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The role of tubulointerstitial inflammation

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The role of tubulointerstitial inflammation.

Background. Exploration of the role of tubulointerstitial inflammation in experimental chronic renal disease (CRD) is an essential step to understanding and finding new treatments for human CRD. Adriamycin nephrosis (AN) is an experimental analogue of human focal glomerular sclerosis and tubulointerstitial inflammation.

Methods. Using murine and rat AN, we have systematically investigated the pathogenic roles of chemokines, costimulatory molecules, and inflammatory cells, such as macrophages and effector and regulatory T lymphocytes. The profile of humoral and cellular mediators was studied in vitro and in vivo. The pathogenic significance of various factors was investigated by DNA vaccination, leukocyte reconstitution and depletion, retroviral transduction, and blockade with monoclonal antibodies.

Results. Renal cortical and tubular cell CC-chemokines, including MCP-1, RANTES, and MIP-1α, were up-regulated via mediation of NFκB, and contributed to disease by attracting inflammatory cells into the interstitium. The role of these chemokines was confirmed by DNA vaccination. CD40-CD40L costimulation signals were involved in expansion and activation of the inflammatory infiltrate, whereas PD-1 signals were inhibitory, and CD28-B7 appeared to have a neutral effect. Macrophage and CD8+ T cells were shown to be effectors of injury, whereas CD4+CD25+ and γδ T cells acted as regulatory cells. FoxP3 transduction was able to convert naïve T cells to CD4+CD25+ regulatory T cells.

Conclusion. There is a broad range of humoral and cellular factors involved in the pathogenesis of experimental CRD, some of which are potential targets for treatment of human CRD.

In virtually all forms of progressive experimental and human chronic renal disease (CRD) there is a prominent inflammatory infiltrate within the interstitial compartment. The extent of the infiltrate and associated areas of fibrosis correlates with progressive decline in renal function. Inflammatory cell populations within this infiltrate are dynamic, with temporal and spatial variations of pathogenic significance. The superficially bland appearance of the infiltrate belies a complex collage of inflammatory cell subtypes (e.g., macrophages, effector and regulatory T lymphocytes, B lymphocytes, dendritic cells, NK cells), each in varying degrees of activation. Basic research over the past decade or so has provided many new insights into the mechanisms underlying the initiation, development, and even demise of the inflammatory infiltrate, and how it damages renal parenchyma and causes progressive loss of function. The implication in these processes of a broad range of specific effector molecules has exposed a number of potential targets for novel strategies to slow disease progression.

Ours is but one of many laboratories worldwide studying basic aspects of tubulointerstitial injury of progressive CRD. This article will describe recent observations from our laboratory, in particular focusing on observations of potential therapeutic promise. Rodent adriamycin nephropathy (AN) was used as the model of CRD in many of these studies, which have investigated the pathogenic roles of chemokines, costimulation pathways, and infiltrating inflammatory cells, including macrophages, and effector and regulatory T lymphocytes.

CHEMOKINES

Chemokines play a central role in CRD by attracting mononuclear inflammatory cells to the interstitium, and by modulating interactions between resident and inflammatory cells [1]. Increased expression of chemokines by tubular epithelial cells, as well as interstitial fibroblasts, has been demonstrated in both human and animal inflamed kidneys [2–5]. The pathogenicity of chemokines and their receptors in various renal diseases has been demonstrated by their blockade with specific antibodies or receptor antagonists [6, 7].

Our laboratory demonstrated that CC-chemokines, MCP-1, RANTES, and MIP-1α were up-regulated in renal cortex of rats with AN [3]. Transcription of those chemokines increased one week after adriamycin (ADR) administration and peaked at week 2. RANTES and MIP-1α then returned to control levels, whereas MCP-1 was sustained at high levels until at least week 4. In

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Table 1. Examples of pro- and anti-inflammatory molecules in adriamycin nephrosis

	Proinflammatory	Anti-inflammatory
Chemokines/ cytokines	MCP-1, RANTES, MIP-1α TNF-α, IL-1, PAF, HGF	IL-10, TGF-β
Costimulatory	CD40-CD40L	PD-1
Regulatory	NFκB, ?AP-1	FoxP3

Table 2. Inflammatory cells in adriamycin nephrosis

Effector	Neutral	Protective
Macrophage	NK cell	CD4+CD25+ T cell
CD8+ T cell	B cell	CD4+ -foxP3 T cell
		γδ Tcell
		? Macrophage

vitro studies revealed that the MCP-1 expression in rat proximal tubular epithelial cells (PTC) was induced by lipopolysaccharide (LPS) and by albumin and transferrin [2]. The induction of MCP-1 transcription was found to be mediated through activation of NFκB. Inhibition of NFκB with the antioxidant pyrrolidine dithiocarbamate reduced LPS-induced MCP-1 and MIP-1α transcription in PTC in vitro [8], and reduced cortical tubulointerstitial injury in proteinuric rats in vivo [9]. In contrast, the antioxidants N-acetylcysteine and quercetin reduced NFκB activation in vitro [8], but failed to do so in vivo and were not protective in rat AN [3, 10].

To establish the importance of CC-chemokines, particularly MCP-1, RANTES, and MIP-1α, to the progression of CRD, a therapeutic strategy targeting those chemokines using DNA vaccination was developed and tested. Advantages of DNA vaccination include that it is not limited by host production of antibodies (Abs) to therapeutic Abs, and that multiple chemokines and their receptors may be targeted. DNA vaccines were made by cloning reverse transcription-polymerase chain reaction (RT-PCR) products of rat MCP-1, RANTES, and MIP-1α into a pTarget vector [11]. Modification of rat MCP-1 sequence, a maneuver which has been purported to increase host immune response in other systems, was achieved by replacing a surface loop region of MCP-1 sequence with P30 tetanus toxoid helper epitope using primer extension PCR.

Rats were vaccinated by 4 weekly injections into the tibialis anterior muscle prior to administration of ADR. DNA vaccine against MCP-1 and RANTES in combination significantly reduced proteinuria and increased creatinine clearance compared with control groups. By morphometric analysis, the severity of glomerular sclerosis was halved, and interstitial infiltration with CD4+, CD8+, and CD25+ cells and macrophages reduced to one third to one tenth by DNA vaccination. Anti-MCP1 and RANTES autoantibodies were detected at higher levels in chemokine DNA vaccinated rats than in control rats, suggesting that this may be the protective mechanism [11].

The modified MCP-1 DNA vaccine has recently been tested in rat AN in our laboratory. Vaccination with modified MCP-1 protected creatinine clearance and reduced glomerular sclerosis and interstitial inflammation, whereas vaccination with unmodified MCP-1 alone was not protective. Modified MCP-1 vaccination significantly reduced interstitial infiltration of macrophages, which are believed to be effector cells in AN. Surprisingly, in vaccinated rats, not only was the titer of anti-MCP-1 antibody significantly increased, but also that of anti-MIP-1α. At present, it is unclear whether this is due to a cross-reaction of the antibody or another mechanism, such as epitope spread resulting from the modification. Thus, DNA vaccination with more than one chemokine simultaneously, or with modified chemokine DNA, protected renal function and structure.

These experiments have provided clear evidence for the importance of CC-chemokines in progression of tubulointerstitial inflammation in AN (Table 1). Antioxidants inhibiting NFκB-dependent chemokine production, or DNA vaccination with these chemokines are but two of several strategies for therapeutic targeting of chemokines in CRD.

COSTIMULATION

Costimulation refers to signals independent of the antigen receptor that are required for full activation of lymphocyte [12]. This represents a bidirectional communication between the antigen presenting cell (APC) and the lymphocyte, which could result in activation or inhibition of the immune response. Costimulatory molecules exist as pairs, with a receptor on T cell and a ligand on APC. In CRD, PTC may serve as "nonprofessional" APC, whose communication with infiltrating lymphocytes may play a role in disease progression. There are two classes of costimulatory receptors on T cells based on sequence homologies: the Ig family including CD28, PD-1, CTLA-4, and ICOS, with their ligands from the B7 family; and the TNF receptor family, including CD40, OX40, 4-1BB, and CD30, with their respective ligands. We have investigated the roles of some costimulatory molecules in murine AN [13].

Ligation of CD40 with its ligand (CD40L) is one of many proinflammatory signals involved in the initiation and maintenance of tubulointerstitial inflammation. Blockade of CD40-CD40L with the monoclonal anti-CD40L antibody MR1 was found in our laboratory to reduce severity of renal injury in the murine AN [13]. CD40 was weakly expressed in tubules of normal mice, but was increasingly expressed by tubules, interstitium, and glomeruli as AN progressed. MR1 was administered

at days 5, 7, 9, and 11 after ADR, and resulted in a significant attenuation of the glomerular and tubular injury seen at day 42. In addition, proteinuria was reduced and creatinine clearance improved by MR1. There was a significant decrease in the number of cortical macrophages at both 14 and 42 days after ADR, and cortical expression of MCP-1 and RANTES was significantly reduced at day 42. The role of CD40-CD40L in AN was also investigated in mice genetically deficient in CD40. CD40 knockout mice showed milder disease in terms of proteinuria, interstitial expansion, and glomerular injury at day 28 after ADR than wild-type mice [14]. Together, these studies of CD40-CD40L provide a strong evidence for its role in the pathogenesis of AN, and suggest a potential therapeutic target.

Another study of CTLA4-Fc fusion protein, which prevents the interaction of CD28 and B7, excluded involvement of CD28-B7 costimulation in AN (unpublished data), but further confirmation is needed by examining the progression of AN using mice genetically deficient in either CD28 or B7.

PD-1 is expressed on a subset of thymocytes, and can be up-regulated on T, B, and myeloid cells upon activation. PD-L1 (B7-H1) and PD-L2 (B7-DC) have been identified as ligands for PD-1, which is believed to be involved in the delivery of inhibitory signals upon engagement of its ligands in later phases of immune response [15].

In a preliminary study of PD-1 expression in murine AN, we found a progressive temporal increase in renal cortical PD-1 mRNA at days 3, 7, 14, and 28. Intensity of renal cortical PD-1 mRNA expression correlated with severity of injury, and minimal PD-1 mRNA expression was found in normal kidney at 4 weeks. Immunohistochemistry showed strong PD-1 staining in the interstitium of AN renal cortex, which was absent in normal controls. The anti-PD-1 monoclonal antibody J43, when administrated on alternate days until day 14, worsened glomerular and tubulointerstitial injury and creatinine clearance at week 4 (Table 1). Anti-PD-1 treatment did not alter the number of infiltrating CD4+ or CD8+T cells, but the number of interstitial macrophages was remarkably increased (unpublished data). When anti-PD-1 antibody was administered only to day 10, it had no effect on the disease course, consistent with PD-1's known activity late in the immune response. If confirmed, these preliminary data emphasize the importance of timing for interactions aimed at boosting PD-1 expression. As further confirmation of the importance of PD-1, PD-L1.Ig will be investigated as a potential treatment for CRD.

EFFECTOR CELLS

The interstitial infiltrate of AN and other forms of CRD consists of a number of different effector cells, including macrophages, CD4+, and CD8+ T cells (Table 2).

Macrophages have long been suspected to contribute to loss of renal function and renal fibrosis in CRD [16]. Specifically, tubulointerstitial macrophage accumulation in CRD correlates with the severity of glomerular and interstitial lesions and the degree of renal dysfunction. Macrophages can mediate tissue injury via release of proteolytic enzymes, reactive oxygen species, vasoactive mediators, and cytokines. Macrophages also contribute to fibrosis by producing fibrogenic growth factors [17]. Nevertheless, much remains to be learned about macrophages in tubulointerstitial injury.

We have examined the role of interstitial macrophages in AN [18]. SCID mice, which lack functional T and B cells, can develop AN of a severity similar to that of immunocompetent mice. Moreover, the number of interstitial macrophages was decreased along with CD8+ cells in immunocompetent mice with AN treated with anti-CD8 [19]. These two observations suggested the macrophage as a cause of interstitial damage in AN. Therefore, macrophage depletion was investigated in AN using a monoclonal antibody ED7 directed against CD11b/CD18 integrin, which is expressed by macrophages. Circulating ED7 positive cells were reduced by approximately one third, and renal cortical macrophages (ED1 positive cells) by almost 50%, whether ED7 was administrated before or after ADR administration. Even so, ED7 reduced renal structural and functional injury only when treatment was commenced prior to ADR administration (unpublished observation). Among several possible explanations for these observations is a temporal change in the predominant macrophage phenotype. If pathogenetic macrophages predominated early and 'protective' macrophages later in the disease course, then only early antimacrophage treatment would be expected to protect against progression.

Recent observations from other investigators support the importance of macrophage phenotype. For example, in mice with unilateral ureteric obstruction reconstituted with marrow of angiotensin II type 1 receptor gene knockout or wild-type mice, infiltrating macrophages were shown to play a beneficial antifibrotic role [20]. Other studies have demonstrated marked macrophage heterogeneity and context specificity, depending on the nature of the injury and location within the kidney [17]. Further studies on possible temporal variations in the phenotype, activation status, and net effect on injury of macrophages should give a better understanding of the complex role of macrophages in CRD.

CD8+ T cells are regarded as the predominant effector cells in a number of forms of renal injury, including nephrotoxic serum nephritis [21], antitubular basement membrane disease [22] and Heymann nephritis [23]. The role of CD8+ cells in murine AN was examined in our laboratory [19]. Anti-CD8+ monoclonal antibody was administered from day 5 after ADR, when overt

proteinuria was established, and mice were sacrificed at week 6. Splenic CD8+ cell levels were reduced to <2% of normal. Anti-CD8+ treatment provided marked protection against structural and functional injury in AN. As mentioned above, interstitial macrophage numbers were also reduced, and so it was not possible to dissect the relative importance of CD8+ T cells and macrophages to disease progression in these particular experiments. Also noteworthy was an apparent, though not statistically significant, increase in the number of interstitial CD4+ cells, which will be discussed further below. Reconstitution of SCID mice with non-CD4+ T cells (predominantly CD8+ cells) exacerbated AN, consistent with an effector role of CD8+ cells.

Given the fact that anti-CD8+ treatment may also reduce numbers of another effector cell, the *NK cell*, its possible role was also investigated. There was no difference in renal injury in AN following administration of anti-asialo GM1 antibody which depleted both peripheral and renal NK cells. This occurred even though the stimulatory NK cell receptor, NKG2D, and its ligand RAE1, were found to be up-regulated in AN. It is possible that the NK activation signal was overwhelmed by an inhibitory signal of MHC class I molecule that was found constitutively expressed in kidney of both normal and AN mice. Supporting the results of NK depletion by antibody, AN in NOD-SCID mice (which lack NK, T, and B cells) showed a similar severity in renal injury to that of SCID mice (which lack T and B cells) [24]. Our findings excluded NK cells from a significant involvement in the tubulointerstitial inflammation of AN (Table 2).

REGULATORY T CELLS

CD4+ T cells constitute a critical component of the adaptive immune system, and are typified by their capacity to help both humoral and cell-mediated responses. However, there is substantial functional diversity among CD4+ T cells, and it is obvious that certain subpopulations hinder rather than help immune responses. The most well characterized example of an inhibitory subpopulation is the CD4+CD25+ cell, which appears to play an active role in down-regulating pathogenic autoimmune responses [25]. CD4+CD25+ T cells are potent immunoregulatory cells that not only suppress T-cell proliferation in vitro, but also have the capacity to suppress immune responses to auto- and alloantigens, tumor antigens, and infectious agents in vivo [26].

We have shown previously that depletion of CD4+ T cells in established AN aggravated glomerular and interstitial injury [27], suggesting their protective role against progression of disease. This was further confirmed by reconstituting SCID mice with CD4+ T cells, which protected against AN. To determine whether the protective effect of CD4+ T cells in AN was due to the subset of CD4+CD25+ T cells, which comprise 5% to 10% of the total peripheral CD4+ T cell population in normal adult mice, we reconstituted SCID mice with CD4+CD25+ T cells after induction of AN. Mice reconstituted with CD4+CD25+ T cells had significantly reduced glomerulosclerosis, tubular injury, and interstitial expansion compared to unreconstituted mice with AN (Table 2). Urine protein levels and serum creatinine were significantly lower, and creatinine clearance significantly higher in reconstituted mice. This protective effect of CD4+CD25+ T cells in murine AN raises the possibility that enhancement of CD4+CD25+ regulatory cell activity may be a useful maneuver for slowing progression of CRD.

γδ T cells are an ancient lineage of T cells that play important roles in antimicrobial immunity, as well as in chronic inflammatory processes [28]. γδ T cells were found to be expanded in kidneys of rats with Heymann nephritis (HN) and AN, and to express a restricted set of Vγ6/Vδ1 TCR genes. High levels of regulatory cytokines, including TGF-β, IL-4, and IL-5, and low levels of IL-2 were expressed by the infiltrating γδ T cells in HN [29], but only high levels of TGF-β in AN. The γδ T cells from both AN and HN kidneys expressed NKG2D. Depletion of γδ T cells by mAb in vivo worsened AN in terms of serum creatinine, glomerulosclerosis, and interstitial inflammation (unpublished data). These results suggest that in these models γδ T cells respond to tissue injury through NKG2D and produce a regulatory response (Table 2).

Recent studies have indicated that the forkhead/winged helix transcription factor gene (Foxp3) is specifically expressed by regulatory T (T_R) cells, and programs their development and function [30, 31]. Currently, we are developing T_R cells by retroviral gene transfer of Foxp3 to convert naïve T cells toward to a regulatory T-cell phenotype for use in CRD. Retroviral vectors expressing Foxp3 and green fluorescent protein (GFP) were transfected into and produced by package cell lines, and then transduced into mouse and rat CD4+ T cells, with an efficiency of 30%. One week after infection, GFP-positive cells, sorted by flow cytometry, were CD25+ with enhanced expression of Foxp3, TGF-beta, and CTLA-4, each a molecule implicated in the regulatory phenotype. Effective generation of rodent T_R from naïve T cells in vitro and in vivo though retroviral Foxp3 transduction brings the prospect of a novel approach for treating CRD.

CONCLUSION

These and other studies suggest that in CRD, proteinuria, and other stimuli may cause up-regulation of CC-chemokines, particularly MCP-1, RANTES, and MIP-1α, by the tubular epithelial cells. The up-regulation of CC-chemokines is mediated particularly through the transcription factor NFκB. In concert with chemokine

secretion, costimulatory molecules such as CD40 are also presented by tubules, interstitium, and glomeruli of AN mice. Blockade of CD40-CD40L by mAb and gene knockout proved that the CD40-CD40L pathway contributes to interstitial inflammation in AN, while the CD28-B7 pathway appeared not to be involved. In contrast, blockade of PD-1 by mAb worsened disease when continued until at least 2 weeks after disease induction, demonstrating that it provides an inhibitory signal in AN. Macrophages and CD8+ T cells were identified as effector leukocytes in AN, while NK cells were excluded from playing any important role. As a whole, the CD4+ T cell population was shown to be protective in AN, a property which resides, at least in part, in the CD4+CD25+ subpopulation. The regulatory role of γδ T cells was demonstrated in rat Heymann nephritis and AN. These observations have revealed a wide range of pathogenic factors involved in tubulointerstitial inflammation, and identified potential targets for novel therapeutic strategies. Unmodified DNA vaccination with multiple chemokines (e.g., MCP-1 and RANTES) or modified DNA vaccination with a single chemokine (e.g., MCP-1) has demonstrated promise as a therapeutic strategy. Another novel approach involving transduction of CD4+ T cells with FoxP3 to develop a functional regulatory phenotype in vitro is under investigation, and could be developed into therapeutic strategy for treating CRD.

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