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Ischemia/Reperfusion-Induced IFN-γ Up-Regulation: Involvement of IL-12 and IL-18[1]

Marc A. R. C. Daemen, Cornelis van't Veer, Tim G. A. M. Wolfs, and Wim A. Buurman[2]

Tissue injury as a consequence of ischemia followed by reperfusion is characterized by early as well as late signs of inflammation. The latter, among others, involves IFN-γ-dependent up-regulation of MHC class I and II Ag expression. Employing a murine model of renal ischemia, we show that renal IL-18 mRNA up-regulation coincides with caspase-1 activation at day 1 following ischemia. IFN-γ and IL-12 mRNA are subsequently up-regulated at day 6 following ischemia. Combined, but not separate, in vivo neutralization of the IFN-γ inducing cytokines IL-12 and IL-18 reduces IFN-γ-dependent MHC class I and II up-regulation to a similar extent as IFN-γ neutralization, suggesting the involvement of functional IL-12, IL-18, and IFN-γ protein. These results reveal a novel relationship between tissue injury of nonmicrobial origin and the induction of IL-12 as well as IL-18. The collaboration observed between endogenous IL-12 and IL-18 in the induction of IFN-γ after renal ischemia/reperfusion, resembles the immune response to bacterial infections. ***The Journal of Immunology,*** **1999, 162: 5506–5510.**

The sequelae of events following renal ischemia and reperfusion (I/R)[3] involves signs of not only an early (1) but also a late (2, 3) inflammatory response. The latter is characterized by enhanced expression of MHC class I and II Ags that are likely to predispose ischemically damaged kidneys to the development of graft rejection (3–5). Enhanced MHC expression after I/R is a direct consequence of the potent MHC-inducing capacities of IFN-γ (6, 7). However, the mechanisms by which IFN-γ is induced after renal I/R still remain to be resolved.

IFN-γ, commonly associated with resistance against pathogens, is produced largely by Th1 cells and large granular lymphocytes. In particular, IL-12 (8), IL-18 (9), and, in an autocrine fashion, IFN-γ (10) are involved in the intricate and tight regulation of IFN-γ expression. Recently, Bohn et al. (11) reported that the LPS-induced IL-12 production by peritoneal macrophages in vitro is inhibited by IL-18. They subsequently showed that this inhibitory effect is suppressed by IFN-γ. In addition, in vitro experiments indicated that IL-12 and IL-18 synergistically enhance production of IFN-γ (12, 13). This synergistic mechanism has been attributed to up-regulation of the IL-18 receptor by IL-12 (14). On the other hand, IFN-γ production is limited by cytokines capable of suppressing Th1 development, such as IL-10 and IL-13 (15). In line with this, we recently demonstrated that endogenously produced IL-10 limits IFN-γ-induced renal MHC class I and II expression after I/R (4). IL-12 and IL-18 are commonly produced in response to pathogens and their products; however, in this study, we investigate the role of IL-12, IL-18, and consequent IFN-γ production triggered by tissue damage resulting from I/R.

We demonstrate that up-regulation of MHC class I and II Ags following renal I/R can be prevented to a similar extent as in mice treated with anti-IFN-γ Abs by combined, but not separate, administration of Abs directed against IL-12 and IL-18. This demonstrates for the first time that ischemic tissue damage initiates production of IL-12 as well as IL-18. Moreover, with respect to the in vivo kinetics of IL-12 and IL-18, we observe a resemblance between renal I/R injury and in vivo immunoactivation by infectious agents.

Materials and Methods

Abs and reagents

The following Abs were used: anti-murine IFN-γ mAb F3, was kindly provided by Hycult Biotechnology (Uden, The Netherlands); anti-murine IL-12 mAb C17.8, was kindly provided by Prof. Trinchieri (Wistar Institute, Philadelphia, PA) (16); mAb GL113, a control rat IgG1, was kindly provided by Dr. Savelkoul (Erasmus University, Rotterdam, The Netherlands); anti-murine MHC class I mAb m1/42.3.9.8 and mAb 5D7-producing hybridomas from the American Type Culture Collection (ATCC; Manassas, VA); anti-murine MHC class II mAb H82-168.10-producing hybridoma was kindly provided by Dr. Pierres (Centre National de la Recherche Scientifique INSERM, Marseille, France); anti-murine IL-18 polyclonal Ab (pAb; purified rabbit IgG) was raised by immunizing rabbits with recombinant murine IL-18, kindly provided by Dr. Kurimoto (Fujisaki Institute, Okayama, Japan). Peroxidase-conjugated goat anti-rat IgG was purchased from Jackson ImmunoResearch (West Grove, PA), Ac-YVAD-amc from the Peptide Institute (Osaka, Japan). All other reagents were purchased from Sigma (St. Louis, MO).

Animal model and protocol

All experiments were approved by the Institutional Animal Care Committee of the University of Maastricht. Renal ischemia was induced as follows. In brief, male Swiss mice (Charles River Breeding Laboratories, Heidelberg, Germany) were anesthetized, and body temperature was maintained at 39°C. After laparotomy, ischemia was induced by clamping the left renal pedicle for 45 min, during which the wound was covered. Subsequent to removal of the clamp, the contralateral kidney was removed and stored. After closing the abdomen, 0.25% bupivacaine was applied topically for postoperative pain management, and mice were supplemented with prewarmed PBS to maintain fluid balance. At the time of sacrifice (1 and 6 days after ischemia), blood was collected, and the left kidney was harvested.

Mice subjected to ischemia were treated 10 min before reperfusion with 0.5 ml PBS i.p. containing 300 μg anti-IFN-γ mAb F3 (n = 20), 1 mg

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[3] Abbreviations used in this paper: I/R, ischemia/reperfusion; BUN, blood urea nitrogen; cDNA, copy DNA; EIA, enzyme immunoassay; MPO, myeloperoxidase; pAb, polyclonal Ab; TMB, 3,3′,5,5′-tetramethylbenzidine.

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anti-IL-12 mAb C17.8 ($n = 20$), 1 mg anti-IL-18 pAb ($n = 20$), a combination of 0.5 mg anti-IL-12 mAb C17.8 and 0.5 mg anti-IL-18 pAb ($n = 20$), or PBS only ($n = 20$). Two additional groups that received isotype-matched control Ab (5D7, a control rat IgG2a mAb; GL113, a control rat IgG1 mAb; $n = 6$ each) when compared with the ischemia/PBS group showed no significant differences (data not shown), indicating that aspecific IgG does not affect the evaluated parameters. Treatment of animals to be sacrificed at day 6 was repeated at day 4 after ischemia. A sham-group ($n = 12$) underwent the same surgical procedure as animals subjected to ischemia, except for clamping of the renal pedicle. Sham-operated animals were only treated with PBS. The capacity of the anti-IL-18 pAb to block biological activity was determined by measuring the ability of the anti-IL-18 pAb to neutralize IL-18-mediated enhancement of IFN-γ production in vitro by stimulated T cells (1 mg IgG/ml inhibits the biological activity of 100 μg of IL-18) (data not shown). Also, the in vivo neutralizing capacity of the anti-IL-18 pAb used was confirmed by its effectiveness in combination with anti-IL-12 mAb as shown by the present results. Dosages of anti-IFN-γ and anti-IL-12 mAb are based on published results (16, 17).

Measurements of renal IL-12, IL-18, and IFN-γ mRNA content

Total RNA was extracted from kidneys and transcribed into copy DNA (cDNA) of which the concentration was subsequently standardized based on the β-actin cDNA fraction. To determine renal IL-12 and IL-18 mRNA content, four 2-fold serial dilutions of cDNA were amplified with specific primers (18, 19). IFN-γ mRNA expression was quantitatively measured by RT-PCR employing a multispecific control fragment as an internal standard (20). Relative amounts of IFN-γ cDNA in specimens were estimated from concentrations of control fragment DNA added to achieve equal amplification compared with IFN-γ cDNA employing IFN-γ-specific primers (20). After separation on 1.5% agarose gel, all PCR products were estimated by imaging the intensity of ethidium bromide luminescence with a computer-controlled display camera (Imagemaster, Pharmacia, Piscataway, NJ) and image analysis with Sigma Gel software (SPSS, Chicago, IL).

Quantification of renal caspase-1-like activity

To quantify renal caspase-1 activity, samples were homogenized, snap frozen, and stored at −70°C in a buffer containing 200 mM NaCl, 10 mM Tris-HCl (pH 7.0), 5 mM EDTA, 10% glycerol, 1 mM PMSF, 0.1 μM aprotinin, 1.0 μM leupeptin, and 5 mM oxidized glutathion. Renal lysates (containing 40 μg total protein) were incubated with 50 μM of the fluorogenic substrate Ac-YVAD-amc (caspase-1-like) in a cell-free system buffer containing 10 mM HEPES (pH 7.4), 220 mM mannitol, 68 mM sucrose, 2 mM NaCl, 2.5 mM KH_2PO_4, 0.5 mM EGTA, 2 mM $MgCl_2$, 5 mM pyruvate, 0.1 mM PMSF, and 1 mM DTT (21). The release of fluorescent 7-amino-4-methylcoumarin was measured for 1 h at 2-min intervals by spectrofluorometry (Cytofluor, Per Septive Biosystems, Cambridge, MA). Data are expressed as the increase in fluorescence as a function of time, normalized against data obtained from the sham-operated group.

Enzyme immunoassay (EIA) and immunohistology for MHC class I and II

For the quantification of MHC class I and II Ags in the kidney, an EIA was employed, as previously described (4). In brief, renal tissue was homogenized in PBS (20 mg/ml) plus 8 nM deoxyribonuclease I, 0.4 mM PMSF, incubated with either mAb m1/42.3.9.8 or mAb H82-168.10, washed, incubated with peroxidase-conjugated goat anti-rat IgG, and washed again. Next, 3,3′,5,5′-tetramethyl-benzidine (TMB) substrate was added in a 96-well sample plate (Costar, Cambridge, MA) to resuspended pellets, and OD was determined at 450 nm. All samples were measured in triplicate. MHC class I and II content were calculated per mg of renal tissue and standardized to a standard horseradish peroxidase titration curve. The obtained MHC class I and II contents are presented relative to the amount of MHC class I and II in the contralateral control kidney.

For immunohistology of renal MHC class I and II, specimens of harvested kidneys were immediately frozen and stored at −70°C. Frozen sections (5 μm) were stained for MHC class I with mAb m1/42.3.9.8 (22) and for MHC class II with mAb H82–168.10 using peroxidase-labeled goat anti-rat IgG as the secondary detection mAb and 3-amino-9-ethylcarbazole as a chromogen, followed by a hematoxylin counterstain. To block aspecific peroxidase activity, sections were pretreated for 10 min with PBS containing 0.03% H_2O_2. No significant staining was detected in slides incubated with control mAb instead of the primary detecting mAb, indicating the absence of significant background staining.

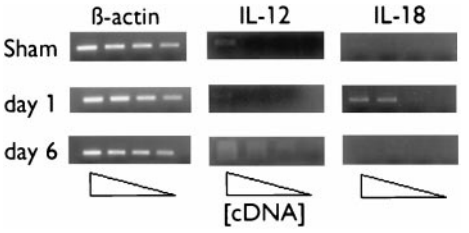

FIGURE 1. Renal IL-18 and IL-12 mRNA levels. Increased renal IL-18 mRNA and decreased IL-12 p40 mRNA expression at day 1 after I/R compared with kidneys obtained from sham-operated controls. At day 6, renal IL-12 p40 mRNA levels increased compared with sham-operated controls. Shown are representative samples of which cDNA was amplified in four 2-fold serial dilutions and calibrated against identical levels of β-actin mRNA.

Assays for renal myeloperoxidase (MPO) and blood urea nitrogen (BUN)

To quantify the extent of renal neutrophil accumulation, renal MPO content was determined (23). In brief, tissue samples were homogenized in 0.5% hexadecyltrimethylammonium bromide in 50 mM PBS (pH 6.0), 0.17 g tissue/ml. After heat incubation (2 h, 60°C) and three freeze-thaw cycles, MPO content was measured in triplicate by incubating supernatants with TMB substrate in a 96-well sample plate (Costar), followed by measurement of OD at 450 nm. MPO activity was calculated per mg renal tissue by comparing OD of samples with a standard titration curve of horseradish peroxidase. Data were standardized with respect to wet:dry ratios of the assayed renal tissue and are presented relative to the amount of MPO present in the contralateral kidney harvested immediately after ipsilateral reperfusion. BUN was measured in serum obtained at the time of sacrifice using a BUN unimate 5 kit in a Cobas Fara autoanalyzer (Roche, Basel, Switzerland).

Statistics

Data are expressed as means ± SEM, and statistical analysis was performed by Student's t test. A value of $p < 0.05$ was taken to denote statistical significance.

Results

Renal I/R induces endogenous IL-18 followed by IL-12, which both mediate IFN-γ-dependent MHC class I and II up-regulation

At day 1 after ischemia, increased levels of kidney-derived IL-18 mRNA were detected (Fig. 1), while IFN-γ mRNA expression remained constitutive (data not shown). Interestingly, at this time point, constitutive IL-12 p40 mRNA levels slightly decreased (Fig. 1). In animals sacrificed at 6 days after ischemia, significant renal IL-18 mRNA levels were undetectable, whereas renal IL-12 p40 mRNA levels increased compared with constitutive levels (Fig. 1). At this time, anti-IL-12 or anti-IL-18 Ab administration neither affected renal IFN-γ mRNA levels (Fig. 2) nor MHC class I and II expression (Fig. 3) when compared with PBS-treated controls. However, the combined administration of anti-IL-12 and anti-IL-18 Ab prevented the increase in renal IFN-γ mRNA levels (Fig. 2), as well as the up-regulation of renal MHC I and II (Figs. 3 and 4) at day 6. In contrast to IL-12, the protein product of IL-18 mRNA translation requires further processing by activated caspase-1 to become mature IL-18 (9, 18). The observed increase in renal caspase-1-like activity (Fig. 5) indicates that the conditions for processing IL-18 protein are met. Indeed, the effectiveness of combined anti-IL-12/18 as well as anti-IFN-γ treatment indicate that IL-12, IL-18, and IFN-γ protein are induced in the present model.

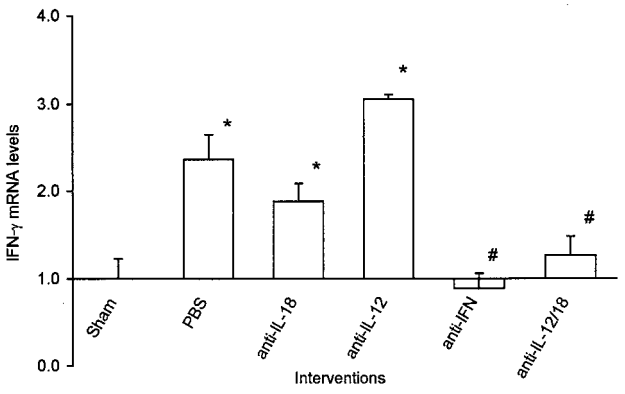

FIGURE 2. Renal IFN-γ mRNA expression. Increased renal IFN-γ mRNA expression at day 6 after ischemia compared with sham-operated animals ($n = 6$), as measured by quantitative RT-PCR is attenuated by anti-IFN-γ Ab ($n = 10$) or combined administration of anti-IL-18 and anti-IL-12 Ab ($n = 10$). IFN-γ mRNA levels are expressed relative to β-actin mRNA levels and normalized to the sham group. Statistical significances compared with sham-operated animals (∗) or compared with PBS-treated animals (#) are indicated. The columns are means ± SEM. ∗ and #, $p < 0.05$.

Delayed IFN-γ-dependent MHC class I and II up-regulation after renal I/R

To explore the mechanisms by which I/R injury initiates IFN-γ production and consequent enhanced MHC class I and II expression, we investigated renal IFN-γ mRNA levels by means of RT-PCR as well as MHC class I and II expression by employing EIA and immunohistology. In line with previous reports (6, 24), the present findings reveal significantly increased levels of IFN-γ mRNA (Fig. 2) coinciding with significant MHC class I and II (Figs. 3 and 4) up-regulation at 6 days after I/R when compared with sham-operated controls. At day 6, tubular epithelium and arterial endothelium stained intensely positive for MHC class I (Fig. 4), whereas MHC class II immunoreactivity was confined mainly to tubular epithelium (data not shown). The immunohistological data additionally showed that the contribution of infiltrating immune cells to I/R-induced MHC class I and II up-regulation was insignificant and that MHC class I and II immunostaining was most prominent in the outer stripe of the outer medulla. As previously reported (6, 24), the observed postischemic renal MHC class I and II up-regulation is an IFN-γ-dependent process, since the present results show a significant reduction in anti-IFN-γ-treated mice when compared with PBS-treated controls (Fig. 3).

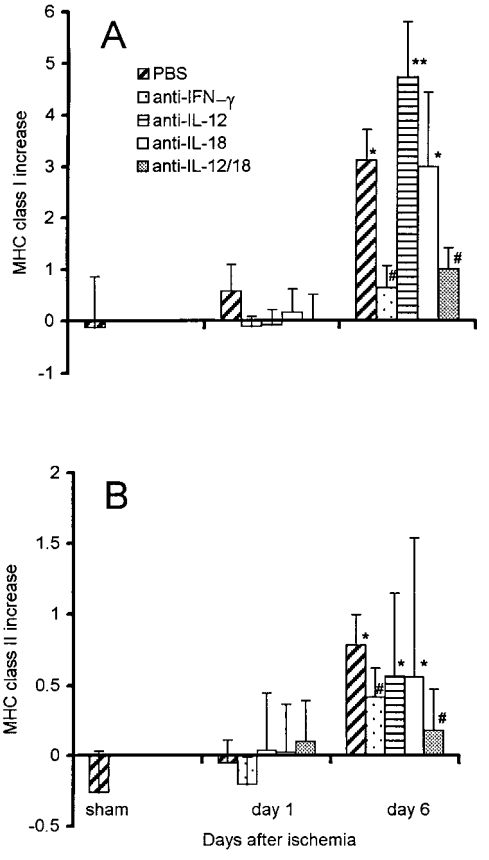

FIGURE 3. Renal MHC I and II Ag expression. Compared with sham-operated animals ($n = 6$), renal expression of MHC class I (*A*) and MHC class II (*B*) at day 6 after ischemia was enhanced, as assessed by EIA. This enhanced expression was equipotentially prevented by anti-IFN-γ ($n = 10$) and combined anti-IL-12 and anti-IL-18 ($n = 10$) treatment. Statistical significances compared with sham-operated animals (∗, ∗∗) or compared with PBS-treated animals (#) are indicated. The columns are means ± SEM. ∗ and #, $p < 0.05$; ∗∗, $p < 0.01$.

Endogenous IL-12 and/or IL-18 do not mediate the early inflammatory response induced by renal I/R

Compared with sham-operated animals, I/R increased kidney neutrophil accumulation and impaired kidney function (Fig. 6) at day 1 after ischemia, as measured by renal MPO and BUN content, respectively. We investigated whether endogenous IL-12 and/or IL-18 mediate these early consequences of ischemic tissue injury.

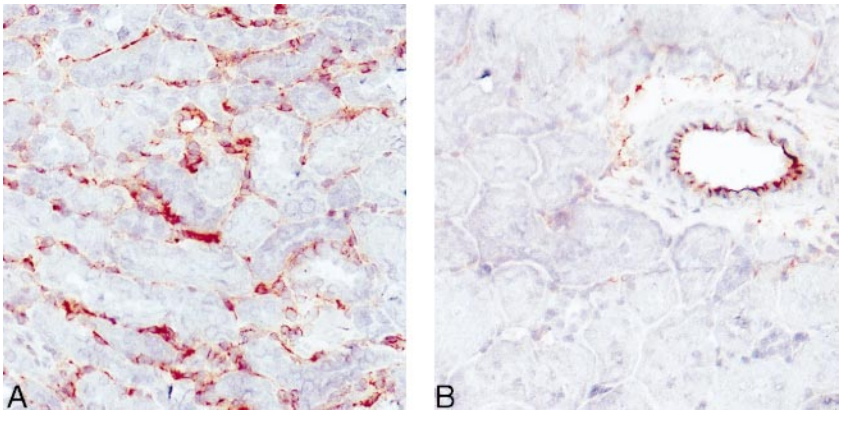

FIGURE 4. Immunohistology for renal MHC class I expression. MHC class I Ag expression, as visualized by employing mAb m1/42.3.9.8 is up-regulated on tubular epithelium at day 6 after I/R in PBS-treated animals (*A*). In contrast, at this time point, only constitutive MHC class I expression was observed on arterial endothelium and tubular epithelium in animals treated with anti-IL-12/18 (*B*), anti-IFN-γ, in contralateral control kidneys or, in kidneys obtained from sham-operated animals. (magnification was ×200).

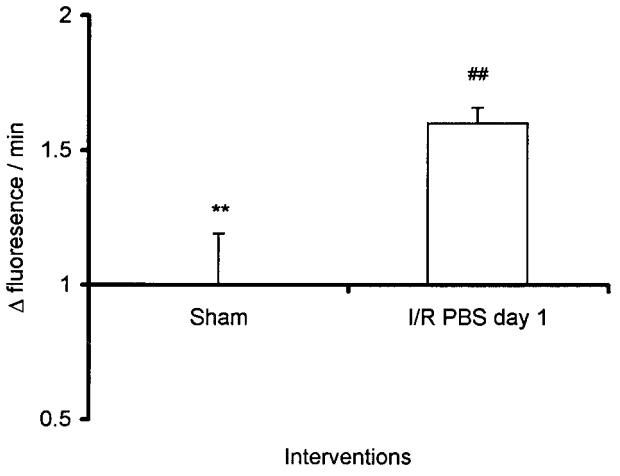

FIGURE 5. Renal caspase-1-like activity. Increased caspase-1-like activity, as determined kinetically in kidneys obtained from PBS-treated animals ($n = 10$) obtained after 1 day of reperfusion in a fluorogenic substrate assay in which Ac-YVAD-amc (caspase-1-like) served as a substrate. Data are expressed as the increase in fluorescence as a function of time, normalized against data obtained from the sham-operated group ($n = 6$). Statistical significance compared with PBS-treated animals was denoted at $p < 0.01$ (**) and compared with kidneys obtained from sham-operated animals at $p < 0.01$ (##). The presented data are means ± SEM.

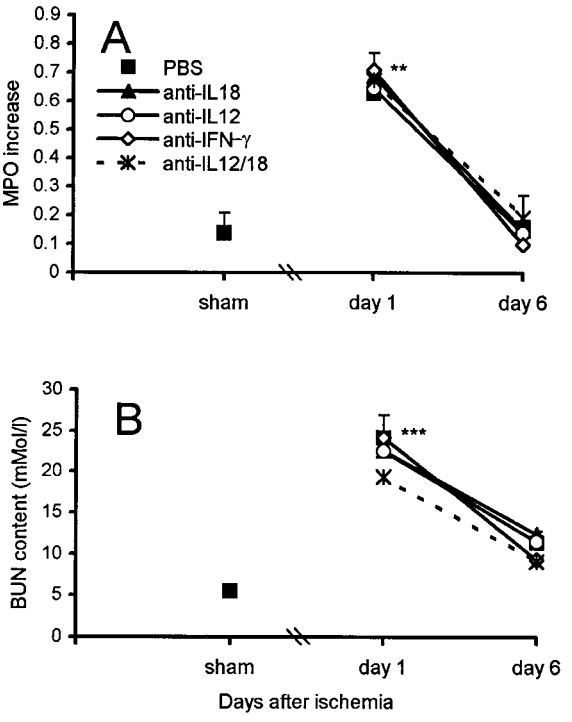

FIGURE 6. Renal neutrophil influx and function. Compared with sham-operated animals ($n = 6$), neutrophil influx (*A*) is up-regulated and renal function (*B*) impaired at day 1 after renal ischemia, as measured by renal MPO increase and BUN content, respectively. Note that all employed treatment regimens fail to attenuate this initial response ($n = 10$ for each group). The statistical significant difference between PBS-treated animals subjected to I/R and sham-operated controls is indicated. The data shown are means (± SEM is included for the PBS-treated group). **, $p < 0.01$; ***, $p < 0.001$.

The administration of anti-IL-12 Ab, anti-IL-18 Ab, or a combination of both regimens failed to attenuate kidney neutrophil accumulation and deterioration of renal function (Fig. 6), suggesting that these cytokines at this stage do not mediate reperfusion injury.

Discussion

IFN-γ is produced by T cells and NK cells following stimulation with IL-12 and/or IL-18. The production of the heterodimeric cytokine IL-12 is tightly controlled by expression of the p40 subunit gene in macrophages, but also in tubular epithelial cells (25) when stimulated with, among others, bacteria or bacterial products (18). IL-18, on the other hand, is an IFN-γ-inducing cytokine expressed by a far wider range of cell types and tissues than IL-12 (18). This may account for the observed renal IL-18 mRNA increase coinciding with the absence of significant IL-12 p40 mRNA induction after I/R. Alternatively, based on experiments with *Yersinia enterocolitica* infected mice, Bohn et al. (11) suggested that IL-18 down-regulates local production of IL-12, which can be overruled by IFN-γ. They showed that low amounts of IL-18-induced IFN-γ, in turn, may activate macrophages to produce IL-12, which subsequently synergizes with IL-18 to produce high amounts of IFN-γ. Interestingly, in the present model, such a mechanism may account for the observed down-regulation of renal IL-12 p40 mRNA expression at day 1 and subsequent up-regulation at day 6 (Fig. 1). Moreover, it may account for our finding that only anti-IFN-γ and combined anti-IL-12/18 treatment prevented I/R-induced enhanced renaI IFN-γ mRNA production (Fig. 2) and subsequent MHC class I and II expression (Figs. 3 and 4).

Local tissue damage as a result of an infection may facilitate penetration of additional pathogens in an infected organ. It is tempting to speculate that IL-12- and IL-18-driven IFN-γ and subsequent MHC up-regulation in response to tissue injury is a functionally important mechanism that primes injured tissue for enhanced immunosurveillance. If microbial-induced cell death would lead to the observed late cytokine alterations and consequent induction of MHC Ags, this mechanism would target infected cells for recognition and elimination by T cells (26, 27). Hence, MHC Ag up-regulation as a consequence of I/R-induced tissue damage may also facilitate the T cell response to invading pathogens. Besides ischemic damage, IFN-γ production as well as MHC up-regulation are associated with poor allograft survival (28).

Previous studies mainly focused on the potential involvement of IL-12- and/or IL-18-induced IFN-γ production in host defense. The present findings for the first time indicate a relationship between tissue injury of nonpathogenic origin and the induction of both IL-12 and IL-18 collaborating in enhancing IFN-γ production in vivo. The similarities observed between mechanisms of IFN-γ induction during bacterial infections and nonmicrobial lesions, such as renal I/R injury, raise the hypothesis that aspecific tissue damage contributes extensively to regulation of IFN-γ production. However, to which extent these findings apply to other (non) microbial stimuli remains to be elucidated.

Acknowledgments

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