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# Phenotyping Renal Leukocyte Subsets by Four-Color Flow Cytometry: Characterization of Chemokine Receptor Expression

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FACS · Antibody · Inflammation · CCR2 · CCR5

Abstract
To investigate mechanisms of cell-mediated injury in renal inflammatory disease it is critical to determine the surface phenotype of infiltrating renal leukocyte subsets. However, the cell-specific expression of many leukocyte receptors is difficult to characterize in vivo. Here, we report a protocol based on flow cytometry that allows simultaneous characterization of surface receptor expression on different subsets of infiltrating renal leukocytes. The described technique combines an adapted method to prepare single cell suspensions from whole kidneys with subsequent four-color flow cytometry. We recently applied this technique to determine the differential expression of murine chemokine receptors CCR2 and CCR5 on infiltrating renal leukocyte subsets. In this article, we summarize our current findings on the validity of the method as compared with immunohistology and in situ hybridization in two murine models of nonimmune (obstructive nephropathy) and immune-mediated (lupus nephritis) inflammatory renal disease. Flow cytometry analysis revealed an accumulation of CCR5-, but not CCR2-positive lymphocytes in inflamed kidneys, compared to the peripheral blood. Particularly renal $CD8^+$ cells expressed CCR5 (79% in obstructed kidneys, 90% in lupus nephritis). In both models, infiltrating renal macrophages were positive for CCR2 and CCR5. These data corresponded to immunohistological and in situ hybridization results. They demonstrate that flow cytometric analysis of single cell suspensions prepared from inflamed kidneys is a rapid and reliable technique to characterize and quantify surface receptor expression on infiltrating renal leukocyte subsets.



## Introduction

Blocking the interaction between leukocyte surface receptors and their specific ligands is an attractive therapeutic approach to reduce infiltration and activation of effector cells in acute and chronic inflammation. To identify potential targets of an anti-inflammatory intervention, the expression of particular receptors on infiltrating leukocytes must be determined in suitable animal models. The widely used techniques for generation of transgenic mice by homologous recombination of embryonic stem cells as well as the availability of many genetically defined strains and mouse-specific immunological reagents renders mice highly suitable for these in vivo studies. A great variety of murine disease models has been described,

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including many forms of glomerular and interstitial nephropathies [1, 2]. However, the cell-specific expression of leukocyte receptors in animal models of kidney diseases is difficult to characterize. Immunohistochemical or immunofluorescence studies are often hampered by the lack of specific and sensitive antibodies which can be used for analysis of frozen or paraffin-embedded tissue. Methods of tissue preparation and antigen retrieval must be carefully optimized for each antibody, and co-staining techniques or evaluation of serial tissue sections are needed to determine cell type-specific expression patterns. To obtain quantitative results, a time-consuming morphometric analysis must be performed. Here, we report an alternative approach using a four-color flow cytometry analysis of single cell suspensions prepared from inflamed murine kidneys to characterize the expression of the chemokine receptors CCR2 and CCR5 on infiltrating renal lymphocytes and macrophages. In combination with in situ hybridization studies to localize the sites of expression within different tissue compartments (e.g. glomerular or interstitial expression), the FACS analysis proved to be a fast and reliable method to determine and quantitate the cell-specific expression pattern of these weakly expressed leukocyte receptors.

The CC chemokines MCP-1/CCL2, MIP1α/CCL3 and RANTES/CCL5 are expressed in different forms of human nephritis [3]. In various animal models of renal disease, these chemokines play an important role in the recruitment of leukocytes to renal sites of inflammation [3]. In parallel, the corresponding CC chemokine receptors CCR2 and CCR5 are upregulated in inflamed kidneys, which suggests a role of these receptors in mediating the chemotactic signal to infiltrating macrophages and T lymphocytes [3–6]. To investigate the cell type-specific expression of CCR2 and CCR5, we have developed rat monoclonal antibodies against murine chemokine receptors CCR2 and CCR5 that were suitable for flow cytometry [7]. In this article, we summarize our findings applying the new protocol in conjunction with these antibodies to analyze expression of CCR2 and CCR5 on infiltrating renal $CD4^+$ lymphocytes, $CD8^+$ lymphocytes and macrophages in murine models of inflammatory renal disease. Our results indicate the potential of the reported technique to characterize weakly expressed surface receptors on different subsets of infiltrating renal leukocytes. It is a powerful tool to identify target molecules for therapeutic strategies that can specifically block recruitment of effector cells mediating inflammatory tissue damage in renal disease.

## Methods

*Animal Models*

Interstitial nephritis was induced by unilateral ureteral obstruction (UUO) in female inbred C57BL/6 mice weighing 20–25 g. Mice were obtained from Charles River (Sulzfeld, Germany) and kept in macrolone type III cages under a 12-hour light and dark cycle. Water and standard chow (Sniff, Soest, Germany) were available ad libitum. Under general ether anesthesia, UUO was performed by ligating the left distal ureter with a 2/0 Mersilene suture through a low midline abdominal incision. Mice were killed 10 days after UUO. Obstructed kidneys were removed for further analysis. Female MRL/MpJ $FAS^{lpr/lpr}$ (MRL/lpr) mice were purchased from the Jackson Laboratories (Bar Harbor, Me., USA) and killed at the age of 14 weeks, when a mesangioproliferative glomerulonephritis and interstitial infiltrates had developed. Before removing kidneys for cell isolation, blood samples were taken from anesthetized mice by retrobulbar puncture, collected in 4 m*M* EDTA and stored on ice until labeling for flow cytometry. All experimental procedures were performed according to the German animal care and ethics legislation and had been approved by the local government authorities.

*Isolation of Renal Cells*

A preparation of isolated renal cells including infiltrating leukocytes was obtained from obstructed kidneys of C57BL/6 mice 10 days after UUO and from MRL/lpr mice at 14 weeks of age, following a method adapted from Cook et al. [8]. Kidneys were cut into small pieces of <1 mm in ice-cold Paris buffer (20 m*M* Tris/HCl, 125 m*M* NaCl, 10 m*M* KCl, 10 m*M* sodium acetate, 5 m*M* glucose). No sieving procedures were applied to isolate glomerular and interstitial tissue fractions as in situ hybridization studies showed that chemokine receptor mRNA expression localized mainly to mononuclear cell infiltrates in the interstitium. After spinning at 1,500 rpm (250 *g*), pellets were resuspended in Hanks' buffered saline solution (1 × HBSS) with calcium and magnesium and washed once. In a first enzyme step, tissue was incubated for 20 min in 5 ml HBSS containing 1 mg/ml collagenase type I and 0.1 mg/ml deoxyribonuclease type III (both from Sigma) at 37°C. Trypsin digestion was omitted to avoid degradation of cell surface antigens. After two washes in 1 × HBSS on ice, tissue was incubated in 5 ml 2 m*M* EDTA, 1 × HBSS (without calcium and magnesium) for 20 min at 37°C and spun at 500 rpm (30 *g*). The supernatant containing isolated cells was removed and kept on ice. In a second enzyme step the remaining pellet was incubated in 5 ml 1 mg/ml collagenase I in 1 × HBSS for 20 min at 37°C. The suspension was passed three times through a 20-gauge needle and settled on ice for 5 min. The supernatant containing free cells was pooled with the first supernatant from the EDTA incubation, washed twice in 1 × HBSS (with calcium and magnesium) and resuspended in 1 ml of the same buffer. After staining with 0.4% trypan blue, cells were counted in a Neubauer chamber. Typically 1–3 × $10^6$ cells/ml could be isolated from one kidney.

*Flow Cytometry*

The specificities of monoclonal antibodies used for flow cytometry are listed in table 1. Rat monoclonal antibodies to detect murine CCR2 and CCR5 expression on isolated leukocytes were generated as described previously [7]. Renal cell suspensions and anti-coagulated full blood samples were incubated with 5 μg/ml of the monoclonal antibodies MC-21, MC-68, or the appropriate isotype control antibody on ice for 60 min. The antibody MC-21 specifically binds to

Table 1. Monoclonal antibodies used in FACS staining

| Target molecule | Clone | Specificity | Reference |
|---|---|---|---|
| Murine L3T4 | RM4–5 | MHC class II-restricted T lymphocytes, including most $CD4^+$ helper cells | 9 |
| Murine Ly-2 | 53-6.7 | MHC class I-restricted T lymphocytes, including most $CD8^+$ suppressor/cytotoxic cells | 10 |
| Murine Mac-1α | M1/70 | $CD11b^+$ monocytes/macrophages, granulocytes, few NK cells | 11 |
| Murine CCR2 | MC-21 | Murine CC chemokine receptor 2 | 7 |
| Murine CCR5 | MC-68 | Murine CC chemokine receptor 5 | 7 |

murine CCR2, the antibody MC68 to murine CCR5. Rat IgG2b (isotype control) were obtained from Pharmingen (San Diego, Calif., USA). After three washing steps, cells were incubated with a biotin-labeled anti-rat polyclonal antibody on ice for 60 min followed by phycoerythrin-labeled streptavidin (both from Dako, Hamburg, Germany). Finally, cells were incubated with a combination of the following directly conjugated antibodies: anti-CD11b fluorescein-isothiocyanate (clone M1/70), anti-CD4 allophycocyanin, and anti-CD8 cy-chrome (all from Pharmingen; table 1). Red blood cells were lysed with FACS-lysing solution (Becton-Dickinson, Franklin-Lakes, N.J., USA), and stained cells were analyzed on a flow cytometer (FACScalibur, Becton-Dickinson). Monocytes/macrophages were identified by their light scatter properties and expression of CD11b, T lymphocytes by expression of CD4 and CD8. The cut-off to define chemokine receptor-positive cells was set according to the staining with the isotype control antibody. For CCR2 and CCR5 expression on monocytes/macrophages, shifts of the mean fluorescence compared to isotype control were evaluated, as some unspecific binding of the IgG2b antibody on activated renal macrophages occurred. Approximately 100,000 events were collected in each analysis. Values are given as mean ± SEM. Data were analyzed by two-sided t-test for paired data. Statistical significance was defined as $p < 0.05$.

*In situ Hybridization and Immunohistochemistry*

Probes for murine CC chemokine receptors CCR2 and CCR5 used for in situ hybridization were prepared by in vitro transcription of subcloned cDNA fragments from the untranslated 5′- and 3′-end, respectively, with low sequence homology. The 153-bp mCCR2 probe corresponds to nucleotides 1736–1888 (GenBank accession No. U47035) and the 220-bp mCCR5 probe represents nucleotides 1384–1604 (GenBank accession No. D83648). Antisense and sense RNA transcripts were labeled with $\alpha^{35}$S-UTP (1,250 Ci/mmol; NEN, Cologne, Germany) to a specific activity of $8 \times 10^8$ cpm/μg and served as hybridization probe and control, respectively. Free nucleotides were separated with a Sephadex G-50 column (quick-spin columns; Roche, Mannheim, Germany). The collected fraction containing labeled probe was precipitated in ethanol and dissolved in prehybridization solution immediately before use. In situ hybridization was performed on 4-μm sections of paraffin-embedded renal tissue as described previously [5]. Following in situ hybridization and washing procedures, immunohistochemistry for $CD3^+$ lymphocytes was performed according to standard protocols omitting autoclaving or microwave pretreatment for antigen retrieval as described previously [5]. A monoclonal cross-reactive rat anti-human CD3 antibody (1:100; Serotec, Oxford, UK) was used.

## Results and Discussion

*Preparation of Renal Single Cell Suspensions and Flow Cytometry*

We have recently established a fast and reliable protocol of renal cell isolation that allows subsequent flow cytometric analysis of weakly expressed surface receptors expressed by leukocytes infiltrating into diseased murine kidneys. Several techniques to obtain infiltrating or intrinsic renal cells have been reported (table 2). Most of these protocols combine mechanical and/or enzymatic disaggregation with additional procedures, e.g. Ficoll separation, Percoll gradient centrifugation, immunomagnetic separation, and propagation in cell culture before characterizing distinct cell populations (table 2). We applied a modified technique based on a protocol previously reported by Cook et al. [8] for rat glomeruli to obtain renal cell suspensions from mouse kidneys for immediate flow cytometry analysis. Whole kidneys were mechanically disaggregated and subsequently subjected to enzymatic digestion with collagenase and DNase. Trypsin digestion had to be omitted as we found a degradation of cell surface antigens, especially CD4 and CD8, when cells were treated with trypsin. On average, $1–3 \times 10^6$ cells per mouse kidney could be isolated with this method, as revealed by trypan blue exclusion microscopy. The cell preparation contained intrinsic renal cells and infiltrating leukocytes.

The use of a four-color flow cytometry approach allowed us to characterize the expression of one surface receptor on three different leukocyte subpopulations simultaneously in each analysis. Directly conjugated antibodies (table 1) were used to identify $CD4^+$ lymphocytes, $CD8^+$ lymphocytes and $CD11b^+$ macrophages. The number of leukocytes isolated from three fourths of each individual kidney was sufficient to perform three independent FACS experiments, e.g. staining with CCR2, CCR5 and isotype control antibodies. Renal cell suspensions prepared from obstructed kidneys after UUO contained

Table 2. Reported techniques for isolation of infiltrating and intrinsic renal cells

| Isolated cell type | Method | Reference |
|---|---|---|
| Mononuclear cells (chicken) | Mechanical disaggregation, Lymphoprep extraction | 12 |
| T lymphocyte clones (mouse) | Mechanical disaggregation, Ficoll centrifugation, propagation in culture | 13 |
| T lymphocytes (mouse) | Mechanical disaggregation, Lympholyte M extraction, propagation in culture | 14 |
| T lymphocyte subsets (human) | Mechanical disaggregation, Percoll gradient centrifugation, Ficoll extraction, immunomagnetic separation | 15 |
| Glomerular single cells (rat) | Mechanical disaggregation, enzymatic digestion with collagenase, trypsin, DNase, propagation in culture | 16 |
| Glomerular dendritic cells (rat) | Mechanical disaggregation, enzymatic digestion with collagenase, trypsin, DNase, propagation in culture, metrizamide gradient centrifugation | 17 |
| Glomerular leukocytes (rat) | Mechanical disaggregation, enzymatic digestion with collagenase, trypsin, DNase | 8 |
| Proximal tubular cells (rat) | Mechanical disaggregation, enzymatic digestion with collagenase, Percoll gradient centrifugation, digestion with trypsin and Dnase | 18 |
| Proximal tubular cells (human) | Mechanical disaggregation, enzymatic digestion with collagenase, Percoll gradient centrifugation | 19 |
| Proximal tubular cells (human) | Mechanical disaggregation, enzymatic digestion with collagenase, Percoll gradient centrifugation, FACS sorting | 20 |
| Distal tubular cells (human) | Mechanical disaggregation, enzymatic digestion with collagenase, Percoll gradient centrifugation, FACS sorting | 21 |
| Tubular cells (human) | Mechanical disaggregation, enzymatic digestion with collagenase, Percoll gradient centrifugation, immunomagnetic separation, propagation in culture | 22 |

an average number of 1,444 $CD4^+$ lymphocytes, 1,424 $CD8^+$ lymphocytes, and 3,688 macrophages per 100,000 gated events (n = 5 mice). Average cell numbers in kidneys of MRL/lpr mice were 680 $CD4^+$ cells, 497 $CD8^+$ cells, and 1,532 macrophages per 100,000 cells (n = 5 mice). One fourth of each kidney could be used for histologic assessment. This allowed simultaneous evaluation by in situ hybridization to localize sites of mRNA expression and FACS analysis to quantitate the cell-specific pattern of protein expression.

The following results indicate the applicability of the presented protocol to characterize chemokine receptor expression on renal T lymphocytes and macrophages in interstitial nephritis induced by UUO, and in kidneys with lupus nephritis. Instead of histology-based techniques that would include laborious and often unsuccessful co-staining procedures, evaluation of serial sections and morphometric quantitation, the flow cytometric analysis provided rapid and reproducible results. Within 1 day preparation of single cell suspensions, cell staining with antibodies and FACS scanning could be performed. In the experiments reported, we investigated $CD4^+$ lymphocytes, $CD8^+$ lymphocytes and $CD11b^+$ macrophages. However, the analysis can be easily extended to various cell types of interest by staining with other directly conjugated antibodies, for example directed against natural killer cells, B cells or dendritic cells. Potentially, also intrinsic renal cells, e.g. tubular epithelial cells, may be analyzed if specific surface antigens and suitable antibodies are available. Thus, the presented technique should be broadly applicable to the study of leukocyte subsets that are preferentially infiltrating renal tissue in various nonimmune or immune kidney diseases.

### *In situ Hybridization and Immunohistochemistry Localizes Renal CCR2 and CCR5 mRNA Expression to Infiltrating Leukocytes*

We previously reported that CCR2 and CCR5 are upregulated in obstructed kidneys after UUO and in murine lupus nephritis [5, 6]. In situ hybridization studies for CCR2 and CCR5 demonstrated an expression of CCR2 and CCR5 mRNA confined to the sites of mononuclear cell infiltration. Obstructed kidneys of C57BL/6 mice 10 days after UUO revealed an exclusively intersti-

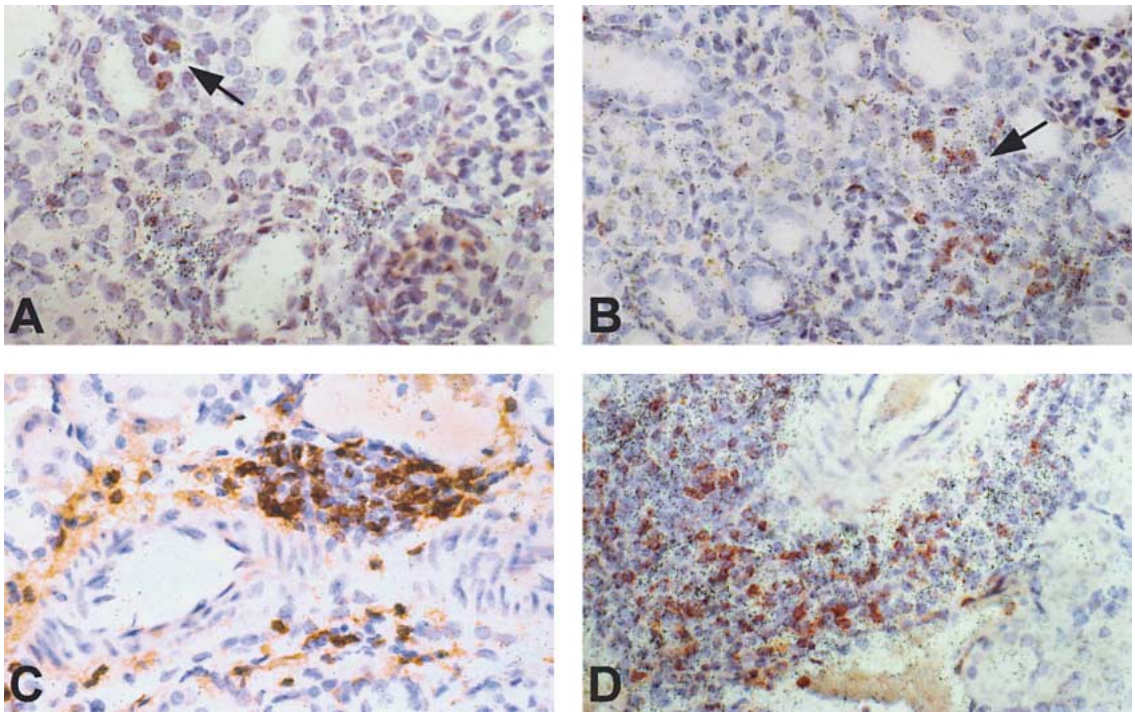


Fig. 1. Combined in situ hybridization for CCR2/CCR5 mRNA and immunohistochemistry for $CD3^+$ lymphocytes. In interstitial nephritis 10 days after UUO no CCR2 (A), but CCR5 (B) transcripts co-localized with $CD3^+$ cells (arrows). Similarly, no co-localization of CCR2 (C), but a positive co-localization of CCR5 (D) mRNA with $CD3^+$ lymphocytes (arrows) could be detected in kidneys of MRL/lpr mice, suggesting a high proportion of CCR5 expressing lymphocytes infiltrating in nephritic kidneys. Hemalaun counterstain (A, B, D × 630; C × 1,000).

tial expression of CCR2 and CCR5. In MRL/lpr mice with lupus nephritis, mRNA transcripts localized to glomerular and mainly to periglomerular and interstitial mononuclear cells [5, 6]. Combination of in situ hybridization for CCR2 or CCR5 mRNA with immunostaining for $CD3^+$ lymphocytes on the same section revealed a co-localization of lymphocytes with CCR5 transcripts in the UUO and lupus model. In contrast, no clear co-localization of $CD3^+$ lymphocytes with CCR2 mRNA transcripts could be seen (fig. 1). The differential mRNA expression of both receptors is particularly obvious when follicular mononuclear cell infiltrates, a characteristic feature in kidneys of MRL/lpr mice, are examined (fig. 1C, D). Thus, infiltrating renal lymphocytes appeared to express predominantly CCR5, but not CCR2 in the two nephritis models investigated. However, both CCR2 and CCR5 mRNA transcripts localized to monocytic infiltrates. This suggested that macrophages would express CCR2, and that CCR5 could be expressed both by infiltrating macrophages and lymphocytes. Unfortunately, we were unsuccessful to combine immunostaining of infiltrating renal macrophages with in situ hybridization, as all specific macrophage antibodies tested only worked in protocols with antigen retrieval thus precluding simultaneous in situ hybridization.

*Expression of CCR5 by Infiltrating Renal T Cells*

There were no antibodies against murine CCR2 and CCR5 available that could be used for immunohistology or immunofluorescence analysis of cell-specific receptor expression, including those developed in our own laboratory. The latter were, however, suitable for FACS analysis [7]. Therefore, we could characterize and quantitate CCR2 or CCR5 membrane expression simultaneously on $CD4^+$ cells, $CD8^+$ T cells and $CD11b^+$ macrophages by flow cytometry. As shown in figure 2, CCR5-positive T cells accumulated in obstructed kidneys after UUO in C57BL/6 mice. While in the peripheral blood only 7 ± 4% of $CD4^+$ and 21 ± 3% of $CD8^+$ cells expressed CCR5, the corresponding numbers were 36 ± 12% ($p = 0.02$

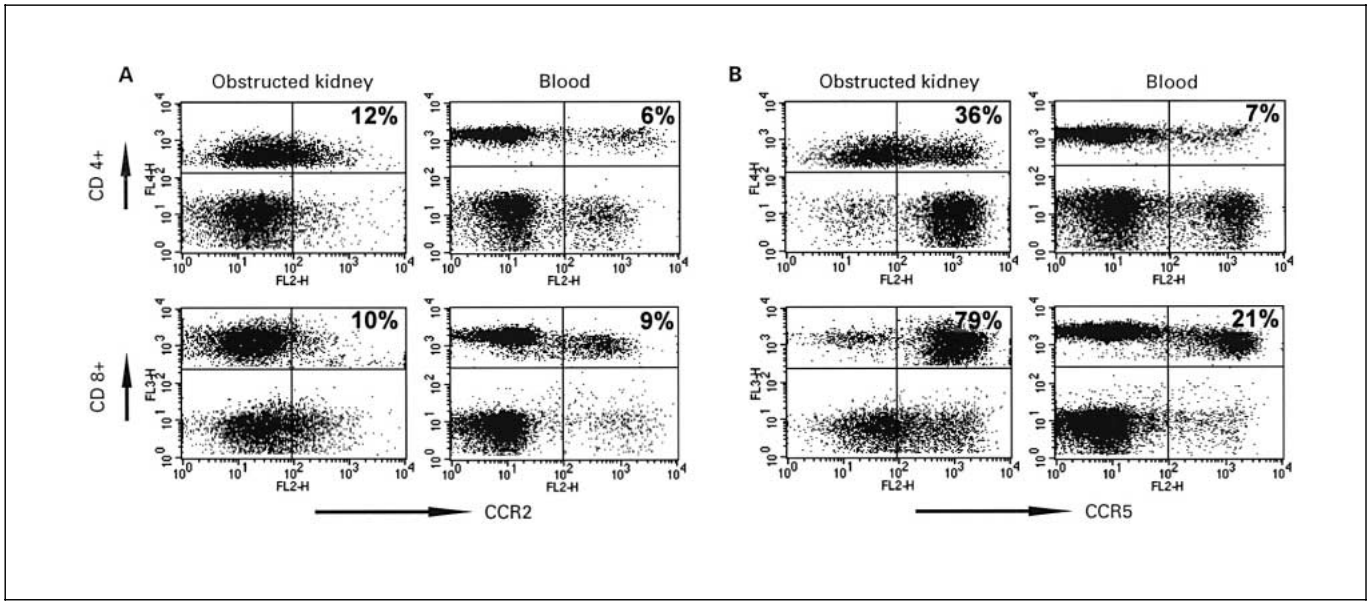


Fig. 2. Flow cytometry analysis of (A) CCR2 and (B) CCR5 expression on infiltrating renal lymphocytes isolated from obstructed kidneys of C57CL/6 mice 10 days after UUO. Only few blood $CD4^+$ and $CD8^+$ lymphocytes expressed CCR2, and low numbers of CCR2-positive lymphocytes were present in obstructed kidneys. In contrast, there was an accumulation of CCR5 expressing $CD4^+$ and particularly $CD8^+$ cells in obstructed kidneys, compared to blood lymphocytes. Dot blots shown are representative for results in 5 mice investigated and percentages given are mean of the five experiments.

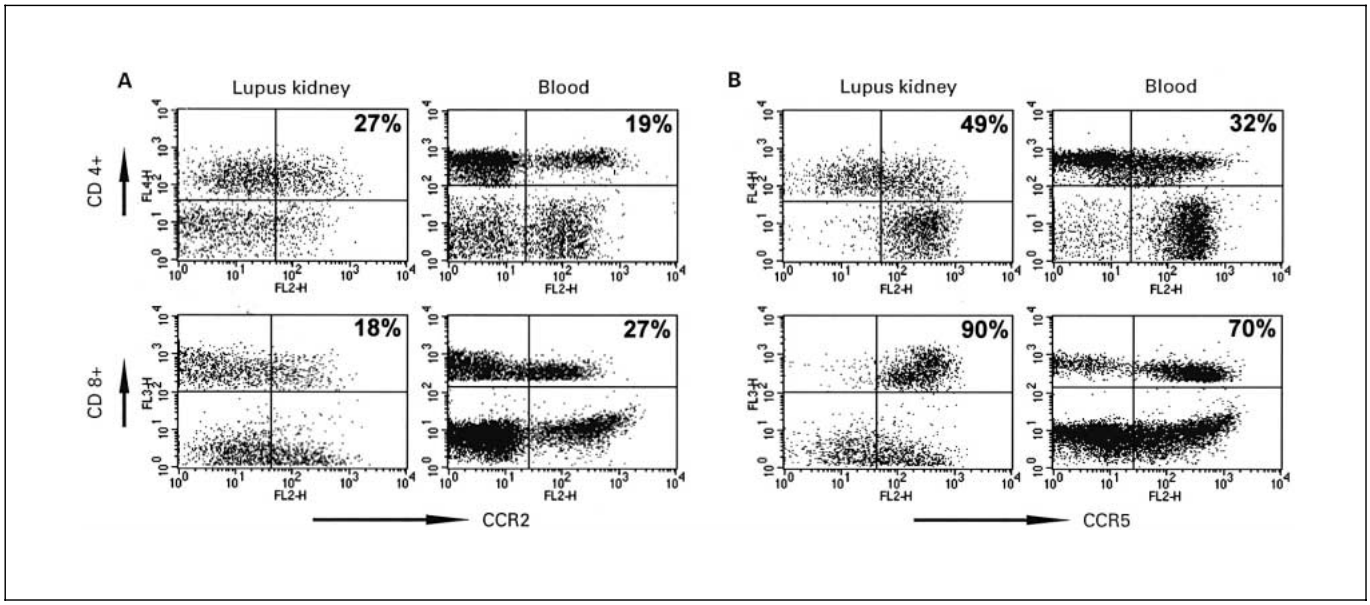


Fig. 3. Flow cytometry analysis of (A) CCR2 and (B) CCR5 expression on infiltrating renal lymphocytes isolated from kidneys of MRL/lpr mice with lupus nephritis at 14 weeks. Compared to C57BL/6 mice a high percentage of CCR2- and CCR5-positive $CD4^+$ and $CD8^+$ lymphocytes was found in the peripheral blood. Similar numbers of renal $CD4^+$ and $CD8^+$ lymphocytes expressed CCR2, whereas a further enrichment of CCR5-positive $CD4^+$ and $CD8^+$ T cells occurred in nephritic kidneys. Dot blots shown are representative for results in 6 mice investigated and percentages given are mean of the six experiments.

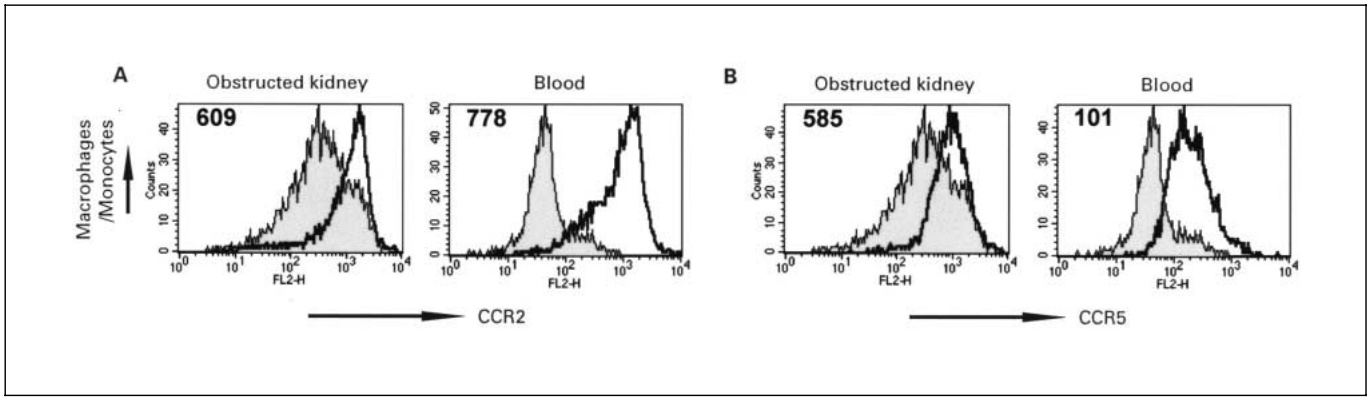


Fig. 4. Flow cytometry analysis of CCR2 (A) and CCR5 (B) expression on infiltrating renal macrophages isolated from obstructed kidneys of C57BL/6 mice 10 days after UUO. Peripheral blood monocytes expressed predominantly CCR2, whereas shifts of the mean fluorescence compared to isotype-matched control IgG (shaded histograms) could be seen both for CCR2 and CCR5 on macrophages isolated from nephritic kidneys. Histograms shown are representative for results in 5 mice investigated. Shift values given are mean of five experiments.

compared to peripheral blood) and 79 ± 10% ($p = 0.0002$) in the kidney infiltrates (fig. 2B). In contrast, no accumulation of CCR2-positive T cells occurred in obstructed kidneys. 6 ± 3 and 9 ± 4% of peripheral $CD4^+$ and $CD8^+$ cells, respectively, expressed CCR2, and a comparable percentage of 12 ± 6 and 10 ± 9% of renal T cells were positive for this receptor (fig. 2A). Interestingly, in MRL/lpr mice with lupus nephritis, we found a much higher percentage of $CD4^+$ and $CD8^+$ lymphocytes in the peripheral blood expressing CCR2 (19 ± 8 and 27 ± 11%, respectively) and CCR5 (32 ± 12 and 70 ± 9%) compared to C57BL/6 mice (fig. 3). Again, a further accumulation of CCR5-positive T cells occurred in nephritic kidneys, with 49 ± 13% of $CD4^+$ cells ($p = 0.1$ compared to peripheral blood) and 90 ± 3% ($p = 0.004$) of $CD8^+$ cells expressing CCR5 (fig. 3B). Similar to the UUO model, CCR2 expression by renal $CD4^+$ and $CD8^+$ cells (27 ± 8 and 18 ± 8%, respectively) mirrored the expression pattern seen in the peripheral blood, without further enrichment of CCR2-positive T cells in the kidney (fig. 3A). Thus, the renal leukocyte infiltrate is characterized by an accumulation of CCR5 but not CCR2-positive T cells, particularly of the $CD8^+$ subtype, in the two models. These data confirmed the results obtained by combined CD3 immunohistology and in situ hybridization for CCR2 and CCR5 performed on the same tissue.

A selective recruitment of CCR5-positive $CD8^+$ T lymphocytes has been reported in a murine model of liver injury in graft-versus-host disease [23]. Several human biopsy studies suggested an accumulation of CCR5 expressing T cells not only in nephritic kidneys [24–26], but also in chronic inflammatory diseases like inflammatory bowel disease [27] and multiple sclerosis [28]. Upregulation of CCR5 may occur in general when infiltrating T cells respond to inflammatory stimuli and are activated. The high percentage of peripheral blood T cells expressing CCR2 and CCR5 in MRL/lpr mice may be the consequence of the *lpr* mutation in these animals allowing autoreactive lymphocytes to escape thymic selection [29]. It is thought that proliferation and autoreactivity of these cells cause the systemic lupus-like disease. On the other hand, circulating T cells of MRL control mice without lupus-like features expressed CCR2 and CCR5 to a similar extent as MRL/lpr mice (data not shown). In comparison to C57BL/6 mice this suggests a highly strain-dependent expression of chemokine receptors.

### *Expression of CCR2 and CCR5 by Infiltrating Renal Macrophages*

Expression of CCR2 and CCR5 on monocytes and renal macrophages was analyzed as shift of the mean fluorescence between the respective anti-CCR antibody in comparison with the isotype control, as some unspecific binding of the IgG2b antibody on activated renal macrophages occurred. In C57BL/6 mice with UUO circulating monocytes in the blood predominantly expressed CCR2 (shift of the mean fluorescence 778 ± 209 arbitrary units (AU)), whereas only a moderate amount of CCR5 expression could be detected (shift 101 ± 82 AU) (fig. 4). In contrast, macrophages isolated from obstructed kidneys

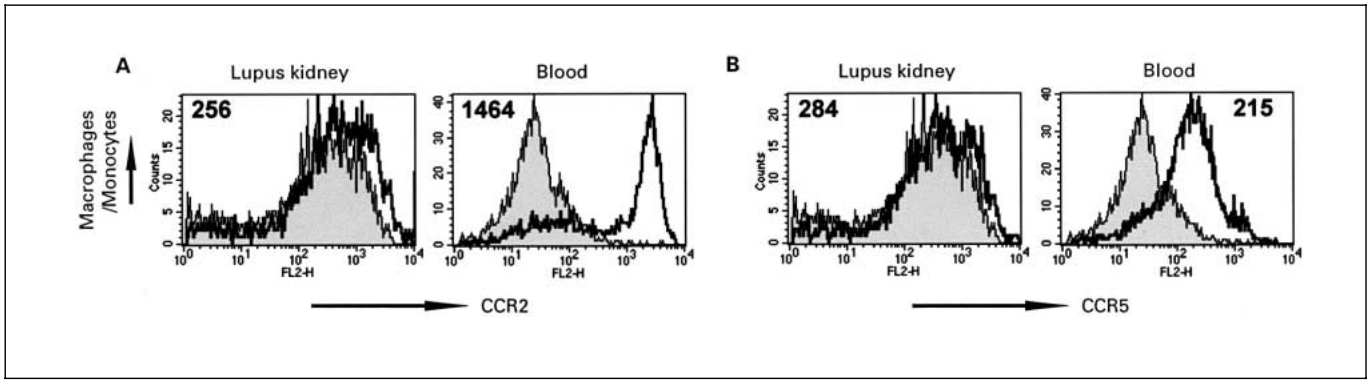


Fig. 5. Flow cytometry analysis of CCR2 (A) and CCR5 (B) expression on infiltrating renal macrophages isolated from kidneys of MRL/lpr mice with lupus nephritis. Macrophages from kidneys with lupus nephritis expressed both CCR2 and CCR5 as indicated by similar shifts of the mean fluorescence compared to isotype-matched control IgG (shaded histograms). In contrast, circulating monocytes of MRL/lpr mice were clearly positive for CCR2, but only few cells expressed CCR5. Histograms shown are representative for results in 5 mice investigated. Shift values given are mean of five experiments.

expressed both CCR2 (shift 609 ± 30 AU) and CCR5 (shift 585 ± 281 AU) (fig. 4). In MRL/lpr mice blood monocytes were clearly positive for CCR2 (shift 1,464 ± 720 AU) and showed a weak expression of CCR5 (215 ± 94 AU). Again macrophages from nephritic kidneys expressed CCR2 (shift 256 ± 137 AU) and, to a similar extent, CCR5 (284 ± 106 AU) (fig. 5). These data indicate that blood monocytes of UUO and lupus mice predominantly express CCR2, whereas in both models accumulating renal macrophages are positive for CCR2 and CCR5 as indicated by similar shifts of the mean fluorescence compared to the isotype control within each model.

This suggests that CCR2 and its ligand MCP-1/CCL2 play a role in the recruitment and activation of renal macrophages. Mice with a targeted disruption of CCR2 have a markedly impaired recruitment of macrophages to sites of inflammation [30–34]. Also MCP-1 deficiency in MRL/lpr mice dramatically reduces macrophage (and T-cell) recruitment, protects kidney, lung, skin and lymph nodes from pathological changes, reduces proteinuria, and prolongs survival of the mice [35]. However, as CCR5 is also expressed by infiltrating macrophages, both receptors may be involved in the recruitment of monocytes/macrophages into inflamed renal tissue.

## Conclusions

We have established a fast and reliable method to investigate weakly expressed surface receptors on infiltrating renal leukocyte subpopulations using four-color flow cytometry. The presented data indicate its potential as a powerful tool in phenotyping renal leukocyte subsets and identifying their possible uses in therapy.

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