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Macrophages Transfected with Adenovirus to Express IL-4 Reduce Inflammation in Experimental Glomerulonephritis

David C. Kluth,* Clare V. Ainslie,* Wayne P. Pearce,* Sian Finlay,* Daniel Clarke,* Ignacio Anegon,† and Andrew J. Rees*

Nephrotic nephritis (NTN) is characterized by acute macrophage-dependent inflammation and serves as a model for human glomerulonephritis. In this study we have transfected rat macrophages with recombinant adenovirus expressing IL-4 (Ad-IL4) and demonstrated that these transfected macrophages develop fixed properties as a result of transfection, as shown by reduced NO production in response to IFN-γ and TNF. Ad-IL4-transfected macrophages localized with enhanced efficiency to inflamed glomeruli after renal artery injection in rats with NTN compared with adenovirus expressing β-galactosidase (Ad-βgal)-transfected macrophages and produced elevated levels of the cytokine in glomeruli in vivo for up to 4 days. The delivery of IL-4-expressing macrophages produced a marked reduction in the severity of albuminuria (day 2 albuminuria, 61 ± 15 mg/24 h) compared with unmodified NTN (day 2 albuminuria, 286 ± 40 mg/24 h; p < 0.01), and this was matched by a reduction in the number of ED1-positive macrophages infiltrating the glomeruli. Interestingly, the injection of IL-4-expressing macrophages into single kidney produced a marked reduction in the numbers of ED1-positive macrophages in the contralateral noninjected kidney, an effect that could not be mimicked by systemic delivery of IL-4-expressing macrophages. This implies that the presence of IL-4-expressing macrophages in a single kidney can alter the systemic development of the inflammatory response. Macrophage transfection and delivery provide a valuable system to study and modulate inflammatory disease and highlight the feasibility of macrophage-based gene therapy. The Journal of Immunology, 2001, 166: 4728–4736.

Macrophages have critical roles in host response to injury and the mechanisms by which it is repaired (1–4). They infiltrate damaged tissues where they adapt to the local microenvironment by developing properties that either cause further injury (such as might be advantageous in defense against infection), or alternatively evolve into cells that promote resolution of inflammation and facilitate tissue repair once the original cause of injury has been eliminated (2). Macrophages also have the potential to cause disease when these natural properties are used inappropriately. Thus, maladapted lymphocytes direct macrophages to cause tissue injury in many autoimmune and inflammatory diseases (5–7); micro-organisms have evolved strategies for subverting macrophage function for their own gain (8), and tumors often induce macrophages to sustain their growth rather than destroy them (9). These natural examples provide a precedent and raise the question of whether it might be possible to develop an analogous strategy designed to manipulate macrophage function equally effectively for therapeutic gain.

We have previously shown that rat macrophages, both cell lines and primary cultures, can be transfected with recombinant adenovirus and that these modified macrophages preferentially localized to inflamed glomeruli of rats with nephrotic nephritis (NTN), an experimental model of glomerulonephritis (10). Surprisingly, localization did not worsen injury and actually caused a small reduction in the level of albuminuria. Transfection of macrophages using recombinant adenoviruses could be used to deliver macrophages with specific functions to areas of injury and provide a more physiological approach to study the effects of cytokines in inflammation. The responses of macrophages to pro- and anti-inflammatory cytokines are well established, and macrophages can be programmed by exposure to specific cytokines. For example, prior stimulation with the anti-inflammatory cytokine such as IL-4 makes macrophages unresponsive to proinflammatory cytokines, including IFN-γ and TNF (11). Thus, transfecting macrophages to express specific cytokines might not only alter the secretory profile of but also, their responses to other signals, and when these transfected macrophages infiltrate the focus of inflammation they will be able to affect surrounding cells involved in injury (12). In a number of autoimmune disease models the administration of exogenous IL-4 has been shown to reduce the severity of injury, including experimental glomerulonephritis (13, 14), collagen-induced arthritis (15), and experimental allergic encephalomyelitis (16). This makes IL-4 an ideal candidate molecule to assess the efficacy of macrophage-mediated gene therapy.

The purpose of the experiments in this paper was 1) to characterize the effect of transfection with adenovirus expressing rat IL-4 (Ad-IL4) on macrophage function and responses to proinflammatory cytokines; 2) to determine whether, after injection into the renal artery, Ad-IL4-transfected macrophages localize to inflamed glomeruli and synthesize IL-4 there; and 3) to analyze the effect of

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3 Abbreviations used in this paper: NTN, nephrotic nephritis; Ad-IL4, adenovirus expressing rat IL-4; Ad-βgal, adenovirus expressing β-galactosidase.
injection of these macrophages on glomerular injury in nephrotoxic nephritis in rats. The results show not only that macrophages transfected with Ad-IL4 localized to inflamed glomeruli, but that they reduce the severity of injury in rats with experimental glomerulonephritis. Surprisingly, the injury is reduced not only in the kidney into which the IL-4-expressing macrophages have been injected, but also in the contralateral kidney. This result cannot be explained by systemic leakage of IL-4 and suggests that cells transiting through glomerular inflammation in the context of high concentrations of IL-4 are able to down-modulate glomerular inflammation at a distant site. If confirmed, this would have profound implications for understanding the control of inflammation in general.

Materials and Methods

Cells and reagents

The rat alveolar macrophage cell line NR8383 was obtained from American Type Culture Collection (Manassas, CA) and cultured in Ham’s F-12 (BioWhittaker, Walkersville, MD) with 100 U/ml penicillin, 100 μg/ml streptomycin, and 10% FCS (Sigma, Poole, U.K.). Rat bone marrow-derived cells were isolated and purified using our standard technique (11). All cells were cultured at 37°C with 5% CO2. Nephrotoxic serum was provided by the nephrology unit at Guy’s Hospital (London, U.K.) and stored at −20°C. Recombinant adenoviruses and transfection

Ad-IL4 was constructed as previously described (17) with control adenovirus provided by β-galactosidase expressing vector (Ad-βgal) or Ad-dl312, a null adenovirus with no insert (18). All adenoviruses were produced in 293 cells with subsequent purification on cesium chloride gradient, dialysis against 10 mM Tris (pH 7.4) buffer with 10% (v/v) glycerol, and storage at −70°C (19). Viral titer was determined by plaque assay on 293 cells and expressed as PFUs, while the absence of replication was confirmed by plaque assay on HeLa cells (19). NR8383 rat on 293 cells and expressed as PFUs, while the absence of replication was confirmed by plaque assay on 293 cells and expressed as PFUs, while the absence of replication was confirmed by plaque assay on HeLa cells (19). NR8383 rat alveolar macrophages were stimulated with 5–10% L929-conditioned medium containing M-CSF for 48 h before transfection to up-regulate α,β1 integrins and enhance transfection efficiency (20). Transfection was performed in standard medium with 2% FCS as previously described (10). Production of IL-4 by transfected macrophages into tissue culture medium was assessed using a capture ELISA for IL-4 (PharMingen, San Diego, CA).

Properties of transfected macrophages

The effect of transfection on generation of NO by NR8383 cells and bone marrow-derived macrophages was assessed by nitrite production, assayed using a Greiss reaction (11). In brief, 48 h after transfection 200-μl aliquots of medium from the cells were removed and incubated with 50 μl of Greiss reagent (0.5% sulphanilamide and 0.05% N-(1-naphthyl)ethylenediamine dihydrochloride in 2.5% phosphoric acid) in 96-well flat-bottom culture plates for 10 min. The ODs of the assay samples were then measured at 540 nm using a solution of phenol red-free DMEM. After transfection macrophages were stimulated with 10–20 ng/ml LPS (Sigma) or IFN-γ (Sigma; 1 U = 3 ng) and TNF R&D Systems, Abingdon, U.K.; 1 U = 3 ng), and the production of NO was assessed 24 h later.

Nephrotoxic nephritis and in vivo delivery of macrophages

Male Sprague Dawley rats (weight, 200–250 g) were purchased from Harlan (Bicester, U.K.). Rats were preimmunized with s.c. injection 1 mg of rabbit IgG (Sigma) in CFA. One week later the rats received an i.v. injection of 1 ml/200 g of rabbit nephrotoxic serum under halothane anesthesia (Zeneca, Macclesfield, U.K.). Transfected and nontransfected macrophages were labeled with red fluorescent membrane label, PKH-26GL (Sigma) as previously described (10) and harvested into serum-free medium immediately before injection. Labeled macrophages were injected directly into the renal artery 5–6 h after induction of NTN. This was performed by anesthetizing rats with fentanyl (Janssen-Cilag, High Wycombe, U.K.) and di-zepam (Zeneca Pharmaceuticals, Gloucester, U.K.), performing a midline laparotomy, and inserting a 27-gauge butterfly needle (Abbott, Dublin, Ireland) was inserted directly into the renal artery, the cells were injected over 1–2 min, and renal blood flow was re-established in <5 min. Animals were left for 1–7 days, and timed urine collections were made from metabolic cages. In specific experiments transfected NR8383 macrophages were injected into the tail vein or into the peritoneal space to compare the effect of route of delivery.

Glomerular visualization and culture

To enable visualization of the fluorescent macrophages within glomeruli, part of the renal tissue was sieved through 250- and 150-μm pore size sieves, and the glomeruli were collected on a 63-μm pore size filter. The isolated glomeruli were labeled with antibody IgG FITC (Sigma), which binds to rabbit IgG from the nephrotoxic serum deposited on the glomerular basement membrane. The whole glomeruli were visualized under fluorescent microscopy, and the number of PKH-26GL-positive fluorescent cells in 100 glomeruli was counted. Isolated glomeruli were also cultured in DMEM with 10% FCS, and the level of IL-4 produced over 48 h was measured.

Albunminuria and urinary IL-4

Timed urine collections were made, and the albumin concentration was determined using Rocket electrophoresis as previously described (10). Albuminuria was then calculated as the total albumin excreted over 24 h. In addition, the concentrations of IL-4 in urine and plasma were measured by ELISA.

Determination of alloreaction to transfected NR8383 cells

Although the NR8383 cell is derived from Sprague Dawley rats, this is an outbred strain of rat, leading to the possibility of an alloreaction. To assess this experimentally, MLR was performed with splenocytes from rats immunized with transfected NR8383 cells. Rats with NTN were injected with 6 × 106 NR8383 cells transfected with either Ad-βgal or Ad-IL4 (to assess whether IL-4 expression altered any immune response). Rats were sacrificed 7 days later, and splenocytes were isolated and cultured in RPMI with 2% FCS supplemented with 2-ME and 1-glutamine (Life Technologies, Paisley, Scotland). The MLR was set up against mitomycin C (Sigma)-treated NR8383 cells transfected with Ad-dl312, and the incorporation of [3H]thymidine was assessed on days 2–5. Various dilutions of NR8383 cells were used (0.3–5 × 106 NR8383 cells) with 1.5 × 106 splenocytes in 2 ml. A positive control was provided by stimulation of splenocytes with 2 μg/ml Con A (Sigma), and results were compared with splenocytes from rats that were not injected with NR8383 cells.

Pathology

Sections of renal tissue were fixed in methyl Carnoy and paraform-embedded, and 3-μm sections were cut before hematoxylin-eosin staining. To assess the infiltration of glomerular tissue with macrophages, unstained sections were stained with ED1 Ab (Serotec, Kidlington, U.K.) and visualized using an alkaline phosphatase/anti-alkaline phosphatase technique with the slides counterstained with hematoxylin. The numbers of ED1-positive foci were counted in the glomeruli at ×20 magnification using Leica Q-win software and were calculated per glomerular area (50,000 μm2). The slides were examined for histological markers of injury, including glomerular endothelial cell swelling and proliferation (scored 0–3), mesangial cell proliferation (scored 0–3), and necrosis (scored 0–2) by an observer blinded to the experimental protocol.

Statistics

Results are presented as the mean ± SE, and differences between groups of cells or animals were tested using paired t test.

Results

Transfection of NR8383 macrophages and bone marrow-derived macrophages with Ad-IL4 in vitro

Expression of IL-4. The rat alveolar macrophage cell line NR8383 was transfected with increasing doses of Ad-IL4 (0–200 PFU/cell), and the concentration of IL-4 in the medium was measured 48 h later by capture ELISA. Macrophage viability, when assessed by trypan blue exclusion, was not affected by the transfection procedure, but transfection with increasing numbers of viral PFU resulted in a dose-dependent increase in IL-4 secreted into the medium (Fig. 1A). Similarly, large amounts of IL-4 were secreted by rat bone marrow-derived macrophages transfected with Ad-βgal (data not shown). As expected, nontransfected macrophages and macrophages transfected with Ad-βgal did not produce...
Thus macrophages can be transfected successfully with Ad-IL4, and the next issue was to determine the effect of such transfection on macrophage function, in particular their responses to proinflammatory cytokines.

**Abrogation of effect of proinflammatory stimuli on NO generation.** NO generation was used as a marker of the ability of transfected macrophages to respond to the proinflammatory stimuli LPS or IFN-γ and TNF-α. NR8383 macrophages were transfected with 200 PFU/cell of Ad-IL4 or with a null adenovirus (Ad-dl312) at the same dose of virus, and the transduced cells were stimulated with either 20 pg/ml LPS or 20 U/ml of both IFN-γ and TNF 24 h after transfection. The quantity of NO was assayed 24 h after stimulation and compared with that in nontransfected macrophages. Similar results were obtained after stimulation with IFN-γ alone (data not shown).

IL-4-expressing NR8383 macrophages have properties similar to those of macrophages treated with exogenous IL-4; in particular, they generated lower levels of NO when activated with LPS or IFN-γ. This is important because macrophages infiltrating inflamed glomeruli in nephrotic nephritis behave operationally as though programmed by IFN-γ and TNF-α, and this provides the rationale for examining the effect of Ad-IL4-transfected macrophages on injury in this macrophage-dependent model of nephritis.

**Ad-IL4-transfected macrophages localize efficiently to inflamed glomeruli**

First it was important to determine how well IL-4-expressing macrophages localize to inflamed glomeruli. NR8383 macrophages were used for these experiments because the efficiency of transfection exceeds 95% (10). Between 2–4 × 10⁶ Ad-IL4-transfected IL4 NR8383 macrophages were labeled with the red fluorescent membrane label PKH-26GL and injected into the renal artery. Large numbers of fluorescent IL-4-expressing NR8383 macrophages were identified within the glomeruli 24 h after injection of the same dose, and the transduced cells were stimulated with either 20 pg/ml LPS or 20 U/ml of both IFN-γ and TNF 24 h after transfection. The quantity of NO was assayed 24 h after stimulation and compared with that in nontransfected macrophages. Stimulation of both nontransfected and null-transfected NR8383 macrophages with LPS (20 ng/ml) or IFN-γ (20 U/ml) and TNF-α (20 U/ml) greatly increased NO generation. However, in NR8383 macrophages expressing IL-4 the NO response to both sets of stimuli was significantly reduced (Fig. 1B). Similar results were obtained after stimulation with IFN-γ alone (data not shown).

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More than 90% of glomeruli contained at least one transfected macrophage, and the median number was 10. Ad-IL4-transfected NR8383 macrophages localized more efficiently than Ad-βgal-transfected macrophages, with almost 3–4 times as many transfected cells within the glomeruli 24 h after injection when the same numbers of transfected macrophages were injected (Fig. 3A). Labeled IL-4-expressing macrophages were seen only exceptionally in the right kidney after injection into the left renal artery and were never observed after injection of untransfected or Ad-βgal-transfected cells. The increased ability of Ad-IL4-transfected macrophages to localize to inflamed glomeruli was confirmed by studies in which they were injected into the renal artery of healthy rats. Far fewer localized to noninflamed glomeruli, and there was no difference in the numbers of Ad-IL4- or Ad-βgal-transfected macrophages present within the glomeruli (Fig. 3A).

Despite the difference in localization efficiency, Ad-βgal- and Ad-IL4-transfected NR8383 macrophages had a similar duration of residence within inflamed glomeruli, with a half-time of approximately 2 days estimated from measurements taken at 1, 4, and 7 days (Figs. 2B and 3A). The duration of residence Ad-IL4-transfected cells was also reflected in the amounts of IL-4 excreted into the urine and released from glomeruli purified from them. Glomeruli isolated 1 day after injection of Ad-IL4-transfected macrophages produced elevated levels of IL-4, which decreased from days 1 to 4. A marked reduction in albuminuria was observed with Ad-βgal or nontransfected macrophages (*, p < 0.01). On day 4 there remained a reduction in the level of albuminuria compared with those in the other three groups (β, p < 0.05). By day 7 there was no statistically significant difference between the groups (values are the mean ± SEM; IL-4 group, n = 10; control NTN, n = 13; Ad-βgal, n = 5; nontransfected macrophages (Mac), n = 5).
with either Ad-IL-4-expressing macrophages had only been injected into the left glomerular macrophages in NTN.

Effect of Ad-IL4-transfected macrophages on the total number of albuminuria, but only for the time that IL-4-expressing macrophages were essential for the effect. Thus, the delivery of IL-4-expressing macrophages to the left kidney caused a marked reduction in albuminuria (Fig. 4B) on both days 1 and 2 compared with 2–4 × 10⁶ Ad-IL4-transfected macrophages compared to splenocytes from rats which had not been exposed to NR8383 cells. Proliferation was represented as cpm. Data is presented for day 4 of proliferation assay with similar results obtained at days 2–5 of the assay (n = 3 rats for each group; mean ± SD).

To assess the potential alloreaction against NR8383 cells, rats with NTN were injected with 6 × 10⁶ NR8383 cells transfected with either Ad-βgal or Ad-IL4 or were unimmunized and splenocytes were isolated 7 days later. The MLR was then set up against various dilutions of NR8383 cells, with Con A acting as a positive control. Proliferation was assessed by measuring uptake of [³H]thymidine on days 2–5. Results were similar at all dilutions of NR8383 cells used and on days 2–5 of the assay, with the data for day 4 presented in Table I being representative. There was the expected proliferation in Con A-treated splenocytes, but no evidence of proliferation on exposure to mitomycin C-treated Adβgal-transfected NR8383 macrophages at various dilutions of cells. Thus, an alloreaction was unlikely to be responsible for the disappearance of transfected macrophages or altering the course of disease.

In vivo delivery of Ad-IL4-expressing macrophages reduces proteinuria in NTN

Injection of Ad-βgal-transfected or nontransfected NR8383 macrophages into the renal artery causes a small and transient reduction in proteinuria (10). By contrast, injection of similar numbers of Ad-IL4-transfected macrophages produced a profound reduction in albuminuria on days 1, 2, and 4 after injection (Fig. 3D). On days 1 and 2 after injection of IL-4-expressing macrophages, the level of albuminuria was reduced to 15–20% of that in untreated nephritic rats with NTN. The reduction of albuminuria was less on day 4, but still statistically significant, and had returned toward control values by day 7. Attenuation of the effect over time paralleled both the reduced numbers of transfected NR8383 macrophages and the decreased production of IL-4 within the glomeruli.

To correct for the enhanced localization of IL-4-transfected macrophages, 8–10 × 10⁶ Ad-βgal-transfected NR8383 cells were injected compared with 2–4 × 10⁶ Ad-IL4-transfected cells, which resulted in similar numbers of transfected cells within the glomeruli after processing on day 2 (Fig. 4A). Renal artery injection of IL-4-expressing macrophages caused a similar profound reduction in albuminuria (Fig. 4B) on both days 1 and 2 compared with that in unmodified disease and Ad-βgal-injected rats. The fact that IL-4-expressing macrophages reduce injury to a greater extent than Ad-βgal-transfected macrophages despite similar numbers of cells localizing to glomeruli indicates that IL-4 expression is essential for the effect. Thus, the delivery of IL-4-expressing macrophages to the left kidney caused a marked reduction in albuminuria, but only for the time that IL-4-expressing macrophages were resident in nephritic glomeruli.

Effect of Ad-IL4-transfected macrophages on the total number of glomerular macrophages in NTN

IL-4-expressing macrophages had only been injected into the left kidney, and labeled cells were rarely detected in the right kidney. Yet albuminuria was reduced by 80%, which suggests that the treatment also affected injury in the contralateral kidney. NTN is a macrophage-dependent form of glomerular inflammation (1); therefore, we compared the number of macrophages infiltrating the right and left kidneys of nephritic rats in whom IL-4-expressing NR8383 cells were injected into the left kidney. Injection of IL-4-expressing macrophages markedly reduced the total number of ED1-positive macrophages within glomeruli from the left kidney on day 1 compared with both unmodified NTN and NTN injected with Ad-βgal-expressing cells (Fig. 5). The effect was seen even without subtracting the Ad-IL4-transfected NR8383 cells that are ED1 positive from the total number of glomerular macrophages. Macrophage numbers were still reduced on day 4 compared with NTN disease controls, but were not significantly different from those of nephritic rats injected with Ad-βgal-transfected macrophages, a time point at which the level of IL4 had been markedly reduced. By day 7 there was no difference in glomerular ED1-positive macrophage numbers between any of the groups (data not shown); however, on day 7 there were differences in the light microscopic appearances. In control rats there was evidence of increased glomerular endothelial cell swelling and proliferation (control, 1.8 ± 0.3; IL-4 treated, 0.7 ± 0.2, scoring out of 3), mesangial cell proliferation (control, 2.5 ± 0.2; IL-4, 0.7 ± 0.3, scoring out of 3), and necrosis (control, 1.1 ± 0.2; IL-4, 0, scoring out of 2) compared with IL-4-treated rats, with all these differences significant (p < 0.01). The most important finding was that the number of ED1-positive macrophages was also reduced in the non-injected right kidney. Thus, injection of IL-4-expressing macrophages into one kidney can reduce inflammation at a distant focus. It is unlikely that this effect could be due to IL-4-expressing macrophages in the contralateral kidney, because only small numbers appeared there (<5% of glomeruli contained cells, with only one or two cells seen in these glomeruli).

Renal artery injection of IL-4-expressing macrophages produces a marked reduction in glomerular inflammation

IL-4 could not be detected in the circulation of rats after injection of Ad-IL4 NR8383 cells into the renal artery, but leakage of IL-4 into the circulation still provides the simplest explanation for the reduced injury in the contralateral kidney, because systemic injection of IL-4 reduces injury in this model of glomerulonephritis (14). This possibility was examined by comparing the results after injecting IL-4-expressing NR8383 cells into different sites. Nephritic rats were injected with 2–4 × 10⁶ Ad-IL4-transfected macrophages via the tail vein (i.v.), the left renal artery, or the peritoneal space (i.p.). Intra-peritoneal injection of IL-4-expressing macrophages had no effect on albuminuria, while injection into the tail vein caused a very transient reduction despite localization of small numbers of labeled macrophages to both glomeruli (5–10%...
of glomeruli contained transfected cells, with one or two cells per glomerulus). By contrast, injection into the left kidney again reduced albuminuria significantly (Fig. 6A) as well as glomerular macrophage infiltration into both kidneys compared with all control groups (Fig. 6B). This demonstrates that the impact of IL-4-expressing macrophages on injury is most profound when they localize to the focus of inflammation, and this effect cannot be mimicked by systemic delivery of macrophages.

**Discussion**

Macrophages are key cells in the evolution of the inflammatory response, and we have previously shown that they can be transfected using recombinant adenovirus and localize to inflamed glomeruli (10). We have now extended this approach to genetically modify macrophages to express high levels of IL-4 and have shown that these cells localize with increased efficiency to inflamed glomeruli and reduce the degree of renal glomerular injury in rats with nephrotoxic nephritis. Most importantly, injection of such macrophages into the left renal artery also reduces injury in the right kidney, whereas systemically injected IL-4-transfected macrophages have no effect.

Our data highlight the fact that macrophage transfection provides an effective method to deliver genes to and express biologically active molecules in glomeruli, a target that has proved difficult using conventional viral and nonviral methods (22). The Sendai virus method (23) has been used to transfect and express TGF-β1 in approximately 50% of glomeruli (24), resulting in glomerulosclerosis. Recently the same approach has been used to express 15-lipoxygenase in glomeruli of rats with NTN, resulting in a reduction in albuminuria but no effect on macrophage infiltration (25). Cell-based systems have proven more successful to deliver genes to glomeruli using either mesangial cells (26) or macrophages (10, 27, 28), with macrophages of particular value in the modification of inflammatory disease (10, 29) due to their ability to infiltrate a focus of injury. Injection of adenovirus-transfected macrophages provides a highly effective method for glomerular gene transfer, with nearly 100% of glomeruli containing transfected macrophages. Adenoviruses have a number of advantages over retroviral transformation of macrophages, including higher levels of expression due to transfection with more than one copy of cDNA, transfection of primary cultures of macrophages (10), and a single-step transfection without need for selection before injection. In addition by transfecting macrophages to express anti-inflammatory cytokines such as IL-4, it prevents macrophages...
Macrophages are important in the development of many forms of inflammation including nephrotic nephritis (1, 30) and infiltrate glomeruli within 24 h of the onset of disease. In our experiments large numbers of NR8383 macrophages localize to inflamed glomeruli when injected 6 h after the induction of nephritis. This property was enhanced in IL-4-transfected macrophages, with 3–4 times as many cells localized compared with either Ad-β-gal-transfected macrophages or nontransfected macrophages (data not shown). The reasons for enhanced localization of IL-4-expressing macrophages is unclear, but was specific to inflamed glomeruli as similar numbers of Ad-IL4- and Ad-β-gal-transfected NR8383 macrophages localized to nondiseased glomeruli. Similarly, IL-4 transfection did not change macrophage morphology or adhesiveness in vitro. This suggests that increased adhesiveness is mediated specifically by up-regulation (or activation) of receptors involved in binding to glomerular endothelium during the course of NTN.

Monocyte infiltration has been extensively studied and shown to be dependent on selectins, VCAM, and ICAM (31); however, in the glomerulus different molecules appear to be involved. Selectins are poorly expressed and do not play an important role in glomerular leukocyte recruitment (32, 33). ICAM-1 is up-regulated on glomerular endothelium in NTN; however, the evidence for its involvement in localization of macrophages to inflamed glomeruli is contradictory depending on the species of rat used (34, 35). IL-4 has been well characterized to increase endothelial expression of VCAM-1 and subsequent monocyte adhesion (36, 37); however, there is little evidence that VCAM is expressed on glomerular endothelium during NTN or that it is involved in macrophage adhesion in NTN (34, 38). In addition there is no evidence that IL-4 alters the expression of or activates integrins including CD18/CD11 (counter-receptor to ICAM1 and ICAM2) or very late Ag-4 (counter-receptor to VCAM) or alters the expression of selectins. Thus, the standard integrin adhesion molecules are unlikely to be responsible for enhanced localization of IL-4-expressing macrophages.

Recent evidence has implicated both fractalkine (39) and Gro-α (40) in the localization of macrophages in NTN. IL-4 has been shown to increase the expression of chemokine receptors CXCR1 and CXCR2, which are counter-receptors to IL-8 and Gro-α, thus making monocyte/macrophages responsive to IL-8 (40). In addition we have recently demonstrated that blockade of Gro-α receptor on the macrophage surface reduces their ability to localize to inflamed glomeruli with NTN within 24 h of the onset of disease (4). Thus, IL-4 transfection gives macrophages a unique ability to localize to inflamed glomeruli, and altered expression of chemoadhesive receptors is the prime candidate for mediating this effect.

Once within the inflamed glomerulus, the transfected NR8383 macrophages produce large quantities of the cytokine, as detected both in urine and in isolated glomeruli containing transfected macrophages. The transfected macrophages remain within the glomerulus with a half-life of approximately 2 days for both the number of cells present and the production of IL-4. The fate of the transfected macrophages is unclear; they may die within the glomerulus, migrate to regional lymph nodes (41, 42), or reverse migrate across the endothelium (43). Clearly migration to local lymph nodes may be crucial in altering the course of the disease and in particular in affecting inflammation in the contralateral kidney.

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**FIGURE 6.** A. Albuminuria following delivery of IL-4-expressing macrophages i.v., i.p., or into the renal artery. Injection of IL-4-expressing macrophages into the renal artery produces marked reduction of albuminuria compared with both i.v. and i.p. delivery of the same cells and compared with renal artery injection of Ad-β-gal-transfected macrophages and unmodified disease (n = 4; results are the mean ± SD); *, p < 0.05 compared with all groups except the i.v. IL4 group (NS); #, p < 0.01 compared with all groups. B. Intra-arterial delivery of IL-4-expressing macrophages reduces ED1-positive macrophages within glomeruli. Compared intra-arterial delivery of Ad-β-gal-transfected macrophages (IA b-gal D2), i.v. delivery of IL-4-transfected macrophages (IV IL4 D2), and unmodified NTN (control NTN D2), renal artery injection of IL-4-expressing macrophages produces a significant reduction in the number of ED1-positive cells within the glomerulus (n = 4; mean ± SD). +, p < 0.05 compared with all groups.

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The NR8383 macrophages are from Sprague Dawley rats, and the experiments have used an outbred strain of Sprague Dawley rats, which raises the possibility that an alloreactive may be involved. This was made unlikely by the short time course we studied (<7 days) before an alloreaction developed and was confirmed by showing the absence of proliferation of splenocytes isolated from rats immunized for 7 days with either Ad-βgal- or Ad-IL4-transfected NR8383 cells on re-exposure to adenovirus-transfected NR8383 cells.

The injection of IL-4-expressing macrophages into a single renal artery has a profound effect on the severity of injury, with a 75% reduction in the level of albuminuria, which is a sensitive indicator of glomerular damage. In addition there was a marked reduction in the number of ED1-positive macrophages. ED1 is pan-macrophage marker, and at this stage of disease they are the principal inflammatory cell and thus represent a sensitive marker of glomerular inflammation before more severe histological injury. Interestingly on day 7 there were histological differences between control and IL-4-treated rats with reduced necrosis and capillary and mesangial cell proliferation. Thus, reduction of macrophage infiltration early on prevents subsequent histological changes. This effect is dependent on the production of IL-4 by the transfected macrophages, as injection of Ad-βgal-transfected macrophages did not have the same impact. IL-4 is capable of reducing the expression of a number of proinflammatory cytokines, particularly in macrophages, including TNF, IL-1β, IFN-γ, and IL-12, which have been implicated in glomerular inflammation (44–46), and increasing expression of antagonists to proinflammatory cytokines such as IL-1R antagonist and IL-1 decoy receptor (reviewed in Ref. 47). Intraperitoneal injection of high doses of IL-4 in this model from before the onset of disease caused a less pronounced reduction in both albuminuria and numbers of ED1-positive macrophages. Thus, IL-4 expression by transfected macrophages is more effective at modifying inflammation than repeated systemic injections.

Gene delivery using IL-4 has been shown previously to alter inflammatory disease. In collagen-induced arthritis in mice adenosine transfection with IL-4 into the joint appeared to enhance syновial inflammation, but reduce cartilage destruction, with suppression of TNF, IL-1β, IL-12, and IL-17 expression (48) and inhibition of osteoclast formation (49). While in a model of inflammatory bowel disease i.p. injections of adenovirus-expressing IL-4 reduced tissue damage, with an increase in systemic IL-4 rather than clear local expression (50). Also delivery of retrovirus-transduced T cells expressing IL-4 reduces the severity of inflammation in EAE in mice (51). Our results further demonstrate the immune modulation achieved by local IL-4 delivery and its anti-inflammatory potential. Ad-βgal-transfected macrophages also resulted in a small reduction in albuminuria as noted in our previous work (10). The most likely mechanisms for this are blockade of entry of endogenous macrophages by NR8383 cells occupying endothelial adhesion molecules or transfected macrophages developing anti-inflammatory properties.

The most surprising observation was that injection of IL-4-expressing NR8383 macrophages into a single kidney reduced the number of infiltrating ED1-positive macrophages in both the injected and noninjected glomeruli. This implies that the presence of disease-modifying macrophages in one inflammatory site can alter inflammation at a distant site. The most obvious explanations are leakage of cells or leakage of cytokine; however, our control experiments effectively exclude both these possibilities. Very few cells leak across to the contralateral, although theoretically these small numbers could be effective. This is made unlikely by the i.v. experiments in which small numbers of IL-4-expressing macrophages were found in both glomeruli and resulted in no sustained reduction in albuminuria or reduction in ED1-positive cells. IL-4 was not detected in the circulation after intra-arterial injection of IL-4-expressing NR8383 macrophages. Similarly, IL-4 was not detected after injection of comparable numbers of NR8383 macrophages either i.p. or i.v., and delivery of cells by either route did not affect glomerular inflammation. Thus, large numbers of IL-4-expressing macrophages in inflamed glomeruli are necessary to reduce inflammation in the contralateral glomeruli. There is a precedent for this from transfection of rabbit knee joints with experimental arthritis. Here transfection with adenovirus-expressing soluble IL-1 and TNF receptors reduced inflammation in both the transfected and nontransfected contralateral kidney (52), with similarly no evidence of systemic leakage. The most probable explanation is trafficking to regional lymph nodes of either the transfected macrophages themselves (41) or other mononuclear cells that have been exposed to IL-4 in the glomeruli and had their phenotype altered (11). Technically these possibilities are difficult to resolve with tracking of transfected and/or infiltrating macrophages not yet possible.

These observations have profound implications, as they suggest that local delivery of IL-4-expressing macrophages could reduce similar inflammation at a distant site. In addition these experiments establish the precedent of macrophage gene therapy for inflammatory disease.

References


