Macrophages from Inflamed but Not Normal Glomeruli Are Unresponsive to Anti-Inflammatory Cytokines

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This study examined the properties and responsiveness to cytokines of macrophages purified from normal and nephritic glomeruli to ascertain whether macrophages activated *in vivo* develop programmed unresponsiveness to cytokines as do bone marrow-derived macrophages *in vitro* when activated by interferon- γ (IFN- γ), tumor necrosis factor (TNF), interleukin-4 (IL-4), or transforming growth factor- β (TGF- β). Macrophages from normal glomeruli did not generate nitric oxide (NO) spontaneously but only after treatment with IFN- γ and TNF- α . NO generation by these macrophages was abrogated by administering IL-4, TGF- β , or TNF- α before but not after IFN- γ treatment. Glomerular macrophages also expressed β -glucuronidase, which was increased by TGF- β and decreased by IFN- γ and TNF. By contrast, glomerular macrophages from rats with nephrotoxic nephritis did not express β -glucuronidase even after exposure to TGF- β . Furthermore, they generated NO spontaneously, and this spontaneous generation of NO was not suppressed by IL-4, TGF- β , or TNF- α . Systemic treatment of nephritic rats with IL-4 reduced NO generation by 40% but did not prevent activation, which is similar to the effect of IL-4 on bone marrow-derived macrophages *in vitro* when given simultaneously with IFN- γ . We conclude that macrophages infiltrating inflamed glomeruli have developed programmed unresponsiveness to activating cytokines. This may enable them to function appropriately in the complex conditions within an inflammatory focus. (*Am J Pathol* 2000, 156:295–301)

Macrophages have many different roles in inflammation, and their function varies with the nature of the injury and its stage.¹ Depending on the circumstances, macrophages can either increase the intensity of inflammation² or promote its resolution.³ They are also critical to angiogenesis⁴ and tissue remodeling and repair.^{5,6} Infiltrating macrophages can perform these different roles because they adapt to the local microenvironment in infected or otherwise damaged tissues by developing coordinated

sets of properties that enable them to perform a particular function.⁷ Knowledge of what controls such macrophage adaptation and dictates (or limits) macrophage activities is essential for understanding how inflammation is regulated.

Early studies by Mackaness,⁸ North,⁹ and their colleagues demonstrated that macrophages elicited into the peritoneal cavity after injection of an irritant and macrophages infiltrating the peritoneum as part of a T cell-mediated response have different properties. These and many similar experiments led to the distinction between elicited and activated macrophages and to the identification of interferon- γ (IFN- γ) as the principal macrophage-activating factor.¹⁰ Since then it has become apparent that these are only two of many states that macrophages can adopt and that macrophages can be alternatively activated by other cytokines, for example by interleukin-4 (IL-4).¹¹ It is now clear that there are many other macrophage activation states^{12,13} and that differently activated macrophages cause tissue injury or facilitate its repair.

As the first step toward understanding these processes, we¹⁴ and others^{15,16} have analyzed the effect of specific cytokines on the development of complex macrophage functions and have shown that IFN- γ , tumor necrosis factor- α (TNF- α), transforming growth factor- β (TGF- β), and IL-4 committed macrophages to sets of nonoverlapping and mutually exclusive properties or programs. In each case the macrophage programming was determined by the first cytokine to which the macrophages were exposed, and an essential component of the program was the development of unresponsiveness to alternatively activating cytokines.¹⁴ This is consistent with reports showing that inhibitory effects of anti-inflammatory cytokines occur predominantly when macrophages are pretreated.^{17,18}

It is important whether programmed macrophages activated *in vivo* are also unresponsive to anti-inflammatory cytokines, because this could provide a mechanism for

Supported by the Deutsche Forschungsgemeinschaft (ER 254/1–1 to L.-P. R.). This work was performed in the context of European Union Contract EMH4B MH4-CT98–3631.1: Chronic Inflammation Leading to Scarring: the Major Cause of Renal Failure.

Accepted for publication September 16, 1999.

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them to operate coherently within the chaotic environment of damaged tissue. Here they are exposed to an extreme variety of (often contradictory) receptor-mediated signals, including those derived from interactions with chemokines and adhesion molecules, immunoglobulins, and complement^{19,20}; or contact with other cells (eg, through CD40, CD80, and CD86) or with the extracellular matrix (eg, via integrins and CD44). Analysis of these interactions requires an appropriate model, and accelerated nephrotoxic nephritis (NTN) in rats is particularly suited for this. It is characterized by acute macrophage-dependent injury,²¹ as demonstrated by macrophage depletion experiments.²² Injury is also attenuated by administration of anti-inflammatory cytokines with effects on macrophage function, such as IL-4, IL-6, and IL-10.^{23,24} The intensity of injury can be quantified functionally and morphologically, and macrophages can readily be purified from the inflamed glomeruli and studied *ex vivo*.

This study was to determine whether macrophages infiltrating glomeruli of rats with NTN become programmed and, if so, whether programming could be influenced by exogenously delivered cytokines. The results show that macrophages from normal glomeruli behave like uncommitted bone marrow-derived macrophages (BMDMs), whereas those from nephritic glomeruli have the characteristics of IFN- γ -primed, TNF-activated macrophages. They generate large amounts of NO spontaneously and do not express β -glucuronidase, properties that cannot be modulated by *ex vivo* incubation with TGF- β or IL-4. Analysis of NO generation by glomerular macrophages from nephritic rats treated systemically with IL-4 suggests that programming occurs after macrophages localize to the nephritic kidney. These results provide a further insight into the function of inflammatory macrophages and have obvious implications for the use of cytokine therapy to modulate acute immunologically mediated inflammation.

Materials and Methods

Reagents

Nephrotoxic serum, rabbit antiserum to rat glomerular basement membrane (GBM), was prepared as described previously.²⁵ Recombinant human TNF- α , recombinant human TGF- β , and recombinant rat IFN- γ were obtained from Boehringer (Ingelheim, Germany), Sigma Chemical Co. (Dorset, UK), and Bradsure Biologicals Ltd. (Loughborough, UK), respectively. Recombinant rat IL-4 was produced in house as described previously,²³ using a Chinese hamster ovary cell line generously donated by Dr. Neil Barclay (MRC Cellular Immunology Unit, Oxford, UK).

Induction of Accelerated Autologous Phase of NTN

Male Sprague-Dawley rats were immunized by subcutaneous injection of 1 mg of normal rabbit immunoglobulin

G (Sigma Chemical Co., St. Louis, MO) in Freund's complete adjuvant (Difco). The rats (weight 180–200 g) were injected intravenously 7 days later with 1 ml of nephrotoxic serum. Groups of rats were killed 48 and 96 hours after induction of nephritis, for histological studies and preparation of glomerular macrophages. Urine was collected by placing rats in metabolic cages over night for 18 hours before induction of nephritis and every subsequent night. Albuminuria was quantified by rocket immunoelectrophoresis as previously described.²⁶

Administration of Recombinant Rat IL-4

Recombinant rat IL-4 (8 μ g in 0.5 ml of cell culture supernatant) was administered intraperitoneally twice daily starting the day before induction of nephritis and continuing until rats were sacrificed on day 4. Control rats received the same volume of cell culture supernatant from the parent Chinese hamster ovary-K1 cell line.

Morphology and Immunohistology

First the kidneys were perfused *in vivo* with phosphate-buffered saline at 4°C through a cannula inserted into the abdominal aorta. Samples of renal tissue were either snap-frozen in liquid nitrogen or fixed in formalin for light microscopy and immunohistochemistry as previously described.^{23,24} Macrophages were identified in formalin-fixed sections by alkaline phosphatase anti-alkaline phosphatase (APAAP) staining with monoclonal antibody ED1 (Serotec, Oxford, UK).

Purification of Glomerular Macrophages

Glomeruli were isolated from kidneys perfused *in vivo* with 50 ml sterile phosphate-buffered saline, by a standard sieving technique. Isolated glomeruli were enzymatically digested to single-cell suspensions with trypsin, collagenase, DNase, and ethylenediaminetetraacetic acid (Sigma) as previously described.²⁷ The macrophages were isolated from the single-cell suspension by adherence in 24-well plates at an approximate density of 1×10^6 macrophages per well. The cells were then counted, and the results were corrected for differences in cell number. In addition all wells that contained less than 8×10^5 or more than 1.2×10^6 cells per well were excluded from the experiments. The purity of the macrophage population always exceeded 90%, and there was no significant mesangial cell or endothelial cell contamination. The macrophages were washed and incubated for 48 hours in Dulbecco's modified Eagle's medium (DMEM) containing 2 mmol/L glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin and 10% heat-inactivated fetal calf serum alone or in medium containing cytokines.

Isolation and Culture of BMDMs

Rat BMDMs were obtained using a technique previously described in detail.²⁸ Briefly, bone marrow cells were

Table 1. NO Generation and β -Glucuronidase Expression of BMDMs and Macrophages Isolated from Normal Glomeruli

Type of isolate	IFN/TNF (20 U + 10 ng/ml)	IL-4 (5 μ l/ml; 8 ng/ml)	TGF (5 ng/ml)	TNF (10 ng/ml)	Control
BMDM*					
NO generation (μ mol/ml)	22 [±] 1	1.95 $\pm$ 0.5	1.7 $\pm$ 0.05	2.49 $\pm$ 0.08	2.2 $\pm$ 0.6
β -Glucuronidase (score)	1.3 [±] 0.2	2.1 $\pm$ 0.2	3.5 [±] 0.1	1.8 $\pm$ 0.3	2.3 $\pm$ 0.2
Glomerular [†] macrophages					
NO generation (μ mol/ml)	19.48 [±] 2.17	1.54 $\pm$ 0.6	2.0 $\pm$ 1.1		3.04 $\pm$ 3.20
β -Glucuronidase (score)	0.9 $\pm$ 0.2 [§]	1.8 $\pm$ 0.5	3.3 [§] $\pm$ 0.4	1.7 $\pm$ 0.4	1.9 $\pm$ 0.3

NO, Nitric oxide; BMDM, bone marrow-derived macrophage; IFN, interferon; TNF, tumor necrosis factor; IL, interleukin; TGF, transforming growth factor

*N = 10; duplicate wells; mean $\pm$ SD.

[†]N = 8; single wells; mean $\pm$ SD.

[‡]P < 0.01 compared with untreated rats.

[§]P < 0.05 compared with untreated rats.

flushed aseptically from the dissected femurs of male Sprague-Dawley rats with a jet of complete medium directed through a 25-gauge needle to form a single-cell suspension. The cells were cultured in 75-mm tissue culture flasks (Corning, NY) adherent to plastic, in DMEM containing 2 mmol/L glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin; 10% heat-inactivated fetal calf serum; and 10% L929-conditioned medium as a source of macrophage-colony stimulating factor. After 7 days in culture, the cells were carefully removed using 1% trypsin-ethylenediaminetetraacetic acid and dispensed into 24-well culture plates (Corning) at a concentration of 1×10^6 cells/well, and they rested for 24 hours in macrophage-colony stimulating factor-free medium before they were washed and incubated with the cytokines. When combinations of cytokines were used, the initial cytokine was given 4 hours before the second cytokine, and the cytokines were not removed from the medium until macrophage function was assessed.

Quantitation of NO Synthesis

Generation of NO was estimated by assaying culture supernatants for nitrite, a stable reaction product of NO. Of each cell-free culture, two 200- μ l aliquots per well of supernatant were incubated with 50 μ l of Griess reagent (0.5% sulfanilamide, 0.05% N-(1-naphthyl) ethylenediamine dihydrochloride in 2.5% phosphoric acid) in 96 flat-bottomed tissue culture plates for 10 minutes at room temperature. The optical densities of the assay samples were then measured at 540 nm, using a solution of phenol red-free DMEM. In most experiments, nitrite was measured after 24 and 48 hours in culture. A nitrite standard was included in all experiments, and the results for a single experiment are the means of four aliquots taken from duplicate wells after 48 hours. The addition of the nonspecific NO synthase-competitive inhibitor L-monomethyl arginine at 100 μ M largely inhibited IFN-induced NO generation in BMDMs and glomerular macrophages.

Quantitation of β -Glucuronidase Expression

β -Glucuronidase is a lysosomal hydrolase that is strongly expressed and released in bacterial infections or when macrophages ingest particulate matter such as group A

streptococcal cell walls, zymosan particles, or β -1-3-glucan. TGF- β primes macrophages to express lysosomal hydrolases in response to particulate stimuli,²⁹ and a series of studies suggests that the induction of lysosomal hydrolase synthesis and secretion may actively contribute to the debridement phase of the inflammatory response.⁷ At higher concentrations, IFN- γ decreases β -glucuronidase expression *in vitro* and abrogates the effects of TGF- β .^{12,13}

β -Glucuronidase was visualized by an enzymatic staining method in which β -glucuronidase catalyzed the reaction of α -naphthol AS-BI β -D-glucuronide into the red-soluble chromogenic naphthol AS-BI-HPR complex.³⁰ Cytospin preparations of macrophages harvested from the 24-well tissue culture plates were fixed in a glutaraldehyde-acetone solution, and the sections were air-dried. They were then stained with β -glucuronidase staining solution and counterstained with methylene blue before being mounted with aqueous medium. Slides were coded, and 1000 cells per slide were scored by our standard system,¹⁴ using the following scale: 0, no staining; 1, equivocal positive staining; 2, weak positive staining; 3, moderate positive staining; 4, strong positive staining. The score represents an overall change in the density of staining.

Statistical Analysis

Differences between the groups in NO generation and scoring of β -glucuronidase were analyzed by the Wilcoxon rank sum test and Kruskal Wallis analysis of variance.

Results

Functional Properties of Macrophages Isolated from Normal Glomeruli

Uncommitted BMDMs do not generate NO spontaneously but can be programmed to do so by administering IFN- γ followed by TNF. Once programmed, NO generation is unaffected by IL-4 or TGF- β .¹⁴ Macrophages isolated from normal glomeruli responded to cytokines in exactly the same way and did not generate NO spontaneously or when stimulated individually with IL-4, TGF- β ,

Figure 1. The NO generation of macrophages isolated from normal and nephritic glomeruli and the effect of *ex vivo* incubation with cytokines. Macrophages were incubated for 48 hours with medium alone (spontaneous) or with IFN (20 U/ml) followed by TNF (10 ng/ml), TGF-β (5 ng/ml), or IL-4 medium (5 μl/ml). Mean plus SD; *n* = 8. **P* < 0.05 versus unstimulated cells.

or TNF-α. However, they generated an amount of NO similar to that of uncommitted BMDMs when incubated with IFN-γ followed 4 hours later by TNF (Table 1).

Like uncommitted BMDMs, glomerular macrophages from normal rats expressed moderate amounts of β-glucuronidase when assayed immediately after isolation (Table 1). Expression increased after incubation with TGF-β and was attenuated by IFN-γ followed by TNF-α; neither TNF-α nor IL-4 alone had any effect (Table 1). These data indicate that normal glomerular macrophages and uncommitted BMDMs respond identically to the cytokines. It is important that they also demonstrate that the isolation procedure neither primed nor activated the macrophages. This validates use of this approach to analyze

the properties of macrophages purified from inflamed glomeruli.

Functional Properties of Macrophages Isolated from Inflamed Glomeruli

Macrophages were purified from acutely inflamed glomeruli of nephritic rats at various times after induction of nephritis. Unlike normal glomerular macrophages, these macrophages generated large amounts of NO spontaneously in culture. This activity was maximal in macrophages harvested on day 2 after induction of nephritis (Figure 1). Incubation with IFN-γ followed by TNF further enhanced NO generation at each of the time points, whereas IL-4, TGF-β, and TNF alone had no effect (Figure 1). These responses are identical to those of BMDMs that have been programmed *in vitro* by IFN-γ and TNF.^{14,31}

β-Glucuronidase was virtually undetectable in glomerular macrophages from nephritic rats killed at 2 and 4 days after induction of NTN, and those harvested at day 7 contained very little β-glucuronidase (data not shown). Neither TGF-β nor IL-4 increased β-glucuronidase expression when incubated with the macrophages for 48 hours (Table 2), which again is similar to results found in BMDMs programmed by IFN-γ and TNF.¹⁴

Thus, macrophages isolated from inflamed glomeruli of rats with accelerated NTN generate NO but do not express β-glucuronidase and are unresponsive to alternative activating stimuli. Next we treated rats with systemically administered rat rIL-4 to determine whether pre-exposure to the cytokine influenced macrophage programming in the inflamed glomerulus.

Effect of in Vivo Injection of IL-4 on Normal and Nephritic Rats

Systemic treatment with IL-4 attenuates injury in rats with NTN,²³ and IL-4 has profound effects on IFN-γ-induced priming of BMDMs *in vitro*, in that IL-4 prevents priming when given before IFN-γ modulates it when given at the same time, but has no effect if given afterward.¹⁴ We therefore assessed the effect of systemic treatment with IL-4 on glomerular macrophages. The IL-4-treated rats with NTN had less severe injury both functionally and morphologically throughout the course of the experiment. Albuminuria was 945.3 ± 307 mg/day on day 2 in controls and 356.4 ± 138 mg/day in IL-4-treated rats, and at

Table 2. β-Glucuronidase Expression of Glomerular Macrophages from Untreated and Treated Nephritic Rats

<i>Ex vivo</i> culture	Control (normal rats)	Day 2 (untreated rats)	Day 2 (IL-4-treated rats)	Day 4 (untreated rats)	Day 4 (IL-4-treated rats)
Spontaneous	2.09 ± 0.2	0.1 ± 0.1	0.2 ± 0.1	0.2 ± 0.1	0.8 ± 0.6*
IFN/TNF (20 U + 10 ng/ml)	1.1 ± 0.2	0	0.1 ± 0.1	0.1 ± 0.1	0.8 ± 0.3*
IL-4 (5 μl/ml)	1.9 ± 0.5	0.2 ± 0.2	0.2 ± 0.2	0.3 ± 0.2	1.0 ± 0.5*
TGF (5 ng/ml)	3.3 ± 0.1	0.4 ± 0.3	0.3 ± 0.2	0.4 ± 0.2	0.9 ± 0.3*

Abbreviations: IL, interleukin; IFN, interferon; TNF, tumor necrosis factor; TGF, transforming growth factor

**P* < 0.05 compared with untreated rats. *N* = 8 for control and untreated nephritic rats and *N* = 5 for IL-4-treated rats.

Table 3. Effect of Systemic Administration of IL-4 on Nephritic Rats

	Pre-nephritis	Day 2	Day 4
Untreated rats			
Albuminuria (mg/day)	1.44 ± 0.4	945.3 ± 307	346 ± 88.6
Percentage of glomeruli with capillary thrombi	0	91.7 ± 5.2	92.7 ± 4.7
ED1-positive cells per glomerulus	1.44 ± 0.6	21.4 ± 3.1	16.13 ± 1.5
IL-4-Treated Rats			
Albuminuria (mg/day)	1.57 ± 0.8	356.4 ± 138.4*	162.44 ± 87.1*
Percentage of glomeruli with capillary thrombi	0	70 ± 19.7	69.5 ± 9.2*
ED1-positive cells per glomerulus	0.76 ± 0.2	5.25 ± 0.4*	5.53 ± 0.2*

**P* < 0.05 compared with untreated rats; *N* = 8 for control and untreated nephritic rats, and *N* = 5 for IL-4-treated rats.

autopsy the percentages of glomeruli with capillary thrombi were $92\% \pm 5.2$ in untreated nephritic rats and $70\% \pm 20$ in IL-4-treated rats (Table 3). There were significantly fewer glomerular macrophages in IL-4-treated rats: 21.4 ± 3.1 ED1-positive cells per glomerulus in untreated nephritic rats and 5.3 ± 0.4 ED1-positive cells per glomerulus in IL-4-treated rats (Table 3).

Macrophages isolated from normal rats treated systemically with IL-4 for 4 days had reduced responses to treatment with IFN- γ and TNF, which results in 60% less NO generation (Figure 2) and a smaller reduction in β -glucuronidase expression than that of macrophages isolated from glomeruli of untreated rats (Table 2). Macrophages isolated from nephritic rats treated continu-

ously with IL-4, starting 14 hours before induction of NTN, generated significantly less NO at days 2 and 4 of NTN compared with macrophages from diseased but untreated rats (Figure 2). However these macrophages were not refractory to treatment. IFN/TNF *ex vivo* still increased NO, albeit to a lesser extent than in untreated nephritic rats, and the maximum amount of NO generated was also significantly less in the IL-4-treated rats. It is interesting that NO generation by the macrophages isolated from the IL-4-treated rats was not altered when the cells were incubated with IL-4, TGF, or TNF *ex vivo* (Figure 2). There was significantly greater β -glucuronidase expression in macrophages isolated from glomeruli of IL-4-treated rats at day 4 of NTN than by untreated nephritic controls (Table 2). These results are both qualitatively and quantitatively similar to those obtained for BM-DMs treated simultaneously with IL-4 and IFN- γ followed by activation with TNF,¹⁴ and they suggest a similar temporal sequence *in vivo*.

Discussion

Macrophages infiltrating an inflammatory focus encounter an extremely complex microenvironment, which results in ligation of numerous different cell surface receptors. Many of these have opposing effects on macrophage function when studied in isolation, and understanding how macrophages operate coordinately in such environments is central to the understanding of inflammation. Our previous *in vitro* studies^{14,31} and those of others^{13,15} suggest that certain cytokines program macrophages and render them insensitive to cytokines with alternative activities. Here we show for the first time that macrophages purified from a focus of immunologically mediated inflammation are programmed *in vivo*, whereas normal tissue macrophages are not.

NTN provided an ideal model for these studies because macrophages can be easily isolated from normal and nephritic glomeruli, and the results showed that the isolation procedure neither activated nor programmed the macrophages, thus demonstrating the suitability of the model for analyzing macrophage activation. Injury in this model is macrophage-dependent, but we cannot exclude that some of the protective effects of IL-4 described here are on neutrophils because injury in this model is also neutrophil-dependent.³² However it is unlikely that the effects of IL-4 on glomerular macrophages

Figure 2. The NO generation of macrophages isolated from IL-4-treated normal and nephritic glomeruli and the effect of *ex vivo* incubation with cytokines. Macrophages were incubated for 48 hours with medium alone (spontaneous) or with IFN (20 U/ml) followed by TNF (10 ng/ml), TGF- β (5 ng/ml) or IL-4 medium (5 μ l/ml). Mean plus SD; *n* = 5. **P* < 0.05 versus unstimulated cells.

reported here can be explained on this basis because the effects were evident well after the glomerular neutrophil infiltration had resolved. NO generation and β -glucuronidase expression were chosen as measures of macrophage function primarily because they are complex functions that have been used to characterize macrophage programming *in vitro*.^{12,14,16,28} Furthermore, the cytokines that induce them *in vitro* are present in inflamed glomeruli,³³ and glomeruli isolated from nephritic rats produce NO *ex vivo*.³⁴ Thus we were able to determine whether macrophages that have infiltrated an inflammatory focus *in vivo* are still able to respond to cytokines.

Macrophages from normal glomeruli responded to cytokines in exactly the same way as uncommitted BMDMs. Both could be programmed to generate NO by incubation with IFN- γ followed by TNF, and, once programmed, NO generation was unaffected by IL-4 or TGF- β . By contrast, macrophages isolated from inflamed glomeruli generated NO spontaneously, and this was not inhibited by IL-4, TGF- β , or TNF—results identical to those of BMDMs activated with IFN- γ followed by TNF.¹⁴ The large quantity of NO that was produced indicated that a substantial proportion of the macrophages contributed to NO generation. Analysis of β -glucuronidase expression confirmed that macrophages from normal and nephritic glomeruli behaved like uncommitted and IFN/TNF macrophages, respectively. This assay has the additional advantage of enabling macrophage function to be examined at the single-cell level, and it showed that the macrophages purified from a given kidney behaved as a single population with little cell-cell variation in β -glucuronidase expression either spontaneously or after exposure to cytokines. Thus, inflammatory macrophages purified from nephritic glomeruli are uniformly programmed and cannot be reprogrammed by anti-inflammatory cytokines *ex vivo*.

In vitro, BMDMs are programmed by the first cytokine they encounter,¹⁴ which raises the question whether administration of cytokines *in vivo* can divert infiltrating macrophages down an alternative programming pathway for therapeutic gain. We investigated this possibility by administering IL-4 starting 14 hours before the induction of NTN. IL-4 was chosen for these experiments first because it attenuates injury in NTN, with fewer infiltrating macrophages expressing the macrophage activation marker sialoadhesin, recognized by the monoclonal antibody ED3^{23,35}; second, because IL-4 has been shown to reduce NO generation by whole glomeruli isolated from rats with NTN³⁶; and, third, because *in vitro* IL-4 prevents priming for NO generation when given 4 hours before IFN- γ , modulates NO generation when given at the same time as IFN- γ , and has no effect when given after IFN- γ . Thus the responses of glomerular macrophages to systemic IL-4 administration would be expected to differ depending on whether monocytes/macrophages were programmed before or after infiltrating the kidney. The results show that IL-4 did not prevent programming because macrophages from treated rats generated NO and were unresponsive to IL-4 and TGF- β *ex vivo*. However the infiltrating macrophages generated significantly less NO and thus behaved like BMDMs si-

multaneously exposed to IFN- γ and IL-4.¹⁴ This implies that circulating monocytes are not susceptible to programming and only become so after localization to the inflamed site. Currently we are conducting experiments to examine this hypothesis, but various differences in responses of monocytes and macrophages to IL-4 and other cytokines have already been described,^{37–39} and adherence is important for facilitating the altered responsiveness.^{40,41} Recently, Bonder et al⁴² showed that cultured human monocytes do not express the IL-2 receptor γ chain, a component of the IL-4 receptor, whereas macrophages do, and that some of the anti-inflammatory responses to IL-4, such as their ability to down-regulate lipopolysaccharide-induced TNF- α production, are lost. This suggests that, under the experimental conditions reported here, the macrophages were programmed in the inflammatory focus and not in the circulation when, as monocytes, they were first exposed to IL-4.

Finally, the results raise the question of how macrophage function alters with time during the resolution of inflammation. This could occur if inflammatory macrophages regained the ability to be programmed once the original programming stimulus had been removed, as occurs *in vitro* when BMDMs are rested for 48 hours after exposure to IL-4.¹⁴ Alternatively, programmed macrophages could be removed from an inflammatory focus either by apoptosis or migration to the local lymph nodes⁴³ and be replaced by newly infiltrating macrophages that are differently programmed. This would require a substantial flux of macrophages through an inflammatory focus even though total numbers within the infiltrate show little change.⁴⁴ Our recent work with β -galactosidase-expressing macrophages and fluorescently labeled macrophages supports the idea of a substantial flux of macrophages through inflamed glomeruli.⁴⁵

In conclusion, our experiments demonstrate how macrophages could maintain a coordinated response to the local environment without being overwhelmed by the multitude of signals impinging on their receptors. If correct, this would have obvious implications for the role of macrophages in the control of inflammation and for the use of cytokine therapy.

Acknowledgments

The authors are indebted to Miss M. Johnson and Mrs. A. Woodger for the preparation of this manuscript.

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