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Functional Characteristics of Macrophages in Glomerulonephritis in the Rat

O_2^- Generation, MHC Class II Expression, and Eicosanoid Synthesis

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Macrophage infiltration is important in the pathogenesis of acute proliferative glomerulonephritis (gn). The state of activation of macrophages during gn may be central to their role in injury. To study this, a method for extracting macrophages from nephritic glomeruli in active* in situ *gn was developed. MHC Class II (Ia) antigen expression, superoxide (O_2^-) generation, and eicosanoid synthesis were compared with thioglycollate elicited peritoneal macrophages (TEM). At the height of inflammation there were 407 ± 83 macrophages/glomerulus. Compared with TEM, Ia expression, and* in vitro *production of O_2^- were enhanced. Synthesis of prostaglandin E_2 was greatly reduced (day 6 gn, 62 ± 10 ng/mg; TEM 663 ± 128 ng/mg cell protein). Thromboxane synthesis was relatively conserved (day 6 gn, 109 ± 28 ng/mg; TEM 201 ± 53 ng/mg). Leukotriene B_4 (LTB_4) was undetectable (day 6 gn, <13 ng/mg; TEM 119 ± 56 ng/mg). This large influx of activated macrophages in glomeruli may be fundamental to pathogenesis of glomerular inflammation. (Am J Pathol 1989, 134:431–437)

In acute proliferative glomerulonephritis (gn) leukocyte influx is part of the inflammation initiated in glomeruli by antigen-antibody complexes. This cellular infiltrate may be predominantly neutrophilic or monocytic depending on the type and phase of injury. To investigate further the role of leukocytes in gn we sought to quantitate the extent of leukocyte accumulation, define the leukocyte types, and study the activation profile of mononuclear phagocytes infiltrating glomeruli during experimental acute proliferative gn.

Macrophages are versatile cells that change their characteristics depending on site, maturation and state of activation. The abilities of macrophages to synthesize a variety of arachidonic acid metabolites, express class II MHC antigens, and produce reactive oxygen species are important activation characteristics that affect the regulation of inflammation and the immune response. These are the three aspects of activation we have studied by developing a method for extracting viable macrophages from nephritic glomeruli.[1] The model we have studied is acute *in situ* immune complex gn in the rat induced by intrarenal perfusion with cationized human IgG,[2] a model that produced a severe acute proliferative gn with a major leukocyte influx.

Material and Methods

Animals

Inbred male Lewis rats from St. Mary's Hospital Medical School were used for all experiments. Operative procedures were carried out with halothane (ICI, Macclesfield, UK) anesthesia.

Induction of Glomerulonephritis

Gn was induced by a modification of the method of Oite et al.[2] Human IgG and cationized human IgG were prepared as previously described.[1] Cationized IgG was of pI > 9.3 as determined by isoelectric focusing. Rats aged 6–10 weeks were immunized three times at monthly intervals with 1 mg human IgG and Freund's complete adju-

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vant. Serum antibody levels were assessed by reverse radial immunodiffusion. Rats with detectable antibody 7–10 days after the last immunization were used. Gn was induced by perfusion of the left kidney with normal saline followed by 400 μg cationized IgG in 0.5 ml normal saline given over 30 seconds as described previously.[1] Immediately after left kidney perfusion the right kidney was removed. Control perfusions with cationized human IgG were performed in unimmunized rats. Urine samples were collected from rats in metabolism cages and urine protein estimated by the sulphosalicylic acid method.[3]

Microscopy

Tissue for light microscopy was fixed in Bouin's fixative and embedded in paraffin wax. Tissue for electron microscopy was processed as described previously.[1]

Isolation of Glomerular Cells

Glomeruli were obtained from saline-perfused left kidneys of normal rats, and of gn rats on various days after induction of gn, and digested to single cell suspensions using trypsin, collagenase, and deoxyribonuclease as described previously.[1] Leukocytes and Ia-positive cells in glomerular cell suspensions (GCS) were enumerated by staining with monoclonal antibodies OX1 (Serotec, Kidlington, UK) to the rat leukocyte common antigen, and OX3 and OX4 (Seralab, Crawley Down, UK) to rat Ia determinants, followed by fluorescein conjugated rabbit $F(ab')_2$ anti-mouse immunoglobulin (Dako, High Wycombe, UK) as described.[1] Positive cells were counted on a fluorescence activated cell sorter (EPICS V, Coulter Electronics, Luton, UK). Cytocentrifuge preparations of GCS were stained with Giemsa for counting neutrophils. In some cases they also were stained with rat monocyte/macrophage specific monoclonal antibody ED1[4] (Serotec) for mononuclear phagocytes. The preparations were fixed in acetone for 10 minutes at room temperature, air dried, and stained with ED1 at 1:2000 in PBS (pH 7.4) for 1 hour. The slides were washed, endogenous peroxidase was blocked with H_2O_2 in methanol, and then they were stained with peroxidase labeled rabbit anti-mouse immunoglobulins (Dako) at 1:40 in PBS with 5% normal rat serum for 1 hour. The peroxidase reaction was developed with diaminobenzidine and H_2O_2.

Isolation of Adherent Glomerular Cells

GCS were plated at 1.5×10^6 in 1 ml in 16-mm plastic tissue culture wells (Nunc, Uxbridge, UK) in Dulbecco's modified Eagle medium (DMEM, Flow Laboratories, Rickmansworth, UK) with 10% fetal calf serum (FCS), penicillin 10 U/ml and streptomycin 10 mg/ml, and incubated at 37 C under 4% CO_2. After 2 hours the cells were washed vigorously three times and then cultured overnight. In some experiments cells were cultured on glass coverslips that were fixed and stained with monoclonal antibody ED1 as described above, after overnight culture.

Isolation of Peritoneal Macrophages

Peritoneal macrophages were obtained by peritoneal lavage with calcium free perfusion buffer (pH 7.4) from normal rats injected intraperitoneally with either thioglycollate broth (Difco, East Molesey, UK)) 10 ml, 6 days previously or with *Corynebacterium parvum* (7 mg/ml, Wellcome Foundation, Beckenham, UK) 0.2 ml, 10 days previously. Peritoneal macrophages were plated at 1×10^6 in 1 ml in 16-mm culture wells in DMEM with 10% FCS, washed at 2 hours, and cultured overnight.

To examine the effect of enzymic digestion on peritoneal macrophages, cells elicited with thioglycollate or *C. parvum* were allowed to adhere for 2 hours and subjected to an identical enzymic digestion as GCS as follows: Trypsin (type III, Sigma, Poole, UK) 0.5 mg/ml, collagenase (type I, Sigma) 1 mg/ml, and deoxyribonuclease (type III, Sigma) 0.1 mg/ml for 20 minutes; 2 mM EDTA without calcium or magnesium for 20 minutes; collagenase 1 mg/ml for 20 minutes (1 ml of enzyme solution per well). After enzyme treatment cells were washed and cultured overnight.

Superoxide and eicosanoid generation were measured in adherent GCS and in peritoneal macrophages with or without prior enzymic treatment. After measurement of superoxide or eicosanoid generation, cell protein was measured in individual wells by the Lowry method using Sigma kit No. 690 with the tartrate reagent added directly to the well (mean GCS 16 μg; peritoneal macrophages 19 μg).

Generation of Superoxide

Production of superoxide was measured as superoxide dismutase (SOD) inhibitable reduction of ferricytochrome c as described.[5] One milliliter of ferricytochrome c (Sigma) in 80 μM in Hank's balanced salt solution (HBSS) was added to adherent cells in culture with either opsonized zymosan (Sigma) 1 mg/ml or PMA (Sigma) 4 μg/ml. PMA was initially dissolved in DMSO at 2 mg/ml. Incubations were performed with or without SOD (ICN Biomedical, High Wycombe, UK) 40 μg/ml. All tests were performed in duplicate. The cells were incubated at 37 C under 4%

CO_2 for 90 minutes; supernatants were removed, centrifuged at 1200*g* for 10 minutes, and absorbance measured at 550 nm using reaction mixture incubated in wells without cells as a blank. Moles of cytochrome c reduced, equivalent to moles of superoxide generated, were calculated from the molar extinction coefficient E_{550} = 21.0 $\times 10^3$/cm.

Generation of Eicosanoids

Adherent cells were washed and 1 ml of serum-free DMEM with or without calcium ionophore A23187 (Sigma) 1 μg/ml was added to each well. Wells were incubated for 5 minutes at 37 C and supernatants were removed and centrifuged at 1200*g* for 10 minutes. All tests were performed in duplicate. TXB_2, PGE_2, and LTB_4 were measured in cell-free supernatants by direct radioimmunoassay without prior extraction or chromatographic purification as described.[6,7] The lower limits of detection of the assays were PGE_2 0.2 ng/ml, TXB_2 0.1 ng/ml and LTB_4 0.2 ng/ml.

Statistics

Results are expressed as mean ± SEM. Statistical analyses are by two-tailed Student's *t*-test with $P < 0.05$ taken as significant.

Results

Leukocyte Isolation in Glomerulonephritis

Quantitation and Identification of Cell Types

All rats in which glomerulonephritis was induced by preimmunization and intrarenal infusion of cationized IgG developed proteinuria and glomerular hypercellularity. Proteinuria was present from day 1 and reached a maximum at 7 days (Figure 1). Histology at 24 hours showed leukocyte infiltration and focal fibrin deposition and endothelial cell necrosis. Leukocyte infiltration increased with time, becoming maximal at 1 week. Electron microscopy showed, in addition, extensive foot process fusion, initially subendothelial deposits, and, by 24 hours, subepithelial electron-dense deposits. Ultrastructurally, the leukocytes were neutrophils and mononuclear phagocytes with the

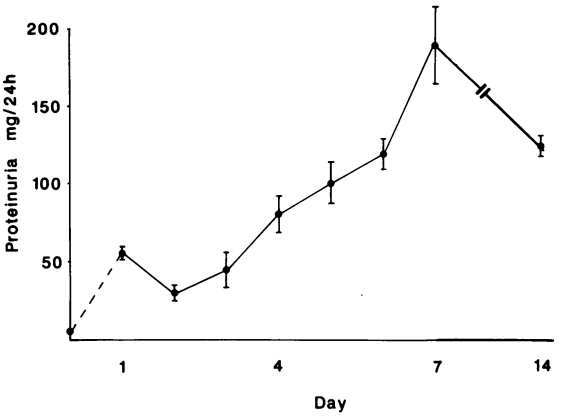

Figure 1. *Proteinuria in acute* in situ *glomerulonephritis (gn). Gn was induced in rats, preimmunised with human IgG, by left renal perfusion with cationized IgG followed by right nephrectomy on day 0.*

latter predominating after 24 hours. In control unimmunized rats given cationized IgG and right nephrectomy there was no glomerular hypercellularity and proteinuria did not rise above 10 mg/24 h.

Isolation of glomerular cells by enzymic digestion (Table 1) showed a progressive rise in the total cells per glomerulus (up to sixfold) in nephritic glomeruli during the first 6 days; the great majority of cells were leukocytes (OX1 positive). At day 7 there was a mean of 534 leukocytes per glomerulus, 407 of which were mononuclear cells; the identity of these as mononuclear phagocytes was confirmed by ED1 staining. There was also a substantial number of neutrophils. The percentage of mononuclear leukocytes expressing Ia antigens (OX3/4 positive) on day 1 was only 15% (normal glomerular monocyte expression 41%) but by day 4 had risen to 42%. By day 14 intraglomerular leukocyte numbers were falling and most were mononuclear cells.

In Vitro *Studies on Glomerular and Peritoneal Macrophages*

After 2-hour adherence and overnight culture of GCS the adherent cells were >90% mononuclear phagocytes as identified by ED1 staining. The Ia expression of recently isolated peritoneal cells was <5% for thioglycollate elicited cells and 34% for *C. parvum* elicited cells.

The results of superoxide generation by isolated adherent glomerular mononuclear phagocytes and peritoneal macrophages treated with identical enzymic digestion are shown in Table 2. Glomerular cells produced more superoxide with either PMA or opsonized zymosan stimulation than peritoneal cells and this difference was statistically significant with zymosan stimulation on day 6 ($P < 0.05$). Superoxide production by both types of peritoneal cells was higher without enzyme treatment (thiogly-

Table 1. *Isolation and Characterisation of Cells from Glomeruli*

	Day*	Cells per glomerulus	LC+†	Ia+†	Neut‡	Monos§ Number	Monos§ %Ia	N
Normal rats		144 ± 13	13 ± 1	5 ± 1.5			41 ± 19	3
GN rats	1	224 ± 30	116 ± 29	7 ± 2	51 ± 9	65 ± 22	15 ± 3	4
	2	280 ± 28						22
	4	714 ± 105	472 ± 77	120 ± 24	165 ± 21	381 ± 64	42 ± 4	9
	6	861 ± 146						14
	7	757 ± 88	534 ± 69	161 ± 57	127 ± 21	407 ± 83	37 ± 8	4
	14	184 ± 17	106 ± 51	27 ± 4	9 ± 1	95 ± 52	37 ± 17	2

Values are the mean ± SE of the number of experimental left kidneys indicated.
* Day after induction of gn.
† Isolated cells were labeled with Ab OX1 for leukocyte common antigen (LC), or OX3/4 for Ia antigen and enumerated by FACS.
‡ Neutrophils were identified by Giemsa staining.
§ Mononuclear phagocytes were identified with Ab ED1.

collate: PMA stimulated, 103 ± 24 nmol/mg, zymosan stimulated, 183 ± 60 nmol/mg; *C. parvum:* PMA stimulated, 78 ± 21 nmol/mg). Glomerular cells also produced H_2O_2 (as measured by horseradish peroxidase-dependent oxidation of phenol red) when stimulated by PMA (day 2, 138 ± 34 nmol/mg/h, N = 4; day 6, 186 ± 73 nmol/mg/h, N = 3).

Eicosanoid production by glomerular cells and enzyme-treated peritoneal cells is shown in Table 3. Ionophore-stimulated PGE_2 generation by glomerular macrophages was significantly less than that of thioglycollate or *C. parvum* peritoneal cells at all times studied ($P < 0.01$). TXB_2 production by glomerular macrophages was significantly reduced compared with *C. parvum* elicited cells on days 1 and 2 ($P < 0.05$) and significantly reduced compared with thioglycollate elicited cells on day 1 ($P < 0.05$). Basal synthesis of PGE_2, but not of TXB_2, was progressively less with time after induction of gn (37.8 ng/mg on day 1 and below detectable limits on day 6), but similar in *C. parvum* and thioglycollate elicited cells. LTB_4 was produced by thioglycollate elicited cells (119 ± 56 ng/mg, N = 3) but was undetectable in glomerular cells at any time point and in *C. parvum* elicited cells (representing <13 ng/mg given a lower limit of detection of 0.2 ng/ml and a mean protein of 16 μg).

Table 2. *Superoxide (O_2^-) Generation by Macrophages Isolated from Nephritic Glomeruli and Peritoneum*

Cell	Day*	O_2^- nmol/mg cell protein PMA	Zymosan	N
Glomerular				
	2	141	444	2
	6	131 ± 34	312 ± 79	4
Peritoneal				
Thioglycollate	6	45 ± 17	89 ± 31	3
C. parvum	10	65 ± 10	ND	3

ND, Not determined.
Values are mean ± SE.
* Day of isolation. For glomerular cells, days after induction of nephritis. For peritoneal cells, day after intraperitoneal thioglycollate or of *C. parvum*.

Enzymic treatment produced a slight, but not statistically significant, reduction in PGE_2 production by thioglycollate elicited peritoneal macrophages (enzyme treated 663 ± 128 ng/mg, N = 3, untreated 726 ± 102) and slight rises in TXB_2 and LTB_4 (TXB_2:enzyme treated 201 ± 53 ng/mg, N = 3, untreated 168 ± 18 ng/mg, *N* = 3; LTB_4 enzyme treated 119 ± 56 ng/mg, N = 3, untreated 62 ± 26 ng/mg, N = 3). No detectable LTB_4 was produced by *C. parvum* elicited cells without enzyme treatment.

Discussion

To examine the macrophages infiltrating glomeruli in gn, we used a method originally designed to obtain populations of intrinsic glomerular cells. We have assessed three aspects of macrophage activation, Ia antigen expression, oxygen radical production, and eicosanoid generation, and compared glomerular macrophages with two types of elicited peritoneal macrophages: peritoneal macrophages elicited by thioglycollate that represent an influx of unactivated circulating monocytes,[8] and those elicited by intraperitoneal *C. parvum* that show a number of characteristics of activation including enhanced tumor cell killing.[9]

Large numbers of viable leukocytes were obtained from nephritic glomeruli in the model of acute proliferative glomerulonephritis studied. Neutrophils were present throughout the times studied but macrophages were the predominant cell. The number of macrophages extracted shows that most previous methods have underestimated the extent of inflammatory cell accumulation in acute hypercellular gn.

Our results for glomerular Ia antigen expression are consistent with an influx of predominantly Ia negative monocytes (less than 5% of circulating rat monocytes are Ia positive) followed by induction of Ia antigen within the glomerulus, so that by 4 days over 40% of glomerular macrophages are Ia positive. This change parallels that

Table 3. *Eicosanoid Synthesis by Macrophages Isolated from Nephritic Glomeruli and Peritoneum*

		Concentration of eicosanoids (ng/mg cell protein)						
		PGE2		TXB2		LTB4		
Cells	Day*	A23187	Basal	A23187	Basal	A23187	Basal	N
Glomerular								
	1	72 ± 21	38 ± 27	50 ± 22	10 ± 4	UN	UN	4
	2	100 ± 5	13 ± 0.7	109 ± 5	16 ± 3	UN	UN	4
	6	62 ± 10	UN	109 ± 28	8 ± 1.8	UN	UN	3
Peritoneal								
Thioglycollate	6	663 ± 128	47 ± 32	201 ± 53	6 ± 0.8	119 ± 56	UN	3
C. parvum	10	442 ± 39	64 ± 46	150 ± 4	5 ± 1.6	UN	UN	3

UN, <0.2 ng/ml – lower limit of detection.
Values are mean ± SE.
* Day of isolation. For glomerular cells, day after induction of nephritis; for peritoneal cells, days after intraperitoneal thioglycollate or *C. parvum*.

seen in normal rats after irradiation, where the restoration of the normal population of glomerular mononuclear phagocytes occurs with subsequent induction of Ia expression.[10] The stimulus to Ia induction is unknown but could be lymphokines generated during inflammation.[11]

Our results show that the macrophages isolated are viable and can be used for functional studies. Two immediate issues are whether these macrophages are representative of those present in glomeruli *in vivo* and whether their function is unaffected by the isolation procedure. The cell numbers obtained correlate well with the histologic appearance of the glomeruli in this model and no residual glomerular cores remain at the end of the digest, so that cell release appears to be complete. The number of leukocytes obtained per glomerulus by this method from normal kidneys is the same as that found by staining whole isolated glomeruli with anti-leukocyte monoclonal antibody.[12] For these reasons it is unlikely that there is a selective release of macrophages by our method. The issue of whether the isolation procedure affects the functional profile of the isolated cells we attempted to answer by treating control populations of macrophages with an identical enzymic regime. In fact it is likely that these cells were exposed to a more concentrated enzymic milieu because, unlike the glomerular cells, they were not partly protected by basement membrane and mesangial matrix. O_2^- production was lower in peritoneal macrophages after enzymic treatment, thus making it unlikely that the high levels produced by glomerular cells were a result of the method of isolation. Slight changes in eicosanoid production were also detected in peritoneal macrophages after enzymic treatment. Enzymic treatment produced a slight reduction in PGE_2 that was not statistically significant and in no way comparable with the sixfold reduction found in glomerular macrophages. Levels of TXB_2 and LTB_4 were actually slightly increased by treatment, whereas in glomerular cells TXB_2 production was low and LTB_4 was undetectable. Thus, the macrophages isolated from glomeruli appear to be representative of those accumulating *in vitro* and the functional profile that we have identified cannot be explained purely on the basis of the method of isolation.

Macrophages isolated from nephritic glomeruli showed enhanced release of O_2^- compared with peritoneal macrophages. Macrophage respiratory burst varies with the state of activation. In the mouse, peritoneal cells elicited with either thioglycollate or *C. parvum* show markedly enhanced release of O_2^- compared with resident peritoneal macrophages and this difference is maintained for 24 hours in culture.[5] Mechanisms by which this occurs include enhanced catalytic efficiency of the NADPH oxidase[13] and increased protein kinase activity.[14] We have found that generation of O_2^- by thioglycollate and *C. parvum* elicited cells in the rat is similar, whereas macrophages from inflamed glomeruli on day 6 of gn produced significantly more O_2^- when stimulated with zymosan than peritoneal macrophages which had undergone an identical enzyme treatment. Reactive oxygen species may be one of the principle ways in which inflammatory cells cause glomerular injury. They have been demonstrated both directly and indirectly to cause glomerular damage; most evidence comes from studies involving polymorph-mediated injury. H_2O_2 infused into the kidney with myeloperoxidase causes proteinuria and endothelial swelling.[15] Catalase prevents the proteinuria associated with intrarenal perfusion of cobra venom factor[16] or phorbol myristate acetate[17] and ameliorates the neutrophil-dependent proteinuria of heterologous phase acute nephrotoxic nephritis.[18] In rabbits proteinuria in a similar model is prevented by desferrioxamine, suggesting that the hydroxyl radical is involved in injury.[19] These experiments have not identified the source of reactive oxygen species in gn: cultured mesangial cells have been shown to produce both O_2^- and H_2O_2[20]; O_2^- generation by mesangial cells was about 30 nmol/10^6 cells/30 minutes, which is on the same order as our results for glomerular macrophages. Our results show that macrophages are a potent source of reactive oxygen species in nephritic glomeruli.

Macrophages synthesize arachidonic acid metabolites that vary in amount and type depending on their state

of activation.[21] This changing eicosanoid profile may be important in the evolution of the inflammatory response. We found that PGE_2 and LTB_4 synthesis are reduced with relative preservation of TXB_2 in glomerular macrophages, findings similar to those on immune activated mouse peritoneal macrophages.[22–24] Granuloma macrophages also have reduced PGE_2 synthesis[22] and become unresponsive to prostaglandin-inducing stimuli.[25] Rat macrophages are a potent source of eicosanoids[26] but little work has been done on cells of this species. We have found that rat peritoneal macrophages elicited by thioglycollate produce approximately three times as much PGE_2 as TXB_2 when stimulated with calcium ionophore and generate appreciable amounts of LTB_4. *C. parvum* elicited cells do not produce LTB_4 although PGE_2 and TXB_2 generation is similar to that of thioglycollate elicited cells.

The causes and effects of eicosanoid down-regulation during macrophage activation are unknown. Diminished activity of phospholipase,[22] cyclooxygenase, and lipoxygenase enzymes[24] have been implicated in down-regulation and gamma interferon (γIFN) has various regulating effects on macrophages including inhibition of arachidonic acid release[27] and inhibition of LTB_4 and LTC_4 production.[28] The effects of eicosanoid down-regulation on the *in vivo* inflammatory response are complex; eicosanoids affect a variety of different leukocyte functions, vascular responses, and activity of other chemical mediators. Reduction of PGE may be pro inflammatory in some situations, as *in vivo* administered PGE_1 suppresses macrophage-dependent inflammation[29] and in particular macrophage Ia antigen expression.[30] *In vitro* PGE_2 inhibits macrophage interleukin-1 production.[31] LTB_4 is a potent chemotactic and activating agent for polymorphonuclear leukocytes[32] and inhibition of LTB_4 may limit further leukocyte recruitment into developing inflammatory lesions.

Our results may partly explain previous findings on eicosanoid generation by whole isolated glomeruli in experimental acute hypercellular gn, particularly in relation to LTB_4. LTB_4 is synthesized in small amounts by normal isolated glomeruli,[33] possibly by resident macrophages, but is either not increased[6] or only transiently increased[34] in acute nephritic glomeruli where leukocyte infiltration greatly increases the macrophage population. This may be explained by our findings that these leukocytes, when extracted, are not producing detectable levels of LTB_4. In addition, in whole nephritic glomeruli, TXB_2 levels are raised several fold, whereas PGE_2 levels are less consistently raised.[6] This may reflect the relative preservation of TXB_2 and down-regulation of PGE_2 we find in glomerular macrophages.

In conclusion we have identified and quantitated a large macrophage influx in glomeruli, in actively-induced proliferative gn. By developing a method for extracting viable macrophages from nephritic glomeruli, we have been able for the first time to study functional changes in these cells during the course of glomerulonephritis. These cells have high Ia expression, high respiratory burst activity, and down-regulation of PGE_2 and LTB_4 production with relative preservation of TXB_2 resembling immune-activated murine macrophages. These results should increase our understanding of the ways in which macrophages participate in glomerular inflammation and suggest lymphokine generation as a possible cause of glomerular macrophage activation in this model.

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