Susceptibility to T Cell-Mediated Injury in Immune Complex Disease Is Linked to Local Activation of Renin-Angiotensin System: The Role of NF-AT Pathway

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Susceptibility to T Cell-Mediated Injury in Immune Complex Disease Is Linked to Local Activation of Renin-Angiotensin System: The Role of NF-AT Pathway

Yusuke Suzuki,†§ Carmen Gómez-Guerrero,* Isao Shirato,‡ Oscar López-Franco,* Purificación Hernández-Vargas,* Guillermo Sanjuán,* Marta Ruiz-Ortega,* Takeshi Sugaya,† Ko Okumura,§¶ Yasuhiko Tomino,‡ Chisei Ra,§ and Jesús Egido*‡

FcR provides a critical link between ligands and effector cells in immune complex diseases. Emerging evidence reveals that angiotensin (Ang)II exerts a wide variety of cellular effects and contributes to the pathogenesis of inflammatory diseases. In anti-glomerular basement membrane Ab-induced glomerulonephritis (GN), we have previously noted that FcR-deficient mice (γ−/−) surviving from lethal initial damage still developed mesangial proliferative GN, which was drastically prevented by an AngII type I receptor (AT1) blocker. We further examined the mechanisms by which renin-angiotensin system (RAS) participates in this immune disease. Using bone marrow chimeras between γ−/− and AT1−/− mice, we found that glomerular injury in γ−/− mice was associated with CD4+ T cell infiltration depending on renal AT1-stimulation. Based on findings in cutaneous delayed-type hypersensitivity, we showed that AngII-activated renal resident cells are responsible for the recruitment of effector T cells. We next examined the chemotactic activity of AngII-stimulated mesangial cells, as potential mechanisms coupling RAS and cellular immunity. Chemotactic activity for T cells and Th1-associated chemokine (IFN-γ-inducible protein-10 and macrophage-inflammatory protein 1α) expression was markedly reduced in mesangial cells from AT1−/− mice. Moreover, this activity was mainly through calcineurin-dependent NF-AT. Although IFN-γ-inducible protein-10 was NF-eB-dependent, macrophage-inflammatory protein 1α was dominantly regulated by NF-AT. Furthermore, AT1-dependent NF-AT activation was observed in injured glomeruli by Southwestern histochemistry. In conclusion, our data indicate that local RAS activation, partly via the local NF-AT mechanism, enhances the susceptibility to T cell-mediated injury in anti-glomerular basement membrane Ab-induced GN. This novel mechanism affords a rationale for the use of drugs interfering with RAS in immune renal diseases.

immune-mediated GN and allograft rejection (13, 14). However, the mechanisms of the beneficial effects of RAS blockade in those diseases are still unclear.

Although the precise mechanisms remain undefined, previous studies have demonstrated that Ab deposition onto GBM strongly activates the intrarenal (15) and systemic RAS (16, 17), inducing hemodynamic changes in a dose-dependent manner. Surprisingly, the AT1 blocker drastically attenuated glomerular injury and accumulation of macrophages in γ−/− GN (3). Furthermore, the activated RAS may participate early in the pathogenesis of this disease. Those findings highlighted a certain role of RAS in immune renal injury.

We hypothesized that RAS activation plays an essential role in the susceptibility of local cellular immune response. In the kidney and lymphocytes, AngII exerts its biological effects mainly via AT1 (18). In rodent, AT1 exists in two isoforms, AT1A and AT1B, regulated by two different genes. The murine AT1A is the isoform predominantly expressed in most tissues (19). AngII via AT1A triggers the proliferation of splenic lymphocytes following systemic cellular immune responses in mice (20). A recent study AT1A triggers the proliferation of splenic lymphocytes following systemic cellular immune responses in mice (20).

AngII also examined the potential mechanisms coupling RAS activation with inflammatory responses, such as chemokine expression and the cellular immune response, such as chemokine expression and T cell recruitment. In addition, we further studied intracellular events involved in cell signaling with special attention to transcriptional factors as mediators of the AngII-induced inflammatory process (11, 12), including NF-κB (22, 23) and NF-AT (24, 25). Our present findings show a novel mechanism in the pathogenesis of IC disease and propose the potential therapeutic interest of RAS blockade in immune renal diseases.

Materials and Methods

Mice

FcRγ-chain-deficient (γ−/−) and AT1AR-deficient (AT1−/−) mice were generated by a homologous recombination method. Construction of target-gene flanking sequences for rabbit and murine IgG, C3, CD4+ T cells, and then stained with FITC-labeled Abs (ICN Pharmaceuticals, Frankfurt, Germany; DAKO, Barcelona, Spain; and BD Pharmingen, San Diego, CA). Mesangial proliferation was evaluated by the numbers of mesangial cells (MC) in one glomerular tuft (score 0, 0–2%; 1, 3–4%; 2, 5–6%; 3, 7–8%; 4, >8%). Glomerular endothelial damage was scored by the percentage of fibrin deposition in one glomerulus (score 0, 0%; 1, 1–25%; 2, 25–50%; 3, 50–75%; 4, >75%). At least 25 glomeruli of one animal and five animals of each group were examined. The mean scores of each group were expressed in Table I as follows: 0–1, (–); 1–2, (+); 2–3, (++; 3–4, (++; 4, ++). For the evaluation of the CD4+ T cells, at least 30 glomeruli per section were examined using a blinded protocol as previously described (26). The results were expressed as cells per glomerular section.

Preparation of murine MC and AngII stimulation

Murine MC (WT and AT1−/−) were cultured from isolated glomeruli by several sieving techniques and different centrifugation as previously described (23), and maintained in RPMI 1640 medium (Life Technologies, Grand Island, NY) containing 10% FCS, 1 mM l-glutamine, and 100 μg/ml penicillin/streptomycin. MC were characterized by phase contrast microscopy and immunohistochemistry (positive staining for desmin and vimentin, and negative staining for keratin and factor VIII Ag) (23, 27). Glutaraldehyde cells between the first and third passages were used for assays.

After a 48-h starvation, both WT and AT1−/− MC were stimulated with AngII 10−7 M in serum-free medium for 3, 6, 12, and 24 h. Supernatants from stimulated MC were collected for chemotaxis assays. For inhibition assays, MC were preincubated with a NF-κB inhibitor, 10 μM parthenolide (Sigma-Aldrich, Madrid, Spain) for 1.5 h (28), or with calcineurin (C41B NF-AT inhibitors, 1 μM cyclosporin A (CsA; Sigma-Aldrich), or 10 μM CdA (an inhibitor polyaromatic hydrocarbon binding, Damron, Ge-
Chemotaxis assays

The chemotactic activity of MC supernatants was evaluated in 24-well Transwell chemotaxis chambers (Costar, High Wycombe, U.K.), as previously described (30). The lower wells were loaded in triplicate with 600 µl of the supernatants and covered with a 5-µm pore-size polycarbonate membrane. Upper compartments were loaded with 100 µl of the cell suspension containing 5 × 10^5 T cells (Jurkat cell; ATCC TIB-152; American Type Culture Collection, Manassas, VA). The chambers were incubated at 37°C for 4.5 h. Chemotaxis of T cells. Migrating cells in the lower compartment were counted by flow cytometry. Specific chemotaxis data represent the fold-increase of the average number of migrated cells with ± SEM. To assess chemotaxis of T cells, migrating cells in the lower compartment were counted by flow cytometry. Specific chemotaxis data represent the fold-increase of the average number of migrated cells with ± SEM.

RNA extraction and mRNA expression analyses

Total mesangial RNA was obtained by the TRIzol method (Life Technologies). One microgram RNA from stimulated MC was reverse-transcribed in the presence of 0.5 µCi [α-32P]dCTP (3000 Ci/mmol; Amersham, Arlington Heights, IL) and 20 pmol specific primers for mouse IFN-γ-inducible protein (IP)-10 (sense, 5′-CAACCAAGTGTGGCC-3′; antisense, 5′-GGGAAATCTCACATGTTGACCA-3′; fragment, 475 bp, ref. AF 227743) (31), mouse macrophage-inflammatory protein (MIP) 1α (sense, 5′-CGCTGCTCCTCTTGCAACT-3′; antisense, 5′-CTGGCGGCTTGGCTGTTA-3′; fragment, 189 bp, ref. NM 002983) (32), and mouse GAPDH (sense, 5′-GCGGGTTCTGAGTGGCAGTG-3′; antisense, 5′-CAGGGTTCTGAGGTCGTGATG-3′; fragment, 289 bp, ref. AK 013857). The amplifications were performed under annealing temperatures of 61°C (IP-10), 62°C (MIP1α), or 59°C (GAPDH). The optimum number of amplification cycles used for semi-quantitative RT-PCR (30, 32, and 25, respectively) was chosen on the basis of pilot experiments (data not shown). In some cases, PCR products of IP-10 and MIP1α were purified from low-melting temperature agarose gel, radiolabeled with Random Primed DNA Labeling kit (Roche, Indianapolis, IN), and used as cDNA probes for hybridization in Northern blot analysis. The expression of GAPDH was used as internal control. Aliquots of each reaction were run on a nondenaturing, 4% acrylamide gel at 100 V at room temperature and stained with ethidium bromide. Soluble materials were precipitated by centrifugation at 12,000 rpm for 30 min at 4°C. Supernatants were dialyzed overnight against a binding buffer containing 20 mM HEPES-NAOH (pH 7.6), 20% (v/v) glycerol, 0.1 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSE, and 1 µg/ml pepstatin A. The homogenate was vigorously shaken, and the insoluble materials were precipitated by centrifugation at 12,000 rpm for 30 min at 4°C. Supernatants were dialyzed overnight against a binding buffer containing 20 mM HEPES-NAOH (pH 7.6), 20% (v/v) glycerol, 0.1 mM NaCl, 5 mM MgCl2, 0.1 mM EDTA, 1 mM DTT, and 0.5 mM PMSE. These dialysates were cleared by centrifugation at 10,000 × g for 15 min at 4°C and stored in aliquots at −80°C until use. Protein concentration was quantified by the bicinchoninic acid method (Pierce, Rockford, IL).

NF-AT consensus oligonucleotides (5′-CGCCCCAAGAGGAAAATTTGTTTCAATA-3′) (Santa Cruz Biotechnology, Santa Cruz, CA) were [32P]-end-labeled by incubation for 10 min at 37°C with 10 U T4 polynucleotide kinase (Promega) in a reaction containing 10 µCi [γ-32P]ATP (3000 Ci/mmol; Amersham), 70 mM Tris-HCl, 10 mM MgCl2, and 5 mM EDTA. The reaction was stopped by the addition of EDTA to a final concentration of 0.5 M. Nuclear proteins (10 µg) were equilibrated for 10 min in a binding buffer containing 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 2 µg poly(dI-dC) for a 20-µl final volume. When competition and supershift assays were performed, the cold probe and Abs (anti-NF-ATc4; 5 µg/Santa Cruz Biotechnology) were added to this buffer 30 and 60 min before the addition of the labeled probe. Labeled probe (0.035 pmol) was added to the reaction and incubated for 30 min at room temperature. The reaction was stopped by the addition of gel loading buffer (250 mM Tris-HCl, 0.2% bromophenol blue, 0.2% xylene cyanol, and 40% glycerol) and run on a nondenaturing, 4% acrylamide gel at 100 V and room temperature in 89 mM Tris-borate, 2 mM EDTA (pH 8.0; TBE) (22).

Southwestern histochemistry

This technique was developed to detect the in situ distribution and DNA-binding activity of transcriptional factors (34). NF-AT consensus oligonucleotide was digoxigenin-labeled with a 3′-terminal transferase (Boehringer Mannheim, Mannheim, Germany). Paraffin-embedded tissue sections were fixed in 0.5% paraformaldehyde and incubated with 0.1 mg/ml DNase I. The DNA binding reaction was performed by incubation with 50 pmol of the labeled DNA probe in buffer containing 0.25% BSA and 1 µg/ml poly(dI-dC). The sections were then incubated with alkaline phosphatase-conjugated anti-digoxigenin Ab, and colorimetric detection was performed as described. Preparations without probe were used as negative controls, and mutant-labeled probe and excess of unlabeled probe were used to test the specificity of the technique.

Assessment of cutaneous DTH

Mice were immunized i.p. with Ag (250 µg goat IgG) emulsified in CFA (8, 35). After 7 days, immunized mice were challenged with the same Ag (250 µg) in the hind footpad. For both induction of anti-GBM GN and DTH in γ−/− mice, those mice were immunized with rabbit IgG 3 days after DTH preimmunization and were injected with 3-fold higher NTS at the same time of the Ag challenge. DTH responsiveness was determined 24-h post-challenge by measuring the dorsal-ventral thickness difference of the Ag-injected left footpad and the saline-injected right footpad, as a control, using a micrometer (Mitutoyo, Kanagawa, Japan).

Table 1. Receptor phenotypes and glomerular lesions in each mouse strain

<table>
<thead>
<tr>
<th>Phenotype of FcR and AT1</th>
<th>Phenotype of Glomerular Lesion</th>
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<tbody>
<tr>
<td></td>
<td>1× NTS</td>
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<tr>
<td></td>
<td>end. damage</td>
</tr>
<tr>
<td>FcR</td>
<td>mes. prolif.</td>
</tr>
<tr>
<td>BMMC</td>
<td>++</td>
</tr>
<tr>
<td>RRC</td>
<td>+</td>
</tr>
<tr>
<td>AT1</td>
<td>+</td>
</tr>
<tr>
<td>γF</td>
<td>+</td>
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<td>γD</td>
<td>+</td>
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<tr>
<td>γW</td>
<td>+</td>
</tr>
<tr>
<td>WA</td>
<td>+</td>
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* 3× NTS, 3-fold higher amounts of NTS; nd, not done, because most of them died; end. damage, glomerular endothelial damage; mes. prolif., mesangial proliferation; crescents, crescental crescents.
Statistical analysis

Results are expressed as mean ± SD and were analyzed by ANOVA (see Fig. 1) and Mann-Whitney test (see Figs. 3–5) for comparison of quantitative variables. Statistical significance was established as p < 0.05 (two-tailed curve).

Results

FcR on bone marrow-derived cells (BMCs) plays a crucial role in acute glomerular injury in anti-GBM disease

We have previously demonstrated that FcR and AT1 are critical molecules in the induction of this disease (3). However, the relevant cell types expressing these receptors remain unknown. Therefore, to clarify this feature, we generated bone marrow chimera between mice strains lacking each receptor. Their receptor phenotypes (FcR and AT1) of BMCs and RRCs are summarized in Table I. We also generated control mice which were transplanted with bone marrow from the same mice strain (WW, γγ, and AA) under same irradiation conditions, and induced this disease. We could not find any significant difference in their disease phenotypes (urinary protein and renal pathology) from mice without transplantation (WT, γ−/−, and AT1−/−, respectively; data not shown), indicating that bone marrows in recipient animals were functionally reconstituted by transplantation and the irradiation condition may not elicit significant alteration of this disease.

Next, we analyzed the evolution of anti-GBM GN in these animals. During the acute phase of the disease, WT, AT1−/−, and WA mice showed severe proteinuria peaking at day 7 (Fig. 1A) with glomerular endothelial damage associated with fibrin deposits (Fig. 2A, a–c) (Table I). However, proteinuria peak in AT1−/− and WA mice was significantly less than in WT (p < 0.05) mice. All WT and most AT1−/− (62%) and WA (67%) mice died with massive ascites before day 35, while γ−/−, γW, and γA mice were completely protected from proteinuria (Fig. 1A) and endothelial damage (Fig. 2A, d–f). These data further confirm a critical implication of FcR on BMCs in the acute glomerular damage of this disease.

AT1-stimulation induces glomerular injury associated with CD4+ T cell infiltration in γ−/− mice

In contrast to that shown above, γ−/− and γW mice injected with higher amounts of NTS (3× NTS) developed moderate proteinuria (Fig. 1B) and glomerular injury characterized by mesangial proliferation, cellular infiltration, and glomerular enlargement with occasional crescents (Fig. 2A, g and h) (Table I). Glomerular injury in γ−/− mice was associated with CD4+ T cell infiltration in a dose-dependent manner (Table II). Interestingly, γA mice were drastically protected from proteinuria (Fig. 1B) and glomerular injury (Fig. 2Aii) with absence of T cell infiltration (Table II) in the 3× NTS model, even though the heterologous (rabbit IgG), autologous (mouse IgG) Ab, and C3 depositions in γA mice were similarly noted in γ−/− (Fig. 2B) or WT (data not shown) mice. These data indicate that tissue AT1 is responsible for T cell-associated glomerular injury in this disease.

Cutaneous DTH response is not attenuated in AT1−/− or γ−/− mice with anti-GBM GN

To investigate whether FcR or AT1 deficiency may affect systemic cell-mediated immune responses, we induced cutaneous DTH, a classical T cell-dependent inflammatory lesion. No difference in DTH responsiveness was noted in WT, γ−/−, and AT1−/− mice (Table III). This finding is consistent with the data of the autologous IgG deposition (Fig. 2B), and suggests that cutaneous DTH response is independent of FcR and AT1.

In certain conditions, AngII participates in the regulation of systemic cellular immune response (20). Therefore, to investigate whether the systemic RAS activation in anti-GBM GN (16, 17) is sufficient to nonspecifically enhance the systemic cellular immune response, we simultaneously induced anti-GBM GN (3× NTS model) and cutaneous DTH response in γ−/− mice. We failed to find any difference in systemic DTH response in γ−/− mice with or without GN (Table III), suggesting that systemic RAS activation in this disease may have no significant role in systemic T cell function.

AngII enhances the chemotactic activity for T cells and the mRNA expression of Th1-associated chemokines in MC through AT1

Based on the above-mentioned in vivo findings from bone marrow chimera and systemic DTH responses, we next postulated that glomerular T cell infiltration may be regulated by RRCs activated by AngII. We especially focused on glomerular...
MC, because they possess both AT1 and AT2, regulate the glomerular blood flow, and release proinflammatory cytokines in response to AngII (11, 12, 33). In addition, mesangial proliferation is abolished in γA mice with 3X NTS. In this study, we examined AngII-induced chemotactic activity for T cells, as a possible mechanism involved in the recruitment of those cells. In the chemotactic assays with T cells, supernatants from WT MC treated with AngII had significantly higher activity (~3.5-fold) than treatment medium alone, reaching a plateau after 12 h (Fig. 3A). By contrast, supernatants from AngII-stimulated AT1−/− MC showed significantly less activity (around basal at 12 h) (Fig. 3A), indicating that the chemotactic activity induced by AngII in MC occurred mainly through AT1. We also noted that AngII by itself presented a low chemotactic activity for T cells (average of migration in control medium vs medium with AngII: 354 ± 90 vs 743 ± 92 cells), consistently with a previous study (36).

Recent data have convincingly demonstrated that nephритogenic T cells associated with crescent GN in this disease are mainly Th1 cells (6). Functional diversity between Th1 and Th2 is partly due to the difference the chemokine receptor phenotypes (37, 38). CXCR3 and CCR5 are preferentially expressed in Th1 cells (37). Therefore, we also studied the regulation of their corresponding ligands (CXCR3, IP-10; CCR5, MIP1α) in MC stimulated by AngII. As noted in Fig. 3B, AngII (10−6 M) significantly up-regulated the mRNA expression of IP-10 and

**FIGURE 2.** FcR on BMCs and tissue AT1 are responsible for distinct glomerular damages. A, Severe endothelial damage with fibrin deposits in acute phase was observed in mice strains having FcR on BMCs (at day 7, a, WT; b, AT1−/−; c, WA), but not in mice strains lacking them (d, γ−/−; e, γW; f, γA). However, higher amounts of NTS (3X NTS) induced mesangial proliferative GN depending on tissue AT1 (at day 14, g, γ−/−; h, γW; i, γA). B, Although γA did not present morphological lesions, no obvious differences in the deposition of heterologous IgG (rabbit IgG) and autologous IgG and C3 (mouse IgG/C3) were noted between γ−/− and γA mice. (Original magnification in each panel, ×100).
MIP1α in WT MC, peaking at 6 h, as determined by semiquan-
titative RT-PCR. These data were confirmed by Northern blot
analyses. As shown in Fig. 3C, AngII induced mRNA expres-
sion of both chemokines in MC with similar kinetics. By both
methods (Fig. 3, B and C), AT1−/− MC showed significantly
less mRNA expression in IP-10 and MIP1α than WT MC, indi-
cating that the expressions of both chemokine genes are
mainly elicited through AT1 stimulation.

**AngII-induced chemotactic activity involves CaN/NF-AT and
NF-κB pathways**

Emerging data reveal that the AngII/NF-κB pathway contrib-
utes to the pathogenesis of inflammatory diseases via regulation
of chemokine production (11). In contrast, although CaN/
NF-AT pathways were firstly reported in T cells (39), their
importance has been recently highlighted in other organs, such
as the heart, vascular system, neurons, and muscles (24, 25,
40–43). Special attention has been paid to the AngII/NF-AT
pathway in the pathogenesis of certain diseases (24, 25). The
activity of NF-AT proteins is tightly regulated by the calcium/
calmodulin-dependent phosphatase CaN (39). Recent studies
suggested the implication of the CaN-mediated activation of
NF-AT in chemokine production (44). Therefore, to ap-
proach possible transcriptional regulations in this mechanism,
we pretreated MC with inhibitors of NF-AT (CsA, 97% of
inhibition at 6 h, 85% at 12 h), but not with CsA (24% at 6 h,
4% at 12 h) (Fig. 4A), even though CsA itself slightly induced
chemotactic activation to MC (around 1.2- to 1.4-fold increase
at 12 h) (Fig. 4). By contrast, parthenolide showed only 24 and 15% inhibition at 6 and 12 h, respectively
(Fig. 4A), suggesting that the CaN-dependent pathway plays a
predominant role in the AngII-induced chemotaxis by MC.

Next, we examined the implication of both pathways in Th1-
associated chemokine production. AngII-induced IP-10 mRNA
was markedly attenuated by pretreatment with parthenolide (75% inhibition at 6 h, 85% at 12 h), but not with CsA (24% at 6 h, 4% at 12 h) (Fig. 4B). In contrast, MIP1α mRNA expression was
inhibited around 40–50% by CsA at 6 and 12 h. The data are
consistent with previous studies that showed the presence of func-
tional NF-AT sites in the MIP1α promoter-enhancer region (47).
Accordingly, these data suggest that AngII did enhance mRNA
expression of both Th1-associated chemokines (IP-10 and
MIP1α), mainly via AT1 on MC, though their transcriptional reg-
ulation may be different.

![FIGURE 3. AngII enhances the chemotactic activity for T cells and the mRNA expression of Th1-associated chemokines in MC through AT1. Supernatants from AngII (10−6 M) -stimulated WT MC (●) showed significantly higher chemotactic activity for T cells than those from AT1−/− MC (○) (A). In WT MC stimulated with AngII (10−6M) (●), mRNA expression of Th1-associated chemokines (IP-10 and MIP1α) was higher than those in AT1−/− MC (○), as determined by RT-PCR (B) and Northern blotting (C). Data are presented as the mean ± SD (n = 4–5 experiments). * p < 0.05 (B), ** p < 0.01 (C) vs AT1−/− MC.](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Table II. <em>Glomerular T cell infiltration</em></th>
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<tr>
<td><strong>Group</strong></td>
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<tr>
<td>-----------------</td>
</tr>
<tr>
<td>γ−/− 1× NTS</td>
</tr>
<tr>
<td>γ−/− 3× NTS</td>
</tr>
<tr>
<td>γA 3× NTS</td>
</tr>
</tbody>
</table>

<sup>a</sup> p < 0.05, vs γ−/− 1× NTS or γA 3× NTS. c/gcs: cells/30 glomeruli cross-sections.

![Table III. *DTH responsiveness of each mouse*](http://www.jimmunol.org/)

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Ag challenge</th>
<th>Control saline</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>3</td>
<td>29.3 ± 4.9</td>
<td>8.3 ± 5.9</td>
</tr>
<tr>
<td>AT1−/−</td>
<td>3</td>
<td>27.7 ± 3.5</td>
<td>6.3 ± 4.2</td>
</tr>
<tr>
<td>γ−/−</td>
<td>3</td>
<td>26.6 ± 2.1</td>
<td>4.0 ± 2.0</td>
</tr>
<tr>
<td>γ−/− + nephritis</td>
<td>3</td>
<td>27.3 ± 4.9</td>
<td>6.7 ± 2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> DTH responsiveness was determined 24-h postchallenge by measuring the in-
crease of footpad size (×0.01 mm).

**γ−/− mice with anti-GBM GN show renal NF-AT activation which is attenuated by an AT1 blocker**

To confirm the implication of the renal CaN/NF-AT pathway in
this disease, we performed EMSA with nuclear proteins from the
renal cortex. WT mice showed an early peak of NF-AT activation at 3 h after the injection of the Ab, and reactivation at 24 h (Fig. 5). \( \gamma^{−/−} \) mice showed basically the same kinetics of NF-AT activation. There was no significant difference in the peak amplitude of NF-AT activation in WT and \( \gamma^{−/−} \) mice. Preincubation with an anti-NF-ATc4 Ab attenuated the NF-AT peak signals, indicating that activated renal NF-AT in this disease involves NF-ATc4 (NF-AT3).

To clarify the relevant cell types of this activation, Southwestern histochemistry with NF-AT oligo probes was done in \( \gamma^{−/−} \) mice. In the acute phase of this disease, NF-AT activation was observed in glomeruli, mainly at MC (Fig. 6, upper panels). Interestingly, in the chronic phase of this disease, \( \gamma^{−/−} \) mice showed activation signals not only in glomeruli, but also in tubuli and interstitial infiltrating cells (Fig. 6, lower panels).

Next, we investigated whether RAS blockade may affect the NF-AT activation in the acute phase of this disease. Surprisingly, treatment with valsartan, an AT1 blocker, drastically attenuated the NF-AT activation at 3 and 24 h in \( \gamma^{−/−} \) mice (Fig. 5), consistently with the decrement of glomerular activation (Fig. 6, upper panels). These data suggest that AngII-induced NF-AT activation in RRCs may contribute to the initiation of this disease. Furthermore, the NF-AT pathway in resident and infiltrating cells could also be involved in the development of this disease.

**Discussion**

FcR on BMCs plays a crucial role in acute glomerular injury in anti-GBM disease

The role of activating FcR in providing a critical link between ligands and effector cells in Ab/IC-mediated inflammation has been well-established (1, 2), but the significance of these receptors on each effector cell type along the disease still remains unclear. In the present study, even though \( \gamma^W \) and \( \gamma^A \) mice have FcR on RRCs, acute lethal damage observed in WT and \( \gamma^{−/−} \) mice was...
completely abolished in these chimeric mice. These findings further confirm the critical implication of FcR in the acute phase of anti-GBM GN (3) and indicate that FcRs, especially on BMCs, are essentially required for an initial inflammatory response after Ab deposition. Consistently, the injury can be induced in WT chimeras (BMCs; WT, RRCs; \( \gamma^c \)) (48). Imasawa et al. (49) recently have suggested that BMCs may have the potential to differentiate into glomerular resident cells. However, in contrast, Mayadas and co-workers demonstrated that FcγRIII on PMN is essentially required for initial recruitment of PMN in anti-GBM GN (50) and, following interaction between FcγR and CD11b/CD18 (Mac 1) on PMN, is also necessary for sufficient PMN spreading on the glomerular capillary wall (51). In fact, the absence of acute glomerular damage in \( \gamma^c \) mice was associated with the lack of PMN influx (3). Although we need to examine the contribution of bone marrow-derived glomerular resident cells in the acute inflammatory settings, our present data further support the idea that FcγR on PMN, but not on RRCs, may play a major role for acute endothelial damage.

**Persistent proliferative GN in \( \gamma^c \) mice is closely linked to glomerular-infiltrating CD4\(^+\) T cells**

Interestingly, \( \gamma^c \) mice developed GN persisting for >5 mo and its severity was dependent on the amount of Ab injected and the number of glomerular-infiltrating CD4\(^+\) T cells. In addition, the morphological lesions are highly analogous to those seen in studies demonstrating T cell-dependent injury of this disease (6). These data indicate that CD4\(^+\) T cell (Th1)-dependent response is pivotal for the development of mesangial proliferative GN in \( \gamma^c \) mice. This hypothesis is further supported by recent findings of our group using mouse strain overexpressing Smad7 (an inhibitory molecule of TGF-β signaling) (26), in which the CD4\(^+\) T cells cannot migrate into the inflammatory sites due to the deregulation of CD62 ligand (L-selectin) expression. In anti-GBM disease, the development of GN, including macrophage infiltration, in these animals was drastically attenuated, suggesting that the development and the persistence of this disease essentially require glomerular-infiltrating CD4\(^+\) T cells.

**Renal RAS activation conducts glomerular T cell response**

T cell-dependent injury in \( \gamma^c \) and WT mice required three times higher amounts of anti-GBM Ab than FcR-mediated endothelial injury, indicating different thresholds for their activation by the same Ab. However, \( \gamma^c \) chimeras were protected from the glomerular T cell response even in the high dose model, emphasizing that AngII action via AT1 on recipients could be responsible for the threshold of the T cell-mediated mechanism. Besides, different T cell responses between WT and \( \gamma^c \) chimeras indicate that their...
bone marrow-derived glomerular resident cells (49) (presumably FcR− but AT1+) may not play an important role for T cell recruitment. In this disease, dose-dependent activation of intrarenal and systemic RAS has been demonstrated (15–17). AngII has some cellular effects on most tissues, mainly via AT1, that may contribute to the disease pathogenesis (11, 19), and also regulates cellular immunity by acting on the proliferation of splenic lymphocytes (20). However, in a cutaneous DTH, AT1 deficiency did not alter the responsiveness, in accordance with a previous study (21), and we failed to find any difference between γ−/− mice with or without nephritis, suggesting that systemic RAS activation in this disease may not play a significant role in general T cell function. Accordingly, the amplitude of intrarenal RAS activation would dominantly conduct the glomerular DTH response in this disease, though we must carefully elucidate the alteration of AT1 expression on T cells by elevated plasma AngII.

**AngII enhances Th1-associated chemokine expression in MC, mainly via AT1**

It is already known that AngII itself is chemotactic for T cells (36). Besides confirming this feature, we noted that supernatants of MC treated with AngII elicited a marked chemotactic activity for T cells, indicating a predominant role of second mediators (presumably chemokines) induced by AngII. The experiments with AT1−/− MC revealed that those AngII actions were exerted mainly via AT1. These findings are consistent with previous studies showing that AngII, acting through both AT1 and AT2, induces T cell-chemokine production (12, 33, 52).

Enhanced expressions of IP-10, MIP1α, and their receptors in kidney have been previously shown in this disease (53), as well as in human mesangial proliferative GN (e.g., IgA nephropathy) (54). The present study shows that the expression of these Th1-associated chemokines in MC is up-regulated by AngII mainly through AT1. MIP1α redundantly cross-reacts with CCR5 and CCR1 as well as other chemokines (53), whereas IP-10 is more selective to CXCR3 (37). Moreover, CCR1 is expressed equally in Th1 and Th2, while CCR5 is not (37, 38). In this regard, interestingly, CCR1-deficient mice with anti-GBM GN showed enhanced Th1 response and glomerular crescents, while not only both chemokines, but also CXCR3 and CCR5, were up-regulated in association with higher CD4+ T cell and macrophage infiltration (53). This evidence suggests that Th1-deviated immune response of this disease may be partially enhanced by chemokine phenotypes produced by RMCs.

Importantly, once T cells and subsequent macrophages are, even if nonspecifically, recruited into the inflamed kidney by local RAS activation, they may orchestrate the autocrine/paracrine-acceleration loop accompanied by locally elevated AngII because they are equipped with all RAS components (55). In fact, significant sources of tissue ACE in human atherosclerotic plaques are regions of clustered macrophages (56). In addition, IL-12, a key cytokine for Th1 response, from mononuclear cells is suppressed by ACE inhibitors (57). In this regard, the role of immunocompetent cells in nonimmune renal diseases further supports this notion (55). Salt-sensitive hypertension after AngII infusion was associated with tubulointerstitial accumulation of AngII-producing lymphocytes and was prevented by the immunosuppresser mycophenolate mofetil coincidentally with a reduction of those cells (38).

**AngII exerts proinflammatory effects in the kidney, partly through the CaN-dependent NF-AT pathway**

Elevated local AngII in this disease may result from physiological responses to the alterations elicited by the specific Ab deposition. Therefore, MC could be one of the major targets of the AngII effect. Indeed, as a consequence of mesangial constriction by AngII, a significant decrease in glomerular plasma flow and single nephron glomerular filtration rate, followed by increased renal vascular resistance, was observed in this model in a dose-dependent manner (15, 17). Consequently, one can postulate that excessively elevated AngII may elicit increased intracellular calcium levels in MC and subsequently a wide variety of cellular responses by a Ca2+-dependent pathway. Some parts of RAS influence on immunological function may be due to such indirect outcome (20, 36, 59). In this sense, it is noteworthy that the chemotactic activity for T cells in AngII-treated MC was largely attenuated by CaN-specific inhibitors in the present study. Although NF-AT3 (NF-AT4) mRNA was previously detected in the kidney (60) and endothelin 1 activates cyclooxygenase 2 expression via NF-AT in cultured MC (61), there are still no studies demonstrating the functional or pathological contribution of NF-AT during kidney disease. NF-AT activity requires the sustained Ca2+ stimulus provided by the Ca2+-release-activated Ca2+ influx channel and Ca-dependent phosphatase CaN (39, 43). Therefore, there is considerable evidence that the Ca2+-release-activated Ca2+ influx in MC is under the control of both protein kinase C and calmodulin, and thus represents a key mechanism for the control of Ca2+-regulated mesangial function (62).

Because a study with synthetic peptides blocking NF-AT activation by CaN postulates CsA-sensitive (presumably CaN-dependent) gene expressions that are not controlled by NF-AT (47), we must carefully elucidate the mesangial CaN/NF-AT pathway with AngII stimulation in future studies. However, our present data in EMSA and Southwestern histochemistry strongly support the notion that AT1-stimulated NF-AT activation may be involved in the pathogenesis of this immune-mediated disease, as it occurs in myocardial hypertrophy (24). It is interesting to note that CsA could directly prevent mesangial proliferative GN independently of its immunosuppressive action (63). AngII regulates cellular immune responses through the CaN-dependent pathway within the lymphoid tissue (20). The present data show for the first time the activation of the local CaN/NF-AT pathway early in this renal disease, and its attenuation by valsartan, an AT1 blocker, suggesting that locally elevated AngII, probably together with inflammatory cytokines (25), may regulate cellular immune responses partly via the local CaN/NF-AT pathway. Furthermore, the distribution of the activated NF-AT, changing from glomeruli to tubulo-interstitium and infiltrating cells along the disease course, suggests its implication in the different stages of the disease and supports the idea of the potential interest in targeting this pathway (24, 47).

Although we and others have already reported the contribution of AngII/NF-κB (11, 33) and IC/NF-κB pathways in the pathogenesis of GN (27, 64) through the chemokine release, NF-κB inhibitors had less effects on the chemotactic activity. Our data indicate that IP-10 expression was mainly regulated by the AngII/NF-κB pathway, while AngII-enhanced MIP1α expression was mainly through CaN/NF-AT. Interestingly, although MIP 1α can be active to resting T cells (65), IP-10 mainly influences on activated T cells (66). Tight regulation of the chemokine receptor expression in T cells can be the reason for the difference (38, 67). Because this cell clone behaves more like “naïve” than “activated” T cells (68, 69), it could be one of the reasons why the good inhibitor of IP-10, parthenolide, had less effects on chemotaxis. In fact, CaN/NF-AT inhibitors showed marked attenuation of chemotaxis, suggesting that AngII-induced NF-AT activation may preferentially contribute to the chemotaxis of inactivated T cells. However, in vivo T cell chemotaxis may be regulated in a more complicated manner (38). Because chemokine/chemokine receptor interaction, for example, contributes to position effector T cells (38, 67), the infiltrating T cell population would shift from more
like “naïve” to “activated” cells along the disease course, as well it occurs in other immune diseases. Indeed, acute activation of NF-AT, peaking at 3 h, and delayed NF-κB activation at 24 h in γ−/− mice (our unpublished data) may support this idea. Therefore, our present data indicate that AngII-activated transcriptional factors and subsequent chemotactic mediators, including MPIo and IP-10, play roles in a process of multistep navigation to T cells in this disease.

Conclusion

In conclusion, the current studies provide evidence for AngII-dependent NFAT pathway in the pathogenesis of IC nephritis. Finally, these results afford a rational basis for the use of RAS antagonists in patients with renal immune diseases.

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References

ROLE OF LOCAL RAS IN T CELL-MEDIATED IC DISEASE


