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IL-4 Is an Endogenous Inhibitor of Neutrophil Influx and Subsequent Pathology in Acute Antibody-Mediated Inflammation

Sohail Saleem,* Zhenhua Dai,* Sandra N. Coelho,† Bogumila T. Konieczny,* Karel J. M. Assmann,‡ Fady K. Baddoura,* and Fadi G. Lakkis2*

IL-4 is an immunoregulatory cytokine that has in vitro and in vivo anti-inflammatory actions. In this study we investigated whether endogenously produced IL-4 modulates inflammatory processes that occur after Abs bind to target tissue by comparing the severity of glomerulonephritis induced by heterologous anti-glomerular basement membrane Abs in wild-type (IL-4+/+) mice to that of glomerulonephritis induced in homozygous IL-4 gene knockout (IL-4−/−) mice. Two hours after Ab injection, IL-4−/− mice had significantly higher intrarenal intercellular adhesion molecule-1 mRNA expression and intraglomerular neutrophil accumulation than the IL-4+/+ group. Treatment of IL-4+/+ mice with recombinant murine IL-4 at the time of disease induction reduced intercellular adhesion molecule-1 expression and neutrophil influx to levels observed in IL-4+/+ kidneys.

Four days after Ab administration, untreated IL-4−/− mice developed significantly greater urinary protein excretion, intracapillary fibrinogen deposits, and glomerular hypercellularity than IL-4+/+ mice. These results demonstrate that endogenous IL-4 suppresses neutrophil influx and limits tissue damage in Ab-induced glomerulonephritis, suggesting that IL-4 is an important regulator of acute inflammatory processes.


Materials and Methods

Animals

Six- to eight-week-old male C57BL/6 wild-type (IL-4+/+) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). C57BL/6 mice homozygous for the disrupted IL-4 genes (IL-4−/−) were provided by Dr. Manfred Kopf (Basel Institute of Immunology, Basel, Switzerland) (7) and were bred at the Atlanta Veterans Affairs Medical Center animal facility.

Abbreviations used in this paper: GBM, glomerular basement membrane; NTS, nephrotoxic serum; NRS, nonimmune rabbit serum; Ur/UrN ratio of urinary protein to creatinine concentration; PAs, periodic acid-Schiff; CAE, chloroacetate esterase; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; ICAM-1, intercellular adhesion molecule-1.
Experimental protocols

Expt. A. To study the effect of IL-4 on Ab-mediated neutrophil influx, anti-GBM nephritis was induced in 8- to 10-wk-old IL-4−/− and IL-4+/+ C57BL/6 mice (n = 6/group) by injecting 1 ml of heat-inactivated nephrotoxic serum (NTS; rabbit anti-mouse GBM) i.p. (20). NTS was provided by Dr. Karel J. M. Assmann (University Hospital, Nijmegen, The Netherlands). An additional group of IL-4−/− C57BL/6 mice (n = 6) received 3000 U of recombinant murine IL-4 (a gift from Dr. Satwant Narula, Schering-Plough Research Institute, Kenilworth, NJ) i.v. at the time of NTS administration. Control IL-4−/− and IL-4+/+ (n = 3/group) received 1 ml of heat-inactivated rabbit serum (NRS; Sigma Chemical Co., St. Louis, MO) i.p. All mice were killed 2 h later, and renal tissue was saved for analysis.

Expt. B. To study the effects of IL-4 on glomerular pathology that occurs subsequent to neutrophil infiltration, anti-GBM nephritis was induced in 8- to 10-wk-old IL-4−/− and IL-4+/+ C57BL/6 mice (n = 10/group) by injecting 1 ml of heat-inactivated NTS i.p. on day 0 (20). Control IL-4−/− and IL-4+/+ mice (n = 3/group) received 1 ml of heat-inactivated NRS (Sigma) i.p. on day 0. Twenty-four-hour urine was collected on days 1 and 4 from individual metabolic cages (Nalgene, Inc., Rochester, NY). All mice were killed at the end of the fourth day following disease induction, and renal tissue was saved for analysis. The urinary protein concentration was quantitated by the Bradford method (Bio-Rad Laboratories, Melville, NY). The urinary creatinine concentration was quantitated by calculating the average number of CAE cells per glomerulus.

Histopathology

Kidney sections from Expt. A and B were fixed in B5 solution (Great Lakes Diagnostics, Troy, MI) followed by 10% neutral-buffered formalin (Fisher Scientific, Pittsburgh, PA) and embedded in paraffin wax. Hematoxylin and eosin, periodic acid-Schiff (PAS), Masson trichrome, and silver staining were performed on 4-μm sections. Light microscopic examination was performed in a blinded fashion by a pathologist (F.K.B.). Glomerular pathology was assessed by examining at least 100 glomeruli/animal. The extent of glomerular hypercellularity is reported as the average number of cells per glomerulus.

Neutrophil enumeration

Kidney sections from Expt. A were fixed in 10% neutral-buffered formalin (Fisher Scientific, Fairlawn, NJ) and embedded in paraffin wax. For neutrophil detection, sections were deparaffinized and stained cytochemically with the esterase substrate, naphthol AS-D chloroacetate (CAE; Sigma) (26). All sections were counterstained with hematoxylin. Positive cells within 100 randomly selected glomeruli per animal were enumerated by light microscopy in a blinded fashion (25). Intraglomerular infiltration was quantitated by calculating the average number of CAE+ cells per glomerulus.

Immunofluorescence

Fresh kidney tissue from Expt. A and B was mounted in OCT compound (Miles Diagnostics, Elkhart, IN) and frozen in liquid isopentane cooled on dry ice, and 4-μm cryostat sections were prepared (Leica, Nussloch, Germany). Direct immunofluorescence studies were performed by fixing frozen sections in acetone in freezing with 10% normal goat serum and addition of FITC-conjugated sheep Ab directed against rabbit IgG (Zymed, South San Francisco, CA), FITC-conjugated rabbit Ab directed against human fibrinogen (Dako, Copenhagen, Denmark), or FITC-conjugated rat anti-mouse CD11b (Harlan/Serotec, Indianapolis, IN) at a 1/100 dilution. After washing with PBS and 1% Tween-20, sections were studied under a fluorescence microscope by a pathologist (F.K.B.) in a blinded fashion. The average number of intracapillary fibrinogen deposits and intraglomerular CD11b+ cells was determined by examining 100 glomeruli/animal (20). A C57BL/6 mouse was used as a measure of proteinuria (25).

Results

Intrarenal IL-4 mRNA expression in murine anti-GBM nephritis

Complete inactivation of IL-4 gene function in IL-4−/− mice was verified by measuring IL-4 protein concentration in supernatants of Con A-stimulated splenocytes. While IL-4−/− splenocytes produced up to 32 pg/ml of the cytokine over 48 h, IL-4 was not detected (<5 pg/ml) in IL-4−/− supernatants. We then studied intrarenal IL-4 mRNA expression before and after NTS injection. As shown in Figure 1, IL-4 mRNA measured by RT-PCR was present in normal IL-4+/+ renal tissue and increased significantly 2 h following induction of nephritis. This is consistent with our previous observation that intrarenal IL-4 mRNA and IL-4 protein are up-regulated in rat anti-GBM nephritis (25). On the other hand, IL-4−/− mice failed to express IL-4 mRNA in their kidneys in either the basal state or after induction of nephritis (Fig. 1).
IL-4 is an endogenous suppressor of neutrophil infiltration in murine anti-GBM nephritis

The acute inflammatory response in anti-GBM nephritis is characterized by intraglomerular neutrophil accumulation that peaks 2 h after disease induction (20). To determine whether IL-4 is an endogenous regulator of this response, intraglomerular neutrophil (CAE<sup>+</sup> cells) counts in IL-4<sup>+/+</sup> and IL-4<sup>−/−</sup> mice were compared 2 h after NTS injection. As shown in Figure 2, NTS induced significantly greater neutrophil infiltration in IL-4<sup>−/−</sup> (mean ± SE = 1.7 ± 0.4 CAE<sup>+</sup> cell/glomerulus) than in IL-4<sup>+/+</sup> glomeruli (0.5 ± 0.2 CAE<sup>+</sup> cell/glomerulus; p < 0.05). Administration of recombinant murine IL-4 at the time of NTS injection reduced intraglomerular neutrophil accumulation in IL-4<sup>−/−</sup> kidneys to levels comparable to those in the IL-4<sup>+/+</sup> group (0.5 ± 0.1 and 0.5 ± 0.2 CAE<sup>+</sup> cell/glomerulus, respectively; Fig. 2). Neutrophil counts before NTS injection (pre-NTS) were similar in IL-4<sup>−/−</sup> and IL-4<sup>+/+</sup> mice injected with NRS. Control IL-4<sup>+/+</sup> and IL-4<sup>−/−</sup> mice injected with NRS did not display significant neutrophil accumulation in their glomeruli.

IL-4 is an endogenous suppressor of ICAM-1 up-regulation in murine anti-GBM nephritis

To investigate the mechanism by which IL-4 inhibits neutrophil migration, we studied intraglomerular ICAM-1 mRNA expression before and 2 h after NTS administration. ICAM-1 plays an important role in neutrophil adherence to the vascular endothelium (28). As shown in three separate animal experiments (Fig. 3A), ICAM-1 mRNA up-regulation following induction of nephritis was significantly greater in IL-4<sup>−/−</sup> than in IL-4<sup>+/+</sup> kidneys. Quantitative densitometric analysis of ICAM-1 mRNA revealed a 3.1 ± 0.3-fold increase over baseline in IL-4<sup>+/+</sup> kidneys compared with a 5.7 ± 0.9-fold increase in the IL-4<sup>−/−</sup> group (mean ± SD; p < 0.05; Fig. 3B). IL-4 administration at the time of NTS injection reduced intrarenal up-regulation of ICAM-1 mRNA in IL-4<sup>−/−</sup> mice to levels observed in IL-4<sup>+/+</sup> mice (3.2 ± 0.4 vs 3.1 ± 0.3-fold increase, respectively; Fig. 3, A and B). Baseline (pre-NTS) expression of ICAM-1 did not differ significantly between the IL-4<sup>+/+</sup> and IL-4<sup>−/−</sup> groups (Fig. 3A).

Increased glomerular pathology in IL-4<sup>−/−</sup> mice after induction of murine anti-GBM nephritis

Functional and morphologic renal abnormalities in murine anti-GBM nephritis develop within several hours to a few days after neutrophil influx into glomerular tissue (20, 21). These abnormalities are neutrophil dependent and include proteinuria, increased glomerular cellularity, and fibrinogen deposits within glomerular capillaries and mesangium (22–24). We, therefore, investigated whether the glomerular lesions of anti-GBM nephritis are exaggerated in the absence of IL-4. There were no deaths following NTS injection. Average body weights in the IL-4<sup>+/+</sup> and IL-4<sup>−/−</sup> groups were similar at the end of the experiment (27 ± 3 and 28 ± 2 g, respectively). Intense linear staining for rabbit IgG could be demonstrated along the GBM after NTS administration and did not differ between IL-4<sup>+/+</sup> and IL-4<sup>−/−</sup> glomeruli (data not shown). Circulating mouse anti-rabbit IgG or intrarenal mouse IgG deposits were not detected. Increased protein excretion was not observed in either group at 2 h. By the fourth day of nephritis, however, IL-4<sup>−/−</sup> mice displayed worse glomerular injury than IL-4<sup>+/+</sup> mice, as measured by the following parameters.

**Proteinuria (U<sub>P</sub>/U<sub>Cr</sub>).** Urinary protein excretion in the IL-4<sup>−/−</sup> group on days 1 and 4 after NTS injection was higher than that in day-matched IL-4<sup>+/+</sup> mice (Fig. 4). The difference in U<sub>P</sub>/U<sub>Cr</sub> ratio on day 4 between the IL-4<sup>−/−</sup> group (mean ± SE = 71 ± 6; n = 10) and the IL-4<sup>+/+</sup> group (41 ± 7; n = 10) reached statistical significance (p < 0.05; Fig. 4). Administration of recombinant murine IL-4 (1000 U i.p., twice per day) to IL-4<sup>−/−</sup> mice starting at the time of NTS injection reduced the day 4 U<sub>P</sub>/U<sub>Cr</sub> ratio to 50 ± 10 (n = 4), which was not significantly different from that in the IL-4<sup>+/+</sup> group. Urinary protein excretion did not increase over baseline in control mice injected with NRS.

**Histopathologic changes.** Four days following NTS injection, the majority of IL-4<sup>−/−</sup> mice (8 of 10) developed diffuse renal pathology characterized by glomerular hypercellularity and narrowing or
Neutrophil accumulation is a hallmark of acute, Ab-induced glomerulonephritis (16). Their transmigration into glomeruli is facilitated by ICAMs expressed on endothelial cells (29). Neutrophil depletion (30, 31) or inhibiting neutrophil migration by blocking their adherence to the endothelium (32, 33) ameliorates experimental Ab-induced glomerulonephritis. In this study we provided direct evidence that IL-4 is an endogenous sup-

Table I. Glomerular histology in IL-4+/+ and IL-4−/−

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<tr>
<td></td>
<td>IL-4+/+ (n = 3)</td>
<td>IL-4−/− (n = 3)</td>
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<td>Total cells/glomerulus</td>
<td>24 ± 3</td>
<td>24 ± 2</td>
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<td>Neutrophils/glomerulus</td>
<td>0.04 ± 0.01</td>
<td>0.08 ± 0.01</td>
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<td>Fibrinogen deposits/glomerulus</td>
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<td>ND</td>
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* One hundred glomeruli per animal were examined in a blinded fashion. Total glomerular cellularity (mean ± SD) was determined by examining PAS-stained, fixed kidney sections. Intracapillary fibrinogen deposits (mean ± SD) were enumerated in frozen kidney sections stained with FITC-conjugated anti-fibrinogen Ab.

p < 0.05 compared to pre-NTS groups and post-NTS IL-4−/− group.

Discussion

The observation that endogenous IL-4 inhibits neutrophil influx is consistent with known pharmacologic effects of this cytokine. Mulligan et al. reported that intratracheal administration of rIL-4 suppresses neutrophil infiltration in a murine model of immune complex-mediated lung injury (14). Similarly, i.v. IL-4 inhibited neutrophil accumulation in a murine air pouch after local application of IL-1β (34). We also observed in this study that IL-4 is an endogenous inhibitor of ICAM-1 mRNA up-regulation following induction of murine anti-GBM nephritis. It is likely that intrarenal ICAM-1 mRNA correlates with ICAM-1 protein expression in this model because NTS administration has been shown to increase ICAM-1 on glomerular endothelial cells (33, 35). Mulligan et al. demonstrated that exogenous IL-4 suppresses immune complex-induced ICAM-1 up-regulation in pulmonary vessels (14). Similarly, in vitro experiments have shown that IL-4 inhibits IL-1β-induced ICAM-1 up-regulation on endothelial cells (36, 37). Taken together, these observations suggest that modulation of ICAM-1 expression is an important mechanism by which IL-4...
inhibits neutrophil influx. It is possible, however, that IL-4 suppresses neutrophil migration by additional mechanisms. For example, IL-4 inhibits IL-8 production by monocytes and neutrophils (11, 38). In vivo neutralization of IL-8 has been shown to prevent neutrophil influx in Ab-mediated glomerulonephritis (39). IL-4 also inhibits leukotriene B4 production by monocytes (40). Leukotriene B4 is a potent neutrophil chemoattractant (41) and may play a role in recruiting neutrophils to the kidney in anti-GBM nephritis (42).

We also observed in this study that IL-4−/− mice develop worse glomerular pathology than the IL-4+/− group. Four days after NTS administration, IL-4−/− mice had significantly greater proteinuria, glomerular cellularity, and intracapillary fibrinogen deposits. Because neutrophils play a central role in murine anti-GBM nephritis (22–24), increased pathology in IL-4−/− mice could have resulted from greater neutrophil influx into their glomeruli. We did not detect significant monocyte (CD11b+ cell) accumulation in either IL-4−/− or IL-4+/− kidneys (less than one cell per glomerulus 4 days after NTS injection). Others have shown that T lymphocytes and monocytes do not contribute significantly to glomerular pathology in this model (43–45). The difference in proteinuria between IL-4−/− and IL-4+/+ mice on the first day of nephritis was less marked than the difference in neutrophil accumulation between the two groups. This discrepancy may be explained by neutrophil-independent mechanisms of proteinuria such as nephrotoxic Abs, which directly alter the glomerular filtration barrier (46, 47). We suspect that glomerular hypercellularity in IL-4−/− mice resulted in part from increased mesangial cell number because mesangial cell proliferation has been observed in experimental Ab-induced glomerulonephritis (48), and IL-4 has been shown to suppress proliferation of mesangial cells in culture (49).

Our results do not exclude that IL-4 can modulate glomerulonephritis by down-regulating the inflammatory functions of endothelial cells and resident macrophages. For example, IL-4 suppresses procoagulant expression by endothelial cells (12) and inhibits nitric oxide production by macrophages (50). It remains to be determined whether procoagulant activity and nitric oxide release are increased in IL-4−/− glomeruli and whether they contribute to intracapillary thrombosis in murine anti-GBM nephritis. IL-4 also stimulates IL-1RA while inhibiting IL-1 and TNF-α production by monocytes (5, 51). In addition, it induces IL-1 decoy receptor synthesis by neutrophils (52). Although a balance between IL-1 and its antagonists may determine the outcome of some forms of experimental glomerulonephritis (53, 54), their role in murine anti-GBM nephritis is not known.

In conclusion, we demonstrated that endogenously produced IL-4 down-regulates ICAM-1 expression, neutrophil influx, and subsequent pathology in murine Ab-induced glomerulonephritis. In contrast to IL-4’s role in promoting IgE-mediated immediate-type hypersensitivity, these results suggest that IL-4 is an inhibitor of acute inflammation induced by IgG Abs.

Acknowledgments

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