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A Role for Mac-1 (CDIIb/CD18) in Immune Complex–stimulated Neutrophil Function In Vivo: Mac-1 Deficiency Abrogates Sustained Fcγ Receptor–dependent Neutrophil Adhesion and Complement-dependent Proteinuria in Acute Glomerulonephritis

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Summary

Mac-1 ($\alpha_m\beta_2$), a leukocyte adhesion receptor, has been shown in vitro to functionally interact with Fcγ receptors to facilitate immune complex (IC)–stimulated polymorphonuclear neutrophil (PMN) functions. To investigate the relevance of Mac-1–FcγR interactions in IC-mediated injury in vivo, we induced a model of Fc-dependent anti–glomerular basement membrane (GBM) nephritis in wild-type and Mac-1–deficient mice by the intravenous injection of anti-GBM antibody. The initial glomerular PMN accumulation was equivalent in Mac-1 null and wild-type mice, but thereafter increased in wild-type and decreased in mutant mice. The absence of Mac-1 interactions with obvious ligands, intercellular adhesion molecule 1 (ICAM-1), and C3 complement, is not responsible for the decrease in neutrophil accumulation in Mac-1–deficient mice since glomerular PMN accumulation in mice deficient in these ligands was comparable to those in wild-type mice. In vitro studies showed that spreading of Mac-1–null PMNs to IC-coated dishes was equivalent to that of wild-type PMNs at 5–12 min but was markedly reduced thereafter, and was associated with an inability of mutant neutrophils to redistribute filamentous actin. This suggests that in vivo, Mac-1 is not required for the initiation of Fc-mediated PMN recruitment but that Mac-1–FcγR interactions are required for filamentous actin reorganization leading to sustained PMN adhesion, and this represents the first demonstration of the relevance of Mac-1–FcγR interactions in vivo. PMN-dependent proteinuria, maximal in wild-type mice at 8 h, was absent in Mac-1 mutant mice at all time points. Complement C3–deficient mice also had significantly decreased proteinuria compared to wild-type mice. Since Mac-1 on PMNs is the principal ligand for ic3b, an absence of Mac-1 interaction with C3 probably contributed to the abrogation of proteinuria in Mac-1–null mice.

Mac-1 ($\alpha_m\beta_2$, CD11b/CD18, and complement receptor type 3), a β_2 integrin present primarily on granulocytes and monocytes, binds intercellular adhesion molecule 1 (ICAM-1),[1] an endothelial leukocyte adhesion receptor, complement C3 fragment C3bi, matrix molecule heparin, and coagulation factors fibrinogen and factor X. It mediates several adhesion-dependent processes in leukocytes, such as adhesion to the endothelium, phagocytosis, superoxide production, and other activation events (1). We have recently demonstrated that mice rendered genetically deficient in Mac-1 are severely compromised in chemoattractant leukotriene B_4 (LTB_4)–induced leukocyte adhesion to the vessel wall in vivo (2). Mac-1–deficient murine polymorphonuclear neutrophils (PMNs), are unable to phagocytose complement-opsonized particles, have reduced spreading and oxygen radical generation compared to normal PMNs, and show an unanticipated defect in PMN apoptosis (2). The role of Mac-1 in these functions presumably contributes to the abnormal adhesion, spreading, phagocytosis and generation of the oxidative burst in PMNs of patients

[1]*Abbreviations used in this paper:* F-actin, filamentous actin; IC, immune complex; ICAM-1, intercellular adhesion molecule 1; GBM, glomerular basement membrane; LTB_4, leukotriene B_4; PMN, polymorphonuclear neutrophil.

Part of this work was previously presented as an abstract (1996. *J. Vasc. Res.* 33[S1]:A393).

with leukocyte adhesion deficiency type 1 (LAD-1), a disease resulting from a congenital deficiency in β_2 integrins (1).

Mac-1 also cooperates with FcγR to mediate a number of neutrophil functional responses after engagement of FcγR with immune complexes (ICs). These include IC-stimulated phagocytosis, adhesion, and tyrosine phosphorylation (3–8). Mac-1 probably mediates these processes by directly interacting with FcγR on the neutrophil surface (9–11). This interaction occurs at a site distinct from the ligand binding A domain of Mac-1, probably through the COOH-terminal lectin-like domain (9). Mac-1 also associates with the cytoskeleton during neutrophil interaction with ICs (5, 7, 10, 12), which may promote IC-stimulated PMN functions.

Although the role of Mac-1 in facilitating FcγR-IgG effector functions has been described in vitro, the in vivo relevance of this interaction has not been previously examined. We therefore assessed the role of Mac-1 in acute, passive, heterologous anti–glomerular basement membrane (GBM) nephritis in which immobilized GBM–anti-GBM ICs trigger rapid glomerular PMN accumulation and PMN-dependent leakage of albumin into the urine (13, 14). Importantly, in this model, glomerular neutrophil recruitment is Fc-dependent, since $(Fab)_2$ fragments of this antibody do not promote neutrophil accumulation (14). Neutrophil accumulation is largely complement-independent since C5a-deficient mice and cobra venom factor–treated animals still exhibit PMN influx (14, 15). PMN accumulation is transient: PMNs remain adherent to the lumen of IC-coated vessels ("frustrated phagocytosis") but then presumably detach and return to the blood stream (16) to meet their fate in the spleen or liver. The observed proteinuria has been ascribed to cathepsin G and elastase released from PMNs accumulated in the glomeruli (17).

In this paper we show that Mac-1 deficiency abrogates the peak PMN accumulation, occurring at 2 h in this model, and protects against proteinuria at all time points. We present in vitro evidence suggesting that the decrease in neutrophil accumulation in Mac-1–deficient mice is due to an absence of Mac-1–FcγR interactions at the neutrophil cell surface which are required for firm attachment and spreading of neutrophils on ICs. We also report that proteinuria is complement dependent. This suggests that the lack of proteinuria in the Mac-1–null mice may result from the lack of Mac-1 interaction with the ic3b fragment of complement C3, a well described ligand for Mac-1.

Materials and Methods

Animals and Experimental Protocols. Mac-1–deficient mice were recently generated by standard gene targeting techniques (2). These mice and their wild-type mates are a mixed strain of 129SV and C57Bl/6. They are bred and maintained in a virus-antibody free facility at the Longwood Medical Research Center (Boston, MA). Experimental animals were 8–14 wk of age and were sex-matched. Both males and females were used. 25% of the experimental mice used at each time point were littermates from heterozygous matings. The remainder were the progeny of wild-type and Mac-1–null homozygous matings. Previously generated mice deficient in ICAM-1 (18) were of a pure C57Bl/6 background strain, were 8 wk old, and were age- and sex-matched with their wild-type mates. Mice deficient in complement C3 and C4 were 7–8-wk-old females of a mixed 129Sv and C57Bl/6 strain. ICAM-1 and complement-deficient mice were bred and maintained in a virus-antibody free facility at Harvard Medical School.

Anti-GBM nephritis was induced essentially as previously described by our group (13). In brief, preimmune urine was collected ~1 h before injection of 0.25 ml anti-GBM antiserum per mouse (14). At 1, 2, 4, 8, and 18 h after the injection, urine and blood samples (by retroorbital bleed) were collected. Animals were then killed and both kidneys were harvested.

Histologic Studies. Kidneys were fixed in 4% paraformaldehyde, paraffin-embedded or frozen in OCT compound embedding medium (Miles, Inc., Elkhart, IN), and sectioned. 5-μM paraffin-embedded sections were stained for dichloroacetate esterase to identify PMNs and tissue was counterstained with nuclear-fast red (19). The number of PMNs in 100 glomeruli (50/each kidney) in cross-sections were counted per animal without prior knowledge of the genotype of the animal. For electron microscopy, glomeruli from one wild-type and one Mac-1–null nephritic animal, 1 h after anti-GBM antibody injection, were examined by standard techniques. 15 wild-type and Mac-1–null intraglomerular capillary PMNs were examined.

Rabbit anti-GBM antiserum deposition in mouse glomeruli was quantitated by immunofluorescence. 4 μM frozen sections prepared from kidneys harvested from wild-type and mutant mice 1 h after anti-GBM injections were incubated with a 1:200 and 1:350 dilution of FITC-tagged secondary antibody (goat anti–rabbit, Vector Laboratories Inc., Burlingame, CA). These dilutions were saturating as determined by the following method: secondary antibody dilutions ranging from 1:100 to 1:5,000 were applied to wild-type kidney sections and the fluorescence images from stained sections were acquired for 0.1 s using Oncor Image Analysis software (Oncor, Inc., Gaithersburg, MD), in combination with a Nikon microphot-FXA microscope and a 12-bit cooled CCD SenSys camera (Photometrics, Tucson, AZ), to obtain digital images. These digital images were then processed using a Optimas Image analysis program. An index of fluorescence specifically associated with the glomeruli was calculated using an Optimas program (designed by Edward Marcus, Ed Marcus Laboratories, Boston, MA) which calculates the following: average luminance (filtered) which is defined as the sum of gray levels/number of pixels in the selected region both within selected glomeruli (Fg), and in the area within the tubules that is designated as the background (Bg). The filter function of the program is applied to smooth small luminance variations, before detection of the primary luminance peak. This was necessary because measurement of the staining intensity within the glomeruli might be affected by FITC-stained glomerular capillaries present within an unstained background. The ratio of Fg over Bg (Fg/Bg) was used as an index of fluorescence specifically associated with the glomeruli. Plotting of these indices obtained for the serial dilutions of secondary antibody assured that secondary antibody dilutions of 1:200 and 1:350 were saturating. Glomerular cross-sections of wild-type and mutant mice were processed with secondary antibody dilutions of both these dilutions and analyzed as described above. These dilutions gave similar results, therefore only data obtained from the 1:200 dilution is shown.

Determination of Urinary Albumin and the Creatinine Concentration. Urinary albumin was determined by a double-sandwich ELISA as previously detailed (13). Urinary creatinine was quanti-

tated spectrophotometrically using a commercially available kit (Sigma Chemical Co., St. Louis, MO). To standardize urine albumin excretion for glomerular filtration rate, proteinuria was expressed as microgram of urinary albumin per milligram of urinary creatinine (20).

Isolation of murine PMNs. Peripheral blood was obtained by retroorbital sampling and was collected in 5 mM EDTA. Blood samples from 6 to 7 mice, typically yielding 6–7 ml of blood, were pooled and layered over a Nim.2 neutrophil isolation medium according to manufacturer's protocols (Cardinal Associates, Santa Fe, NM), and referred to as one experimental sample. Red cells contaminating the neutrophil preparation were lysed with ice-cold 0.2% NaCl. Neutrophils were resuspended in RPMI medium.

Adhesion Assay. BSA–anti-BSA ICs and BSA were immobilized on glass coverslips. According to the manufacturer's technical information, the BSA (a 35% solution, ICN Pharmaceuticals, Inc., Costa Mesa, CA) was heat-shocked, and contained no vitronectin or fibrinogen. Glass coverslips (12 × 12 cm) were treated with 0.1 mg/ml poly-D-lysine (mol wt ≥ 70,000, Sigma Chemical Co.) for 1 h, treated with 2.5% glutaraldehyde for 15 min, and washed extensively in ddH_2O followed by two PBS washes. Coverslips were then coated with 1 mg/ml BSA for 30 min, washed twice in PBS, incubated with 0.1 M glycine for 2 h to quench aldehyde groups, and then incubated with 20 μg of rabbit anti-BSA IgG (Sigma Chem. Co., St. Louis, MO) in 0.5 ml of PBS for 1 h. This concentration of anti-BSA IgG has been previously shown to yield maximum adhesion of human PMNs to ICs and LTB_4 release from these cells (21). All these procedures were done at room temperature.

Isolated murine PMNs ($3–5 \times 10^5$cells/well, in 0.6 ml RPMI) were placed in 12-well dishes containing coverslips and incubated in a 5% CO_2 incubator at 37°C. At indicated time points, coverslips were washed twice in PBS, fixed with 1.25% glutaraldehyde, stained with Giemsa, and mounted on slides with permount. Adherent PMNs were quantitated as follows: the total number of adherent PMNs in 10 high (×40) powered fields (HPF) was determined and expressed as number of adherent cells per HPF. This number was then converted to the percentage of control binding (set at 100%), which was that observed for wild-type PMNs incubated with IC-coated coverslips for 5 min. Therefore, adherent cells (% control) = (total bound cells per HPF)/(total bound cells per HPF at 5 min for wild-type) × 100%. Spread PMNs were identified as cells with abundant cytoplasm and a flattened nucleus and the percentage of spread was determined as explained above for determining percentage of adherent PMNs. This conversion to percentage of control was necessary since the number of murine PMNs isolated from wild-type and mutant mice varied between individual experiments, although within the same experiment equal numbers of wild-type and mutant PMNs were used. Adherence of mouse PMNs to IC and BSA was very efficient. For example, for wild-type PMNs, after a 5 and 40 min incubation the number of PMNs adherent to IC-coated coverslips (144 mm^2) was $1.9 \pm 0.4 \times 10^5$ and $3.2 \pm 0.2 \times 10^5$, respectively. This represents ~44 ± 4 and 82 ± 21% of the total number of PMNs applied to the coverslip. The number of wild-type PMNs adherent to coverslips coated with BSA alone after 5 and 40 min incubations was $2.1 \pm 0.5 \times 10^5$ and $2.8 \pm 0.5 \times 10^5$, respectively, and represented 40 ± 4 and 59 ± 9% of the total number of PMNs applied to the coverslip.

F-Actin Staining. PMNs that adhered to IC-coated glass coverslips for 5 or 40 minutes were stained with rhodamine phalloidin (Molecular Probes, Inc., Eugene, OR) according to manufacturer's protocols. In brief, coverslips were washed in PBS, fixed in 3.7% formaldehyde/PBS for 10 min, and extracted for 3–5 min on ice with 0.1% Triton X-100 buffer. Coverslips were then washed in PBS and incubated with 160 nM rhodamine phalloidin in PBS for 20 min at room temperature. Coverslips were mounted with Cytoseal (Stephens Scientific, Riverdale, NJ) and stored at 4°C.

Quantitation of LTB_4. LTB_4 levels in media collected from PMNs adherent to IC-coated surfaces were quantitated using an ELISA assay for murine LTB_4 (PerSeptive Diagnostics, Cambridge, MA). According to manufacturer's data, the ELISA assay has a detection limit of 8.9 pg/ml. Its cross-reactivity with other major arachidonic acid products, e.g., LTC_4, LTD_4, LTE_4, 5-HETE (hydroxy eicosatetraenoic acid), 12-HETE, and 15-HETE, is less than 1%, as is its cross-activity with LTB_4 degradation products 20-carboxy-LTB_4 and 20-hydroxy-LTB_4.

At the indicated time points, medium was collected into ice-cold eppendorf tubes and spun at 750 *g* (4°C) for 10 min to remove cells. The supernatants were transferred to fresh ice-cold tubes and the ELISA assay was performed in duplicate following the manufacturer's protocol.

Statistics. Data are expressed as mean ± SEM. Unpaired student's *t* tests were performed for data and $P < 0.05$ was considered statistically significant.

Results

Mac-1 Is Required for Glomerular PMN Accumulation and Proteinuria in Anti-GBM Nephritis. We injected anti-GBM antibody into Mac-1–deficient mice and their wild-type counterparts and measured glomerular neutrophil accumulation and proteinuria. 1 h after anti-serum injection, neutrophil glomerular accumulation was the same in null and wild-type mice, suggesting that initial PMN recruitment is unaffected by Mac-1 deficiency. In contrast, at 2 h, the peak of PMN accumulation in wild-type animals, there was a significant reduction in the number of glomerular PMNs present in null mice compared with wild-type. The difference in glomerular PMN counts between wild-type and mutant mice persisted at 4 h but by 18 h the counts declined to baseline levels in mice of both genotypes (Fig. 1 *A*). The peripheral blood PMN counts were similar in wild-type and null mice at each experimental time point (data not shown).

The PMN accumulation in the glomeruli of wild-type mice was associated with significant proteinuria. Proteinuria appeared at 4 h in wild-type animals and peaked at 8 h. In contrast, Mac-1–deficient mice failed to develop proteinuria at any of the time points examined (Fig. 1 *B*). Proteinuria is transient in this model and declined in the wild-type mice by 18 h. At this time point, proteinuria in the mutant mice was still negligible and was significantly reduced compared to wild-type.

The decreased PMN accumulation in null mice cannot be attributed to reduced binding of anti-GBM antibody in the glomeruli of these mice since a quantitative immunofluorescence analysis of anti-GBM deposition in the glomeruli revealed no differences between wild-type and null mice (Table 1). This conclusion is also supported by the

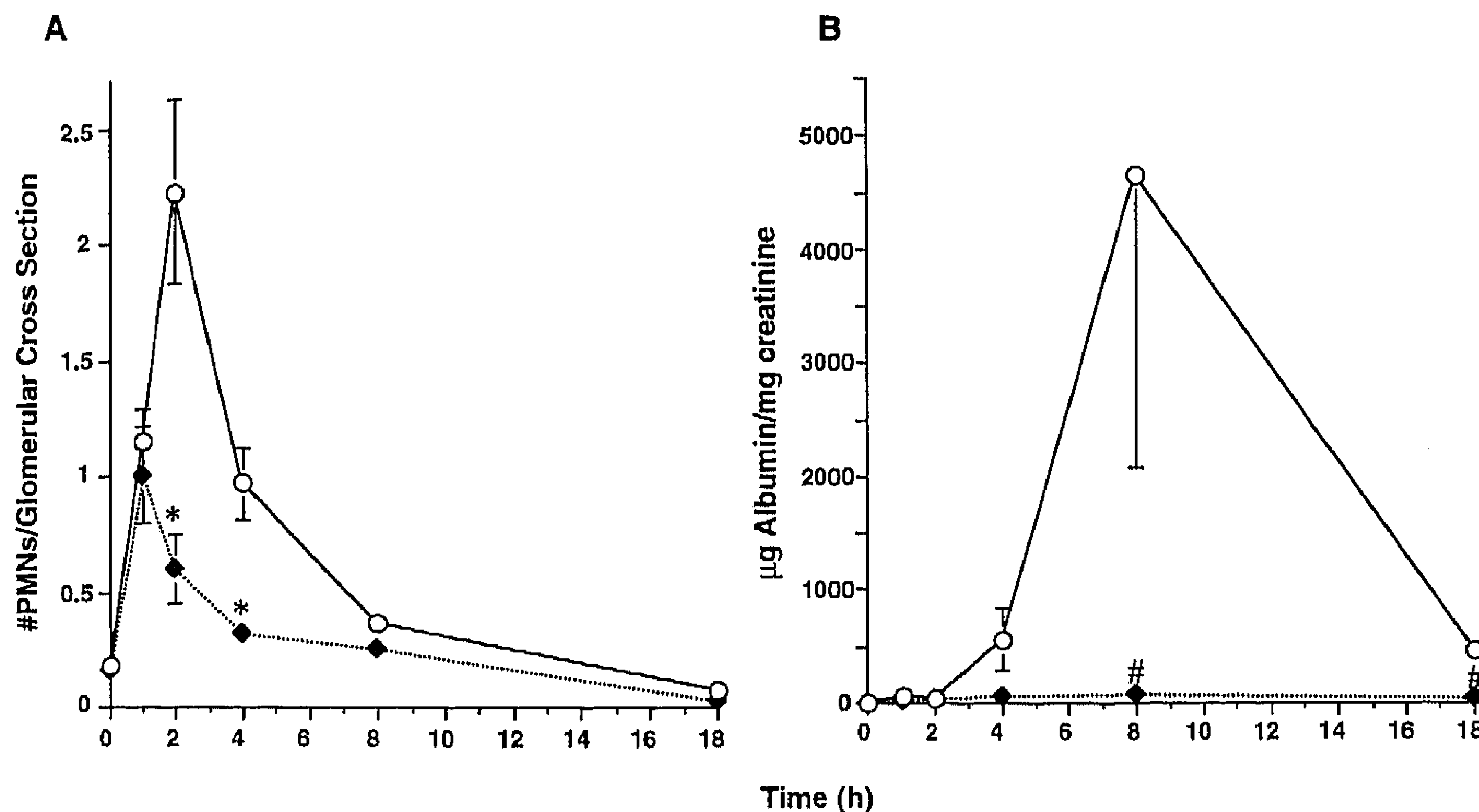


Figure 1. Time course of glomerular PMN accumulation and proteinuria. The number of PMNs per glomerular cross section in Mac-1–null (*diamonds*) and wild-type (*circles*) mice were assessed on esterase stained sections (*A*). Urine albumin excretion (µg) was determined and expressed per mg of urinary creatinine to standardize for glomerular filtration rate (*B*). Glomerular PMN accumulation in wild-type and null mice was comparable at 1 h, and had increased in wild-type mice by 2 h but decreased significantly at 2 and 4 h in the Mac-1–null mice. Strikingly, proteinuria was absent in Mac-1–null mice at all time points. *n* = 7–10 mice/genotype at time points 0–8 h and *n* = 4 mice/genotype at the 18 h time point. $^{*}P < 0.005$, $^{\#}P < 0.05$ compared to wild-type mice.

fact that neutrophil recruitment at 1 h after anti-GBM injection (Fig. 1 *A*) was similar in wild-type and mutant mice.

Despite the Absence of Proteinuria, Endothelial Damage Is Evident in Glomeruli of Mutant Mice. Glomeruli of kidneys were examined by electron microscopy 1 h after anti-GBM injection. In mice of both genotypes, PMNs were observed either unattached in the capillary lumen or making contacts of varying extents with the GBM. The latter was associated with denudation of the fenestrated endothelium (Fig. 2), suggesting that Mac-1 deficiency does not protect against endothelial denudation by PMNs and that such damage is not responsible for the proteinuria in this model. However, the lack of proteinuria in Mac-1–deficient mice observed in Fig. 1 *B* suggests that mutant neutrophils are incapable of inducing functional GBM damage leading to proteinuria, which is characteristic of wild-type mice.

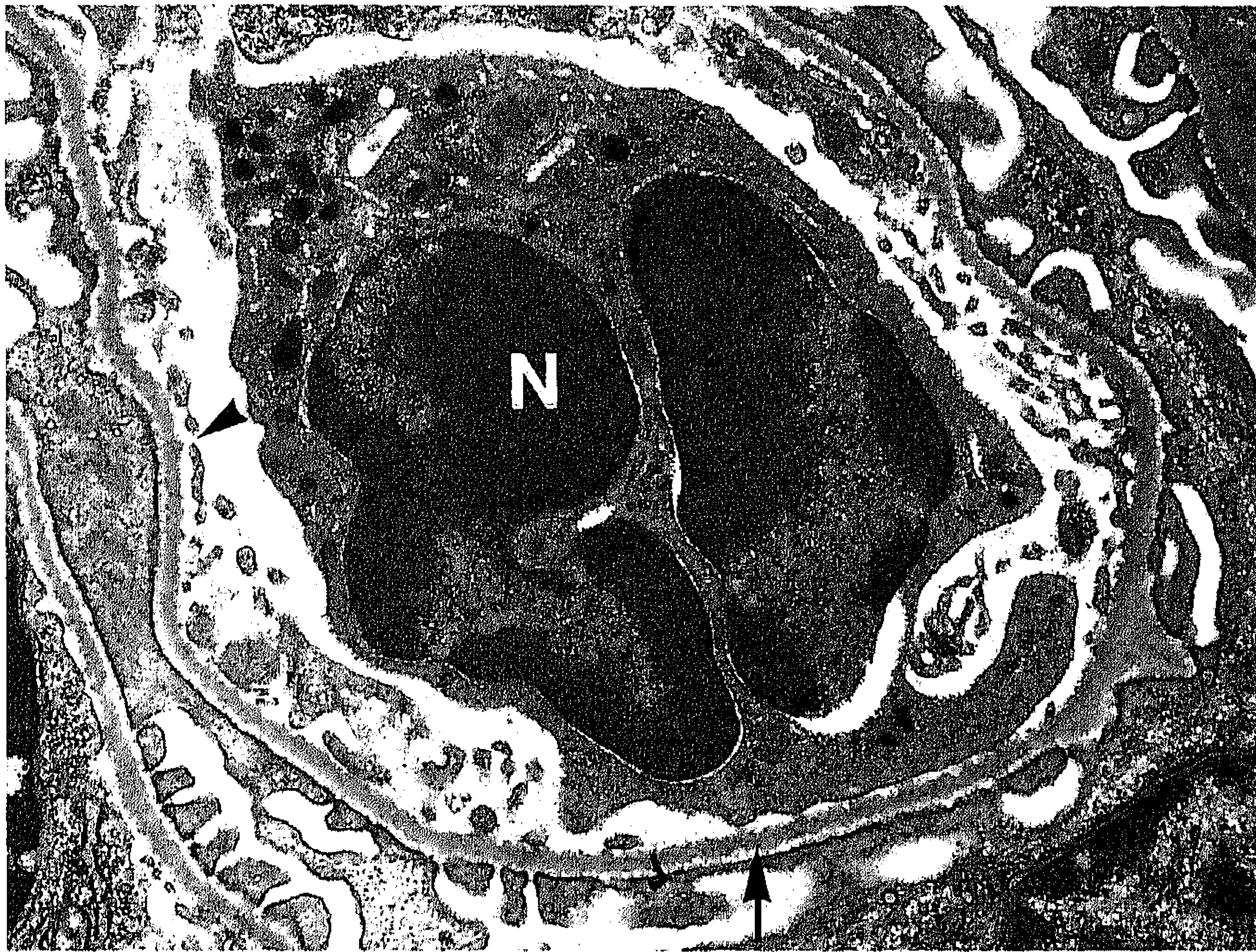
Table 1. *Anti-GBM Antibody Deposition in Wild-type and Mac-1–deficient Glomeruli*

Genotype	Binding index	Average ± SEM
Wild-type		
WT1	14.4	
WT2	10.7	
WT3	8.7	
		11.3 ± 1.7
Mac-1–deficient		
KO1	15.7	
KO2	13.7	
KO3	12.2	
		13.9 ± 1.0

Anti-GBM antibody binding index 1 h after intravenous injection of anti-GBM antiserum. Frozen kidney sections from wild-type and mutant mice were incubated with saturating concentration of FITC-tagged goat anti–rabbit secondary antibody. Fluorescent images were digitalized and processed with an Optima program. The binding index was defined as the average fluorescence associated with the glomeruli divided by that associated within the tubules. Data are averaged measurements from two sections, four to six glomeruli/animal. There was no significant difference in the index associated with glomeruli of wild-type and mutant mice.

Mac-1 Ligands, Complement C3, and ICAM-1 Are Not Responsible for the Reduced Glomerular PMN Accumulation in Mac-1–null Mice. Divalent cation-dependent Mac-1 adhesion to ICAM-1 and iC3b occurs through the A domain of Mac-1 (1). To determine whether the engagement of Mac-1 with complement or ICAM-1 contributes to the reduction in PMN accumulation in Mac-1–null mice 2 h after anti-GBM antiserum injection, groups of mice genetically deficient in C3 (the central component in complement activation), C4 (the classical pathway of complement activation triggered by antigen–antibody complexes), or ICAM-1 were examined 2 h after injection of anti-GBM antiserum. We observed no significant differences in glomerular PMN counts between ICAM-1–deficient mice and their wild-type mates, or C3- and C4-deficient mice and wild-type mice (Fig. 3). These data confirm that neutrophil accumulation is complement-independent (14, 15) and suggest that known Mac-1 ligands that are most likely to support neutrophil adhesion to the vessel wall do not play a role in this process.

However, proteinuria in the C3-deficient mice was significantly reduced compared to their wild-type counterparts at the 8 h time point (Fig. 3). We have previously shown that Mac-1 is the predominant receptor for C3bi since phagocytosis of complement opsonized particles was completely absent in neutrophils isolated from Mac-1–defi-



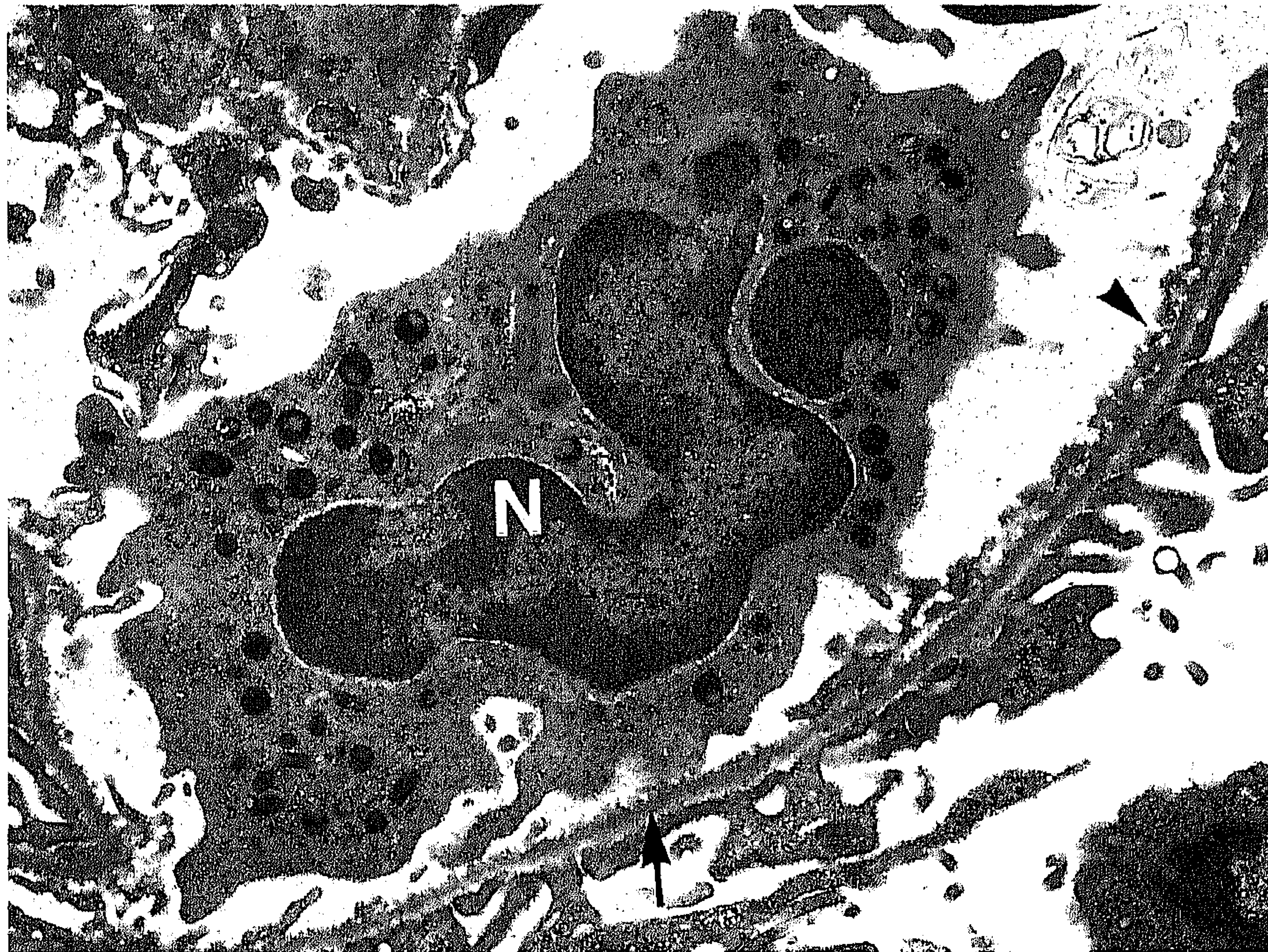


Figure 2. Electron micrographs of nephritic wild-type and Mac-1–null mice kidneys 1 h after the induction of anti-GBM nephritis. Representative pictures of intraglomerular capillary wild-type (*top*) and Mac-1–null (*bottom*) neutrophils are shown. Both sets of glomeruli showed denudation of the fenestrated endothelium and had PMNs directly in contact with the GBM. *Arrowhead*, fenestrated endothelium; *arrow*, GBM; N, Neutrophils. Original magnification: WT, ×15,500; Mac-1–null, ×13,500.

cient mice (2). This suggests that interaction of Mac-1 on PMNs with complement fragment ic3b leads to proteinuria.

The next series of in vitro experiments were undertaken to address the following hypothesis: equal glomerular PMN accumulation in wild-type and mutant mice at 1 h is driven by neutrophil Fc receptors engaging immobilized IC, since this model is Fc-dependent, but Mac-1 may be required for subsequent sustained adhesion and elaboration of chemokines that promote further PMN recruitment in wild-type animals at the 2 h time point (Fig. 1 *A*).

Sustained Neutrophil Interaction with IC-coated Surfaces Requires Mac-1. We examined adhesion and spreading of wild-type and Mac-1–null PMNs on IC-coated surfaces. To mimic the GBM–anti-GBM interactions in vitro, slides were coated with BSA–anti-BSA IgG complexes. The IC-coated surfaces were incubated with resting peripheral blood PMNs isolated from wild-type and null mice (under static conditions) in the absence of serum, thus eliminating potential effects of complement in the assay. The total number of adherent cells was measured at 5, 12, 25, and 40 min. A similar analysis was conducted on PMNs incubated with BSA-coated surfaces alone to discern effects due to BSA from those that were IC-specific. A time course of total binding of PMNs to IC-coated surfaces revealed that initial adhesion to IC, within 5 and 12 min after addition of PMNs, was similar in mutant and wild-type mice (Fig. 4).

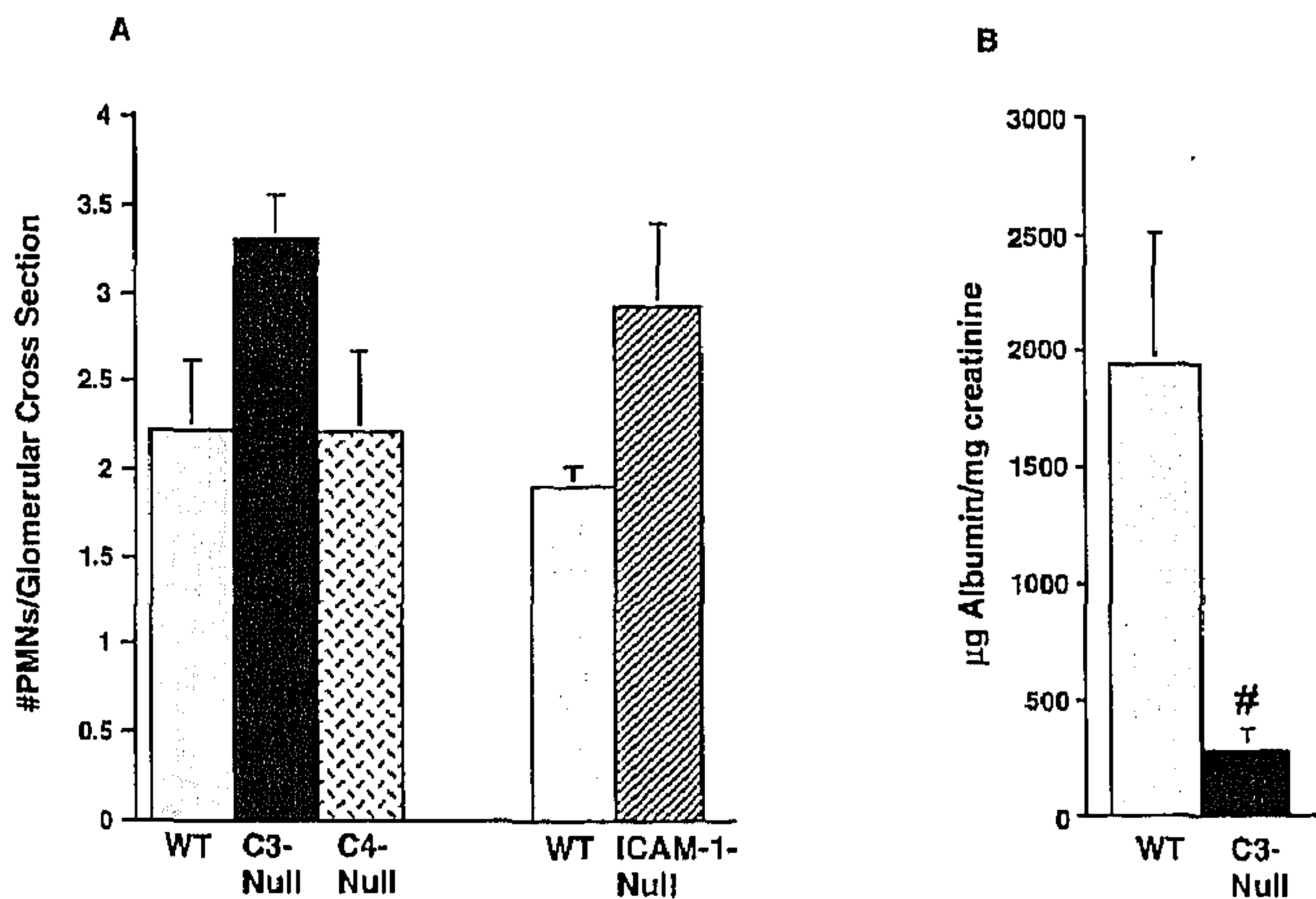


Figure 3. Glomerular PMN accumulation in mice deficient in ICAM-1 and complement C3 or C4 (*A*), and proteinuria in C3-deficient mice (*B*). Anti-GBM nephritis was induced in the aforementioned mice and their wild-type counterparts, and glomerular PMN accumulation was determined 2 h after the induction of nephritis. The number of PMNs per glomerular cross-section was not significantly different between any group of animals and their controls. Proteinuria was examined in C3-deficient mice and their wild-type counterparts 8 h after induction of nephritis. C3-null mice had significantly less proteinuria than wild-types. $n = 4$ for each set of wild-type and complement knockout mice. $n = 6$ for wild-type and ICAM-1–null mice. #$P < 0.05$.

At 25 and 40 min, wild-type PMNs continued to accumulate on the IC, leading to a >50% increase over that seen at 12 min. In Mac-1–null PMNs, the number of IC-adherent PMNs remained the same over a similar time course. Incubation times >40 min did not lead to further enhancement in numbers of adherent PMNs in either wild-type or mutant samples (data not shown). The number of wild-type and mutant PMNs adherent to BSA at 5 or 40 min was similar in wild-type and null cells, suggesting that the observed Mac-1–mediated effects are specific for IC-coated surfaces (Fig. 4).

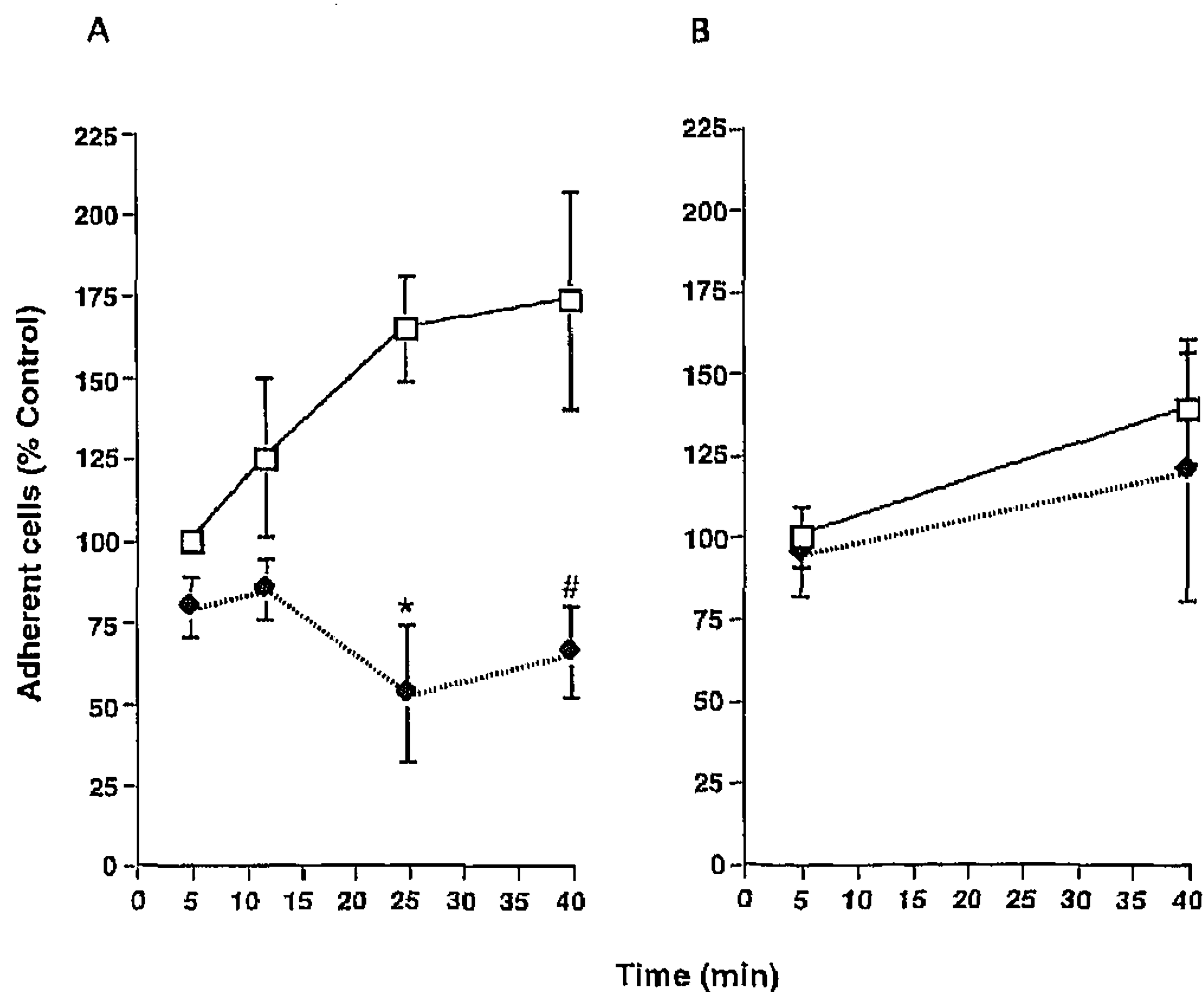


Figure 4. PMN adhesion to ICs. Isolated wild-type (*squares*) or Mac-1–null (*diamonds*) PMNs were incubated with BSA–anti-BSA ICs (*A*) or BSA alone (*B*), and fixed and stained with Wright-Giemsa. The percentage of adhesion (i.e., the percentage of control that was set as 100% for the adhesion to IC present at 5 min in wild-type cells) was similar in cells of both genotypes at 5 and 12 min but decreased significantly in Mac-1–null cells at 25 and 40 min. No difference was seen between the percentage of wild-type and null cells adherent to BSA alone. $n = 7$ experiments. *$P < 0.005$, #$P < 0.05$ compared to wild-type cells.

Sustained PMN Spreading and Filamentous Actin Reorganization on IC Requires Mac-1. To determine whether spreading of mutant PMNs on IC was compromised, we determined the percentage of spread cells in the samples generated in Fig. 4. Initial spreading on ICs was the same for wild-type and Mac-1–null cells at 5 min. The percentage of spread cells increased over time in wild-type cells (Fig. 5 *A*), consistent with the increased numbers of PMNs adherent to the IC at these later time points as seen in Fig. 4. On the other hand, the percentage of mutant PMNs spread on IC declined rapidly over time and was <10% of wild-type levels at 40 min (Fig. 5 *A*) and <0.03% at 120 min (data not shown). On BSA-coated surfaces, a difference in spreading was observed between wild-type and null cells at 5 min and remained constant up to 40 min (Fig. 5 *B*). This is consistent with previous studies that indicate a role for Mac-1 in spreading on BSA (1). The equivalent spreading on ICs at 5 min versus the difference in spreading seen on BSA-coated surfaces at this time point again distinguishes Mac-1–mediated effects on these two substrates.

To pursue the underlying cause for the decrease in PMN spreading in mutant neutrophils, we examined actin distribution by staining the IC-adherent PMNs for filamentous actin (F-actin) (Fig. 5 *C*). At 5 min, F-actin staining was observed in the periphery of both wild-type and mutant neutrophils. By 40 min, wild-type PMNs had extended protrusions, increased cell size (spreading), and had a polarized as well as central punctate F-actin staining. In contrast, Mac-1–null PMNs at this time point were contracted and were much smaller than wild-type PMNs (which indicates a lack of spreading). Several of the rounded Mac-1–null PMNs detached from the dish during processing of the sample for rhodamine phalloidin staining. The few that remained at 40 min were the small percentage of spread cells that had an actin distribution identical to PMNs adherent to IC only for 5 min. We conclude that the actin redistribution associated with sustained adhesion is compromised in Mac-1–deficient PMNs.

PMN LTB_4 Production Is the Same in Wild-type and Mutant PMNs Adherent to IC. To assess whether the decrease in PMN accumulation at 2 h is caused by inhibition of secretion of a chemoattractant by mutant PMNs, we examined the release of an arachidonic acid metabolite LTB_4, since a previous study had shown that β_2 integrins are required for IC-stimulated LTB_4 production (21). In addition, LTB_4 has been linked to the activation and morphologic polarization of PMNs and therefore may be required for sustaining PMN spreading. We measured LTB_4 secreted by wild-type and mutant PMNs bound to IC-coated surfaces by an ELISA assay for murine LTB_4. In wild-type PMNs, LTB_4 was detected in the media at 5 min after neutrophil interac-

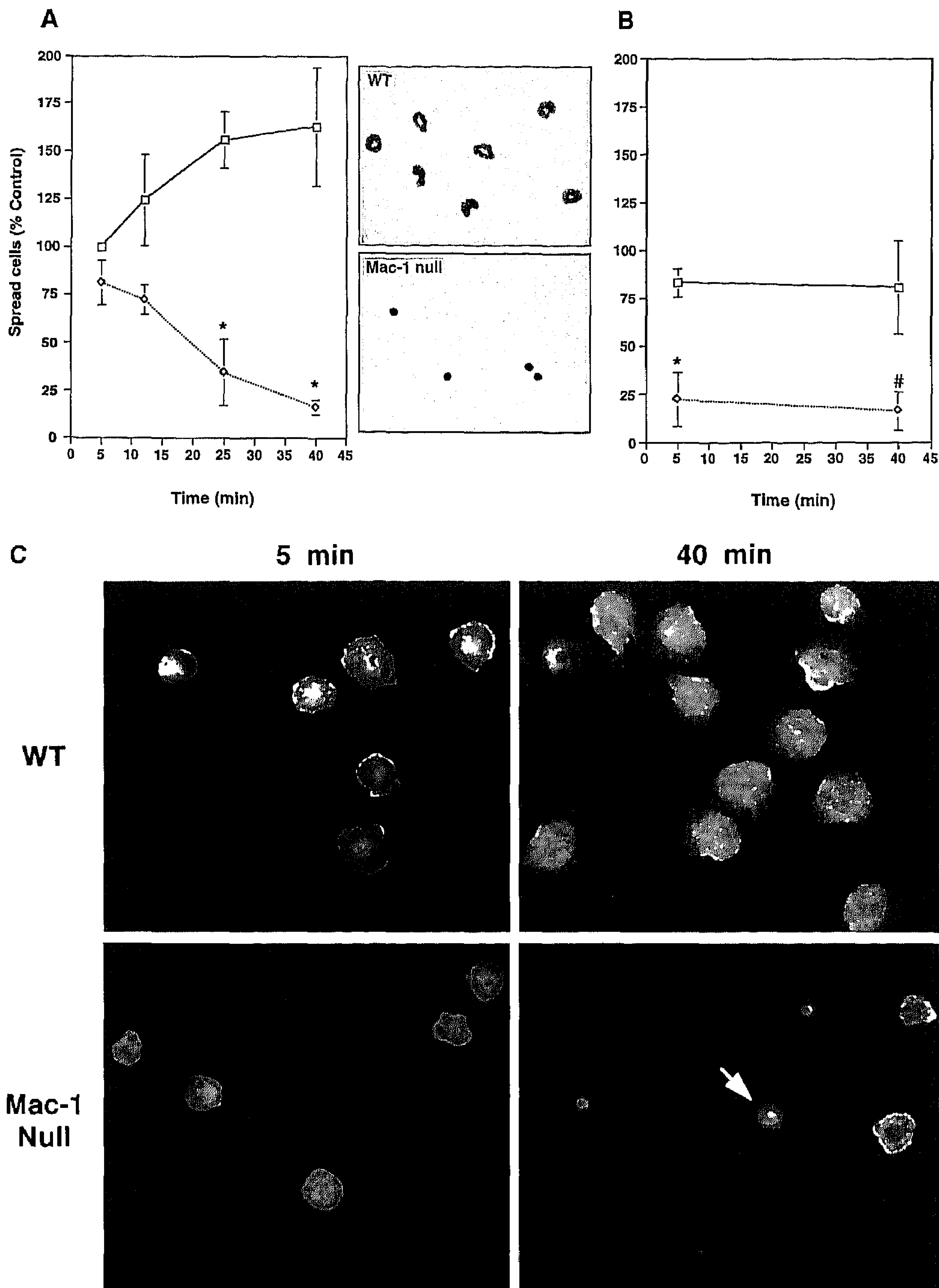


Figure 5. PMN spreading on ICs. Samples analyzed in Fig. 4 were also analyzed for PMN spreading on ICs (*A*) or BSA alone (*B*) and parallel samples were analyzed for F-actin distribution (*C*). The spreading in Mac-1–null cells (*diamonds*) and wild-type cells (*squares*) was equivalent at 5 min, but was dramatically reduced at 25 and 40 min in null cells. Representative Wright Giemsa–stained slides from the 40 min time point are shown. Spreading on BSA was significantly different between wild-type and null PMNs at 5 min and remained so at 40 minutes. The difference in profiles of PMN spreading on ICs and BSA alone indicates the Mac-1 effects on PMN spreading that are IC-specific. *P <0.005, #P <0.05 compared to wild-type cells. (*C*) Wild-type (WT) and Mac-1–null cells adherent to ICs for 5 and 40 min were stained with rhodamine phalloidin to identify F-actin. At 5 min, cells of both genotypes initiated actin polymerization at the periphery of the cells. By 40 min, wild-type PMNs had developed an actin-rich surface with a punctate cytoplasmic pattern and had visible projections. In contrast, mutant cells had not progressed to this stage and several of them had rounded up (*arrow*). Original magnification: ×473.

tion with IC-coated surfaces and had increased 25-fold by 40 min (Fig. 6). We detected no significant differences in the amount of LTB_4 released by wild-type and mutant PMNs at all time points tested, suggesting that Mac-1 is not required for LTB_4 production.

Discussion

This study demonstrates an important role for Mac-1 in glomerular PMN accumulation and proteinuria following the formation of ICs in the GBM. This is consistent with conclusions of previous studies using functional blocking antibodies to Mac-1 in acute glomerulonephritis models in rats (22, 23). Importantly, in our work, a study of the kinetics of PMN accumulation revealed that Mac-1 is not required for the initial PMN influx at 1 h but is essential for further PMN accumulation and PMN-dependent proteinuria. Furthermore, since this model is Fc-dependent (14), our results suggest a role for Mac-1 in events downstream of the initial Fc-dependent neutrophil recruitment. These downstream events are probably not related to the ability of Mac-1

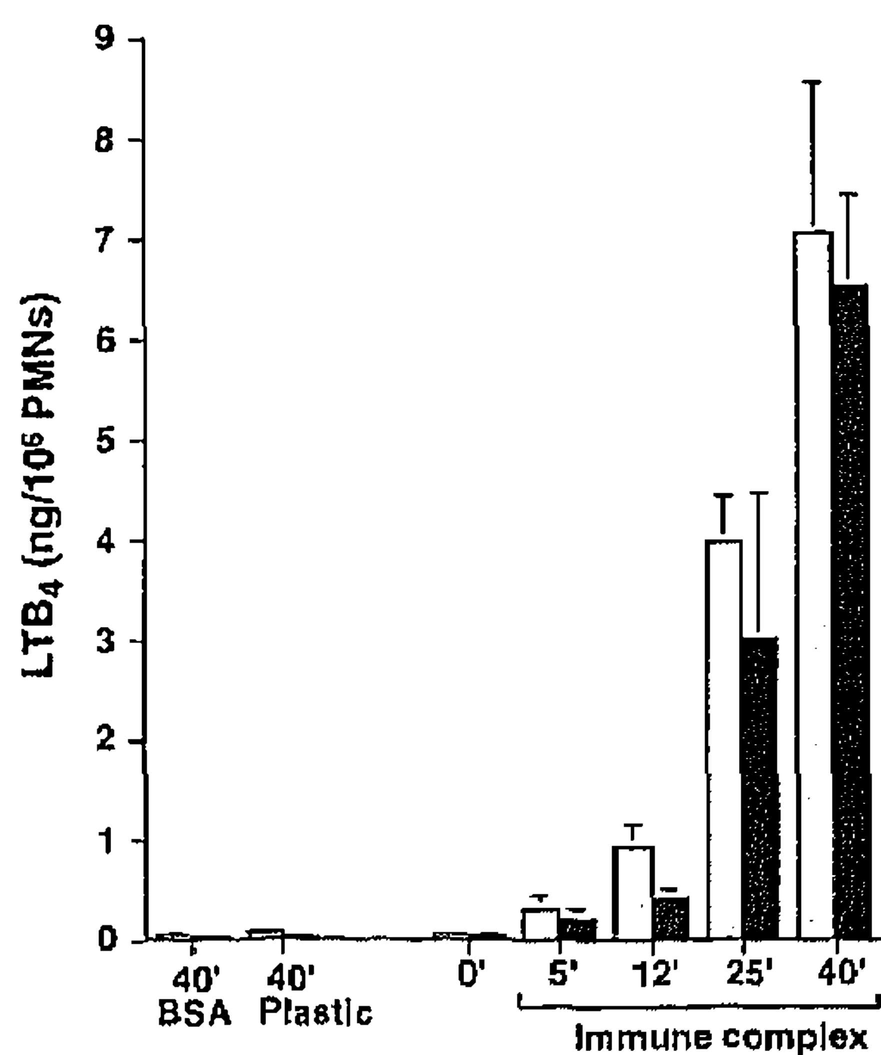


Figure 6. Release of LTB_4 from PMNs incubated with immobilized ICs. The amount of LTB_4 released by wild-type (□) and Mac-1–deficient (■) PMNs incubated with ICs was assessed over the time course used for PMN adhesion and spreading. LTB_4 was rapidly released (within 5 min) from PMNs adherent to ICs and accumulated over time. PMNs on ice, adherent for 40 min to plastic or BSA (12–40 min) did not release LTB_4, demonstrating that its release is stimulated specifically by ICs. There was no significant difference in LTB_4 release between wild-type and null cells incubated with ICs. n = 3–5 experiments/time point.

to function as an adhesion receptor for obvious ligands: mice deficient in Mac-1 ligands, ICAM-1, or complement had glomerular PMN accumulation comparable to wild-type mice. This is consistent with other studies which show that PMN adhesion to the glomerular endothelium under nonstatic conditions does not require Mac-1 (24). It is not possible to make analogies of the FcγR–Mac-1–mediated PMN recruitment model in glomerular capillaries with the multistep paradigm of selectin-induced PMN rolling, which is most relevant at high shear stress (25, 26), followed by firm integrin–dependent adhesion seen in venules. This is because the hemodynamic forces in the glomerular capillaries are different than in venules. Additionally, these forces are known to be significantly altered during glomerulonephritis, with no measurements of shear stress available under these conditions. Finally, leukocyte rolling has not been directly investigated in glomerular capillaries.

The in vitro study suggests that the decrease in neutrophil accumulation in glomeruli of mutant mice at 2 h is due to an inability of mutant PMNs to sustain Fc-mediated adhesion to the immobilized ICs. In vivo this presumably leads to their detachment into the bloodstream. Since complement C3 is not involved in PMN accumulation in vivo, the in vitro adhesion assays to ICs were performed in the absence of serum, thus eliminating potential effects of complement in the assay. In vitro, we have shown that Mac-1–deficient PMNs can initially accumulate and spread on immobilized ICs at wild-type levels, but have a defect in subsequent adhesion and spreading compared to wild-type PMNs. Therefore, Mac-1 is not essential for initial FcγR attachment to ICs but is required for stabilizing PMN interactions with these complexes. We also observed a lack of F-actin redistribution in Mac-1–deficient PMNs contacting ICs. This indicates a role for Mac-1 in actin regulation after engagement of ICs, which may be necessary for PMN spreading. In fact, Mac-1 has previously been implicated in regulating F-actin assembly; Mac-1 is required for tyrosine phosphorylation of the cytoskeletal protein paxillin after neutrophil adhesion to ICs (27). Phosphorylation of paxillin, known to provide a binding site for the SH3 domains of *src* family tyrosine kinases, may play a key role in IgG-induced signaling (8).

The defect in sustained spreading of mutant PMNs on ICs (<10% of wild-type at 40 min) had no effect on release of LTB_4 from these cells, suggesting that spreading is not required for this process. Release of LTB_4, a potent neutrophil chemoattractant produced during PMN interaction with ICs, was previously shown to require the β_2 integrins (21). Together, these studies suggest that one of the other β_2 integrins present on neutrophils, LFA-1 or p150,95, may be required for LTB_4 release. The release of other chemoattractants may be affected in the absence of FcγR–Mac-1 interactions, thus accounting for the increase in PMN accumulation in wild-type mice, but not in the mutant mice, at 2 h. The potential chemoattractants that could be responsible for this effect are numerous and were not investigated.

In vivo, a striking absence of PMN-dependent proteinuria in Mac-1–null mice was observed despite the wild-type levels of PMN accumulation 1 h after induction of nephritis. This is even more significant given that, in electron micrographs, the glomeruli of mutant mice at this time point showed similar endothelial denudation as that of wild-types, and PMNs were in intimate contact with the GBMs. This would suggest that Mac-1–null PMNs are able to activate IC-stimulated functions that lead to endothelial injury. Indeed, IC-triggered superoxide production (21, 28) and rise in intracytoplasmic calcium in PMNs (21) do not require the β_2 integrins. However, events leading to GBM damage and subsequent increased permeability to albumin are lacking in mutant PMNs. Our results in the Mac-1–deficient mice are strikingly similar to those obtained in beige mice subjected to the same model of anti-GBM nephritis (17). Beige mice, which lack cathepsin G and elastase in their azurophilic granules, exhibit PMN accumulation and glomerular endothelial denudation, as assessed by electron microscopy, but develop no proteinuria. Similarly, in Mac-1–deficient mice, release of azurophilic granules containing elastase and cathepsin G may not occur in the mutant PMNs and may account for the absence of proteinuria in these animals. We also demonstrate that complement C3–deficient mice have decreased proteinuria despite accumulating wild-type levels of glomerular PMNs. Importantly, PMN phagocytosis of complement C3b–opsonized zymosan leads to the release of elastase which is Mac-1–dependent (29), and we have previously shown that Mac-1–deficient PMNs are unable to phagocytose complement-opsonized particles (2). Therefore, the interaction of Mac-1 on PMNs with complement C3 is probably necessary for the release of azurophilic granules in our anti-GBM nephritis model and explains the lack of proteinuria in both Mac-1–deficient and complement C3–deficient mice. In addition, another group has shown that PMNs extravasated into the peritoneum of Mac-1–deficient mice after thioglycollate-induced peritonitis have increased β-glucuronidase content, suggesting a deficit in release of azurophilic granules by activated Mac-1–null PMNs (30). Although we propose that an interaction of Mac-1 with complement is responsible for proteinuria in this model, a separate role for C3 in

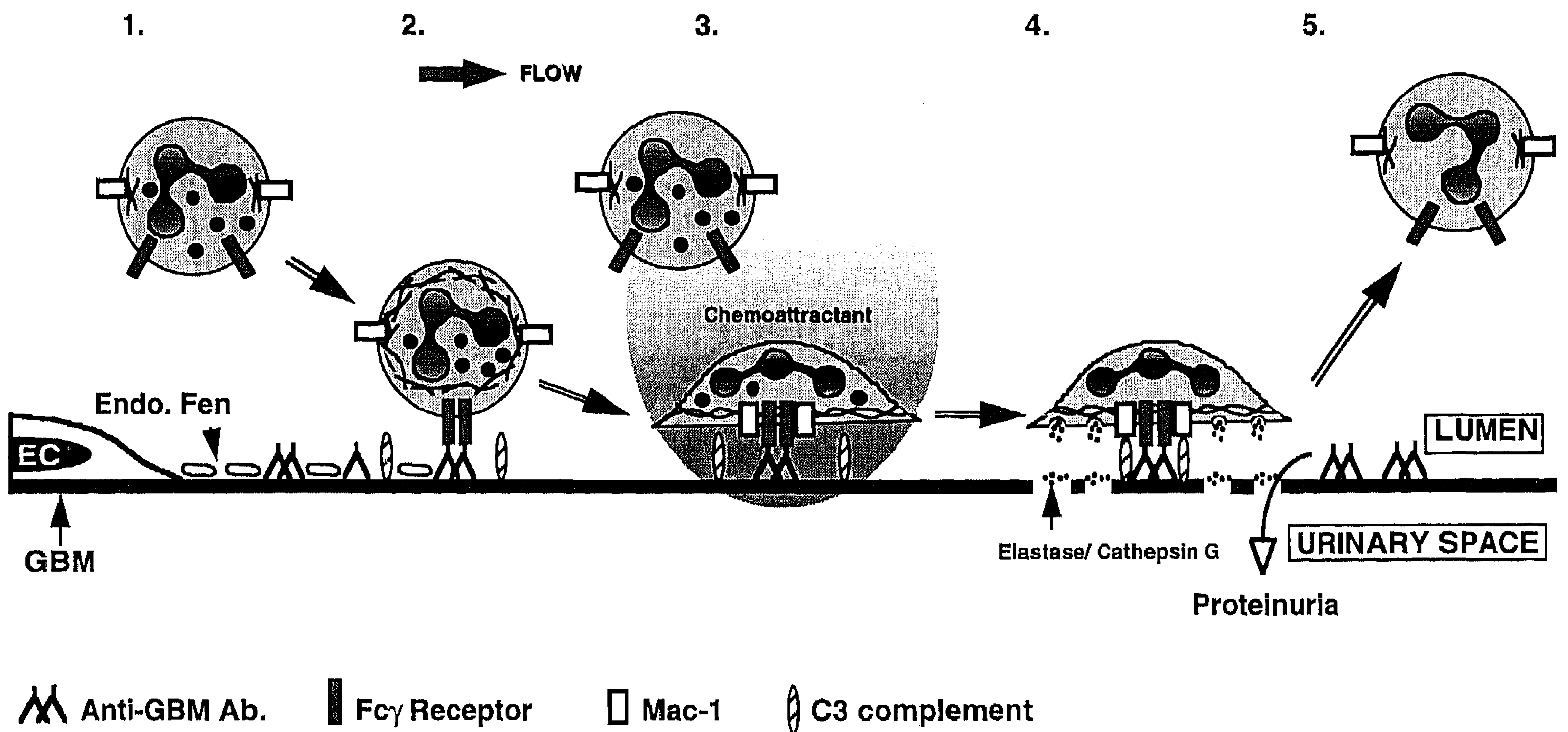


Figure 7. Model of putative role of Mac-1 in IC-stimulated glomerular PMN recruitment and proteinuria. (*1*) Anti-GBM antibody interacts with the GBM forming immobilized ICs. (*2*) ICs are recognized by FcγR on PMNs. The adherent neutrophils have actin polymerization occurring at the periphery. This process does not require Mac-1. (*3*) PMNs adhere to immobilized ICs via the FcγR and require Mac-1 for reorganization of the actin cytoskeleton which promotes sustained spreading on the IC, allowing the PMNs to resist detachment into the flowing blood. Sustained spreading also promotes release of chemoattractants, which enhances neutrophil influx. (*4*) Mac-1 interaction with complement C3 leads to the release of azurophilic granules containing cathepsin G and elastase, which are responsible for GBM damage. (*5*) PMNs detach and return to the bloodstream. In the absence of Mac-1, PMNs adherent to ICs in step 2, detach, and return to the circulating blood as depicted in step 5 and Mac-1/C3–dependent proteinuria does not occur. Endo.Fen, endothelial fenestrate; EC, endothelial cell.

generation of a membrane attack complex leading to proteinuria cannot be ruled out and was not explored further in this work.

These results suggest the following model for the role of Mac-1 in IC-triggered neutrophil accumulation in this form of acute nephritis (Fig. 7). The initial attachment of neutrophils to ICs in the glomerular capillary wall occurs via Fc interactions with FcγR on the surface of neutrophils, and does not require Mac-1. Thus the early neutrophil accumulation is equal in both mutant and wild-type mice. However, Mac-1, through interactions with, or signals from, FcγR, is required for the F-actin reorganization necessary for sustained neutrophil spreading on ICs as shown in our in vitro studies. The functional interaction may be required for elaboration of mediators that signal further neutrophil influx, accounting for the increase in neutrophil accumulation in wild-type mice at 2 h, which does not occur in mutant mice. We have not identified the specific chemoattractants that are effected by Mac-1 deficiency. Production of LTB_4, a powerful neutrophil chemoattractant, is not affected by Mac-1 deficiency or PMN spreading. Interaction of Mac-1 on PMNs with complement iC3b deposited on the vessel wall leads to the release of azurophilic granules, leading to protease-induced damage to the GBM and proteinuria, which is abrogated by Mac-1 or C3 deficiency. The glomerular PMN accumulation in this model is transient. There is evidence to suggest that the PMNs detach from the vessel wall, return to the bloodstream (16), and are probably cleared in the liver or spleen.

In conclusion, this study is the first to suggest a role for Mac-1 in FcγR-dependent neutrophil responses in vivo, thus providing pathophysiological relevance for Mac-1 interactions with FcγR. It also demonstrates a crucial role for Mac-1 in complement-dependent increased permeability, which leads to proteinuria.

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