination of TNF-α, IL-1β, and IFN-γ did not show any change in expression of TNFR1 upon poly (I:C) treatment either (Fig. 3D), whereas the induction of TNFR2 by poly (I:C) was significantly increased by cytokine pretreatment beginning at a stimulation time of 3 h. The maximal and, compared with MC grown under standard conditions, the nearly fivefold higher induction of TNFR2 by poly (I:C), was seen after 12 h. Interestingly, cells solely prestimulated with the cytokine combination showed an increase in TNFR2 over time as well, which led to TNFR2 expression levels higher than in poly (I:C)-stimulated cells after 24 h (Fig. 3E).

TNFR subtype-dependent signaling in TNF-α-mediated induction of IP-10. With human MC showing a higher basal expression of TNFR1 compared with TNFR2 but a subtype 2-restricted receptor upregulation upon stimulation with pro-
inflammatory cytokines and poly (I:C), we analyzed the contribution of the receptor subtypes to TNF-α-mediated induction of IP-10. To allow for viral receptor-mediated upregulation of TNFR, cells were incubated with poly (I:C) for 24 h and then stimulated with TNF-α for different time intervals (6, 12, 24 h). As expected, incubation with neither poly (I:C) nor TNF-α led to an induction of TNFR1 (Fig. 4A), while the increase in TNFR2 expression upon poly (I:C) stimulation was further
enhanced by subsequent incubation of MC with TNF-α. The maximal upregulation of TNFR2 in MC prestimulated with poly (I:C) was reached at 12 h of additional TNF-α treatment (Fig. 4B). IP-10 expression was induced upon incubation with poly (I:C) for 24 h, an effect lost continuously during the following 24 h. Paralleling the time course of TNFR2 induction, a significant upregulation of IP-10 was found starting at 6 h of stimulation with TNF-α and reaching a maximum after 12 h (Fig. 4C).

Specific role of viral receptors in poly (I:C)-mediated induction of mesangial TNF-α and TNFR. To test which of the viral receptors is involved in poly (I:C)-mediated induction of
TNF-α and TNFR2. MC were transfected with siRNA specific for TLR3 or RIG-I as described and stimulated with poly (I:C) for 24 h. The poly (I:C)-induced increase in expression of TNF-α was blocked by siRNA specific for TLR3. siRNA for RIG-I as well as unspecific RNA as a negative control had no effect (Fig. 4D). As expected, expression of TNFR1 was unaffected by poly (I:C) stimulation and transfection with siRNA for TLR3, RIG-I, or control RNA (data not shown). The increase in TNFR2 expression induced by poly (I:C) was not influenced by transfection of MC with siRNA specific for TLR3 or RIG-I. Therefore, additional knockdown experiments with siRNA specific for other viral RNA and DNA receptors (MDA5, NALP3, and AIM2) were performed. None of these siRNAs blocked the poly (I:C)-induced upregulation of TNFR2, indicating a signaling pathway not dependent on these known viral receptors (Fig. 4E).

Effect of poly (I:C) and IP-10 on expression of cytokines, chemokines, and type I interferons. Having shown previously the ability of human MC to upregulate cytokines and chemokines including IP-10 in response to poly (I:C), we next compared the effect of poly (I:C) and IP-10 on the expression of IL-6, MCP-1, and the type I interferons IFN-α and IFN-β.
MC were stimulated with poly (I:C) (10 μg/ml) or IP-10 (100 ng/ml) for different time intervals (3, 6, 9, 12, 24 h), and expression of the selected cytokines, chemokines, and interferons was analyzed. Both IL-6 and MCP-1 were induced by poly (I:C) (Fig. 5, A and C), whereas IP-10 had no effect on their expression (Fig. 5, B and D). Neither poly (I:C) nor IP-10 influenced the low basal expression of INF-α (data not shown). Basal expression of INF-β was very low, too, but was strongly increased by poly (I:C), with an early maximum after 3 h (Fig. 5E). In MC incubated with IP-10, a significant upregulation of INF-β expression was seen after a stimulation time of 9 h, reaching a maximum after 12 h (Fig. 5F).

**Effect of HCV RNA on expression of TLR3, TNF-α, TNFR2, and IP-10 in human MC.** To confirm the relevance of the experimental results obtained upon stimulation of human MC with poly (I:C) in HCV infection, MC were transfected with Lipofectamine and stimulated with HCV RNA isolated as described for different time intervals (3, 6, 12, 18 h); expression of TLR3, TNF-α, TNFR2, and IP-10 was analyzed. An increase in TLR3 expression was observed after 6 and 12 h and reached statistical significance at 18 h of HCV RNA stimulation (Fig. 6A). Upregulation of TNF-α expression by HCV RNA was significant compared with controls from 6 h on and maximal at 18 h (Fig. 6B). Expression of TNFR2 was signif-

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**Fig. 5.** Effect of IP-10 and poly (I:C) on mesangial expression of cytokines, chemokines, and type I interferons. MC were cultivated under standard conditions (basal) or stimulated with poly (I:C) (10 μg/ml) or IP-10 (100 ng/ml) for different time intervals (3, 6, 9, 12, 24 h); expression of IL-6 (A and B), monocyte chemotactic protein (MCP-1; C and D), and IFN-β (E and F) was analyzed by RT-PCR. Values are means ± SE of 2 experiments done in parallel for each condition, and rRNA served as the reference gene. Comparable results were obtained in 2 series of independent experiments. *P < 0.05.
significantly increased after 12 and 18 h of stimulation with HCV (Fig. 6C). An induction of IP-10 expression by HCV RNA was seen only at 18 h (Fig. 6D).

**Effect of cytokines, poly (I:C), and IP-10 on MC proliferation.** Last, proliferation of human MC in response to the proinflammatory cytokines TNF-α, IL-1β, and IFN-γ, poly (I:C) alone, or poly (I:C) with cytokine pretreatment of the cells and IP-10 was assessed by the MTT proliferation assay as described. Neither the cytokines TNF-α, IL-1β, and IFN-γ alone nor the combination of TNF-α and IFN-γ or of all three cytokines had a significant effect on MC proliferation after 24 h (Fig. 7A). MC prestimulated with cytokines and then incubated with poly (I:C) (10 μg/ml) for 24 h showed a significantly reduced cell proliferation compared with control cells pretreated with cytokines and then left in culture medium. This poly (I:C)-dependent decrease in proliferation was reproducible in MC not pretreated with cytokines (Fig. 7B). Stimulation of human MC with different concentrations (10, 100, 250 ng/ml) of IP-10 for 24 h led to a significant, concentration-dependent increase in cell proliferation, as previously published (21) (Fig. 7C).

**DISCUSSION**

A pivotal task of the immune response to viral infections including HCV infection is restriction of viral spread and virus elimination, which requires recruitment of T lymphocytes to infected tissues. To elucidate mechanisms of development of chronic inflammation as seen in hepatitis C-associated GN, which can result both from an ineffective primary immune response with persisting antigenemia and from the virally induced chemokine and cytokine burst with potential induction of autoimmune phenomena and tissue damage, we analyzed the regulation of IP-10 and TNF-α, known to be key players in T lymphocyte recruitment, respectively, tissue inflammation in a variety of kidney diseases, upon activation of the viral receptor TLR3 in resident glomerular cells.

Data on chemokine patterns and the course of inflammatory responses in acute HCV infection are limited. In acutely infected chimpanzees, a significant upregulation of IP-10 has been shown and seems to be important for protective immunity. In the case of an ineffective viral clearance, however, IP-10 continues to recruit inflammatory cells to the liver that perpetuate liver cell injury (11). The same phenomenon has been observed in mice infected intracerebrally with the mouse hepatitis virus; if there is no viral resolution during the acute phase of infection, upregulation of IP-10 persists and leads to myelin destruction by infiltrating inflammatory cells (7). TNF-α, similarly, is supposed to have different effects in early and late phases of inflammation as well as exhibiting receptor
subtype-specific signaling, which under physiological conditions results in a balance between its proinflammatory and immunosuppressive functions. Serum TNF-α levels are described to be elevated early in hepatitis C infection (16) and have been found to correlate with the degree of hepatic inflammation in chronic hepatitis C (17), but detailed data regarding time response and relevance of TNF-α for viral clearance and progression from acute to chronic infection as well as data regarding hepatitis C-associated GN are lacking. Interestingly, in a mouse model of nephrotoxic serum nephritis, TNFR1-deficient mice exhibited a delay in disease onset and a reduced adaptive humoral response. At later time points, the glomerular

Fig. 7. Effect of cytokines, poly (I:C) and IP-10 on proliferation of human MC. A: MC were cultivated under standard conditions (basal) or stimulated with TNF-α, IL-1β, or IFN-γ alone or in combination (comb) for 24 h, and cell proliferation was analyzed by the MTT assay. B: MC were prestimulated with TNF-α, IL-1β, or IFN-γ alone, a combination of TNF-α and IFN-γ, or a combination of all 3 cytokines for 24 h, and subsequently incubated with poly (I:C) (10 μg/ml) for 24 h. C: MC were exposed to increasing concentrations of IP-10 (10, 100, 250 ng/ml) for 24 h. IP-10 stimulation led to a dose-dependent increase in MC proliferation. Each bar represents the mean ± SE of 8 parallel incubations for each condition.Comparable results were obtained in two series of independent experiments. Statistically significant differences from control: *P < 0.05, **P < 0.01.
damage was similar to that in wild-type mice except for a marked increase in lymphocytic infiltration. TNFα-deficient mice, however, were completely protected against disease despite a preserved humoral response, indicating an important role of TNFR2 in immune-mediated GN (23). Evidence for a possible mutual dependence of IP-10 and TNF-α results from in vitro studies on dendritic cells known to require an inflammatory stimulus as TNF-α for maturation; in response to TNF-α, mature cells secrete several cytokines and chemokines which include, again, TNF-α, IL-1β, and IP-10. Furthermore, stimulation of human monocytes with TNF-α induces IP-10 even more effectively than stimulation with IFN-γ (18).

We have previously shown that MC, although being primarily nonimmune cells, express the viral receptors TLR3 and RIG-I. A significant upregulation of their low basal expression levels is induced by stimulation with a combination of TNF-α, IL-1β, and IFN-γ, chosen to mimic an inflammatory milieu comparable to the one found in HCV-associated GN. Proinflammatory cytokines thus allow for an actual response of MC to viral infection by upregulation of TLR3 and RIG-I. Furthermore, the enhancement of TLR3 expression by ligand binding which we have shown previously in poly (I:C)-stimulated MC, was confirmed in HCV-infected cells; therefore, viral infection itself is able to potentiate receptor-mediated effects by induction of TLR3.

Upon activation of the viral receptors, exemplified for in vitro experiments by stimulation with poly (I:C), a significant upregulation of the proinflammatory cytokines TNF-α and IL-1β, both relevant to immune response and establishment of inflammation, is seen. Compared with cells treated with poly (I:C), however, MC prestimulated with cytokines exhibit significantly higher expression levels of both TNF-α and IL-1β and, even more interestingly, the level of cytokine induction in prestimulated cells is reduced once they are additionally incubated with poly (I:C). This phenomenon may well be relevant in limiting inflammatory responses.

With NF-κB representing an integral component of the downstream signaling pathway of TLR3, we next analyzed the regulation of the NF-κB-dependent chemokines and cytokines IP-10, MCP-1, and IL-6 upon activation of the viral receptors. Starting from a negligible basal expression, IP-10 is significantly upregulated in MC stimulated with poly (I:C), herein showing a definite time response and a dose dependency. Induction of IP-10 by poly (I:C) is specifically mediated by TLR3, as shown in receptor knockdown experiments and is reproducible in MC infected with HCV. The upregulation of TLR3 in MC incubated with IP-10 does not reach statistical significance, and RIG-I expression remains unchanged, so that IP-10 itself does not seem to be able to facilitate virus binding and receptor signaling. Analysis of the effects of proinflammatory cytokines involved in GN on IP-10 expression shows a significant early upregulation of IP-10 in MC stimulated with the combination of TNF-α, IL-1β, and IFN-γ; TNF-α alone induces IP-10, too. However, with the expression of both TNF-α and IL-1β remaining unchanged in MC treated with IP-10, there is no evidence for a mutual regulation of IP-10 and TNF-α.

For IP-10 the same phenomenon is seen as described previously for TNF-α and IL-1β; that is, MC prestimulated with a combination of proinflammatory cytokines exhibit higher expression levels of IP-10 than those treated with poly (I:C), and incubation with poly (I:C) reduces IP-10 induction in prestimulated cells. Since the increase in IP-10 expression upon stimulation with the cytokine combination occurs at an earlier point in time than the induction of TLR3 by the same cytokines, we conclude an additional, not fully TLR3-dependent cytokine effect on IP-10. As shown before to upregulate IP-10, we considered TNF-α to be the most obvious candidate for mediating this effect.

Human MC are found to constitutively express both TNF-α receptor subtypes. TNF-α itself as well as IL-1β and a combined stimulation of MC with TNF-α, IL-1β, and IFN-γ are able to induce significantly the very low basal expression levels of TNFR2, whereas not having any effect on the expression of TNFR1. Furthermore, a significant upregulation of TNFR2 is seen in response to poly (I:C); this effect can be potentiated by prestimulating cells with the cytokine combination specified above. Again, results can be reproduced in MC infected with HCV. Thus, in the setting of viral infection, the ligand-induced upregulation of TNFR2 is further increased by activation of viral receptors. Very interestingly, knockdown experiments show that the induction of TNF-α upon stimulation with poly (I:C) is specifically mediated by TLR3. The upregulation of TNFR2 by poly (I:C), however, does not depend on any of the known viral receptors for RNA and DNA viruses, namely, TLR3, RIG-I, MDavaş, NALP3, or AIM2, as confirmed in knockdown experiments. Therefore, we observed an alternative yet unidentified signaling pathway mediating TNFR2 induction by poly (I:C) in human MC. Based on our observation of a subtype-specific regulation of TNFR in response to cytokines and poly (I:C) in human MC and the findings concerning immune-mediated GN in TNFR-deficient mice discussed earlier, we next analyzed the relative contribution of signaling via TNFR1 and TNFR2 to the upregulation of IP-10. In human MC prestimulated with poly (I:C), TNF-α is found to induce IP-10 in parallel with upregulating TNFR2. Therefore, in an inflammatory setting involving viral infection, induction of TNFR2 seems to be essential for IP-10 induction by TNF-α.

Finally, revisiting the similarly NF-κB-dependent MCP-1 and IL-6, a significant upregulation of their expression levels in MC upon activation of viral receptors by poly (I:C) is shown as expected. Furthermore, an induction of the type I interferon IFN-β...
at a very early time point is seen, with IFN-α expression levels being unchanged. Very interestingly, we can show that IP-10, while not having any effect on the expression of MCP-1 and IL-6, strongly induces IFN-β. As type I interferons are known to be relevant not only for early antiviral responses (12) but IFN-β has been shown to exert antiproteinuric effects (20), a protective effect of IP-10 in early viral infection by induction of IFN-β can be postulated.

In summary (Fig. 8), besides confirming the amplification of inflammatory responses by activation of TLR3, in this study we show for the first time a specifically TLR3-mediated, time-, and dose-dependent induction of IP-10 and TNF-α expression in human MC infected with HCV. We provide evidence for the expression of functional TNFR of both subtypes on human MC and a primarily TNFR2-mediated induction of IP-10 in the setting of viral infection; the TNFR2-restricted signaling of TNF-α supports the hypothesis of its paracrine effect. Furthermore, poly (I:C) is shown to induce TNFR2 via an yet unidentified pathway. As discussed previously, this newly described TLR3-dependent signaling cascade is supposed to be relevant for virus elimination in early infection; once chronic inflammation has occurred, both IP-10 and TNF-α could well mediate tissue damage. Therefore, these findings are of particular interest from a therapeutic point of view in chronic HCV infection and disease manifestations as vasculitis and GN. Blockade of CXCR3 with TAK-779, a synthetic nonpeptide antagonist of CCR5 and CXCR3, and monoclonal antibodies to CXCR3 has been used with promising results in animal models of different autoimmune diseases, and mean-while five inhibitors of TNF-α have been approved for therapy and successfully adopted in small clinical trials, including for patients with lupus nephritis, resistant primary focal segmental glomerulosclerosis, and antineutrophil cytoplasmic antibody-associated vasculitis (4, 9, 10). Anti-TNF-α treatments may also lead to the induction of autoimmunity, although based on the observed protection from tissue injury in receptor-deficient mice and its compartment-specific expression induced by inflamma-tory cascade, it has been proposed to focus on a selective receptor blockade with TNFR2 in kidney diseases (6). The results of our study support this recommendation. Finally, as elevated IP-10 levels have been associated with acute kidney injury and glomerular pathologies of autoimmune origin, a TLR3-dependent regulation of IP-10 conceivably might occur as well by nuclear acid fragments originating from cell debris.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

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