Pre-Existing Glomerular Immune Complexes Induce Polymorphonuclear Cell Recruitment Through an Fc Receptor-Dependent Respiratory Burst: Potential Role in the Perpetuation of Immune Nephritis

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Pre-Existing Glomerular Immune Complexes Induce Polymorphonuclear Cell Recruitment Through an Fc Receptor-Dependent Respiratory Burst: Potential Role in the Perpetuation of Immune Nephritis

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In immune complex (IC) diseases, FcR are essential molecules facilitating polymorphonuclear cell (PMN) recruitment and effector functions at the IC site. Although FcR-dependent initial tethering and FcR/integrin-dependent PMN accumulation were postulated, their underlying mechanisms remain unclear. We here addressed potential mechanisms involved in PMN recruitment in acute IC glomerulonephritis (nephrotoxic nephritis). Since some renal cells may be recruited from bone marrow (BM) lineages, reconstitution studies with BM chimeras and PMN transfer between wild-type (WT) and FcR-deficient mice (γ−/−) were performed. Severe glomerular damage was induced in WT and Wγ chimeras (BM from WT to irradiated γ−/−), while it was absent in γ−/− and γW chimeras (γ−/− BM to WT). Moreover, WT PMN transfer, but not γ−/− PMN, reconstituted the disease in γ−/−, indicating that FcR on resident cells is not a prerequisite for PMN recruitment in this disease. Surprisingly, transferred WT PMN were recruited coincidentally with NF-κB activation and TNF-α overexpression even in glomeruli with preformed IC (nephrotoxic Ab administered 3 days previously), suggesting that PMN can initially be recruited via its own FcR without previous chemotacticant release. Furthermore, H2O2 inhibition by catalase attenuated the acute WT PMN recruitment and the induction of NF-κB and TNF-α much more than integrin (CD18) blockade, indicating a role for the respiratory burst before integrin-dependent accumulation. In coculture experiments with IC-stimulated PMN and glomeruli, PMN caused acute glomerular TNF-α expression predominantly via FcR-mediated H2O2 production. In conclusion, glomerular IC, even preformed, can cause PMN recruitment and injury through PMN FcR-mediated respiratory burst during initial PMN tethering to IC. The Journal of Immunology, 2003, 170: 3243–3253.

Immune complex (IC) deposition is considered a pathological hallmark in a variety of inflammatory diseases, including systemic lupus erythematosus, rheumatoid arthritis, hypersensitivity pneumonitis/alveolitis, vasculitis, and glomerulonephritis (GN). Growing evidence has shown that genetic polymorphisms of FcR and consequent alteration of their function may affect the prognosis of these IC diseases (1–3). In the last decade experimental studies, particularly with different knockout strains of animals, have revealed that FcR are crucial molecules providing a critical link between ligands (IC) and effector cells in IC-mediated inflammatory cascade (4, 5). Although this evidence highlights the clinical relevance of FcR in chronic IC diseases, effector cell types expressing FcR and their roles in each disease are not yet completely elucidated.

Leukocytes are well-established effector cells in IC diseases. Their emigration from microcirculation and activation in the focus of IC are key events in pathological conditions. In general, their adhesion and transmigration in venules are determined largely by multistep process of sequential engagement of distinct receptor molecules on the leukocytes and endothelial surfaces (6). The selectin family and its ligands mediate the initial contact between the circulating leukocytes and the vascular endothelium (“rolling”). Subsequently, rolling leukocytes encounter activating stimuli that trigger activation-dependent adhesion required for integrin-mediated firm arrest. In IC diseases, it is viewed that de novo synthesis of many structurally diverse inflammatory mediators (e.g., C5a, leukotriene B4, platelet-activating factor) from tissue-resident cells associated with IC formation may trigger hemodynamic alterations and an initial chemotaxis to endothelium of postcapillary venules adjacent to the site of IC (6–8). Those mediators may also enhance selectin expression, leading to initial leukocyte recruitment (6, 8).
Although convincing evidence showed the requirement for integrins, studies addressing the role of selectins have yielded conflicting data in some models of IC GN (8–13). Glomerular leukocyte recruitment was not attenuated in these diseases by anti-P- or E-selectin Abs (9–11) or oligosaccharide (FF7)-blocking L- and P-selectin (12) or in P-selectin-deficient mice (13). Interestingly, blockade of selectin by FF7 may affect postglomerular capillary venules, but not glomerular capillaries, in IC GN (12). This evidence reveals redundant roles for selectin in some IC diseases and indicates that different activation mechanisms on endothelial cells may underlie the regulation of the initial leukocyte recruitment.

A recent paper showed a new paradigm in leukocyte recruitment to the IC site (14). It demonstrated that the selectin-independent, but FcγRIII-dependent, contact formation (tethers) of leukocytes is involved in its initial recruitment at sites of IC deposition under physiological flow. Previous studies also demonstrated that cooperation between FcγR and β2 integrins, particularly Mac-1 (CR3, CD11b/CD18), is required for polymorphonuclear cells (PMN) accumulation on IC (15, 16). In fact, these ideas are compatible with our previous findings (17) in acute passive anti-glomerular basement membrane GN (anti-GBM GN) in which immobilized GBM-anti-GBM IC trigger rapid glomerular PMN accumulation and PMN-dependent damage. In FcγR-chain-deficient mice (γ−/− mice) lacking functional FcR (FcγRI, FcγRIII, FcεRI) (4), lethal endothelial damage is completely abrogated because of the absence of acute PMN influx in this disease (17). These findings allow the possibility that intracellular signaling of FcR in PMN during its FcR-dependent tethering may be involved in and initiate the inflammatory cascade leading to its own accumulation (firm adhesion). This idea could be important for the interpretation of preformed IC injury, since this mechanism may not require soluble mediators and endothelial activation in advance of the initial leukocyte recruitment. In addition, it may suggest that the phenotype of FcR in effector cells (e.g., expression level) may influence the susceptibility to IC diseases (4). To address this hypothesis, in the current work we employed anti-GBM GN. We looked for the contribution of renal resident cells in the initial recruitment, since those cells, including resident macrophages, also possess functional FcR (18–20), and some are probably recruited from bone marrow (BM) lineage (21, 22). We therefore performed in vivo reconstitution by BM chimeras and specific myeloid lineage (PMN) transfer between γ−/− mice and their wild-type (WT) littermates, and additionally examined the leukocyte recruitment onto preformed IC. PMN are thought to damage glomeruli, including in this disease, as a combination of reactive oxygen species and proteolytic enzymes (7). Previous studies with this model in cathepsin G/elastase deficient-mice (23, 24) and an injection of a cysteine protease inhibitor (25) demonstrated that only early proteinuria (within day 1) was dependent on proteinase, but PMN influx and development of glomerular injury were not. Thus, we postulated that the FcR-mediated PMN respiratory burst may be a rapid and powerful effector mechanism in the early cell recruitment. Additionally, we here focused on early tissue NF-κB activation, a regulator of many proinflammatory mediators, and TNF-α expression. Our present findings further extend the implication of FcR in the pathogenesis of IC diseases and support the idea that FcR may be a potential therapeutic target in the management of these conditions.

Materials and Methods

Mice.

γ−/− mice were generated by a homologous recombination method with BL6/J ES cells. Construction of targeting vectors and generation of this strain have been previously described in detail (26). In all in vivo experiments, we used female animals weighing 18–23 g. γ−/− mice have a C57BL/6 background. Although WT littermates (γ+/+) and C57BL/6 mice were analyzed in this disease, no significant differences were found in the kinetics of proteinuria and glomerular/interstitial damage, as noted in our previous studies (17). Thus, the results for C57BL/6 mice matched for age were shown as representative controls of WT.

Generation of BM chimeras

BM transplantation was performed on 6- to 8-wk-old female γ−/− and WT mice. BM cells were collected from tibias and femurs of each mouse strain, treated with Gay’s solution to exclude RBC contamination for protection against vascular (thrombotic) injury, and then i.v. transplanted (3×107 BM cells) to mice that had been irradiated with 1000 or 600 rad of x-ray with renal protection by lead plates. Since they had the same genetic background, there were no symptoms of graft-vs-host disease in any of them. In the next 4 wk, transplanted animals were kept in air-conditioned clean cages. We generated two different BM chimeras as follows: WW (BM from WT to γ−/− irradiated WT) and Wγ (WT BM to γ−/−). As controls, WW (WT BM to WT) and γγ (γ BM to γ−/−) mice were also generated.

Genotype exchange in peripheral blood of each BM chimera (WW and Wγ) was determined by PCR with purified genomic DNA from peripheral blood and tissue (tail) before and 5 wk after BM transplantation (QIAamp Blood Kit: Qiagen, Hilden, Germany). Primers used for the murine FcγR chain were as follows: specific primer for exon 3 (5′-GAA TTC GGAT GCCT CGGAC CCCTGAGAT T-3′) and exon 2 (5′-GAA TCTCGTG CTGC TTGAGCT-3′) and for the created neo γ-chain (26) in which exon 2 was replaced (5′-GCCA CCCTACT GCTG GTAGAG T-3′) was simultaneously performed with these primers under the following conditions: 94°C for 1 min, 57°C for 1 min, and 72°C for 1.5 min for 33 cycles. After confirmation of the genotype exchange, these chimeras were subjected to the general experiments.

Experimental protocol for anti-GBM GN

The method for preparation of nephrotoxic serum (NTS) has been previously described (17). Anti-GBM GN was induced by i.v. injection of NTS through the tail vein in mice that had been preimmunized with rabbit IgG and CFA 4 days before NTS administration and followed until day 100. Since a preliminary study showed that NTS at a dose of 20 μg/20 g BW was sufficient to cause proteinuria and severe renal damage in WT mice, we employed this dose in WT and γ−/− mice and their chimeras. No mice developed anaphylactic symptoms until 1 wk after the injection of NTS. Urinary protein was determined by Knight’s method, as previously described (17). Kidneys were perfused with cold saline and removed under general anesthesia.

For evaluation of the effect of reactive oxygen species, γ−/− mice were injected i.p. with bovine liver catalase (534,000 U/20 g body weight; Sigma-Aldrich, Madrid, Spain) (27) or anti-CD18 neutralizing Ab (250 μg/20 g body weight; BD PharMingen, Heidelberg, Germany) (11, 28) three times at 16, 8, and 2 h before the injection of PMN.

Preparation of PMN from BM

For in vitro differentiation of PMN, collected BM cells were incubated overnight in DMEM supplemented with 20% FCS, 15% cell culture supernatant derived from Wehi-3b cells (ATCC TIB-68; American Type Culture Collection, Manassas, VA), 1% glutamine, and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin) in 5% CO2 at 37°C as previously described (29). Before each experiment, PMN were analyzed for the expression of CD11b and Gr-1 (BD PharMingen), a marker of mature PMN, using a flow cytometer.

For in vivo transfer, we prepared 5×106 (high dose) or 5×105 (low dose) PMN and injected them through the tail vein 3 h before or 3 days after the injection of NTS. Preliminarily, we confirmed that no mice developed anaphylactic symptoms until 1 wk after the injection of PMN alone.

Renal histopathologic studies

Kidney sections fixed in 4% paraformaldehyde were stained with periodic acid-Schiff (PAS) reagent or Masson’s Trichrome in 4-μm-thick sections to assess histological changes by light microscopy. We first examined the diameter of representative glomeruli in the renal cortex of mice with nephritis at 2 h (0.11 ± 0.014 mm; n = 6). The sizes of the glomeruli were not significantly different from those in normal mice of the same age (0.097 ± 0.03 mm; n = 3). Thus, glomeruli with a diameter larger than 0.1 mm (>15 glomeruli) were employed for the evaluation of PMN influx, and more than three animals per group were examined. The results were expressed as cells per glomerular cross-section.
Frozen renal sections were used for immunofluorescence for rabbit IgG and murine C3 and then stained with FITC-labeled Abs (Cappel/ICN, Aurora, OH; DAKO, Barcelona, Spain). The extent of glomerular immunostaining of IgG and C3 was semiquantitatively determined by means of a scale from 0 (none) to 4+ (very intensive), as previously described (30).

**RNA extraction and RT-PCR**

Total RNA was obtained by the TRisol method (Life Technologies, Gaithersburg, MD). One microgram of RNA was reverse transcribed and then amplified with a commercial kit (Promega, Southampton, U.K.), with the use of 0.5 μCi of [α-32P]dCTP (3000 Ci/mmol; Amersham International, Little Chalfont, U.K.) and 20 pmol of specific primers for mouse TNF-α (sense, 5′-CCGACGGTGAACCTGGCAGAG-3′; antisense, 5′-GGTGAC AAACCATGGCCCTGGCA-3′; fragment, 384 bp) (31) and mouse GAPDH (sense, 5′-CCGGTCGCTGATGTAGTGTGC-3′; antisense, 5′-CAGCTTC TCTGAGTGGCAGT-3′; fragment, 289 bp; reference no. AK 013857). The amplifications were conducted with annealing temperatures of 63°C (TNF-α) or 57°C (GAPDH). The optimum number of amplification cycles used for semiquantitative RT-PCR (in vivo, 30 and 25; in vitro, 32 and 25, respectively) was chosen on the basis of pilot experiments (data not shown). The expression of GAPDH was used as an internal control. Aliquots of each reaction were run on 4% acrylamide-bisacrylamide gels. The gels were dried and exposed to X-OMAT AS films (Eastman Kodak, Madrid, Spain). Autoradiograms were quantified by scanning densitometry (Molecular Dynamics, Sunnyvale, CA). The density of each gene was compared after individual correction by density of GAPDH.

**Preparation of substrate-coated coverslips**

BSA-anti-BSA IgG IC were immobilized on tissue culture plastic coverslips (ThermoShandon, Miles Scientific, Evanston, IL) with mild modulations of a previous report (14). Round coverslips (13 mm in diameter) were 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-Aldrich) in 0.5 ml of PBS for 1 h. This concentration of anti-BSA IgG has been previously shown to yield maximal attraction of murine PMN to IC and the leukotriene B4, released from these cells (15). In addition to the BSA plate without the Ab, we prepared BSA-anti-BSA (Fab) (Fab IC) coverslips as a control to confirm the involvement of FcR. Fab were prepared by the digestion of anti-BSA IgG with papain (40/1 mg ratio, 30 min, 37°C). Undigested IgG and papain were subsequently removed by filtration through protein A-Sepharose and BSA-agarose, and Fab were dialyzed against PBS.

**Respiratory burst assay**

PMN from each mouse strain were incubated with BSA-IC coverslips for 30, 60, and 120 min. Concentrations of hydrogen peroxide (H₂O₂) in aqueous solutions were measured by Peroxi Detect (Sigma-Aldrich), which is based on the fact that peroxides at acidic pH convert Fe³⁺ to Fe⁴⁺. The Fe⁴⁺ ion forms a colored adduct with xylenol orange detectable at 560 nm. The standard of 100 μM H₂O₂ was prepared by observing the absorbance at 240 nm (10 mM 0.436 OD₂₄₀). A standard curve of nanomoles of H₂O₂ was plotted by 0, 10, 20, 40, 60, and 80 μl of the 100 μM standard H₂O₂. The nanomoles of peroxide in the sample were calculated from the standard curve: nmol peroxide/ml = [A 560 sample - A 560 blank] × dilution factor/[A 560 1 μM peroxide] × (vol of sample).

To examine the effect of IC-induced PMN respiratory burst on glomerular gene expression, a Transwell chamber (Costar, High Wycombe, U.K.) with 0.4-μm pore-sized membrane (24 mm in diameter), where PMN can not pass through, was used. The lower wells were loaded in triplicate with 2 × 10⁶ glomeruli isolated by a sieving method as previously described (33). IC-, Fab IC-, or BSA alone-coated coverslips (13 mm in diameter) were put on the membrane of the upper chamber. Then a 1 × 10⁶ PMN suspension was loaded into the upper chamber. Glomeruli from WT and γ⁻⁻ mice were incubated for 2 h and then collected for RNA. In some cases PMN or glomeruli were pretreated with 1 × 10⁴ U/ml catalase 30 min before loading into the chamber.

**Statistical analyses**

Results are expressed as the mean ± SD and were analyzed by ANOVA (Figs. 1 and 2) and Mann-Whitney test (Figs. 3–6) for comparison of quantitative variables. Statistical significance was established as p < 0.05 (two-tailed curve). For statistical analysis of survival rate (Fig. 1d), Yates’s correction was employed.

**Results**

**BM-derived cells expressing FcR play a crucial role in the induction of anti-GBM GN**

To clarify the relevant cell types expressing FcR, we generated BM chimeras between mice lacking functional FcR (γ⁻⁻) and their WT littermates. We also generated control mice that were ally reconstituted by transplantation, and the irradiation condition was mainly characterized by endothelial swelling and hematuria around day 10 (Fig. 1a). Renal lesions, mainly characterized by endothelial swelling and fibrin deposition, were observed in WT mice (Fig. 1b). On the other hand, although there were no significant differences in the extent of glomerular anti-GBM IgG/C3 deposition as in WT mice (WT vs γ⁻⁻)–rabbit IgG, 3 ± 0.71 vs 3.2 ± 0.45 (p = 0.61); mouse C3, 2 ± 0.71 vs
1.8 ± 0.84 (p = 0.69)), all γ−/− mice survived without pathological proteinuria and obvious renal lesions even on day 100 (Fig. 1a and Fig. 2, a and b). CD4+ T cell-dependent injury, characterized by mesangial proliferation as described in our recent study (34), was not induced at this dose of NTS in γ−/− mice.

At 5 wk after BM transplantation (day 0), the genotypes of peripheral blood in Wγ and γW chimeras were replaced with that of WT or γ−/− mice, respectively (Figs. 1c and 2c). These Wγ chimeras showed massive proteinuria (Figs. 1a and 2a) and glomerular lesions characterized by severe endothelial injury and fibrin deposition (Fig. 1b) to the same extent as in WT mice. Although we detected no significant difference in the peak proteinuria between WT and Wγ chimeras (Fig. 1a), Wγ chimeras survived significantly longer than WT mice (day 21, p = 0.003056; day 49, p = 0.0000389; Fig. 1d). In contrast, γW chimeras as well as γ−/− mice (γW/1000 rad) were protected from glomerular injury (Fig. 1a and Fig. 2, a and b). These findings confirm that BM-derived cells expressing FcR are responsible for the induction of this disease.

Glomerular damage can be induced coincidentally with the recovery of recipient WT BM in γW/600 rad BM chimeras

To determine whether FcR+ BM-derived cells can induce glomerular injury in response to preformed IC, we manipulated the recovery of the recipient (WT) BM in γW chimeras with this disease by lower levels of irradiation (600 rad). As shown in Fig. 2c, the genotypes of peripheral blood in both groups of γW chimeras irradiated with 1000 or 600 rad were replaced with that of γ−/− mice at the beginning of this experiment (day 0). Although the genotype in γW/1000 rad chimeras did not change during the disease course, γW/600 rad chimeras showed a heterogeneous genotype (γ−/− and WT) in peripheral blood after day 40, indicating that recipient WT BM gradually recovered and delivered certain amounts of FcR+ circulating cells. Interestingly, coincident with the recovery, moderate proteinuria and glomerular lesions with endothelial damage appeared even after day 40 following the injection of NTS and were sustained until day 100 (Fig. 2, a and b). This suggests that recovered FcR+ BM-derived cells can induce considerable severe glomerular damage even 40 days after IC formation.

PMN recruitment and renal injury in γ−/− mice are reconstituted by FcR-positive PMN transfer with both acute (freshly formed) and preformed IC

Recent papers have suggested that a certain population of glomerular cells is recruited from BM-derived precursor cells (21) and may contribute to the pathogenesis of GN (22). Therefore, we next prepared in vitro differentiated PMN from BM cells and transferred them in vivo to determine the potential contribution of intrinsic or BM-derived FcR+ renal cells to this disease. After the incubation of BM cells in the specific conditioned medium as previously described (29), we evaluated their differentiation to PMN by staining of surface Ags on surviving cells with anti-Gr-1 and anti-CD18 Abs. Gr-1 Ag is directly correlated with granulocyte differentiation and maturation (35), while CD18 can be expressed not only in granulocytes, but also in NK, T, and B cells (36). With this protocol, we obtained a double-positive (Gr-1+CD18+) cell population (88.8 ± 3.0; n = 5) by flow cytometric analysis and employed those positive cells for in vivo transfer and in vitro assays.

Then we explored the reconstitution of the disease, as seen in Wγ and γW/600 rad chimeras, by WT PMN transfer to γ−/− mice. However, it is known that i.v. cell administration induces nonspecifically transient hematuria and proteinuria, partially due to the systemic activation of complement. We also found transient urinary alterations even in the case of PMN transfer to normal mice (data not shown) until 12 h, but not after day 1. Therefore, we analyzed proteinuria after day 1. Transferred WT PMN followed by serum administration (PMN→Ab) caused proteinuria after day 1 in a dose-dependent manner (Fig. 3a). PMN influx was also detected 2 h after serum injection (Fig. 3c). γ−/− PMN transfer to γ−/− mice failed to induce proteinuria after day 1 or sufficient PMN influx (~71% less influx than when transferred with WT PMN; Fig. 3, a and c). Those data indicate that pathological proteinuria is caused by renal injury associated with the FcγR-dependent PMN influx and confirm that FcR on renal resident cells are not prerequisites for glomerular PMN recruitment in this disease. Consistently, WT PMN transfer to γ−/− mice 3 days after NTS
through the activation of NF-κB. γW/1000 rad chimeras did not show proteinuria (a) or renal lesions (b) during the disease course, nor did γ−/− mice, whereas γW/600 rad chimeras gradually revealed proteinuria (a) and glomerular lesions with endothelial swelling (b) accompanied by the restitution of recipient (WT) BM (c). This injury was sustained and developed until day 100 (b). *p < 0.01; **p < 0.05 (a vs γ−/− or γW/1000 rad chimeras; n = 6–13 animals).

FIGURE 2. Glomerular damage can be induced coincidentally with the restitution of FcR−/− BM. γW/1000 rad chimeras do not show proteinuria (a) or renal lesions (b) during the disease course, whereas γW/600 rad chimeras gradually revealed proteinuria (a) and glomerular lesions with endothelial swelling (b) accompanied by the restitution of recipient (WT) BM (c). This injury was sustained and developed until day 100 (b). *p < 0.01; **p < 0.05 (a vs γ−/− or γW/1000 rad chimeras; n = 6–13 animals).

administration (Ab→PMN) induced glomerular damage associated with PMN influx at 2 h, but not γ−/− PMN transfer (~69% less influx than WT PMN transfer; Fig. 3, b and c). This result further suggests that PMN can be recruited into glomeruli via their own FcR, presumably without de novo synthesis of soluble mediators by IC deposition.

Early induction of inflammatory mediators (NF-κB and TNF-α) is markedly attenuated in γ−/− mice

To address potential mechanisms in the initial PMN recruitment, we analyzed renal expression levels of inflammatory mediators, such as the cytokine TNF-α and the transcription factor that regulates its expression (NF-κB). As shown in Fig. 4a, a marked renal NF-κB activation was noted after 2 h in WT mice, but not in γ−/− mice. WT mice also showed overexpression of TNF-α, peaking at 2 h, that was dramatically attenuated in γ−/− mice (Fig. 4b). These data confirm that early induction of these mediators is critical for the evolution of this disease.

We next examined these mediators in transfer models. In both models (PMN→Ab or Ab→PMN in γ−/− mice), the transferred WT PMN induced significantly greater renal NF-κB activation (Fig. 5a) and TNF-α expression (Fig. 5b) than the transferred γ−/− PMN. Southwestern immunohistochemistry showed that WT PMN transfer, but not γ−/− PMN transfer, induced a marked increase in NF-κB activation, not only in glomerular PMN but also in glomerular capillary endothelial cells (Fig. 5c), suggesting that the PMN recruitment via FcR is able to trigger inflammation through the activation of NF-κB and regulated cytokine production.

Pretreatment with catalase attenuated glomerular PMN influx, NF-κB activation, and TNF-α overexpression

The next series of in vivo experiments was undertaken to explore the potential role of the respiratory burst in the early induction of renal NF-κB and TNF-α, especially in the transfer model with preformed IC, since soluble mediators associated with acute IC formation may also influence it. In addition, we evaluated the relative contribution of the integrin-mediated cascade. As shown in Fig. 5c, H2O2 blockade by catalase reduced PMN recruitment at 2 h (51.5 ± 3.2% reduction) much more than integrin blockade by an anti-CD18 neutralizing mAb (30.8 ± 7.3% reduction). Similar protective effects were found in renal NF-κB activation and TNF-α expression (reductions: catalase, 47.1 ± 5.9 and 59.7 ± 12.6%; anti-CD18, 17.7 ± 7.7 and 40.1 ± 11.5%, respectively). These data indicate that local H2O2 production by PMN transfer may play an important role in its own further recruitment, leading to integrin-dependent adhesion and early activation of inflammatory mediators.

Glomerular TNF-α expression can be enhanced by PMN FcR-mediated respiratory burst

To mimic the in vivo GBM-anti GBM IC interaction with PMN, some slides were coated with BSA-anti BSA IgG IC and incubated with resting BM-derived PMN from WT and γ−/− mice in the absence of serum to eliminate the potential effects of complement. H2O2 production was measured at 0, 30, 60, and 120 min. WT PMN incubated with IC-coated coverslips showed, at 60 and 120 min, a significantly higher H2O2 production than γ−/− PMN (Fig. 6a), although γ−/− PMN with IC- or BSA alone-coated coverslips...
weakly produced H₂O₂. Pretreatment of WT (Fig. 6a) and γ<sup>−/−</sup> PMN (data not shown) with catalase reduced IC-induced H₂O₂ production to <0.1 nmol/ml, confirming the extracellular release of H₂O₂ by BM-derived PMN. These findings suggest that under these conditions FcR were a major effector molecule for PMN H₂O₂ release.

Next, we examined whether H₂O₂ can directly stimulate glomerular TNF-α expression. As shown in Fig. 6b, both WT and γ<sup>−/−</sup> PMN incubated with BSA alone-coated coverslips increased glomerular TNF-α expression at 2 h compared with that in coverslips without BSA coating. However, WT PMN incubated with IC-coated plates elicited significantly higher TNF-α expression in both WT and γ<sup>−/−</sup> glomeruli than that by γ<sup>−/−</sup> PMN. Pretreatment with catalase reduced WT PMN-induced TNF-α expression (40.5 ± 7.2% reduction). The same tendency in glomerular TNF-α expression was found between IC- and Fab IC-coated plates (fold increase to Fab IC-coated plates (n = 4): WT PMN vs γ<sup>−/−</sup> PMN on the IC-coated plate, 1.55 ± 0.18 vs 0.98 ± 0.1 (p < 0.01) with γ<sup>−/−</sup> glomeruli; 1.59 ± 0.19 vs 0.87 ± 0.07 (p < 0.01) with WT glomeruli). Together these data demonstrate that FcR-mediated H₂O₂ from PMN can directly enhance the early TNF-α expression in glomeruli.

Discussion

Convincing evidence indicates that FcR are one of the critical molecules in the development of acute inflammatory response and tissue damage in IC-mediated diseases (4, 5). In our previous and current works we specifically corroborated this idea within the context of anti-GBM GN, an acute model of IC-mediated injury.
As observed, FcR may play an essential role in glomerular PMN accumulation, resulting in acute endothelial damage. In the present study we further clarify the prerequisite of FcR Engagement for PMN accumulation, resulting in acute endothelial damage. In fact, FcR on renal resident cells expressing FcR may contribute to the progression of this disease through other mechanisms, such as the interaction with heterologous Ab deposited in the mesangial area.

It has been considered that hemodynamic alterations and soluble mediators, such as C5α and leukotriene B4, associated with glomerular IC formation may provoke the initial leukocyte contact (17). We do, however, note that transferred WT PMN, but not γ−/− PMN, were recruited into glomeruli with preformed IC in γ−/− mice and induced glomerular damage as well as glomeruli with acute (freshly formed) IC. Consistently, γW/600 rad chimeras also showed glomerular injury in accordance with the restitution of recipient WT BM after day 40. Although glomerular injury in both cases showed relatively less severity than that in WT mice, the present data suggest that PMN initially can encounter IC exposed at the open endothelial fenestrae, predominantly via their FcR, and trigger their margination in glomerulus with neither chemotactic mediators, such as C5a and leukotriene B4, associated with glomerular capillary may be different from those in venules, and these forces can be altered in IC-deposited glomerulus. In addition, this idea is compatible with previous findings that C3, C4, and C5α deficiency have no significant influence on glomerular PMN accumulation in this disease (15, 39, 40). In humans, neutrophils express FcγRIIA (41) and glycoprophosphatidylinositol-linked FcγRIIB (14), which are adherent to IC under flow conditions, but only the latter receptor has been proven to mediate the adherence under physiological conditions (2.0 dynes/cm²). The topographic localization of receptors on microvilli is critical for contact formation (tethering) (14, 42). In fact, FcγRIIB of human leukocytes is present on the microvilli (14). On the other hand, murine neutrophils do not express these receptors (4), but our current findings underscore that γ-chain-associated FcγR, presumably FcγRIIA, may be responsible for the tethering to both acute and preformed IC in the murine system. Consistently, initial PMN recruitment onto acute IC is absent in FcγRIIIA-deficient mice as well as in γ−/− mice (14, 17). Therefore, this evidence indicates the microporous localization of murine FcγRIIIA and suggests that the spatial proximity at microvillous protrusions may favor ligation of FcγRIIIA, inducing effector functions during initial tethering.

Firm adhesion of leukocytes is known to be mainly dependent on integrins (6). The β2 leukocyte integrins share a common β-chain (CD18). Three α subunits are noncovalently associated
FIGURE 5. WT PMN transfer, but not $\gamma^{-/-}$ PMN, reconstitutes the enhanced glomerular NF-κB activation and TNF-α expression. WT PMN transfer to $\gamma^{-/-}$ mice induced significantly higher NF-κB activation (a) and TNF-α expression (b) at 2 h in both cases with acute (freshly formed; PMN→Ab) and preformed IC (Ab→PMN) than $\gamma^{-/-}$ PMN transfer. In the case of preformed IC, Southwestern immunohistochemistry with NF-κB-specific oligo probe (c) revealed strong NF-κB activation in glomerular PMN (arrow) and endothelial cells (arrowhead) of $\gamma^{-/-}$ mice. Catalase treatment reduced NF-κB activation much more than anti-CD18 Ab treatment. Lane C in a denotes the competition of the kidney extracts from WT PMN transfer to $\gamma^{-/-}$ mice (WT→$\gamma^{-/-}$). Data are expressed as the fold increase vs control and are the mean ± SD of four to six independent experiments. a: *, $p < 0.01$; b: *, $p < 0.001$; **, $p < 0.05$ (vs WT PMN→$\gamma^{-/-}$).
glomerular TNF-α/H9251 Transwell membrane, showed that WT PMN induced significant H₂O₂ production (lower chamber) and IC-stimulated PMN (upper chamber), separated by a WT incubated with an IC-coated plate, but not those from γ−/− mice in the case of WT PMN transfer. In addition, preconditioning of adherent PMN by TNF-α, such as activation of several specific kinase pathways, is required for their Mac-1-mediated effector function (45, 46). Therefore, those findings suggest that the selectin- and CD18-independent, IC-mediated respiratory burst in this acute phase may play a certain role in endothelial activation leading to integrin-dependent accumulation. TNF-α is the predominant cytokine regulating PMN recruitment via the expression of endothelial adhesion molecules from upstream of the cascade in IC diseases, including anti-GBM GN (6, 8, 47). Under the present experimental conditions without physiological flow, γ−/− PMN also bound to the IC-coated surface and produced H₂O₂. This is probably due to the interaction with BSA or aldehyde moieties incompletely quenched. However, WT PMN showed a markedly higher production of H₂O₂ and a stronger induction of glomerular TNF-α than γ−/− PMN, indicating a predominant contribution of FcR-mediated signaling. Accordingly, our present findings indicate that recruited (tethered) PMN, even transferred, may rapidly cause NF-κB activation in the same cells and in the kidney through FcR-induced respiratory burst. Then, up-regulation of NF-κB-dependent inflammatory mediators in glomeruli, such as TNF-α, may facilitate additional PMN recruitment, presumably via increased adhesion molecule expression. Moreover, Mac-1 on PMN enhances FcγR-IgG effector function (15, 48) and elicits further respiratory burst with interaction of GBM matrix component accompanied by TNF-α (49), suggesting that an acceleration loop by FcγR and Mac-1 may be involved in the chain of events after this initial activation in transferred PMN. Indeed, we initially observed PMN accumulation after 2 h, similar to the findings of our previous study (17), in both WT PMN transfer models (data not shown). The remaining PMN recruitment, NF-κB activation, and TNF-α expression were also found in γ−/− PMN transfer models. Therefore, we have to carefully elucidate some other adhesion receptors (e.g., αβ₅) (50) or other mediators (e.g., angiotensin II) (34) in addition to some alteration in the in vitro differentiated PMN.

In summary, the present study demonstrates that glomerular IC, even preformed, can cause PMN recruitment and glomerular damage in anti-GBM GN, partly by an FcR-mediated respiratory burst in a PMN-dependent, but resident cell-independent, manner. Importantly, once it occurs, even though this mechanism cannot induce full development of glomerular damage, inflamed endothelial surface may alter the disease course, as seen in γW/600 rad BM chimeras, presumably through the facilitation of sensitized-lymphocyte infiltration, and therefore contribute to the perpetuation of proteinuria were lacking in Mac-1-deficient mice with this disease (15), because Mac-1, through interactions with or signals from FcγR, is required for the filamentous actin reorganization necessary for stabilizing PMN interactions with IC. However, in this mutant, an initial FcR-dependent PMN influx accompanied by denudation of fenestrated endothelium as their activation outcome were observed in WT mice as well (15), indicating some other selectin-independent mechanisms activating endothelial cells before or overlapping the integrin activation cascade. The present results revealed that early PMN recruitment into glomerulus with preformed IC was attenuated by catalase treatment (which degrades H₂O₂ to H₂O) much more than by anti-CD18 neutralizing Ab. This protective effect by catalase was also found in NF-κB expression and TNF-α expression. It is known that renal TNF-α expression is regulated through NF-κB activation (43), and that NF-κB activation in endothelial cells is critically involved in TNF-α-induced adhesion molecule expression (44). In this sense it is noteworthy that Southwestern immunohistochemistry showed strong activation of NF-κB not only in recruited PMN, but also in endothelial cells of γ−/− mice in the case of WT PMN transfer. In addition, preconditioning of adherent PMN by TNF-α, such as activation of several specific kinase pathways, is required for their Mac-1-mediated effector function (45, 46). Therefore, those findings suggest that the selectin- and CD18-independent, IC-mediated respiratory burst in this acute phase may play a certain role in endothelial activation leading to integrin-dependent accumulation. TNF-α is the predominant cytokine regulating PMN recruitment via the expression of endothelial adhesion molecules from upstream of the cascade in IC diseases, including anti-GBM GN (6, 8, 47). Under the present experimental conditions without physiological flow, γ−/− PMN also bound to the IC-coated surface and produced H₂O₂. This is probably due to the interaction with BSA or aldehyde moieties incompletely quenched. However, WT PMN showed a markedly higher production of H₂O₂ and a stronger induction of glomerular TNF-α than γ−/− PMN, indicating a predominant contribution of FcR-mediated signaling. Accordingly, our present findings indicate that recruited (tethered) PMN, even transferred, may rapidly cause NF-κB activation in the same cells and in the kidney through FcR-induced respiratory burst. Then, up-regulation of NF-κB-dependent inflammatory mediators in glomeruli, such as TNF-α, may facilitate additional PMN recruitment, presumably via increased adhesion molecule expression. Moreover, Mac-1 on PMN enhances FcγR-IgG effector function (15, 48) and elicits further respiratory burst with interaction of GBM matrix component accompanied by TNF-α (49), suggesting that an acceleration loop by FcγR and Mac-1 may be involved in the chain of events after this initial activation in transferred PMN. Indeed, we initially observed PMN accumulation after 2 h, similar to the findings of our previous study (17), in both WT PMN transfer models (data not shown). The remaining PMN recruitment, NF-κB activation, and TNF-α expression were also found in γ−/− PMN transfer models. Therefore, we have to carefully elucidate some other adhesion receptors (e.g., αβ₅) (50) or other mediators (e.g., angiotensin II) (34) in addition to some alteration in the in vitro differentiated PMN.

In summary, the present study demonstrates that glomerular IC, even preformed, can cause PMN recruitment and glomerular damage in anti-GBM GN, partly by an FcR-mediated respiratory burst in a PMN-dependent, but resident cell-independent, manner. Importantly, once it occurs, even though this mechanism cannot induce full development of glomerular damage, inflamed endothelial surface may alter the disease course, as seen in γW/600 rad BM chimeras, presumably through the facilitation of sensitized-lymphocyte infiltration, and therefore contribute to the perpetuation of
IC diseases. This potential mechanism also postulates that the expression level of FcR on leukocytes may influence on the susceptibility and perpetuation of IC disease or its flare-up. This would partly explain clinical evidence that bouts of the disease are frequently associated with unspcific upper respiratory tract infection, because cytokines related to common infection, such as IFN-γ, are known to enhance the expression levels of FcR on leukocytes (51). Although systemic blockade of FcR confers beneficial effects in acute IC glomerulonephritis (52), FcR exert physiological effects during the infection. Thus, tissue-specific blockade of the FcR-mediated effector mechanism would probably be a better approach for the management of chronic IC diseases.

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References


