CC Chemokine Ligand 5/RANTES Chemokine Antagonists Aggravate Glomerulonephritis Despite Reduction of Glomerular Leukocyte Infiltration

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The chemokine CC chemokine ligand (CCL)5/RANTES as well as its respective receptor CCR5 mediate leukocyte infiltration during inflammation and are up-regulated early during the course of glomerulonephritis (GN). We tested the effects of the two CCL5/RANTES blocking analogs, Met-RANTES and amino-oxypentane-RANTES, on the course of horse apoferritin (HAF)-induced GN. HAF-injected control mice had proliferative GN with mesangial immune complex deposits of IgG and HAF. Daily i.p. injections of Met-RANTES or amino-oxypentane-RANTES markedly reduced glomerular cell proliferation and glomerular macrophage infiltration, which is usually associated with less glomerular injury and proteinuria in HAF-GN. Surprisingly, however, HAF-GN mice treated with both analogs showed worse disease with mesangiolysis, capillary obstruction, and nephrotic range albuminuria. These findings were associated with an enhancing effect of the CCL5/RANTES analogs on the macrophage activation state, characterized by a distinct morphology and increased inducible NO synthetase expression in vitro and in vivo, but a reduced uptake of apoptotic cells in vivo. The humoral response and the Th1/Th2 balance in HAF-GN and mesangial cell proliferation in vitro were not affected by the CCL5/RANTES analogs. We conclude that, despite blocking local leukocyte recruitment, chemokine analogs can aggravate some specific disease models, most likely due to interactions with systemic immune reactions, including the removal of apoptotic cells and inducible NO synthetase expression.

Proliferative immune complex glomerulonephritis (GN) is characterized by infiltration of macrophages into the glomerulus. Glomerular macrophages secrete proinflammatory cytokines, reactive oxygen radicals, and NO, which are critical mediators of further glomerular damage and proteinuria, as macrophage depletion with antisera reduces cytokine production and proteinuria (1–3). Therefore, drugs that selectively inhibit glomerular leukocyte infiltration might be an option to reduce the inflammation of GN (4).

Glomerular up-regulation of CC chemokines such as CC chemokine ligand (CCL)2/monocyte chemoattractant protein-1 and CCL5/RANTES mediate the infiltration of leukocytes into the glomerulus in rat anti-Thy1.1 and rat and mouse nephrotic serum nephritis (NSN) as well as in murine models of lupus nephritis (5–9). Chemokine blockade with specific Abs or chemokine antagonists has been shown to reduce monocyte influx and proteinuria in some of these models (7, 10, 11). Therefore, chemokine antagonism is thought to be a promising therapeutic approach for proliferative GN as well as for many other inflammatory diseases (12–16).

However, chemokines are involved in the regulation of systemic immune responses at multiple levels, e.g., immune cell differentiation and migration to lymphoid organs, proliferation, and T cell polarization, all of which have been shown to influence the outcome of renal disease (17, 18). Therefore, before approaching clinical studies, chemokine antagonism must be carefully evaluated in various disease models, including those involving systemic immune responses, such as immune complex GN.

Horse apoferitin (HAF)-induced GN is a model of mesangio-proliferative immune complex GN in mice that is characterized by an early glomerular expression of CCL5/RANTES followed by proteinuria and infiltration of CCR5-positive macrophages into the glomerulus (19). Therefore, we intended to study the role of CCL5/RANTES in HAF-GN by using the two CCL5/RANTES analogs, Met-RANTES and amino-oxypentane (AOP)-RANTES. Both CCL5/RANTES analogs have been shown to block CCL5/RANTES binding to its respective human chemokine receptor CCR5 and to the murine CCR5 and to block leukocyte chemotaxis in vitro (20). Treatment with Met-RANTES decreased leukocyte infiltration in rat renal transplant rejection and in murine NSN (10, 21, 22). AOP-RANTES reduced the early influx of monocytes into the glomerulus after injection of the anti-Thy1.1 Ab in rats (23). However, as both antagonists have been shown to induce partial agonistic effects on CCL5/RANTES receptors, these analogs may have additional effects in other disease models (20).
Therefore, we hypothesized that Met-RANTES and AOP-RANTES may reduce glomerular macrophage infiltration and improve the murine HAF-GN, but may have additional effects on macrophage function, T cell polarization, and the humoral immune response in this model. Surprisingly, we found that, despite substantial reduction of glomerular macrophage accumulation, both CCL5/RANTES analogs induced aggravation of GN. The analysis of potential mechanisms responsible for the unexpected deterioration revealed that CCL5/RANTES antagonists do not cause a shift in the Th1-Th2 balance but induce a certain phenotype of macrophages that is characterized by a foam cell-like morphology and increased inducible NO synthetase (iNOS) expression in vitro and in vivo. Furthermore, Met-RANTES treatment of mice reduced subsequent uptake of apoptotic cells by macrophages. These novel findings could help to explain why the CCL5/RANTES antagonists can aggravate HAF-GN. These novel findings also point to novel functions of chemokines in monocyte/macrophage biology such as iNOS expression and illustrate the complexities of the use of chemokine antagonists in immunological diseases.

Materials and Methods

**Animals and experimental protocol**

Female inbred BALB/c mice (18 to 21 g body weight) were obtained from Charles River (Sulzfeld, Germany) and were kept in macrolone type III cages under a 12-h light and dark cycle. Water and standard chow (Sniff, Soest, Germany) were available ad libitum. Mice were sacrificed by cervical dislocation. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

Seven groups of six to seven mice each were studied. In group I (saline), mice were injected with 200 μl of normal saline i.p. once daily. In group II (MR40), mice received 40 μg of Met-RANTES in 100 μl of normal saline once daily i.p. on 14 consecutive days. In group III (AR10), mice received 10 μg of AOP-RANTES in 100 μl of normal saline once daily i.p. on 14 consecutive days. In group IV (HAF), GN was induced by daily i.p. injections of 5 μg of HAF in 100 μl of 0.1 M sodium chloride (Sigma-Aldrich, Steinheim, Germany) for 14 consecutive days, as previously described (19). Once daily, all mice of this group received an additional injection of 100 μl of normal saline i.p. In group V (HAF-MR10), HAF-GN was induced as in group IV, with an additional injection of 10 μg of Met-RANTES in 100 μl of normal saline once daily i.p. In group VI (HAF-MR40), mice were injected as in group IV using a Met-RANTES dose of 40 μg once daily. In group VII (HAF-AR10), HAF-GN was induced as in group IV, with an additional injection of 10 μg of AOP-RANTES in 100 μl of normal saline once daily i.p.

**Evaluation of immune complex GN**

Spot urine samples were collected from each animal at the end of the study. Urine albumin concentration was measured using a mouse albumin ELISA kit (Albowell M: Excocell, Philadelphia, PA). Albumin excretion was expressed as urine albumin-creatinine ratio (micrograms-milligrams). Urinary creatinine concentrations were determined using a previously described method (19).

**Spot urine samples**

Light and electron microscopical evaluation

Renal tissue was fixed in 4% buffered formalin, processed, and embedded in paraffin. Three-micrometer-thick sections were cut and stained with periodic acid–Schiff stain. For the quantitation of obstructed glomerular capillary, 30 cortical glomeruli were analyzed from each section using the following scoring system: 0, no capillaries obstructed; 1, <25% of the capillary tuft obstructed; 2, 25–50% of the capillary tuft obstructed; 3, 51–75% of the capillary tuft obstructed; and 4, >75% of glomerular capillaries obstructed. A piece of cortical tissue from one kidney pole was fixed in glutaraldehyde and embedded in araldite for electron microscopic analysis as described (19).

**Immunofluorescence**

Renal tissue was snap frozen in liquid nitrogen and stored at −80°C. HAF deposits were detected by a fluorescein-conjugated anti-horse ferritin Ab (1/100; Jackson ImmunoResearch Laboratories, West Grove, PA). IgG deposits were detected by a Cy3-conjugated anti-mouse IgG Ab (1/100, Dianova). Other Abs used included anti-fibrinogen (rat, FITC, 1/200; Nordic, Hamburg, Germany) and anti-complement factor 3 (rabbit, FITC, 1/200; ICN Pharmaceuticals, Frankfurt, Germany).

**Immunohistology**

Paraffin-embedded sections were processed as described (19). The following rat and rabbit Abs were used as primary Abs: anti-CD45 (leukocytes; 1/100; BD Pharmingen), anti-Mac3 (1/100; macrophages; BD Pharmingen), anti-Mac2 (1/50; macrophages; Cederlane, Ontario, Canada), anti-CD3 (1/50; lymphocytes; Serotec, Oxford, U.K.), anti-Ki-67 (rabbit, 1/25; cell proliferation; Dianova), anti-Met-RANTES (rat, 1/50; see Ref. 24), anti-iNOS (rabbit, 1/2000; obtained from J. Pfeilschifter (University of Frankfurt, Frankfurt, Germany)). Staining for lgs was performed on acetone-fixed frozen section using anti-lg (P260; rat, 1/400; DAKO), anti-IgG1 (rabbit, 1/50; Dianova), anti-IgG2a (rabbit, 1/100; Dianova) as detection Abs. All sections except for Ki-67 staining were counterstained with hemalum.

**Apoptosis assay**

To assess the amount of apoptotic cells within the glomerulus, sections of paraffin-embedded renal tissue were stained by a TUNEL method according to the instructions of the supplier (TdT FragEl kit; Oncogene, San Diego, CA). DNase-treated cells were used as a positive control for assay performance.

**Quantitative analysis**

For glomerular cell counts, intraglomerular cells were counted in at least 15 cortical glomeruli per section, selected by random sampling, from each animal. Only cells within the glomerular tuft were counted. Glomeruli were assessed only if >10 capillary loops were present to exclude evaluation of pole cuts. For the evaluation of apoptotic cell bodies, all glomeruli of a single section (81 ± 16 glomeruli per section) were analyzed and expressed as a ratio (number of apoptotic cells to all glomeruli per slice). For the assessment of glomerular Ig or complement deposits, 15 cortical glomeruli were analyzed from each section. Glomerular signals were scored using a semiquantitative index as follows: 0, no signal; 1, low signal intensity; 2, moderate signal intensity; and 3, strong signal. Staining for iNOS of glomerular cells was scored accordingly.

**Real-time quantitative RT-PCR on microdissected renal tissue**

Ten glomeruli were manually microdissected for each animal and underwent mRNA isolation and reverse transcription as reported (19). Real time RT-PCR was performed on a TaqMan ABI 7700 sequence detection system (PE Biosystems, Weiterstadt, Germany) using a heat-activated TaqDNA polymerase (AmpliTaq Gold; PE Biosystems) as described (19). Controls consisting of double distilled H2O were negative for target and housekeeper gene GAPDH. Primers and probes for mouse CCL5/RANTES were obtained as predesigned assay reagents from PE Biosystems. Primers for murine GAPDH of the following sequences were used: forward, 5′-CATGGCGTCCTGTTGTCACA-3′; reverse, 5′-ATGCTGGTTCACCACTCTTTC-3′; internal fluorescence labeled probe (VIC), 5′-CCTTTGCGTGCCGTGGGTGTTGACGC-3′; murine iNOS, forward, 5′-GTCAGCGACAAACTGACTCTCCAG-3′; reverse, 5′-GCATCGGCTCTTCTCCTGGT-3′; and internal fluorescence labeled probe (FAM), 5′-TGGAATCTACAGCTTACCGTACCG-3′.

**Measurement of IFN-γ production by splenocytes**

Spleens were removed under aseptic conditions and placed in RPMI 1640 plus 10% FCS on ice. After manual dissection, centrifugation, and an additional washing step, the single-cell suspension (3 × 106 cells/ml) was incubated in RPMI 1640 plus 10% FCS and 10 μg/ml PHA at 37°C and 5% CO2 in plastic six-well tissue culture plates. Culture supernatants were removed after 48 h and stored at −80°C. IFN-γ concentrations were determined using a commercial ELISA kit following the instructions of the supplier (BD Pharmingen).

**Phagocytosis assay**

The effect of the CCL5/RANTES analogs on the uptake of apoptotic cells by macrophages was determined by a previously described method (25). In brief, apoptosis was induced with incubation of murine thymocytes with 1 μM dexamethasone for 3 h before labeling with TAMRA (25 μg/ml for 20 min). TAMRA-labeled apoptotic cells (1 × 106) were injected i.p. into BALB/c mice 4 days after induction of asperic peritonitis by i.p. injection of 0.5 ml of brewer’s thyglocylate (Sigma-Aldrich). Before the i.p. instillation of apoptotic cells, the mice were pretreated three times with either 10 μg of Met-RANTES or saline i.p. for 36 h at 12-h intervals.

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Peritoneal macrophages were harvested 30 min after injection of the apoptotic cells and subjected to flow cytometry to determine the percentage of TAMRA-positive macrophages as an index of the uptake of apoptotic cells.

**In vitro studies with murine mesangial cells (MC)**

A murine MC line was used as previously described (26). Responses to cytokines and expression of chemokines, growth factors, and adhesion molecules of these MC are comparable with those of primary cultures of mouse MC (26). The cells were maintained in DMEM (Biochrom, Berlin, Germany) supplemented with 5% bovine serum (Serum Supreme; Bio-whittaker, Walkersville, MD) and 1% penicillin-streptomycin (100 U/ml and 100 μg/ml; Biochrom). To assess the proliferation of MC, the MTT assay (Sigma-Aldrich) was used. Aliquots of 20 × 10^3 cells in 100 μl of medium were cultured in 96-well microtiter plates for 24 h under standard conditions to yield firmly attached and stably growing cells. After discarding the supernatants, 100 μl of medium containing chemokines as indicated were added, and the cells were incubated for 24 h before performing the MTT assay. For each experiment, at least six wells were analyzed per experimental condition.

**In vitro studies with murine J774 macrophages**

J774 cells (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 containing 1 mM HEPES, 10% heat-inactivated bovine serum, and 1% penicillin-streptomycin. J774 cells were incubated for 12 h with 3 μM either CCL5/RANTES or Met-RANTES or standard medium without supplements. After stimulation, cells were harvested by trypsinization, and total RNA was prepared as described (16). In a similar experiment, J774 cells were incubated for 36 h, harvested, and prepared for immunostaining with an Ab specific for murine iNOS as described in Immunohistology.

**Statistical analysis**

Data were expressed as mean ± SD. Comparison of groups was performed using univariate ANOVA. Posthoc Bonferroni’s correction was used for multiple comparisons. Paired Student’s t test was used for the comparison of single groups (RT-PCR and in vitro studies). A value of p < 0.05 was considered to indicate statistical significance.

**Results**

**Functional and histopathological parameters of HAF-GN**

Daily HAF injections for 14 days resulted in mesangio-proliferative GN with mesangial hypercellularity and matrix expansion compared with saline-injected controls (Figs. 1, A and D). Glomerular cell proliferation is illustrated by an increase of Ki-67-positive cells (Table I; Fig. 2A). Proliferating cells were located to areas of mesangial matrix expansion and not to endothelial cells or podocytes and should represent MC and macrophages. As a sign of increased glomerular cell turnover during HAF-GN, apoptotic cells were detected within the glomerulus (Fig. 2C). No apoptotic cells were present in glomeruli of saline-injected controls (not shown). Glomerular infiltration of Mac-3- and Mac-2-positive macrophages was prominent compared with the mild increase of CD3-positive cells. Only very few resident macrophages or lymphocytes were present in glomeruli of saline controls (Table I; Fig. 2E). Consistent with the diffuse prominent mesangio-proliferative GN, HAF-injected mice developed albuminuria compared with saline-injected control mice (HAF, 153 ± 32, vs saline, 18 ± 9 μg albumin/mg creatinine; p < 0.05).

**Met-RANTES and AOP-RANTES reduce leukocyte infiltration and cell proliferation but increase the number of apoptotic cells in the glomerulus**

In mice with HAF-GN, Met- and AOP-RANTES reduced the amount of glomerular macrophages by 50% (Table I; Fig. 2, E and F). This reduction was independent of the two Met-RANTES doses administered. The phenotype of glomerular macrophages in Met-RANTES- and AOP-RANTES-treated mice was different from those of HAF controls. Large Mac-3-positive cells with prominent vacuoles were present within the mesangium, resembling foam cells. The presence of lymphocytes was not affected by both antagonists (Table I). The amount of proliferating cells in the glomerulus was dramatically reduced by both CCL5/RANTES antagonists (Table I; Fig. 2, A and B). In contrast, a significant increase of glomerular apoptotic cell bodies was noted in Met-RANTES-treated mice with HAF-GN (HAF-MR40, 0.24 ± 0.9, vs HAF, 0.08 ± 0.02 apoptotic cells/glomerulus; p < 0.05; Fig. 2, C and D) corresponding to one apoptotic cell per 7 glomeruli vs one per 20. Healthy mice treated with Met-RANTES (group II) or AOP-RANTES (group III) alone did not develop any histopathological abnormalities or albuminuria compared with saline-injected controls of group I (Table I).

**Met-RANTES and AOP-RANTES aggravate HAF-GN**

Surprisingly, HAF-GN mice of the groups treated with Met-RANTES or AOP-RANTES (groups V to VII) developed severe histopathological changes associated with massive albuminuria (Table I). In the kidneys of the treated mice, almost all glomeruli showed mesangiolysis and hyaline material in single capillaries (Figs. 1 and 3). Electron microscopy of mice treated with the antagonists confirmed mesangiolysis with necrotic MC and some cells undergoing apoptosis (Fig. 4A). Mesangial, subendothelial, and subepithelial dense deposits were present (Fig. 4B). Some capillaries were markedly dilated and

**FIGURE 1.** Renal pathology of HAF-GN. After 14 days of daily HAF administration, diffuse mesangio-proliferative GN was present in group IV (A) with several mesangial and infiltrating cells in mesangial fields (inset). Met-RANTES (40 μg)-treated mice with HAF-GN (B) and AOP-RANTES-treated mice (C) revealed mesangiolysis (large arrows and inset in B) and thrombus-like material within single glomerular capillaries (small arrows). Mice treated with Met-RANTES or AOP-RANTES (not shown) alone did not develop histopathological changes compared with saline-injected controls (D; periodic acid-Schiff; original, ×1000).
glomerular cells (arrows) was present (Fig. 1). Treatment with 40 μg Met-RANTES (B) reduced glomerular cell proliferation to the low levels of saline-injected controls (not shown; anti-mKi-67; original, ×630). Single glomerular apoptotic cell bodies as detected by staining for DNA fragments were found in higher amounts in Met-RANTES-injected mice (D) compared with controls with HAF-GN (C; TdT FragEl stain; original, ×630). HAF-injected mice developed glomerular macrophage accumulation (E) which was attenuated by Met-RANTES (40 μg) treatment (F). The remaining macrophages were larger and filled with lipid droplets (anti-mCD45; original, ×1000).

Table I. Serum, urinary, and histological findings in HAF-GN

|                         | Group                                |
|-------------------------|--------------------------------------|
|                         | I (saline)                           |
|                         | II (saline + 40 μg Met-RANTES)       |
|                         | III (saline + 10 μg AOP-RANTES)      |
|                         | IV (HAF)                             |
|                         | V (HAF + 10 μg Met-RANTES)           |
|                         | VI (HAF + 40 μg Met-RANTES)          |
|                         | VII (HAF + 10 μg AOP-RANTES)         |
| Albuminuria             |                                      |
| Uₐ₁₈/Uₑₐ₈ (μg/mg)ᵃ       | 18 ± 9                               |
| Humoral response        |                                      |
| Serum titers            |                                      |
| Anti-HAF Ig             | 12 ± 4                               |
| Anti-HAF IgG₁           | 11 ± 1                               |
| Anti-HAF IgG₂a          | 9 ± 3                                |
| Glomerular deposits     |                                      |
| HAF                     | 0 ± 0                                |
| Ig                      | 0 ± 0                                |
| IgG₁                    | 0 ± 0                                |
| IgG₂a                   | 0 ± 0                                |
| C3                      | 0 ± 0                                |
| Cellular response       |                                      |
| Glomerular leukocytes   |                                      |
| MAC3                    | 0.2 ± 0.2                            |
| CDS³                    | 0.1 ± 0.1                            |
| Proliferation and apoptosis | 0.4 ± 0.2                      |
| Ki-67                   | 0.0 ± 0.0                            |
| Apoptotic cells         | 0.0 ± 0.0                            |

ᵃ Urine albumin-creatinine ratio.

*, p < 0.01; group IV (HAF-GN) vs group I (saline); †, p < 0.01, groups V, VI, and VII (HAF-GN plus CCL5/RANTES analogs) vs group IV (HAF-GN).

Met-RANTES and AOP-RANTES do not affect the humoral response to HAF

To examine the underlying mechanisms for the worsening disease observed with the antagonist treatment, we evaluated whether Met- and AOP-RANTES altered the humoral immune response during...
HAF-GN. Serum titters of anti-HAF IgG were elevated to the same extent in all groups of HAF-injected mice (groups IV to VII, Table I). The amount of mesangial immune complex deposits assessed by immunofluorescence staining for HAF and IgG as well as of mesangial complement factor 3 deposits were also unaffected by both antagonists (Table I). Because CCR5 is a known marker for T cells associated with Th1-type reactions, we questioned whether CCL5/RANTES antagonists altered the Th1/Th2 balance during HAF-GN. We measured serum anti-HAF IgG1 and IgG2a titters, mesangial IgG1 and IgG2a deposits, and splenocyte IFN-γ and IL4 secretion as markers for the type of immune response. Met-RANTES and AOP-RANTES did not induce a subclass switch of serum anti-HAF IgG or of the respective mesangial IgG isotype deposits (Table I). Levels of IFN-γ in the supernatant of stimulated splenocytes were also unaffected by Met-RANTES treatment of mice with HAF-GN (HAF plus Met-RANTES, 45.2 ± 6.2, vs HAF plus saline, 50.8 ± 14.2 pg/ml). IL-4 levels in supernatants of stimulated splenocytes of all groups were below the detection level of the ELISA system used. Together, these data indicate that the CCL5/RANTES antagonists did not alter the serum Abs and IFN-γ production, arguing against a major shift in the Th1/Th2 balance.

As blocking the CCL5/RANTES receptors could potentially aggravate GN by positive backsignaling on glomerular CCL5/RANTES secretion, we also examined CCL5/RANTES mRNA expression in microdissected glomeruli isolated from mice of group IV (HAF) and group VI (HAF-MR40) using quantitative real-time RT-PCR (Fig. 5). There was no difference in glomerular CCL5/RANTES expression in both groups, indicating that the administration of the CCL5/RANTES analogs had no effect on local CCL5/RANTES production.

Met-RANTES and AOP-RANTES do not affect MC proliferation in vitro

To study the potential effects of both CCL5/RANTES antagonists on MC proliferation, we used the MTT assay in cultured murine
MC. Stimulation of murine MC in vitro with increasing concentrations of either CCL5/RANTES, Met-RANTES, or AOP-RANTES (0.01, 0.05, 0.1, 0.25, 1.0, and 2.0 μg/ml) did not affect MC proliferation (not shown).

RANTES analogs increase iNOS expression of glomerular macrophages

Because glomerular macrophages in Met-RANTES- and AOP-RANTES-treated mice revealed morphological differences compared with those of saline-treated controls, we intended to characterize these cells on a more functional level. As a marker of macrophage activation, we assessed glomerular iNOS protein expression by immunohistochemistry. Control mice with HAF-GN had only faint iNOS signals in occasional leukocytes within the glomeruli. In contrast, Met- and AOP-RANTES-treated mice with HAF-GN had a significantly higher number of iNOS-positive infiltrating cells per glomerulus (Fig. 6). Semiquantitative assessment of iNOS staining intensity showed that Met- and AOP-RANTES-treated mice with HAF-GN had increased staining intensity of glomerular cell infiltrate (Fig. 6).

To evaluate whether CCL5/RANTES and its analog Met-RANTES might have direct effects on macrophage iNOS expression, J774 macrophages were incubated with either saline, CCL5/RANTES, or Met-RANTES in vitro. Met-RANTES and CCL5/RANTES markedly increased iNOS mRNA expression after 12 h compared with saline controls (Fig. 7). The Met-RANTES- and RANTES-induced iNOS mRNA expression correlated with the respective expression of iNOS protein on the J774 macrophages as assessed by immunocytochemistry after 36 h of incubation in vitro compared with saline controls (Fig. 7).
Met-RANTES reduces uptake of apoptotic cells by peritoneal macrophages

Because both CCL5/RANTES antagonists increased the number of glomerular apoptotic cells, we determined whether Met-RANTES treatment in vivo affects the uptake of apoptotic cells by macrophages, especially as enhanced iNOS expression could alter apoptosis. Quantification of the uptake of TAMRA-labeled apoptotic cells by peritoneal macrophages was performed in BALB/c mice that were pretreated with Met-RANTES or saline. Met-RANTES pretreatment reduced the percentage of peritoneal macrophages that had ingested TAMRA-positive apoptotic cells in relation to all gated macrophages from a lavage fluid of a single animal (n = 5 per group; each experiment conducted in duplicate).

Discussion

Our data show that two CCL5/RANTES antagonists, i.e., Met- and AOP-RANTES, reduce glomerular macrophage infiltration in a model of murine immune complex GN. However, both Met- and AOP-RANTES failed to improve the disease process of HAF-GN, but rather aggravated glomerular damage and albuminuria. To evaluate the possible mechanisms of this observation, we examined the characteristics of the cellular and the humoral immune response during HAF-GN. Whereas the latter was unaffected by the antagonists, our data indicate that treatment with both CCL5/RANTES antagonists is accompanied by glomerular accumulation of a certain macrophage phenotype. These macrophages show a foam cell-like appearance with enhanced iNOS expression and decreased uptake of apoptotic cells. This particular proinflammatory phenotype of tissue macrophages may—irrespective of the lower number of macrophages—contribute to glomerular damage and albuminuria.

Met-RANTES and AOP-RANTES reduce macrophage infiltration in HAF-GN

The finding of a decrease in glomerular macrophage accumulation in HAF-GN correlates with the effects of Met-RANTES observed in murine NSN and during renal transplant rejection in rats (10, 21, 22). AOP-RANTES reduced glomerular macrophage accumulation in the rat anti-Thy 1.1 GN model (23). Treatment with Met-RANTES reduces monocyte arrest on activated human endothelium that presents CCL5/RANTES on the luminal surface, a mechanism that could be mediated by blocking multiple chemokine receptors (27). Studies using cells that overexpress human CCR1, CCR3, or CCR5 have demonstrated that AOP- and Met-RANTES can block ligand-induced chemotaxis via all of these receptors (20). However, we have previously demonstrated that CCR3 is not expressed in kidneys during HAF-GN, suggesting that activation of CCR3 does not mediate macrophage infiltration in this model (19). CCR1 is expressed in the kidney during HAF-GN, and may well be involved in mediating leukocyte infiltration (19). However, in contrast to the human system, murine CCL5/RANTES does not bind to murine CCR1 (18), and thus, Met-RANTES could not block glomerular macrophage infiltration via CCR1 in our mouse model. Therefore, the observed reduction of glomerular macrophages relates to impaired macrophage infiltration by blocking CCR5 or some other yet-unknown CCL5/RANTES receptors. The accumulation of the remaining macrophages may be independent of CCL5/RANTES and mediated by other chemotactic molecules, e.g., monocyte chemoattractant protein-1, and/or local macrophage proliferation (28).

Met-RANTES and AOP-RANTES aggravate albuminuria and glomerular damage

One would have expected that the reduced macrophage infiltration would lead to less glomerular damage in HAF-GN. Surprisingly, we observed the contrary. In spite of fewer glomerular macrophages, treatment with Met- and AOP-RANTES induced nephrotic-range albuminuria and aggravation of histopathological damage, indicating that CCL5/RANTES effects other than chemotaxis may be involved in HAF-GN. At first, we excluded CCL5/RANTES antagonist-mediated direct toxic effects by showing that, in control animals, the antagonists did not cause any histological or proteinuric effects. Furthermore, in vitro studies with MC provided no evidence for direct cytotoxicity of the CCL5/RANTES antagonists. Thus, the mesangiolysis in antagonist-treated mice with HAF-GN is unlikely to be a direct effect of the antagonists on MC. We then studied whether the CCL5/RANTES modulated the humoral immune response, as HAF-GN is an immune complex GN that relies on a specific B cell response to HAF, mesangial immune complex deposition, and complement activation. However, neither serum HAF Ab titers nor mesangial deposits nor glomerular complement activation was affected by either antagonist, arguing against increased immune complexes remaining in the glomeruli of mice treated with CCL5/RANTES analogs in HAF-GN.

A shift in Th1-Th2 immune response can influence experimental GN (29). Therefore, we compared serum anti-HAF IgG isotype levels, mesangial IgG isotype deposits, and splenocyte cytokine production as indices of the Th1-Th2 response between CCL5/RANTES antagonist- and saline-treated mice. However, we found no differences in these parameters between the experimental groups. The CCL5/RANTES antagonists also did not affect glomerular CCL5/RANTES expression. Thus, these data suggest that the Met-RANTES- and AOP-RANTES-induced aggravation of HAF-GN is not associated with changes in the humoral immune response to HAF or in the local deposition of immune complexes.

CCL5/RANTES antagonists induce a certain phenotype of glomerular macrophages

Aggravation of proteinuria and histological damage in the presence of fewer glomerular macrophages could be a consequence of a different phenotype of glomerular macrophages (30). Cells of the monocyte-macrophage lineage can appear in proinflammatory or anti-inflammatory subtypes and different activation states during tissue destruction, wound repair, angiogenesis, or Ag presentation (31). The specific macrophage phenotype is determined by the surrounding milieu of cytokines, lipid mediators, and chemokines, resulting in specific programming of macrophages (30, 32–34).
For example, it was recently shown that the chemokine CCL5/RANTES induced MHC class II expression in macrophages, whereas the CX3C chemokine fractalkine had the opposite effect (33). In our study, Met-RANTES-treated mice revealed a different phenotype of glomerular macrophages characterized by an altered morphological appearance. Although the antagonists could have selectively prevented the infiltration of an anti-inflammatory macrophage phenotype that would induce a bias toward proinflammatory glomerular macrophages, our data demonstrate that the CCL5/RANTES antagonists have direct effects on macrophages (35). CCL5/RANTES and Met-RANTES both induced iNOS expression in murine J774 cells in vitro, and Met-RANTES induced iNOS expression in glomerular macrophages in vivo, which indicates a novel function of CCL5/RANTES in vivo, with all its consequences on, for example, apoptosis, bactericidal activity, and vasodilatation. These findings are similar to the report that CCL5/RANTES enhances the trypanocidal activity of macrophages by increased iNOS generation in a previous study (36). These agonistic effects of CCL5/RANTES antagonists could relate to their interaction with CCL5/RANTES receptors, because these agents can have both agonistic as well as antagonistic activities (20). In humans, CCL5/RANTES can activate CCR1, CCR3, and CCR5. However, CCL5/RANTES does not activate CCR1 in mice (18), and we did not find CCR3 expressed in HAF-GN (19). Therefore, CCL5/RANTES-induced iNOS expression should relate to agonistic effects on CCR5. Activation of CCR5 by CCL5/RANTES can generate two different signals via two different intracellular signal transduction pathways. After binding, CCL5/RANTES can induce early responses of cell function, such as Ca^{2+} influx, receptor dimerization, tyrosine phosphorylation, via G_{p} protein and the JAK/STAT pathway within the first 5 min after receptor activation (37). In addition, CCL5/RANTES does also induce late signals such as cell polarization, activation, and association of p125^{FAK} kinase to the chemokine receptor. It has been shown that AOP-RANTES does effectively block these late responses that are required for migration or chemotaxis (38). In contrast, the early responses are not affected by AOP-RANTES, and activation of the JAK/STAT pathway does also mediate iNOS expression in macrophages (39). Together, our in vivo studies suggest that both CCL5/RANTES receptor-related mechanisms of macrophage function are involved in the pathogenesis of HAF-GN and that Met-RANTES and AOP-RANTES effectively block chemotaxis but induce iNOS expression in macrophages.

Macrophage-derived NO is an important mediator of glomerular damage in immune complex GN (40). Macrophage-derived NO inhibits MC proliferation and induces MC apoptosis (41, 42), two mechanisms that we observed in CCL5/RANTES-treated mice with HAF-GN and that may contribute to CCL5/RANTES antagonist-induced mesangiosis in this model. Furthermore, the increased number of apoptotic cells in glomeruli noted after Met-RANTES treatment of HAF-GN mice could also result from a reduced uptake of apoptotic cells by glomerular macrophages. In fact, our results with reduced uptake of apoptotic cells by peritoneal macrophages from mice pretreated with Met-RANTES would support such a mechanism. The recognition and ingestion of apoptotic cells in vivo protect tissues from the toxic content of dying cells and modulate macrophage regulation of an excessive inflammatory and immune response (43). In GN, the uptake of apoptotic cells is an important suppressor of glomerular macrophage activity (30, 44), and reduced phagocytic activity of glomerular macrophages is associated with progression of immune complex GN (45). Therefore, reduced uptake of apoptotic cells by activated macrophages seems to block an important suppressor mechanism for a proinflammatory phenotype of macrophages in HAF-GN.

This effect of Met-RANTES on phagocytosis is a novel finding. Obviously such a new function of RANTES would fit with the chemokine’s role to attract and activate macrophages to the sites of tissue injury. This potential novel aspect of chemokine function deserves further exploration.

Taken together, CCL5/RANTES antagonists seem to modulate glomerular injury in HAF-GN via multiple mechanisms: the induction of glomerular NO secretion by glomerular macrophages (1) followed by an increase of glomerular apoptotic cells (2) that were not adequately removed by glomerular macrophages (3). The decreased uptake of apoptotic cells by glomerular macrophages diminishes a suppressive effect on macrophage activation (4), which may also contribute to a particular aggressive functional and morphological phenotype of glomerular macrophages with increased iNOS expression. This vicious circle would lead to worsening of glomerular injury, in spite of a reduced total number of glomerular macrophages. Apart from pointing toward unexpected detrimental effects of chemokine antagonists in immune-mediated diseases, our results indicate potentially novel functions of CCL5/RANTES in iNOS expression and in the clearance of apoptotic cells by macrophages. Furthermore, CCL5/RANTES-mediated control of the glomerular macrophage phenotype seems to play an important role in modulating the inflammatory disease process of immune complex GN.

**Acknowledgments**

We thank J. Pfießlchter for providing the rabbit anti-mouse iNOS Ab. Part of the data has previously been reported in abstract form (46). Parts of this project were prepared as a doctoral thesis at the Faculty of Medicine, University of Munich, by M. Frink.

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