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# *Ex vivo* programmed macrophages ameliorate experimental chronic inflammatory renal disease

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**Macrophage infiltration of the kidney is a prominent feature associated with the severity of renal injury and progressive renal failure. To determine the influence of macrophages in renal disease models in the absence of endogenous T and B cells, we performed adoptive transfer of macrophages into severe combined immunodeficient (SCID) mice. In this study, macrophages were isolated from the spleens of BALB/c mice and stimulated with lipopolysaccharide to induce classically activated M1 macrophages or with interleukin-4 (IL-4) and IL-13 to induce alternatively activated M2 macrophages. These macrophages were then infused into SCID mice with adriamycin nephropathy; an *in vivo* model of chronic inflammatory renal disease analogous to human focal segmental glomerulosclerosis. Mice infused with M1 macrophages had a more severe histological and functional injury, whereas M2 macrophage-induced transfused mice had reduced histological and functional injury. Both M1 and M2 macrophages localized preferentially to the area of injury and maintained their phenotypes even after 4 weeks. The protective effect of M2 macrophages was associated with reduced accumulation and possibly downregulated chemokine and inflammatory cytokine expression of the host infiltrating macrophages. Our findings demonstrate that macrophages not only act as effectors of immune injury but can be induced to provide protection against immune injury.**

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In human and animal renal disease, macrophage accumulation correlates with the degree of histological and functional injury, suggesting that macrophages play a role in renal injury.[1–3] Adriamycin (ADR)-–induced nephropathy (AN) is a robust experimental analog of human focal segmental glomerulosclerosis, characterized by changes in both tubulointerstitial and glomerular compartments. This animal model of AN was initially established in rat. We previously developed a model of AN in BALB/c mouse, which was similar to AN in rat. More recently, a model of AN was developed in severe combined immunodeficiency (SCID) mice which are homozygous for an autosomal recessive mutation leading to the absence of lymphocytes. In spite of the absence of lymphocytes, SCID mice developed functional and histological changes similar to those of immunocompetent mice, yet with increased sensitivity to ADR-induced injury.[4] Antibody depletion of macrophages (using the monoclonal antibody ED7) resulted in reduced renal injury in AN.[5] Ablation of macrophages in murine crescentic glomerulonephritis also reduced renal injury and improved renal function.[6] Our recent studies in SCID mice (deficient in T and B cells) and NOD/SCID mice (deficient in T, B, and NK cells) have shown that neither lymphocytes nor NK cells are necessary for induction of this model, consistent with a primary role for macrophages as key effectors in AN.[7]

Other studies have instead shown a beneficial role for macrophages, which depends on their phenotype and location and the nature of injury.[8–11] In mice with unilateral ureteric obstruction (a model of chronic renal injury) reconstituted with bone marrow of angiotensin II type 1 receptor gene knockout mice, infiltrating macrophages were shown to play an antifibrotic role.[12] Macrophages transfected with adenovirus to express IL-4 have been shown to reduce renal inflammation in rats with nephrotoxic nephritis.[13]

The capacity to modify macrophage function has been demonstrated in a number of studies *in vitro*.[14–21] Lipopolysaccharide and proinflammatory stimuli such as interferon-$\gamma$ or tumor necrosis factor-$\alpha$ (TNF-$\alpha$) induce classically activated macrophages (M1 macrophages), characterized by antimicrobial and cytotoxic properties, which underlie their role in host responses to infection or autoimmune disease, whereas exposure to IL-4 and IL-13 results in alternative

activation of macrophages (M2 macrophages). Recent studies have shown that these alternatively activated cells produce several components involved in the synthesis of extracellular matrix, suggesting that their primary role may involve tissue repair rather than microbial killing.[22] M2 macrophages have been studied extensively *in vitro* with respect to their suppressive activity, secretion of anti-inflammatory cytokines and ability to modulate wound healing and angiogenesis.[23] However, adoptive transfer of cytokine-programmed M2 macrophages has not been explored *in vivo*.

We hypothesized that macrophages could be polarized *in vitro* in a way that would result in a persistent *in vivo* functional phenotype. Using SCID mice, we evaluated the effect of different macrophage phenotypes on tissue injury *in vivo* in the absence of T and B cells. Transfusion of M1 macrophages not surprisingly exacerbated AN, whereas M2 macrophages were shown to protect against *in vivo* renal injury in the presence of host macrophages suggesting they could exert a dominant protective effect.

## RESULTS

### Macrophage purity and phenotype

Macrophages were isolated from the spleen of BALB/c mice using a magnetic bead isolation kit with CD11b beads. The fraction of CD11b-positive cells from fresh spleens following purification was 96%, whereas CD11c (dendritic cell marker), CD19 (B-cell marker), and CD49b (NK-cell marker)-positive cells accounted for $<1\%$ of the purified suspension (Figure 1).

There was strong expression of CCL3, inducible nitric oxide synthase (iNOS), TNF-$\alpha$, CD86, and major histology complex class II by M1 macrophages, but not by M0 (nonstimulated macrophages) or M2 macrophages. In contrast, expression of CCL17, mannose receptor, and secretion of IL-10 were seen with M2 macrophages, but not M1 macrophages. Trace expression of mannose receptor was seen with M0 macrophages (Figure 2).

### Renal function

We sought to investigate the effect of adoptive transfer of *ex vivo*-modulated macrophages in established renal injury. AN was induced in SCID mice by tail-vein injection of 5.2 mg/kg ADR. Macrophages (M0, M1, or M2 – see Materials and Methods) were infused by tail-vein injection 5 days after ADR. Serum creatinine and urine protein were assessed at days 14, 21, and 28 after ADR, and were significantly worse following transfusion with M1 macrophages, significantly better with M2 macrophages, and unchanged with M0 macrophages compared with AN alone at day 28 (Figure 3). Transfusion of culture medium from final stages of M0, M1, or M2 macrophage preparation (before their transfusion) had no significant effect on kidney function or structure in normal or AN mice (data not shown).

### Renal histology

Marked renal injury was seen at week 4 in AN, characterized by glomerular sclerosis, tubular atrophy, and interstitial fibrosis with significant mononuclear cell infiltration. Renal injury was increased greatly in AN mice transfused with M1 macrophages compared with control AN mice. Conversely, injury was significantly reduced in mice transfused with M2 macrophages, whereas there were no significant changes in mice transfused with M0 macrophages. The number of points within trichrome-positive vs trichrome-negative cortical fields were counted at $\times 200$ magnification from randomly selected 10 fields, and expressed as a percentage. In parallel with the tubulointerstitial injury findings, M1 macrophage transfusion induced more fibrosis ($45.4 \pm 4.5\%$; $P<0.01$) as compared with AN control ($29.2 \pm 5.1\%$) and AN transfused with M0 macrophages ($27.7 \pm 3.9\%$), and M2 macrophage transfusion significantly suppressed the development of interstitial fibrosis ($15.1 \pm 3.1\%$; $P<0.01$ vs other three groups) (Figure 4a–e).

### Immunohistology of macrophages

Tissue sections were double stained with immunofluorescent antibodies F4/80 for macrophages and Ki67 for proliferative cells. Very few double-positive cells were observed. Single immunohistochemical staining with F4/80 and Ki67 showed an increase in the number of total F4/80-positive cells and Ki67-positive nuclei in the M1 transfused mice and a significant reduction in the M2 transfused mice compared

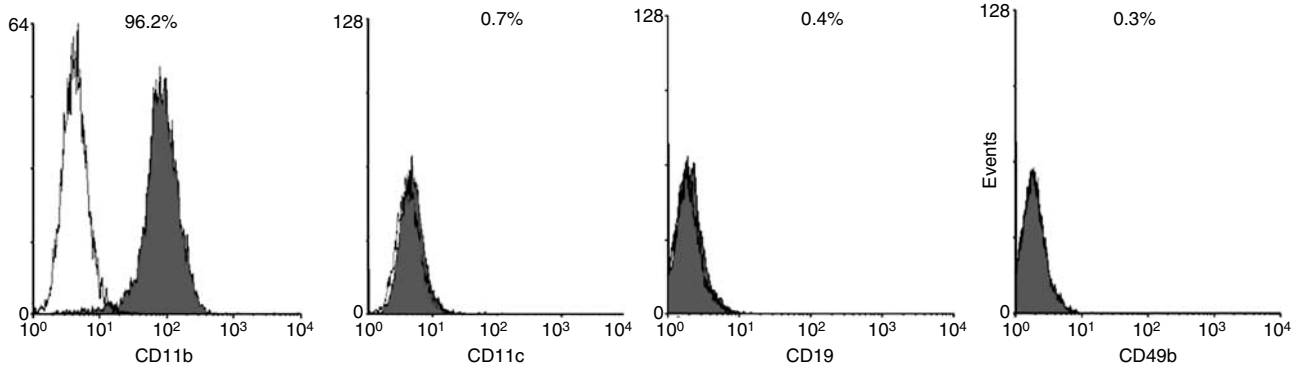


**Figure 1 | Purity of isolated macrophages separated from fresh spleen.** This shows a representative flow cytometry analysis of CD11b, CD11c, CD19, and CD49b-positive cell populations after antibody-coated magnetic beads separation. Data shown are from one of three independent experiments.

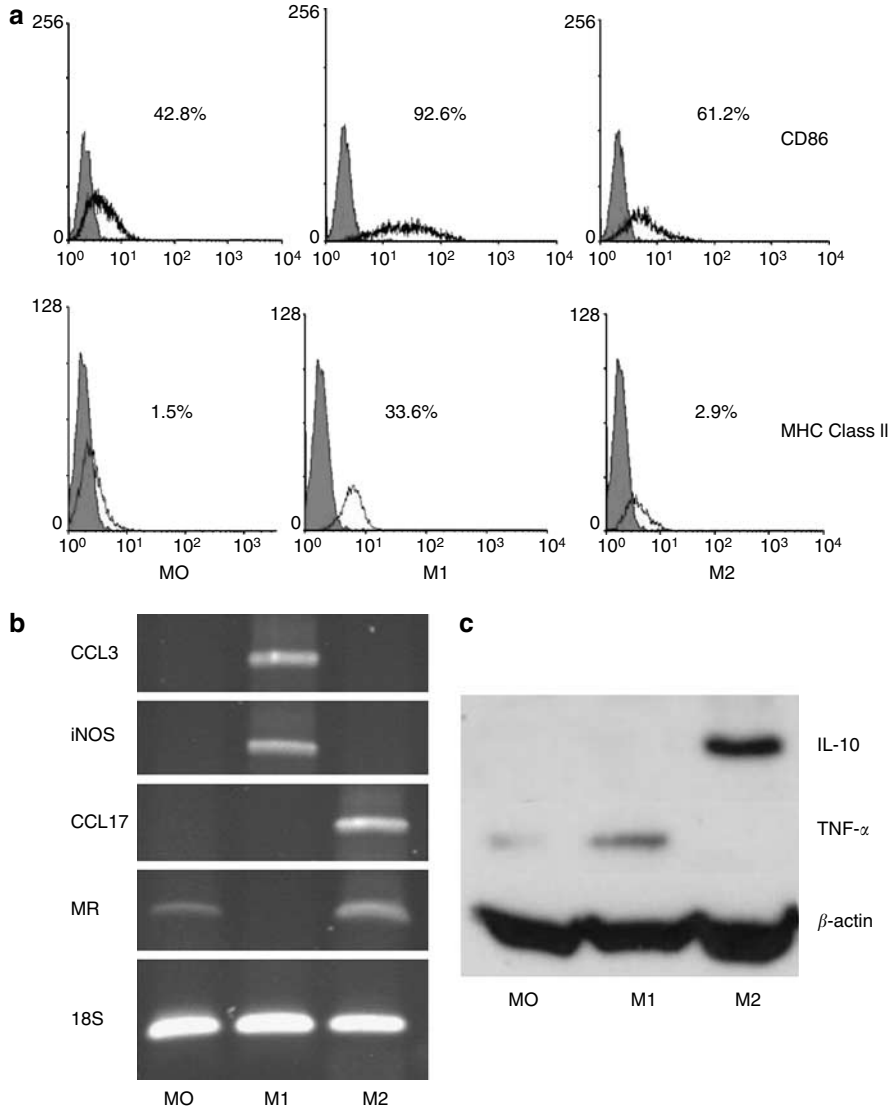


**Figure 2 | Macrophage phenotypes.** Macrophage phenotypes under different incubation conditions were confirmed in all experiments by (**a**) FACS analysis of CD86 and major histology complex class II; (**b**) polymerase chain reaction for mRNA expression of iNOS, CCL3, CCL17, and mannose receptor; and (**c**) western blot of IL-10 and TNF-α.

with the M0 transfused mice (Figure 5). The quantitation of these positive cell numbers is shown in Table 1.

### *In vivo* tracking of transferred macrophages

One million fluorescently labeled M0, M1, or M2 macrophages were injected via tail vein at day 5 after ADR or into control normal mice. Fluorescently labeled cells were distributed to kidney, spleen, and liver of AN mice 24 h after transfer (day 6). Interestingly, there was no trace of fluorescently labeled cells in lung and heart 24 h after transfusion. Few of these cells were seen in spleen and liver at days 14 and 28 (Figure 6a), whereas transfused macrophages accumulated progressively in kidneys up to day 28 after ADR. A reduction of native macrophage infiltration was observed in mice transfused with M2 macrophages. (Figure 6b–f).

### Persistence of macrophage phenotype after prolonged culture *in vitro*

The above *in vivo* experiment demonstrated renal accumulation of transfused macrophages and especially M1 macrophages as long as 4 weeks after transfusion. The number and viability of macrophages were examined. The cell numbers decreased to around half those of the initial seeding (from $1.3 \times 10^7$ to $6.5 \times 10^6$) after 28 days culture *in vitro*. The viability of the cells as defined by trypan blue staining was approximately 85% after 28 days of *in vitro* culture. Similarly, the proportion of apoptotic cells after 28 days increased to 18–25% by annexin V staining. Therefore, we next tested the stability of macrophage phenotype over time. We compared the expression of CCL3 and iNOS mRNA by freshly separated M1 to that of M1 macrophages cultured for 4 weeks, using real-time polymerase chain reaction (PCR). *In vitro* expres-

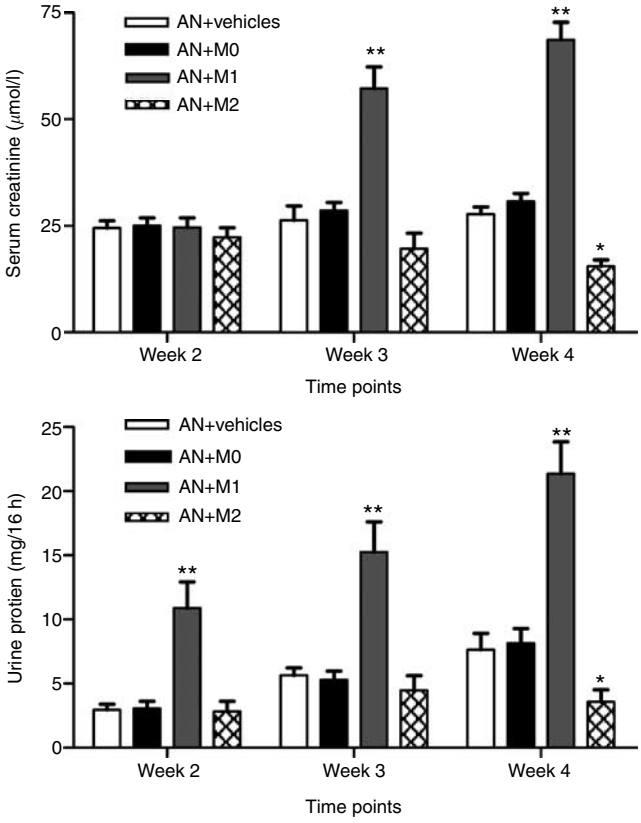


**Figure 3 | Serum Creatinine and 16 h urine protein at day 28.** The values represent the mean ± s.e.m. of evaluations from each group ($n = 7$ per group). *$P < 0.05$ vs other three groups, **$P < 0.01$ compared with other three groups.

sion of CCL3 and iNOS by M1 macrophages was maintained for 4 weeks. Similarly, the expression of CCL17 and mannose receptor mRNA by M2 macrophages was maintained for 4 weeks (Figure 7).

### *In vivo* properties of kidney macrophages after transfusion

Macrophages were isolated from AN kidneys at day 28, using saline perfusion and CD11b beads. Macrophage iNOS, CCL3, CCL17, mannose receptor, CCL2, and TNF-α mRNA expression were assessed. There was a trend toward decreased total macrophage mRNA expression of CCL2 and TNF-α after M2 macrophage transfusion. In contrast, renal macrophage mRNA expression of iNOS, CCL2, and TNF-α expression was significantly upregulated after M1 macrophage transfusion. Renal macrophage mRNA expression of CCL17 at day 28 was upregulated after M2 macrophage transfusion (Figure 8). No difference was seen in expression of mannose receptor and CCL3 among all groups (data not shown).

## DISCUSSION

In this study we examined the effect of transfused macrophages programmed *ex vivo* by cytokines, in a model of chronic inflammatory renal disease (AN). Transfusion of M0 macrophages had no significant effect on renal injury. M1 macrophages promoted both histological damage and functional impairment. However, M2 macrophages strongly protected renal structure and function. Once modified to a M1 or M2 phenotype, macrophages maintained their properties *in vitro* for up to 4 weeks. This provides proof of the potential of macrophages cultured with IL-4 and IL-13 as a treatment for chronic renal disease.

The harmful effect of preactivated macrophages has been demonstrated previously, in models of kidney disease. Ikezumi *et al.*[24] demonstrated that adoptive transfer of macrophages incubated with interferon-γ caused a twofold increase in the degree of proteinuria and glomerular cell proliferation compared with transfer of unstimulated macrophages in a cognate model of renal disease, antiglomerular basement membrane nephritis. This finding is in line with our results that demonstrated an augmentation of renal damage by M1 macrophages in AN in SCID mice, a model of innate immune renal injury.

Ikezumi *et al.*,[25] in a separate study, observed induction of proteinuria and mesangial cell proliferation by adoptive transfer of M0 macrophages during the acute stage of rat accelerated antiglomerular basement membrane disease. The absence of such an effect of M0 macrophages in AN may relate to the primary cellular nature of the inflammation in AN vs the antibody-driven pathology of antiglomerular basement membrane disease where even M0 macrophages may be activated through Fc receptors by pathogenic antibodies.

The most striking finding in these studies is that M2 macrophages protected against both structural and functional damage in AN, a protective effect which has only previously been demonstrated *in vitro*.[10,26,27] Stability of macrophage phenotype after initial cytokine exposure has been reported previously and parallels that found in lymphocyte polarization to Th1 and Th2 cells.[28,29] Other studies have suggested that microenvironment can reprogram the functional phenotype of macrophages.[30] Our results provide *in vivo* support for the suggestion that macrophage phenotype persists despite the microenvironment.

We found that both M1 and M2 macrophages trafficked to inflamed kidneys in AN. This suggests that the expression of chemokine and other trafficking receptors by these macrophages has the potential to deliver them to sites of inflammation. We have previously shown in several models of renal disease the expression of a variety of chemokines including the CCL2, a monocyte recruiting chemokine. This targeting to the inflamed organ enhances efficacy relative to the number of cells transferred and provides promise for potential clinical studies.

Other strategies to ameliorate impaired renal function in animal models have included direct cytokine gene transfer, chemokine blockade, or macrophage manipulation.[31–36] Renal adoptive transfer of macrophages genetically modified to express either IL-4, IL-10, or IL-1 receptor antagonist reduced macrophage infiltration and renal injury in animal nephritis models.[13,37,38] More recently, macrophages in which

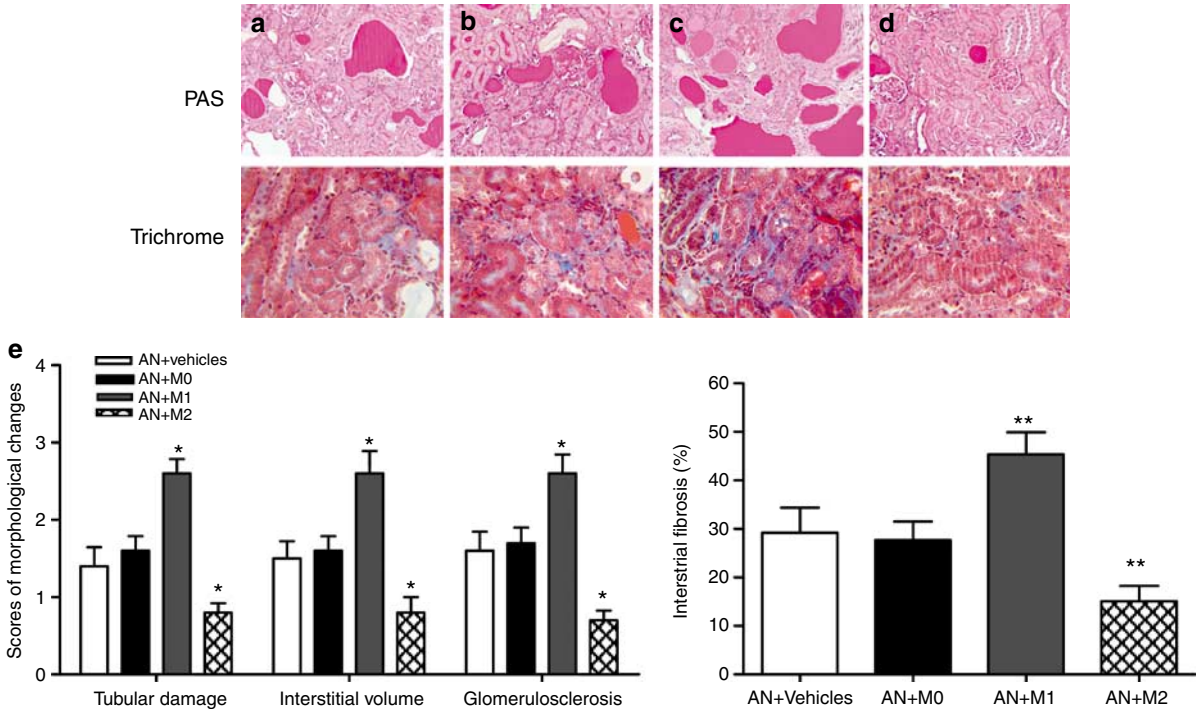


**Figure 4 | Effect of M0, M1, and M2 macrophages transfusion on histological injury and development of renal fibrosis.** (**a**–**d**) Representative periodic acid–Schiff- and trichrome-stained sections of renal cortices at day 28 (original magnification $\times 200$). (**a**) Saline-injected AN mice, (**b**) AN mice transfused with M0 macrophages, (**c**) AN mice transfused with M1 macrophages, (**d**) AN mice transfused with M2 macrophages. (**e**) Kidney injury was assessed semiquantitatively (periodic acid–Schiff) and for fibrosis by point counting (trichrome). Each evaluation represents the mean $\pm$ s.e.m. $^*P < 0.05$ vs other three groups ($n = 7$ per group).

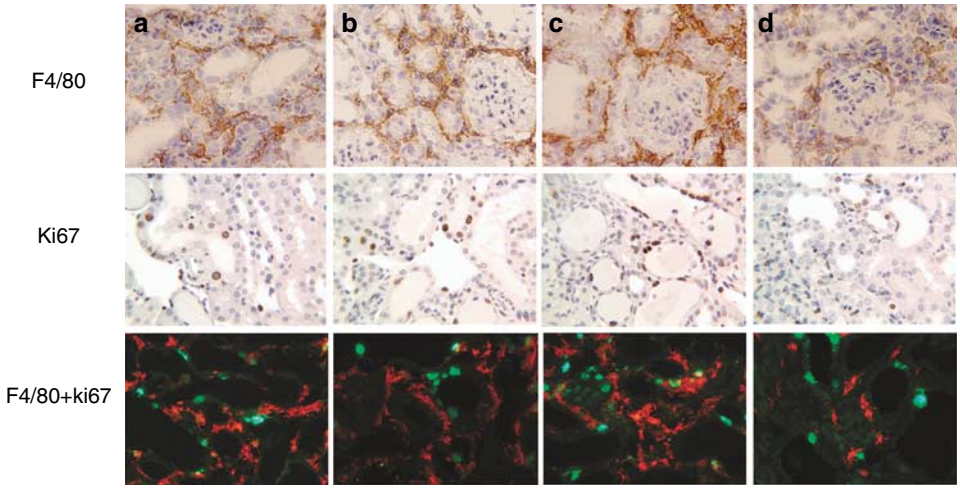


**Figure 5 | Representative double immunofluorescence staining of F4/80 and Ki67 and single immunohistological staining of F4/80 and Ki67 on kidney tissue frozen sections at day 28 (original magnification $\times 400$).** Numerous cells expressing Ki67 + (green nucleus) or F4/80 + (red membrane) cells and very few cells co-expressing F4/80 + Ki67 + (green nucleus surrounded by red membrane) were observed on double immunofluorescence staining sections for all four groups. Single staining of F4/80 or Ki67 on immunohistological staining sections disclosed a relationship of positive cell numbers and renal injury severity. (**a**) Saline-injected AN mice, (**b**) AN mice transfused with M0 macrophages, (**c**) AN mice transfused with M1 macrophages, (**d**) AN mice transfused with M2 macrophages. The quantitation of these positive cell numbers is shown in Table 1 ($n = 7$ per group).

the nuclear factor-$\kappa$B pathway was inhibited by transduction with dominant-negative inhibitory $\kappa$B developed anti-inflammatory properties and were able to reduce glomerular injury in nephrotoxic serum nephritis.[39] Cytokine manipulation of macrophages to the M2 phenotype provides many of the advantages of gene transduction without the associated risks. The advantages of this method include the simplicity of *in vitro* macrophage modulation by cytokines, and the

**Table 1 | Quantitative analysis of F4/80-positive and Ki67-positive cells**

| | AN+vehicle | AN+M0 | AN+M1 | AN+M2 |
|---|---|---|---|---|
| F4/80-positive cells | 84.17 ± 7.73 | 86.67 ± 8.89 | 126.71 ± 17.46[a] | 50.83 ± 6.35[b] |
| Ki67-positive cells | 15.37 ± 2.18 | 16.98 ± 2.73 | 22.68 ± 3.54[b] | 9.23 ± 1.56[b] |

The values represent the mean ± s.d. of positive cells/ × 400 field.
[a]$P < 0.01$ compared with other three groups.
[b]$P < 0.05$ compared with other three groups.

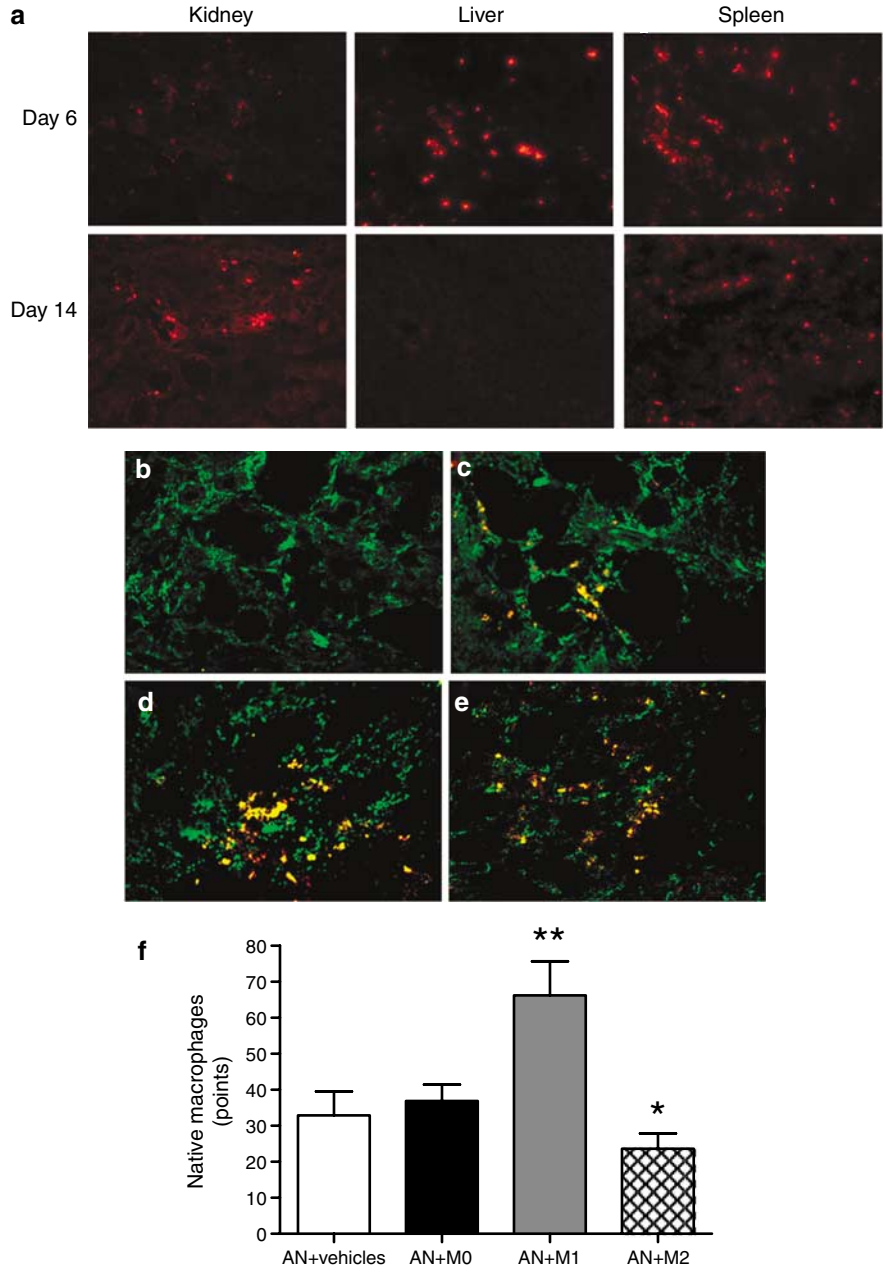


**Figure 6 | Tracking of transfused macrophages *in vivo* during progression of AN (original magnification × 200).** (**a**) Representative fluorescence photomicrographs showing M1 macrophage localization in kidney, spleen, and liver at day 6 and day 14 after ADR, following transfusion with M1 macrophages at day 5. (A similar distribution of M0 and M2 macrophages was seen after their transfusion; not shown.) (**b**) Representative immunofluorescence staining of macrophages on kidney sections at day 28. Cells fluorescing orange are double positive for fluorescent dye and F4/80, whereas cells fluorescing green are F4/80-positive cells, stained with immunofluorescent F4/80 FITC. (**b**) Saline-injected AN mice, (**c**) AN mice transfused with M0 macrophages, (**d**) AN mice transfused with M1 macrophages, (**e**) AN mice transfused with M2 macrophages ($n = 7$ per group). (**f**) The area fluorescing green representing F4/80-stained macrophages was assessed quantitatively by point counting. Each evaluation represents the mean ± s.e.m of averaged number of points per field from each animal. *$P < 0.05$ vs other groups ($n = 7$ per group), **$P < 0.01$ vs other groups ($n = 7$ per group).

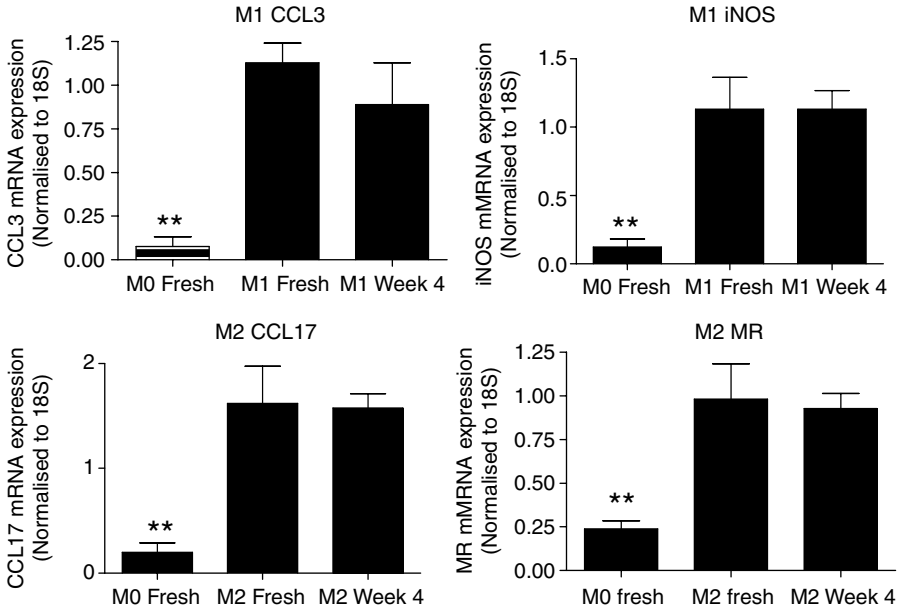


**Figure 7 | Persistence of macrophage phenotype after prolonged culture *in vitro*.** Expression of CCL3 and iNOS mRNA by M1 macrophages (freshly separated or cultured for 4 weeks) and CCL 17 and MR mRNA by M2 macrophages (freshly separated or cultured for 4 weeks) was assessed. $^{**}P<0.01$ the control vs other two groups. Experiments were repeated in triplicate.

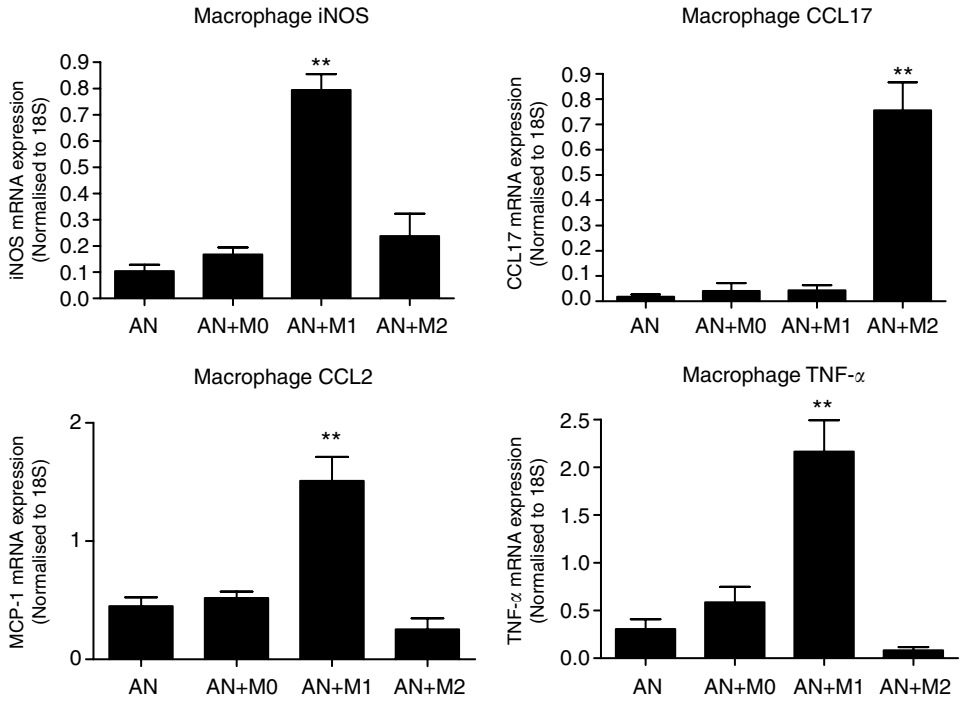


**Figure 8 | *In vivo* properties of kidney macrophages in AN at day 28.** The expression of iNOS, CCL17, CCL2, and TNF-α mRNA was assessed from kidney macrophages ($n=7$ per group). $^{**}P<0.01$ vs other three groups.

effectiveness of intravenous administration without the need for specialized delivery. Most important is the fact that it avoids the limitations of gene therapy including gene delivery and risks of the viral vector.[40,41]

Previous studies of renal macrophage phenotype have studied the composition of the macrophage population. In the anti-Thy1.1 model of proliferative nephritis, 70% of macrophages infiltrating glomeruli demonstrated a M1 phenotype and 30% behaved as if activated by transforming growth factor-$\beta$.[42] Our study was able to do this in a very informative way. Having demonstrated that short-term induction of M1 or M2 phenotype led to a persistence of

these characteristics for up to 4 weeks in culture, we evaluated the effect of transfer of these cells on host macrophages. M1 transfused mice exhibited skewing of expression of host macrophage-related molecules to that of the transfused population. However, M2 macrophage transfusion caused an apparent (nonsignificant) decrease in expression of CCL2 and TNF-α and a significant increase in expression of CCL17 in total macrophages suggesting that in addition to their possible effects on other cells M2 macrophages could skew the host macrophage phenotype. M1 macrophages are known to have direct effects on renal parenchymal cells via the release of NO and other mediators,[43] whereas it is unknown whether M2 macrophages can directly affect the parenchyma. Thus, it is possible that the protective effect of M2 macrophages may involve a direct or indirect effect on renal parenchymal cells, in particular tubular epithelial and mesangial cells.

In conclusion, this study provides the direct evidence that macrophage modulation *ex vivo* by cytokines to an anti-inflammatory phenotype (M2), is an effective strategy for treating experimental chronic inflammatory renal disease. The regulatory effects of M2 macrophage were shown to be target-specific, sustainable, and dominant.

## MATERIALS AND METHODS

### AN murine model

Six- to eight-week-old male SCID and BALB/c mice obtained from the Animal Resources Centre (Perth, Australia) were used in this study. The Animal Ethics Committee of Westmead Hospital approved all procedures. Dose-finding studies defined an optimal dose of 5.2 mg/kg body weight of ADR (Pharmacia & Upjohn Pty Ltd., Australia). ADR was injected once via the tail vein of each non-anesthetized SCID mouse.[44]

### Macrophage separation from spleen and *ex vivo* culture conditions for M1 and M2 macrophages

BALB/c mice splenocytes were harvested and washed in ice-cold RPMI 1640 medium (Invitrogen, Mount Waverly, Australia). Tissue was triturated with the sterile syringes, and the resulting cell suspension was filtered through 40-μm nylon mesh (BD Biosciences, North Ryde, Australia) and then incubated at 37°C for 30 min. The adherent cells were harvested and purified by MACS CD11b+ MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany, Bergisch Gladbach). These spleen-derived macrophages were rinsed three times with RPMI 1640 medium and further processed to become M0, M1, and M2. Macrophages cultured in the normal medium for 48 h were defined as M0; macrophages undergoing a 2-h incubation with lipopolysaccharide (2.5 μg/ml) and then cultured in normal medium for another 46 h were defined as M1; and macrophages undergoing the incubation with IL-4/13 (10 ng/ml each) for 48 h were defined as M2. Lipopolysaccharide was purchased from Sigma-Aldrich (Castle Hill, Australia), and IL-4 and IL-13 were purchased from Invitrogen.

### Analysis of macrophage apoptosis

Apoptosis was analyzed by flow cytometric detection of fluorescein isothiocyanate (FITC)-labeled annexin V (BD, North Ryde, Australia) according to the company's manual. In brief, collected macrophages were washed twice with cold phosphate-buffered saline and then cells were resuspended in 1 × binding buffer at a concentration of $1 \times 10^6$ cells/ml. Then 100 μl of the solution ($1 \times 10^5$ cells) was incubated for 15 min at room temperature (25°C) in the dark after adding 5 μl of annexin V-FITC and 5 μl of propidium iodide. After that, 400 μl of 1 × binding buffer was added to each tube. Flow cytometric analysis was performed with a FACS Calibur flow cytometer (BD, Mountain View, CA, USA) and 10 000 events were acquired from each sample. Data were collected and analyzed with CellQuest 3.1 software (BD).

### Isolation of infiltrated macrophages from mouse kidney

Warm saline (37°C) was perfused to the right heart atrium under anesthesia to remove peripheral blood within mouse kidney. Kidney tissue was then triturated in RPMI 1640 medium with the sterile syringes, and the resulting cell suspension was filtered through 40-μm nylon mesh. Mononuclear cells were separated using a step-gradient sucrose separation procedure (Lymphoprep, Pharmacia, Uppsala, Sweden). Purification of macrophages was performed by positive selection using MACS CD11b+ MicroBeads.

### Flow cytometry

Isolated macrophages were suspended in FACS wash buffer (phosphate-buffered saline with 1% fetal calf serum) and Fc block (eBiosciences, San Diego, CA, USA). Cells were stained with FITC-conjugated anti-mouse CD11b, FITC-conjugated anti-mouse CD 49b, FITC-conjugated anti-mouse CD11c, PE-conjugated anti-mouse CD19, PE-conjugated anti-mouse major histology complex class II, and PE-Cy5 anti-mouse CD86 (eBiosciences, USA). All the samples were then analyzed by flow cytometry on a Beckton Dickinson FACScan (BD Biosciences). At least $1 \times 10^5$ events were collected per sample.

### Macrophage labeling and adoptive transfer to SCID mice

For *in vivo* study, three different phenotypes of macrophages were labeled with red fluorescent membrane label, DiI (Invitrogen) according to the manufacturer's instructions, and harvested into serum-free medium immediately before injection. DiI is a lipophilic membrane stain that diffuses laterally to stain the entire cell. It is weakly fluorescent until incorporated into membranes. This red fluorescent dye, which is spectrally similar to tetramethylrhodamine, is often used as a long-term tracer for neuronal and other cells.[45] In brief, 50 μg DiI was dissolved into 50 μl pure ethanol. To a one –million-cell suspension in 1 ml RPMI 1640, 4 μl DiI was added for 10 min incubation at 37°C followed by 15 min incubation at 4°C. Macrophage viability and phenotype did not change with labeling. One million cells were transferred into treated SCID mice by a single tail-vein injection at day 5 after ADR. Twenty-one mice in each of groups A (ADR + saline), B (AN + M0 macrophages), C (AN + M1 macrophages), and D (AN + M2 macrophages) were caged according to their groups. Seven mice in each group were killed on days 6, 14, and 28 after ADR.

Then kidney sections stained with F4/80 FITC fluorescence were visualized under fluorescence microscopy (magnification × 200). The green fluorescent cells represent F4/80 FITC staining macrophages and the orange fluorescent cells were considered to be transfused macrophages. F4/80 FITC-stained macrophages were assessed and quantified by point counting using Image J software (NIH, USA). Randomly, 10 nonoverlapping cortical fields from each kidney section of seven separate animals per group were selected for analysis.

### Histology and immunohistochemistry

Kidneys were rapidly removed on days 6, 14, and 28. Coronal sections of renal tissue were immersion-fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections 5-$\mu$m thick were stained with periodic acid–Schiff or trichrome. The remaining cortex of the same kidney was frozen in liquid nitrogen and used for RNA analysis and immunohistochemistry. To evaluate the degree of renal injury, 20 fields of view/section at a magnification of $\times 200$ were assessed and the degree of renal injury was estimated by evaluating the percentage of renal injury per field and was graded on a scale of 0–4:0, normal glomeruli, tubules, and interstitial volume; 0.5, small focus of glomerular and tubular injury and interstitial volume; 1, involvement of $<10\%$ of the cortex; 2, involvement of up to 25% of the cortex; 2.5, involvement of 26–50% of the cortex; 3, 51–75% of the cortex; and 4, extensive damage involving $>75\%$ of the cortex.[46] Quantitative analysis of glomerulosclerosis was performed using a modification of the technique described by Saito *et al.*[47] The degree of sclerosis was scored from 0 to 4 in each of 20 glomeruli from periodic acid–Schiff-stained sections and averaged for each mouse. Interstitial fibrosis was assessed and quantified on trichrome-stained sections by point counting using Image J software in each of 10 nonoverlapping cortical fields from the cortical region for analysis. Points falling within blue areas (fibrosis) were considered as positive. Scores derived from 10 fields per kidney section were averaged, and mean scores were from seven separate animals per group.

For immunohistochemical staining of macrophages, rat anti-mouse F4/80 (eBiosciences) or rabbit anti-Ki67 (Lab Vision, Fremont, USA) was used as the primary antibody and biotinylated rabbit anti-rat Ig (Dako Corporation, Botany, Australia) was used as the secondary antibody. Kidney sections were placed in OCT (Sakura Fintek Inc., Torrance, USA). Sections of 5 $\mu$m were cut, dried overnight, and fixed in cold acetone for 8 min. Endogenous peroxidase activity was blocked by 0.3% (v/v) $H_2O_2$ solution for 15 min when incubating the slides. Biotin Blocking System (Dako, Botany, Australia) was used to block endogenous avidin-binding activity. Normal rat immunoglobulin was used for control sections. Sections were incubated with secondary antibodies, ABC, and 3,3-diaminobenzidine substrate-chromogen solution (Dako) were applied and then washed. Slides were counterstained with hematoxylin (Sigma, Castle Hill, Australia). For assessment of interstitial infiltration, positively stained cells located in the tubular interstitial area only were counted from more than 20 random cortical fields (magnification $\times 400$) in each section, and the numbers averaged for each section, as described previously.[44]

Immunofluorescence staining for macrophages was performed on frozen slides prepared as for immunohistochemical staining. FITC-conjugated anti-Rabbit or phycoerythrin-conjugated anti-Rat antibody (eBiosciences) was used as the second antibody.

### Western blot analysis

Cells were lysed in sodium dodecyl sulfate sample buffer (2% sodium dodecyl sulfate, 10 mM Tris–HCl (pH 6.8), and 10% (v/v) glycerol) and equal amounts of proteins from various treatments were subjected to immunoblotting with the IL-10 (1:1000 dilution; R&D, Gymea, Australia), TNF-$\alpha$ (1:2500 dilution; R&D), and $\beta$-actin (1:4000 dilution; Sigma). Immunoreactivity was determined by horseradish peroxidase-conjugated donkey anti-goat antibody and an enhanced chemiluminescence reaction (Pierce, Rockford, USA).

**Table 2 | Real-time PCR primers and products**

| Gene | Primer sequence (5′…3′) | Product |
|---|---|---|
| CCL3 (F) | tgcccttgctgttcttctct | 124 |
| CCL3 (R) | gatgaattggcgtggaatct | |
| iNOS (F) | caccttggagttcacccagt | 170 |
| iNOS (R) | accactcgtacttgggatgc | |
| CCL17 (F) | tgcttctggggacttttctg | 147 |
| CCL17 (R) | catccctggaacactccact | |
| Mannose receptor (F) | caaggaaggttggcatttgt | 111 |
| Mannose receptor (R) | cctttcagtcctttgcaagc | |
| TNF-$\alpha$ (F) | gctgagctcaaaccctggta | 118 |
| TNF-$\alpha$ (R) | cggactccgcaaagtctaag | |
| CCL2 (F) | cccaatgagtaggctggaga | 125 |
| CCL2 (R) | tctggacccattccttcttg | |

iNOS, inducible nitrogen oxide synthase; PCR, polymerase chain reaction; TNF-$\alpha$, tumor necrosis factor-$\alpha$.

### Renal function

All urine and blood specimens were analyzed by the Institute of Clinical Pathology and Medical Research (Westmead Hospital), using a BM/Hitachi 747 analyzer (Tokyo, Japan).

### Isolation of total RNA, synthesis of cDNA, reverse transcription-PCR, and quantitative real-time PCR

Cells were homogenized and the total RNA was isolated using RNeasy Mini Kit (Qiagen, Australia). cDNA was synthesized with a reverse transcriptase reaction using a standard technique (Superscript™ First Strand Synthesis System for RT-PCR; Invitrogen). The PCR primer sequences used in this study were designed using Oligoperfect software (Invitrogen website) and are presented in Table 2. Primers were designed to span intron–exon boundaries. For reverse trancription-PCR, a 0.2 $\mu$g portion of cDNA was used in a 25 $\mu$l PCR mixture with 2 ng/$\mu$l of each primer and 2 U of *Taq* DNA polymerase (Roche, Dee Why, Australia). Housekeeping gene 18S was used as the endogenous control. PCR products after amplification were analyzed by 1.5% agarose gel electrophoresis. For real-time PCR, mRNA levels were quantified using the Rotogene-3000 Real-Time Thermo cycler (Corbett Research, Mortlake, Australia). PCR mixtures containing 0.3 $\mu$M primers in 25 $\mu$l final SYBR mastermix (Invitrogen) were cycled for 2 min at 50°C, 2 min at 95°C followed by 40 cycles of 15 s at 95°C, 30 s at 55°C, and 30 s at 72°C. The normalized value for mRNA expression in each sample was calculated as the relative quantity of relevant primers divided by the relative quantity of the housekeeping gene 18S.

### Statistical analysis

Renal functional data (serum creatinine and urine protein) were log-transformed before analysis to stabilize the variance. The statistical package SPSS for windows version 14 was used to analyze the data. Analysis of variance was used to test for an association between the log-transformed outcome and treatment (one-way analysis of variance). The pairwise comparisons between treatments were adjusted for multiple comparisons using the Bonferroni technique. Other data analyses were performed directly by one-way analysis of variance for multiple comparisons of parametric data, and the Kruskal–Wallis test for nonparametric data. Results are expressed as the group mean $\pm$ s.e.m. A $P<0.05$ was considered statistically significant.

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