Heterogeneity of Macrophage Activation in Anti-Thy-1.1 Nephritis

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Macrophages infiltrating glomeruli in telescoped nephrotoxic nephritis are programmed. The purpose of this study was to assess whether macrophages infiltrating glomeruli of rats with passively induced injury become similarly programmed, and to determine whether macrophage commitment is an early event. Glomerular macrophages isolated from rats with resolving and proliferative anti-Thy-1 nephritis were examined for nitric oxide (NO) generation and expression of lysosomal hydrolases. After a single injection of Thy-1 antibody the cells generated large amounts of NO that was attenuated *ex vivo* by transforming growth factor- β and other anti-inflammatory cytokines. In contrast macrophages infiltrating glomeruli immediately after a second injection of Thy-1 antibody generated NO spontaneously and were unresponsive to alternative activation. β -Glucuronidase expression was used as a second independent assay for macrophage activation and the results confirmed the observations made for NO. Furthermore, macrophages infiltrating the glomerulus after the second antibody injection exhibited a striking dichotomy in that 70% of the cells behave as programmed by interferon- γ and 30% by transforming growth factor- β . The results show that macrophage commitment occurs very early after monocyte migration and that infiltration itself does not invariably induce macrophage programming. It demonstrates that macrophages infiltrating inflamed glomeruli at the same time do not respond uniformly, but are capable of engaging different activation programs. This emphasizes the critical importance of the underlying disease process for macrophage functional development in an inflamed environment. (*Am J Pathol* 2003, 163:2033–2041)

Macrophages are multifunctional cells that infiltrate damaged tissue where they influence all phases of the response to injury.¹ Depending on the setting they cause further injury, for example as part of the host response to infection or autoimmune disease,² or develop into cells

that promote resolution of inflammation with tissue remodeling and repair.^{3,4} These properties are exemplified by studies of renal injury in glomerulonephritis: macrophage infiltration is prominent in all types of severe glomerulonephritis both in patients and in experimental models,⁵ in which depletion and repletion studies demonstrate the injury is macrophage-dependent.⁶ However, other studies show that inhibiting specific pathways for macrophage infiltration aggravates glomerular injury.⁷ Thus macrophages can promote resolution and tissue remodeling as well as causing injury. Possible mechanisms include the removal of unwanted neutrophils⁸ and the control of mesangial cell numbers.⁹ Understanding what dictates the properties of macrophages entering an inflammatory focus is an important challenge for inflammatory cell biologists.

Cytokines provide an early and specific signal for activation of infiltrating macrophages. Interferon (IFN)- γ is the classical cytokine responsible for macrophage activation for tissue destruction.¹⁰ It induces expression of large numbers of genes while suppressing activities of others.¹¹ By contrast other cytokines such as transforming growth factor (TGF)- β and interleukin (IL)-4¹² induce and suppress different sets of genes to produce macrophages with different properties. This has led to the concept of alternative macrophage activation and the extensive characterization of macrophages activated by the T_H2-type cytokines IL-4 and IL-13.¹ However, macrophages can be activated in many different ways and other types of specifically activated macrophages have been described, ie, the type 2-activated macrophage that drives Th2-like responses after a switch in phenotype induced by Fc γ R ligation.¹³ Many of these cytokines (and other activating factors) co-exist within the inflammatory environment but relatively little is known about how macrophages integrate these multiple and sometimes contradictory signals *in vivo*. However, *in vitro* studies have shown that certain activating cytokines, including IFN- γ , IL-4, and TGF- β , commit bone marrow-derived macrophages to defined sets of properties that include pro-

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grammed unresponsiveness to alternatively activating cytokines.¹⁴ Thus macrophage properties are determined primarily by the first activating factor they encounter, and that there is a hierarchy of responsiveness when they are challenged simultaneously by two activating factors: IFN- γ for example dominates over IL-4 and TGF- β .^{14,15} Critically, studies show that macrophages activated in the much more complex environment *in vivo* display similar programmed responses.¹⁶

Glomerulonephritis has proved a particularly good model for studying macrophage activation *in vivo*. Macrophages have been purified from normal and nephritic kidneys for functional studies,¹⁷ including analysis of macrophage surface receptor expression and eicosanoid synthesis,¹⁸ and more recently to examine macrophage programming.¹⁶ Glomerular macrophages from normal rats are uncommitted and can be activated in different ways by IFN- γ , IL-4, and TGF- β both *in vivo*¹⁹ and *ex vivo*.¹⁶ By contrast, macrophages isolated from glomeruli of rats in the acute stage of telescoped nephrotoxic nephritis (tNTN) behave uniformly as though programmed by IFN- γ and are unresponsive to alternative activating factors.¹⁶ This is consistent with the IFN- γ dependence of tNTN that is caused by the host immune response to foreign IgG planted on the glomerular basement membrane. It is now important to know whether macrophages display similar programmed behavior when infiltrating glomeruli in settings not dependent on active immune responses, and the anti-Thy-1.1 model of acute mesangial proliferative glomerulonephritis provides an opportunity for such studies.

Anti-Thy-1.1 nephritis is induced by intravenous injection of antibodies against Thy-1.1, a *trans*-membrane glycoprotein on mesangial cells. This leads to immediate severe complement-dependent injury characterized by mesangial cell death.²⁰ The initial phase is followed by mesangial cell proliferation and matrix expansion that eventually resolves with a return to normal glomerular cell numbers and matrix. Intravenous administration of a second dose of anti-Thy-1.1 antibody during the recovery phase results in a second wave of mesangial cell death that is followed by chronic progressive disease. Interestingly, MCP-1 is released by the damaged mesangial cells within minutes of injection of anti-Thy-1.1 antibodies in both resolving and progressive models of the disease.²¹ This is associated with a rapid but transient macrophage infiltration with few neutrophils.²² Our present study took advantage of this to analyze the behavior of recently migrated macrophages in this model.

The purpose of the present study was to determine whether infiltrating glomerular macrophages in passively induced injury become programmed, and to determine whether macrophage commitment is an early event. The results show that macrophage commitment occurs within the first hour after monocyte migration and that the infiltration itself does not invariably induces macrophage programming. Importantly, they also show that newly emigrated macrophages can be programmed in different ways. This provides an important insight into how macrophage functional development is influenced by the un-

derlying disease process and points to new ways of manipulating macrophage function for therapeutic gain.

Materials and Methods

Reagents

Recombinant human TGF- β and recombinant rat IFN- γ were obtained from Sigma Chemical Co. (Dorset, UK) and Bradsure Biologicals Ltd. (Loughborough, UK) respectively. Recombinant rat IL-4 was produced in-house as described previously²³ using a Chinese hamster ovary cell line generously donated by Dr. Neil Barclay (MRC Cellular Immunology Unit, Oxford, UK).

Preparation of Mesangiolytic Serum

The OX7 antibody specifically recognizes the Thy-1.1 antigen expressed on the glomerular mesangial cell surface, and was prepared from the ascitic fluid of pristane-primed BALB/c mice intraperitoneally injected with an OX7-producing hybridoma line. A technique based on the protocol of Morita and colleagues²⁴ was used for purification of the OX7 antibody.

Induction of Mesangio-Proliferative Glomerulonephritis

Acute mesangio-proliferative glomerulonephritis was induced in male Lewis rats, weighing 175 to 200 g, with a single intravenous injection of 1 mg of the monoclonal anti-Thy-1.1 antibody OX7. Littermate controls received a single dose of an irrelevant isotype-specific monoclonal antibody using an identical schedule.

A second form of mesangio-proliferative glomerulonephritis was induced in male Lewis rats, weighing 175 to 200 g, however, here two intravenous injections of 1 mg each were administered precisely 1 week apart. Littermate controls similarly received two injections of irrelevant mouse monoclonal antibody. Rats were killed at 1 and 4 hours and 1 week after administration of the first dose of OX7, and 1 and 4 hours and 7 days after the second administration of OX7. At time of death, serum was obtained by cardiac puncture, and portions of kidney were snap-frozen for immunofluorescence and RNA extraction, or fixed in methyl Carnoy's for immunohistochemistry or in buffered formalin for light microscopy.

Urine protein excretion was measured on timed overnight samples collected from individual rats in metabolic cages at intervals during the course and assayed by the sulfosalicylic method²⁵ as an index of the severity of the nephritis.

Immunofluorescence

Immunofluorescence microscopy was performed on tissue previously snap-frozen in isopentane sitting in a bed of dry ice. Experimental and control kidneys were sectioned at 4 μ m, fixed in acetone, and stained by direct or

indirect means with fluorescein isothiocyanate-conjugated mouse and rat IgG and rat C3. The tissue was examined at $\times 400$ magnification using a Leitz Ortholux II microscope (Wetzlar, Germany) equipped for epifluorescence and photographed with a Leitz Orthomat.

Immunohistochemistry

Renal samples, fixed in methyl Carnoy's at 4°C , cold processed, and embedded in paraffin wax were stained for ED1, ED2, and ED3 using indirect immunoperoxidase (Serotec, Oxford, UK). Polymorphonuclear leukocyte (PMN) infiltration was assessed in renal tissue stained by the dichloroacetate esterase reaction as previously described.²⁶ Data from at least 50 glomeruli in at least two animals per time point were collected from both control and experimental animals. These data were evaluated as a mean of positive cells per glomerular cross-section.

Glomerular Macrophage Isolation

Glomerular macrophages were obtained from control and nephritic rats as described previously.¹⁶ Briefly, the kidneys of control and nephritic rats were perfused *in vivo* with 50 ml of sterile phosphate-buffered saline then glomeruli were purified by a standard sieving technique. Individual glomeruli were enzymatically digested to a single cell suspension using trypsin, collagenase, DNase, and ethylenediaminetetraacetic acid (Sigma). Macrophages were then isolated from the single-cell suspension by adherence in 24-well plates at a density of 1×10^6 macrophages per well. The macrophages were washed and incubated in medium alone or medium containing cytokines for 24 hours.

Quantitation of Nitric Oxide (NO) Synthesis

Generation of NO was estimated by assaying culture supernatants for nitrite, a stable reaction product of the oxidation of NO, in aqueous solution. Aliquots (200 μl) of each cell-free culture supernatant were incubated with 50 μl 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 Dulbecco's modified Eagle's medium. Densitometrical determination of nitrite was performed using a technique we have previously described.¹⁵ In most experiments, nitrite was measured after 24 and 48 hours in culture.

Quantitation of β -Glucuronidase Expression

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.²⁷ Cytospin preparations of macrophages harvested from the 24-well

tissue-culture plates used above were fixed in 4% glutaraldehyde/50% acetone and air-dried. The cytospin preparations were then stained with β -glucuronidase and counterstained with methylene blue. Slides were coded and scored in a blinded manner using the following scale: 0, no staining; 1, equivocal positive staining; 2, weak positive staining; 3, moderate positive staining; 4, strong positive staining as previously described.¹⁶

Statistical Analysis

Differences in proteinuria between control and experimental rats were analyzed by Student's *t*-test for unpaired data. Analysis of immunofluorescence was accomplished using the Mann-Whitney rank sum test for two different samples. At least 50 glomeruli from each sample were ascribed a value of between 0 and 4 with intermediate levels of fluorescence-ascribed values according to their level of brightness. Differences between the groups in NO generation and scoring of β -glucuronidase were analyzed by Wilcoxon rank sum test and Kruskal-Wallis analysis of variance for comparisons of multiple groups. A probability value of less than 0.05 was considered to be statistically significant.

Results

Severity of Glomerulonephritis

Rats injected with a single dose of OX7 uniformly developed proteinuria within 7 days (35.5 ± 14.3 mg/24 hours compared to 14.9 ± 4.9 for controls). The increase was transient and returned to control values by day 14 (17.8 ± 7 mg/24 hours for the nephritic rats and 17.7 ± 4.1 for controls) where it remained for the rest of the experiment. The experimental rats also had obvious morphological injury (Figure 1). By 24 hours, their glomeruli had focal and segmental mesangiolysis with ballooned capillary lumina, which were engorged with red cells, leukocytes, and plasma proteins. By day 7, the glomeruli were hypercellular, both as a result of increased numbers of mesangial cells and because of infiltrating cells. Murine IgG (OX7) was detected in the mesangial areas during the first 24 hours, presumably bound to Thy-1.1.

Administration of a second dose of OX7 was followed by a further increase in proteinuria (48.3 ± 19.9 mg/24 hours, $n = 12$) compared to the OX7-treated rats not given a second dose (8.4 ± 4.4 , $n = 12$). Again the proteinuria was short lived and returned to control levels within 7 days (13.3 ± 4.3 mg/24 hours in six experimental rats and 11.6 ± 3.8 in six controls). There was prominent focal mesangiolysis with capillary dilatation in segments of the glomerular tuft. Nearly all glomeruli had mesangial hypercellularity and increased mesangial matrix. Rats treated with two doses of OX7 also had glomerular deposition of host IgG, host complement (C3), and OX7 during the first 24 hours. However, OX7 was undetectable 1 week after its administration in either model.

Figure 1. The histological changes observed in glomeruli at 4 hours after the first (**b**) and second (**d**) injection of OX7 and relevant controls (**a**, **c**) are illustrated.

Leukocyte Infiltration

The first injection of OX7 induced a transient glomerular neutrophil (PMN) influx that was evident at 1 hour and maximal at 4 hours. The PMN infiltration had primarily resolved by 24 hours but remained marginally elevated throughout the study (Table 1). The second injection of OX7 provoked another transient PMN influx albeit of lesser intensity (Table 2).

Surprisingly, ED1-positive macrophages were also detectable in glomeruli 1 hour after OX7 was administered and were much more abundant than PMNs. Macrophage infiltration increased at 4 hours before substantially declining at 24 hours (Table 1). ED1 (which recognizes a lysosomally expressed molecule) was used to enumerate the total macrophage infiltrate. ED2 and ED3 expression was assessed as immunohistological indicators of macrophage activation. The molecules recognized by ED2

and ED3 have been identified as CD 163 (hemoglobin-haptoglobin complex receptor) and CD169 (Siglec-1 or sialoadhesin) but so far there have been no systematic studies to determine their expression by macrophages activated in different ways. There was a much smaller but more persistent increase in numbers of ED2-positive macrophages (Table 1). There was also an increase in ED3-positive cells, which was again evident within the first hour of disease induction (Table 1).

The second injection of OX7 also induced a comparable increase in ED1-positive macrophages (Table 2). Once again, ED1-positive cells were maximal at 4 hours before substantially declining at 24 hours (Table 2). The number of ED2-positive cells also increased following a similar pattern and again ED3-positive cells were detected within the first hour of disease induction.

Table 1. Cell Numbers per Glomerular Cross Section after the First Injection of OX7

	PMN	ED1	ED2	ED3
Normal	0.16 ± 0.001	1.06 ± 0.08	0.18 ± 0.09	0.048 ± 0.06
Isotype control	0.72 ± 0.39	1.53 ± 0.28	0.18 ± 0.04	0.25 ± 0.02
1 hour	3.76 ± 0.88 [†]	10.64 ± 0.19*	0.36 ± 0.03 [†]	1.64 ± 0.46 [†]
4 hours	5.9 ± 0.16*	15.4 ± 0.47*	0.38 ± 0.05 [†]	1.68 ± 0.41 [†]
24 hours	1.05 ± 0.07	9.39 ± 0.77*	0.34 ± 0.14	0.87 ± 0.17
7 days	0.54 ± 0.47	3.16 ± 0.2 [†]	0.5 ± 0.01 [†]	0.94 ± 0.08 [†]

N = 6, mean ± SD.

**P* < 0.01 compared to relevant normal control.

[†]*P* < 0.05 compared to relevant normal control.

Table 2. Cell Numbers per Glomerular Cross Section after the Second Injection of OX7

	PMN	ED1	ED2	ED3
Control	1.38 ± 0.11	1.78 ± 0.34	0.23 ± 0.01	0.41 ± 0.01
1 hour	2.16 ± 0.73	6.69 ± 0.84 [†]	0.31 ± 0.07	1.06 ± 0.09*
4 hours	2.07 ± 0.35	11.98 ± 0.17*	1.0 ± 0.22 [†]	1.30 ± 0.56
24 hours	1.08 ± 0.99	2.2 ± 0.13*	0.13 ± 0.03 [†]	0.35 ± 0.1

N = 6, mean ± SD.

**P* < 0.01 compared to relevant normal control.

[†]*P* < 0.05 compared to relevant normal control.

Table 3. NO Generation by Macrophages Isolated from Control and Nephritic Rats after First Injection of OX7

NO generation in umol/ml	Spontaneous	IFN- γ + TNF (20 U + 10 ng/ml)	IL-4 (8 ng/ml)	TGF- β 1 (10 ng/ml)
Control	4.65 ± 0.7	17.73 ± 1.9*	3.93 ± 0.9	3.28 ± 1
1 hour	11.93 ± 1.4	18.65 ± 2.9 [†]	6.43 ± 2.2 [†]	5.7 ± 1.5 [†]
4 hours	15.1 ± 2.5	27.6 ± 3.1 [†]	6 ± 1.3 [†]	7.35 ± 0.8 [†]
7 days	3.48 ± 1.7	23.25 ± 1.1 [†]	3.97 ± 1.8	3.65 ± 2.4

N = 6 for control rats and *N* = 4 for nephritic rats; mean ± SD.

**P* < 0.05 compared with untreated controls.

[†]*P* < 0.05 compared with macrophages from untreated nephritic rats.

In summary, macrophages infiltrate damaged glomeruli immediately after both injections of OX7 and interestingly the leukocyte infiltration data shows that there are fivefold more ED1-positive cells than neutrophils. Furthermore, the infiltrating macrophages are heterogeneous with regards to ED2 and ED3. This makes the anti-Thy-1.1 model ideally suited for functional studies of early macrophage influx.

NO Generation of Glomerular Macrophages

We have previously shown that glomerular macrophages from normal rats do not generate NO spontaneously but can be activated to do so by IFN- γ and TNF- α . By contrast, macrophages purified from rats 2 days after induction of nephrotoxic nephritis behave operationally as though already programmed by IFN- γ and TNF- α in that they generate large amounts of NO that is not suppressed by IL-4 or TGF- β .¹⁶ The next experiments were designed to determine whether glomerular macrophages in anti-Thy-1.1 were similarly programmed, and if so to ascertain how early they became committed to a particular program.

Macrophages were purified from normal rats and from nephritic rats 1 hour, 4 hours, and 7 days after the first injection of OX7. As in our previous experiment, the normal glomerular macrophages behaved like uncommitted bone marrow-derived macrophages (Table 3). Glomeru-

lar macrophages from nephritic rats at both 1 hour and 4 hours after injection of the first dose of OX7 generated moderate amounts of NO spontaneously. NO generation was augmented by IFN- γ and TNF, and suppressed by incubation with either IL-4 or TGF- β (Table 3). Macrophages purified from nephritic rats 7 days after disease induction did not generate NO spontaneously, but were primed to produce significantly more NO when incubated with IFN- γ and TNF than normal glomerular macrophages (Table 3). After the second injection of OX7 infiltrating macrophages also generated large amounts of NO that was augmented by exposure to IFN- γ and TNF- α . However, this could not be suppressed by IL-4 and TGF- β (Table 4). Taken together these results show that macrophages infiltrating glomeruli in response to the first injection of OX7 are not programmed and remain responsive to alternative activation. By contrast, macrophages infiltrating after the second injection are programmed and behave identical to those isolated at days 2 and 4 of nephrotoxic nephritis¹⁶ and bone marrow-derived macrophages that have been programmed *in vitro* by IFN- γ and TNF.¹⁴ NO generation is only one of many programmable macrophage activities and we went on to assess β -glucuronidase expression, which has the additional advantage of being able to assess responses of individual macrophages.¹⁹

Table 4. NO Generation by Macrophages from Control and Nephritic Rats after Second Injection of OX7

NO generation in umol/ml	Spontaneous	IFN- γ + TNF (20 U + 10 ng/ml)	IL-4 (8 ng/ml)	TGF- β 1 (10 ng/ml)
Control	3.9 ± 0.6	18.49 ± 1.8*	3.48 ± 1	3.22 ± 1.1
1 hour	23.95 ± 3.7	34.63 ± 0.9 [†]	23.55 ± 3.6	27.37 ± 4.1
4 hours	34.62 ± 2.8	47.1 ± 1.2 [†]	33.1 ± 1.3	35.67 ± 2

N = 6 for control rats and *N* = 4 for nephritic rats; mean ± SD.

**P* < 0.05 compared with untreated controls.

[†]*P* < 0.05 compared with macrophages from untreated nephritic rats.

Table 5. β -Glucuronidase Expression of Macrophages Isolated from Glomeruli of Rats Killed after the First Injection of OX7 and Healthy Controls

	Control	1 hour	4 hours
Spontaneous	2.0 ± 0.4	$1.3 \pm 0.2^*$	$0.9 \pm 0.1^*$
IFN/TNF 20 U + 10 ng/ml	$0.9 \pm 0.2^*$	$0.6 \pm 0.3^{*\dagger}$	$0.3 \pm 0.3^{*\dagger}$
IL-4 5 μ l/ml	1.8 ± 0.6	$1.2 \pm 0.5^*$	$1.1 \pm 0.4^*$
TGF- β 1 5 ng/ml	$3.4 \pm 0.3^*$	$2.6 \pm 0.3^{*\dagger}$	$2.9 \pm 0.5^{*\dagger}$

* $P < 0.05$ compared to untreated rats.

$^\dagger P < 0.05$ compared to spontaneous β -glucuronidase expression at 1 hour.

$^\ddagger P < 0.05$ compared to spontaneous β -glucuronidase expression at 4 hour.

β -Glucuronidase Expression of Glomerular Macrophages

Uncommitted bone marrow-derived macrophages contain modest amounts of β -glucuronidase that is increased by exposure to TGF- β and decreased by IFN- γ . Glomerular macrophages from normal rats also expressed moderate amounts of β -glucuronidase and behaved similarly when exposed to TGF- β and IFN- γ : this is consistent with our previous reported results (Table 5).^{16,19} One hour after the first injection of OX7 glomerular macrophages uniformly expressed slightly reduced amounts of β -glucuronidase but they responded appropriately to TGF- β and IFN- γ (Table 5 and Figure 2). Macrophages isolated after 4 hours expressed less β -glucuronidase, but again responded to IFN- γ and TGF- β (Table 5 and Figure 1). These results confirm that macrophages infiltrating glomeruli immediately after the first dose of OX7 were not programmed.

Glomerular macrophages isolated 7 days after induction of disease were different. They were strikingly heterogeneous and their β -glucuronidase expression was highly polarized in that 32% stained strongly for β -glucuronidase (score, 3.6 ± 0.3) whereas staining was virtually undetectable in the remaining 68% (score, 0.3 ± 0.2)

(Figure 3). These proportions were remarkably similar in all of the animals studied, which raised the important question of what dictates the different functions of the day 7 macrophages. Possible explanations include their time of recruitment and duration of residence; their localization to glomerular segments at different stages of injury and thus their exposure to a different microenvironment; and that activation is a stochastic event in which individual infiltrating macrophages respond differently to a common microenvironment. The analysis of macrophage infiltrates after a second dose of OX7 was then used to provide evidence for and against these propositions. β -Glucuronidase was again almost undetectable (score, 0.4 ± 0.3) in 70% of these newly emigrated cells and strongly positive (score, 3.6 ± 0.2) in the remainder at both 1- and 4-hour time points (Figure 3). Incubation with TGF- β made no difference to the proportions that were positive or negative (data not shown). Thus all macrophages infiltrating the kidney after the second dose of OX7 were programmed but not in the same way, and the proportions of β -glucuronidase high and low macrophages was identical to those of macrophages present in glomeruli before the antibody was injected. This excludes time of residence as an explanation and strongly suggests that macrophages that localize to common microenvironments in an inflamed focus adopt different functional programs.

Figure 2. Shows β -glucuronidase expression of individual macrophages isolated from glomeruli 1 and 4 hours after a single injection of OX7. Mean + SD, $n = 5$.

Figure 3. Shows β -glucuronidase expression of individual macrophages isolated from glomeruli 7 days after a single injection of OX7 and 1 and 4 hours after a second injection of OX7 on day 7. Mean + SD, $n = 5$.

Discussion

Macrophage programming has been repeatedly demonstrated *in vitro*,¹⁴ and the ability to isolate macrophages directly from glomeruli of rats with experimental nephritis¹⁶ and the retina in uveitis²⁸ have been used to show that infiltrating macrophages are similarly programmed *in vivo*. Extending our observations to the anti-Thy-1.1 model of nephritis has enabled us to make three entirely novel observations: first, localization itself does not induce programming; second, all macrophages infiltrating an appropriate environment become programmed shortly after localization; and third, macrophages infiltrating glomeruli at the same time can be programmed in different ways. These findings have important implications for the role of macrophages in renal injury and were critically dependent on the characteristics of the anti-Thy-1.1 model.

Injury in anti-Thy-1.1 nephritis is caused directly by binding of administered antibody to Thy-1.1 on the mesangial cell surface, which results in complement-dependent cell death in which both necrosis and apoptosis are prominent.²⁹ The earliest phase is accompanied by a marked influx of ED1-positive cells. Substantial numbers of macrophages have already localized 1 hour after injection of OX7 and this is consistent with the very early glomerular expression of MCP-1.²¹ Macrophage infiltration provoked by a second injection of OX7 on day 7 is also transient and has very similar kinetics, even though it occurs in the context of host immunity to the foreign IgG injected 1 week previously. The early macrophage influx together with the paucity of glomerular neutrophils enabled us to use NO generation to compare macrophage programming in these two settings and to ascertain how early macrophages acquired commitment to a particular activity.

NO generation has been used repeatedly to analyze macrophage programming.^{14,30} Fixed production of large amounts of NO has been shown to be characteristic of IFN- γ -programmed macrophages,^{14,28} and glomerular macrophages in tNTN behave uniformly as though programmed in this way.¹⁶ Infiltrating macrophages also generate NO in the anti-Thy-1.1 model.³¹ However, here we show that their responses are more complex (than in NTN). Macrophages infiltrating the kidney after the first injection of OX7 produce large amounts of NO without being programmed, and thus infiltration itself does not induce programmed NO generation. Macrophages infiltrating after the second injection generate even larger quantities of NO and are unresponsive to alternative activation. Therefore macrophage programming only occurs within the appropriate microenvironment, such as in experimental autoimmune uveitis or tNTN^{16,28} and after the second injection of OX7 in the present model. Programmed NO generation could be demonstrated at 1- and 4-hour time points, which clearly demonstrates that macrophages can be committed to a particular program very soon after they enter an appropriate microenvironment. Our previous work suggests that macrophage programming occurs after mononuclear cells localize to inflamed tissue and that a competence signal is required to

make the cells susceptible to macrophage programming.¹⁶ However more recent unpublished observations from our laboratory show that freshly isolated monocytes can be programmed when exposed to specific stimuli *in vitro*.

Glomerular macrophage NO generation was increased after both injections of OX7 but was only programmed after the second of these. This suggests that NO generation is regulated in different ways in the two phases. The demonstration of deposits of host IgG after the second injection demonstrates that infiltration on day 7 occurred in the context of an active immune response analogous to that seen in tNTN. Accordingly, it is not surprising that the macrophages were similarly programmed. In contrast, macrophage infiltration induced by the first dose of OX7 occurred in the absence of active immunity and is likely to be a direct result of cellular injury. Macrophage NO generation can be triggered by many signals other than IFN- γ . For example, IL-1 and lipopolysaccharide induce NO generation without programming in that the cells remain responsive to alternative activation. Lipopolysaccharide-induced NO generation is dependent on its binding to Toll-like receptor-4 (TLR-4) that utilizes a very similar signaling pathway to the IL-1 receptor resulting in activation of nuclear factor- κ B.³² Interestingly, heat shock protein-60 (HSP-60), which is released by damaged or dying cells also binds to TLR-4 and this provides a possible explanation for the induction of a nonprogrammed NO response in the first phase of Thy-1.1 nephritis, provided that HSP-60 was released from dying mesangial cells.

The depth of knowledge about macrophage NO generation is a great advantage when using it as a tool to study macrophage programming. The disadvantage is that it can only be used to study populations rather than individual cells and accordingly the NO results cannot determine whether all of the infiltrating macrophages display programmed behavior or just a proportion of them: this required the use of a second programmable macrophage function β -glucuronidase. The lysosomal enzyme β -glucuronidase is strongly expressed when macrophages ingest particulate matter such as group A streptococcal cell walls, zymosan particles, or β -1-3-glucan. Laszlo and colleagues³³ first showed it displays programmed behavior and that its expression is decreased by IFN- γ and increased by TGF- β .³³ Furthermore, our own unpublished observations and work by Hartner and colleagues³⁴ show that TGF- β isoforms 2 and 3 are up-regulated in anti-Thy-1 nephritis. β -Glucuronidase can be quantified in individual cells histochemically, and we have validated this technique for studying programming *in vitro*¹⁴ and *in vivo*.¹⁶ The β -glucuronidase results independently confirm the conclusions from the NO data. Neither glomerular macrophages from healthy rats nor those with day 1 nephritis displayed programmed behavior whereas two-thirds of those infiltrating after the second dose of OX7 had low and fixed β -glucuronidase expression similar to IFN- γ -activated macrophages.

These experiments revealed another striking level of macrophage heterogeneity. Macrophages isolated 7 days after the first injection, when looked at as a bulk

population, have similar functional properties as macrophages isolated from normal glomeruli. They generated little NO when cultured *ex vivo*, which suggested that not only was the histological injury resolving but also that the number and properties of glomerular macrophages was returning to normal. However, these cells remained primed to produce significantly more NO than macrophages isolated from normal glomeruli when exposed to IFN- γ *ex vivo*. More importantly analysis of β -glucuronidase expression demonstrated a remarkable dichotomy in that 32% of the cells are strongly positive whereas 68% express none or very little. Thus approximately one-third of glomerular macrophages appear programmed by TGF- β whereas the remaining two-thirds had β -glucuronidase levels similar to that seen after exposure to IFN- γ .

Macrophage heterogeneity has previously been described for different areas of an inflamed tissue, ie, interstitial *versus* glomerular macrophages³⁵ and in cells isolated from different types of glomerular injury.³⁶ Here we extend these observations and showed for the first time that macrophages within a particular environment at the same time can be programmed in different ways. Obvious explanations for this include the time of residence or the possibility that the macrophages originate from different glomerular segments at different stages in the evolution of the disease. Alternatively, macrophage programming is a stochastic event in that the chances of macrophages being programmed in a particular way depend on the characteristics of individual components of the programming environment.¹⁴ The analysis of freshly infiltrating macrophages induced by a second dose of OX7 allowed us to distinguish between some of the possibilities. Macrophages isolated 1 and 4 hours after the second dose of OX7 were similarly heterogeneous in that 30% were strongly positive for β -glucuronidase and 70% did not express any. This is remarkable considering that the vast majority of the cells had just localized to the inflamed glomerulus. This eliminates the possibility that time of residence is responsible for the observed heterogeneity in macrophage programming and furthermore makes it unlikely that the localization within the glomerulus is critical. It strongly suggests that commitment to a particular program is determined by the properties of the tissue the macrophages infiltrate and that the program the cells adopt is a stochastic event critically dependent on the local microenvironment.

These observations raise question about the factors that induce macrophage activation and programming at early stages of the inflammatory disease and its consequences for the outcome of the inflammatory process. It provides an important mechanistic insight into how macrophage functional development is influenced by the underlying disease process. The differences in macrophage function after the first and second injection of OX7 in our model suggest that macrophage activation is profoundly different in passive injury and immunologically mediated inflammation. Provocatively, our study provides important *in vivo* support for Matzinger's³⁷ suggestion that control of immune responses is driven by and tailored to the tissue in which injury occurs, rather than

being primarily a function of the cells of the adaptive or innate immune system.

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