Stimulation of the PD-1/PDL-1 T-cell co-inhibitory pathway is effective in treatment of experimental autoimmune glomerulonephritis

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Abstract
Background. Experimental autoimmune glomerulonephritis (EAG) can be induced in Wistar–Kyoto (WKY) rats by immunization with the recombinant NC1 domain of the α3 chain of type IV collagen [α3(IV)NC1]. EAG is characterized by circulating and deposited anti-α3(IV)NC1 antibodies, accompanied by focal necrotizing glomerulonephritis with crescent formation. Programmed death-1 (PD-1) receptor is preferentially expressed on activated T cells and binds two known ligands present on antigen presenting cells, PDL-1 and PDL-2. Engagement of PD-1 by its ligands results in a negative regulatory effect, with inhibition of downstream cellular signalling events and diminished cellular proliferation.

Methods. In order to investigate the role of the PD-1/PDL-1 co-inhibitory pathway in development of EAG, the in vivo effects of a stimulating PDL-1/Fc fusion protein were examined after the onset of disease.

Results. Stimulation of PD-1 led to a significant reduction in albuminuria, serum urea, serum creatinine, crescent formation and tubular damage compared with controls. There was also a reduction in numbers of glomerular macrophages, CD4+ T cells, CD8+ T cells and PD1+ cells compared with controls. No reduction was observed in levels of circulating or deposited antibodies.

Conclusions. These results demonstrate that PDL-1/Fc fusion protein is effective in treatment of glomerulonephritis and confirm the importance of the PD-1/PDL-1 T-cell co-inhibitory pathway in development of EAG. Strategies designed to stimulate this pathway may provide a novel approach to treatment of human glomerulonephritis.

Keywords: alpha 3 chain of type IV collagen; co-stimulatory molecules; experimental autoimmune glomerulonephritis; glomerular basement membrane; WKY rat

Introduction
Glomerulonephritis is one of the commonest causes of kidney failure worldwide and is responsible for ~20% of patients requiring dialysis or transplantation. The exact cause of most types of glomerulonephritis is not known, but immunological mechanisms are clearly involved. Goodpasture’s or anti-glomerular basement membrane (anti-GBM) disease is an autoimmune disorder, which presents with rapidly progressive glomerulonephritis with or without lung haemorrhage [1, 2]. Although uncommon, this condition has been extensively studied and is one of the few human autoimmune diseases in which the autoantigen, the non-collagenous domain of the α3 chain of type IV collagen [α3(IV)NC1], has been well characterized [3, 4]. In order to investigate mechanisms of autoimmunity and inflammation in glomerulonephritis and to test new therapeutic strategies in vivo, an animal model of Goodpasture’s disease known as experimental autoimmune
glomerulonephritis (EAG) has been developed in susceptible strains of rats and mice by immunization with preparations of glomerular basement membrane (GBM) [5–7] or recombinant 3(IV)NC1 [8–11]. The disease is characterized by the development of circulating and deposited anti-GBM antibodies, with focal necrotizing crescentic glomerulonephritis and variable lung haemorrhage. EAG in the rat shares many features in common with the human disease, in that the renal pathology is very similar and that the autoimmune response involves anti-GBM antibodies with the same specificity for the main target antigen, 3(IV)NC1 [12].

As in Goodpasture’s disease, there is compelling evidence for the role of both humoral and cell-mediated immunity in the pathogenesis of EAG. The pathogenicity of anti-GBM antibodies has been confirmed in several passive transfer studies. Transfer of EAG has been demonstrated using antibodies pooled from the serum of nephritic mice [7], antibodies purified from the urine of nephritic rats [13], monoclonal antibodies derived from rats with EAG [14, 15] and antibodies eluted from the kidney of nephritic rats [16]. There is also increasing evidence to support a role for T cells in the pathogenesis of EAG. T cells are present from an early stage in the glomeruli of mice and rats with EAG [7, 12]; T cells from nephritic animals proliferate in response to 3(IV)NC1 [11, 17] and can transfer EAG to naïve recipients [7, 18]; glomerular T cells in EAG show restricted T-cell receptor (TCR) CDR3 spectratypes, suggesting an antigen-specific T-cell response [19]; an immunodominant T-cell epitope from the N-terminus of rat 3(IV)NC1 is capable of inducing severe crescentic nephritis [20–23]; mucosal tolerance can be induced by nasal administration of autoantigen [24] or immunodominant peptide [25] and anti-T-cell immunotherapy can prevent or ameliorate disease [26–28].

Antigen-specific T-cell activation is generally regulated by a two-signal pathway. The first signal is provided by engagement of the TCR with the antigen peptide–MHC molecule complex on antigen presenting cells (APC). However, this interaction alone is insufficient to induce optimal T-cell activation without secondary co-stimulatory signals, which are provided by binding of specific receptors on T cells with their ligands on APC [29, 30]. Two of the best-characterized primary co-stimulatory pathways are firstly, the interaction between CD154 (CD40 ligand) on T cells and CD40 on APC and secondly, CD28 on T cells which interacts with CD80/CD86 (B7.1/B7.2) on APC. Blockade of the CD154-CD40 co-stimulatory pathway with a monoclonal antibody directed against CD154 [27] and blockade of the CD28-CD80/CD86 co-stimulatory pathway by the fusion protein CTLA4-Ig [28] have both been shown to be effective in the prevention of EAG. However, these well-defined co-stimulatory pathways appear to be less important in the generation and maintenance of effector and/or memory T-cell functions [29, 30]. Other more recently described co-stimulatory and co-inhibitory pathways may regulate the second signals for complete T-cell activation [31, 32].

One such novel pathway involves programmed death-1 (PD-1) receptor, a member of the CD28 superfamily which is preferentially expressed on activated T cells and binds two known ligands, PDL-1 and PDL-2 [31, 32]. PDL-1 and PDL-2 are found on professional APC and certain parenchymal cells as well as on a subpopulation of T and B cells. In a similar manner to CTLA-4, a well-established inhibitor of T-cell responses, engagement of PD-1 by its ligands results in a negative regulatory effect, with inhibition of downstream cellular signalling events, diminished cellular proliferation and reduced cytokine production [29, 30]. Furthermore, PD-1 deficiency in mice (as with CTLA-4 deficiency) results in autoimmune phenomena, including splenomegaly, B-cell expansion with increased serum immunoglobulins and lupus-like glomerulonephritis and arthritis by 14 months of age [33]. Thus, after T-cell activation, PD-1 is up-regulated and provides a negative signal for T-cell responses, therefore playing an important role in the maintenance of tolerance. Recent studies have shown that blocking the PD-1/PDL-1 pathway with a monoclonal antibody directed towards PD-1 worsens disease in experimental autoimmune encephalomyelitis (EAE) in C57BL/6 mice [34] and exacerbates autoimmune diabetes in the non-obese diabetic (NOD) mouse [35], demonstrating a critical role for this pathway in the regulation of autoimmunity. It has also been reported that stimulating the PD-1 pathway with a PDL-1/Ig fusion protein can promote cardiac allograft survival and modulate T and B-cell-dependent pathogenic immune responses in vivo [36]. More recent studies have demonstrated the efficacy of PDL-1/Fc administration in a mouse model of collagen-induced arthritis [37].

In this study, we investigate the role of the PD-1/PDL-1 T-cell co-inhibitory pathway in the development of EAG by examining the effect of stimulating PD-1 with a PDL-1/Fc fusion protein. We demonstrate, for the first time, that in vivo administration of PDL-1/Fc fusion protein is effective in the treatment of autoimmune glomerulonephritis. The results from this study confirm the importance of the PD-1/PDL-1 pathway in autoimmune disease and suggest that strategies stimulating this pathway may provide a novel approach in the treatment of human glomerulonephritis.

Materials and methods

Experimental animals
Female Wistar–Kyoto (WKY) rats, aged 6–8 weeks and weighing 120–150 g, were purchased from Charles River (Margate, Kent, UK). All animals were housed in standard conditions and had free access to normal laboratory diet and water. All experimental procedures were conducted in accordance with the UK Animals (Scientific Procedures) Act.

PDL1-Fc fusion protein
A fusion protein of mouse PD-1 ligand (PDL-1), fused with the Fc portion of human IgG (PDL-1/Fc; Chimerigen Inc., Allston, MA), was used to stimulate the PD-1/PDL-1 T-cell co-inhibitory pathway.

Recombinant rat 3(IV)NC1
Recombinant rat 3(IV)NC1 was produced in human embryonic kidney (HEK) 293 cells and purified by FLAG affinity chromatography, as previously described [25]. Briefly, a complementary DNA fragment encoding rat 3(IV)NC1 was subcloned into a pEBT expression vector, and the resultant plasmid was transfected into HEK293 cells. Recombinant rat 3(IV)NC1, secreted into the HEK293 cell supernatant, was then purified on an anti-FLAG affinity column (Sigma–Aldrich Company Ltd, Poole, Dorset, UK).

Induction of EAG
EAG was induced in WKY rats by a single intramuscular injection of recombinant rat 3(IV)NC1 in an equal volume of Freund’s complete
adjuvant (FCA; Sigma–Aldrich Company Ltd), at a dose of 100 μg per rat [10, 25]. Serial blood samples were taken by tail artery puncture under light anaesthesia (Isoflurane), and 24-h urine specimens were obtained by placing animals in metabolic cages.

Assessment of EAG

Albumin excretion. Urinary albumin concentrations were measured in 24-h collections from experimental animals at Day 28 by rocket immunoelectrophoresis (Amersham Bioscience UK Ltd), as previously described [6, 12]. Briefly, urine samples from experimental animals were subjected to immunoelectrophoresis at 60 V in an electrophoresis tank containing Barbitone buffer (BDH Laboratory Supplies, Dorset, UK), pH 9.5, for 6 h, using a 1% agarose gel (BDH Laboratory Supplies) containing rabbit anti-sera to rat albumin raised in our laboratory. Results were calculated using rat serum albumin standards (which were run at the same time) and expressed in milligram per 24 hours.

Serum creatinine and urea. Levels of creatinine and urea were measured in sera of experimental animals at Day 28 on an Olympus AU2700 analyzer (Olympus Diagnostics, London, UK), as previously described [25]. Creatinine was measured by a kinetic Jaffé method (alkaline picrate) and urea was measured using an enzymatic method (urease and glutamate dehydrogenase). All samples were analysed in a single batch, to enable comparison between different groups.

Light microscopy. Kidney tissue was fixed in 10% neutral buffered formalin, processed and embedded in paraffin wax for light microscopy by standard techniques (Histopathology Department, South Kensington Campus, Imperial College London). Briefly, 3-μm sections were stained with haematoxylin and eosin and periodic acid–Schiff and then assessed and graded by a ‘blinded’ observer. Glomerular damage was assessed by counting 50 consecutive glomeruli in cross section and graded as normal, abnormal (segmental necrosis and/or extracapillary proliferation affecting <50% of glomerulus) or severe (segmental necrosis/crescent formation affecting >50% of glomerulus) and expressed as a percentage of glomeruli examined [6, 12]. Tubular damage was assessed by counting 50 consecutive quadrants in cross section through the interstitium and graded as 0, normal; 1, 1–10% affected; 2, 11–25% affected; 3, 26–50% affected or 4, >50% affected [38].

Immunohistochemistry. Kidney sections were stained for macrophages, T cells, CD4+ T cells, CD8+ T cells and PD1+ cells using a standard avidin–biotin complex immunoperoxidase staining technique. Briefly, formalin-fixed paraffin-embedded kidney sections were stained with monoclonal antibodies: ED1 for macrophages (Serotec Ltd, Kidlington, UK), W3/13 for T cells (Serotec Ltd), W3/25 for CD4+ T cells (Serotec Ltd), OX8 for CD8+ T cells (Serotec Ltd) and RMP1-14 for PD1+ cells (e-biosciences Ltd, Hatfield, UK). Numbers of glomerular macrophages, T cells, CD4+ T cells and CD8+ T cells were detected using biotinylated goat anti-mouse IgG secondary antibody (Dako Ltd, Cambridge, UK) and glomerular PD1+ cells using biotinylated goat anti-hamster IgG (e-biosciences Ltd), followed by an avidin–biotin complex (Dako Ltd). The cellular infiltrate of each cell type was assessed by a blinded observer by counting the number of positively stained cells per 50 consecutive glomeruli in cross section [12, 25].

Direct immunofluorescence. Deposits of IgG within the glomeruli were detected by direct immunofluorescence (IF), as previously described [6, 12]. Kidney tissue was embedded in OCT II embedding medium (Miles Inc., Elkhart, IN) on cork discs, snap frozen in isopentane (BDH Laboratory Supplies) pre-cooled in liquid nitrogen and stored at −80°C. Cryostat sections were cut 5 μm thick and were incubated with fluorescein isothiocyanate-labelled rabbit anti-rat IgG (Serotec Ltd). The degree of IgG deposition was assessed by a blinded observer, by grading the intensity of immunostaining from 0 to 3+ per 50 consecutive glomeruli in cross section.

Enzyme-linked immunoabsorbent assay (ELISA). Circulating anti-α3IV/NC1 antibody concentrations were measured in sera of experimental animals at Day 28 after immunization by a direct solid-phase enzyme-linked immunoabsorbent assay (ELISA), as previously described [10, 28]. Briefly, recombinant rat α3IV/NC1 was coated on to microtitre ELISA plates (Life Technologies, Paisley, UK) at a concentration of 5 μg/mL by overnight incubation at 4°C. Sera from experimental animals were applied at a dilution of 1/100, 1/300, 1/1000, 1/3000 and 1/10 000 for 1 h at 37°C. Bound anti-GBM antibody was detected by alkaline phosphatase-conjugated sheep anti-rat IgG (Sigma–Aldrich Company Ltd) and developed using the substrate p-nitrophenyl phosphate (Sigma–Aldrich Company Ltd). The absorbance for each well was read at 405 nm using an Anthos Multiskan ELISA plate reader (Lab Tech International, Uckfield, UK) and the results calculated as mean optical density for each triplicate sample.

Experimental protocol

Groups of animals (n = 9) immunized with recombinant rat α3IV/NC1 in FCA were given PDL-1/Fc fusion protein from Day 18 after immunization (after onset of proteinuria) at a single dose of 1 mg intravenous followed by doses of 0.4 mg intraperitoneal every 2 days until Day 28. A positive control group immunized with rat α3IV/NC1 in FCA (n = 7) and a negative control group immunized with FCA alone (n = 5) were given an equal volume of saline instead of the PDL-1/Fc fusion protein. Our previous studies in EAG showed that administration of a control fusion protein, as compared with saline, had no effect on the development of disease [28].

Statistical analysis

Differences between data were determined by non-parametric Kruskal–Wallis test followed by a Dunn post hoc test.

Results

Assessment of EAG after treatment with PDL-1/Fc fusion protein

Albuminuria. All positive control animals immunized with recombinant rat α3IV/NC1 in FCA showed an increase in the level of albuminuria at Day 28 after immunization. Animals treated with PDL-1/Fc fusion protein from Day 18 showed a significant reduction in the level of albuminuria, when compared to positive control animals. Negative control animals injected with FCA alone did not develop albuminuria. Results are shown in Figure 1a.

Serum creatinine. All positive control animals showed an increase in the level of creatinine in the sera at Day 28 after immunization. Animals treated with PDL-1/Fc fusion protein from Day 18 showed a significant reduction in the level of creatinine, when compared to positive control animals. Negative control animals injected with FCA alone did not show any increase in serum creatinine. Results are shown in Figure 1b.

Serum urea. All positive control animals showed an increase in the level of urea in the sera at Day 28 after immunization. Animals treated with PDL-1/Fc fusion protein from Day 18 showed a significant reduction in the level of urea, when compared to positive control animals. Negative control animals injected with FCA alone did not show any increase in serum urea. Results are shown in Figure 1c.

Glo merular damage. All positive control animals developed extensive segmental necrosis of the glomerular tuft with crescent formation at Day 28 after immunization. Animals treated with PDL-1/Fc showed a marked reduction in the degree of segmental necrosis and/or crescent formation. Negative control animals showed normal glomerular architecture. Results are shown in Figure 2a and illustrated in Figure 3a and b.

Tubular damage. All positive control animals developed extensive tubular damage in the interstitium and mild
interstitial inflammation, at Day 28 after immunization. Animals treated with PDL-1/Fc showed a significant reduction in the degree of tubular damage in the interstitium. Negative control animals showed normal tubular architecture. Results are shown in Figure 2b.

Glomerular cellular infiltrate. All positive control animals showed a significant increase in the number of glomerular macrophages, T cells, CD4+ T cells, CD8+ T cells and PD1+ cells. Animals treated with PDL-1/Fc showed a significant reduction in the number of glomerular macrophages, total T cells CD4+ T cells, CD8+ T cells and PD1+ cells, when compared to positive controls. Negative control animals showed no significant cellular infiltrate. Results for macrophage infiltration are shown in Figure 2c and illustrated in Figure 3c and d. Results for glomerular T-cell and PD1+ infiltration are shown in Figure 4a and d and illustrated in Figure 5.

Deposits of IgG on the GBM. Direct IF for IgG on kidney tissue at Day 28 after immunization revealed that positive control rats showed strong linear deposits of IgG along the GBM. Animals treated with PDL-1/Fc showed no significant reduction in deposits of IgG on the GBM, when compared to positive controls. Negative control animals showed no IgG deposition. Results are shown in Figure 6a.

Circulating antibody responses. All positive control animals showed a high level of circulating anti-α3(IV)NC1
**Fig. 3.** Kidney tissue at Day 28 in WKY rats with EAG showing (a) marked segmental necrosis of the glomerular tuft with crescent formation in a positive control animal; (b) marked reduction in the severity of glomerular abnormalities in an animal treated with PDL-1/Fc fusion protein; (c) large numbers of macrophages in a cellular crescent in a positive control animal and (d) marked reduction in the number of glomerular macrophages in an animal treated with PDL-1/Fc fusion protein. Magnification, ×300.

**Fig. 4.** Effect of PDL-1/Fc fusion protein in groups of WKY rats (n = 5–9) after the onset of EAG on the number of glomerular (a) T cells; (b) CD4+ T cells; (c) CD8+ T cells and (d) PD1+ cells. Results shown represent the value of each individual animal in each group at Day 28 after immunization (*P < 0.01, **P < 0.0007; ***P < 0.0002; ****P < 0.0001, positive control (POS) versus PDL-1/Fc treated).
antibodies by Day 28 after immunization. Animals treated with PDL-1/Fc showed no significant reduction in circulating antibody levels at dilutions of 1/100, 1/300, 1/1000, 1/3000 or 1/10 000, when compared to positive controls. Negative control animals did not develop circulating antibody. Results are shown in Figure 6b.

**Discussion**

Although the importance of the CD28-CD80/CD86 and CD154-CD40 T-cell co-stimulatory pathways is well established in primary T-cell activation, these molecules appear to be less important in the generation and maintenance of effector and/or memory T cells. The more recently described co-stimulatory molecules present on activated T cells may have greater effects in established immune responses [29–32]. Recent studies have shown that blocking the PD-1/PDL-1 T-cell co-inhibitory pathway with a monoclonal antibody directed toward PD-1 exacerbated the development of EAE in C57BL/6 mice [34] and accelerated the onset of diabetes in the NOD mouse [35], demonstrating that the PD-1/PDL-1 pathway plays a critical role in the regulation of autoimmunity by limiting the expansion of CD4+ and CD8+ autoreactive T cells [39, 40]. Although stimulating the PD-1 pathway with PDL-1/Ig fusion protein has been shown to promote cardiac allograft survival in mice [36] and to treat collagen-induced arthritis in mice [37], to our knowledge, no in vivo therapeutic studies using a PDL-1/Ig fusion protein have been reported in an experimental model of glomerulonephritis. We therefore examined the effect of stimulating the PD-1/PDL-1 pathway with a PDL-1/Fc fusion protein in the treatment of EAG in the WKY rat.

In this study, we were particularly interested in the effect of administering the PDL-1/Fc fusion protein after the onset of disease since this would be more clinically applicable to the treatment of human disease. We therefore administered the PDL-1/Fc fusion protein from Day 18 after immunization, at which time point, the animals had a significantly increased level of albuminuria, serum creatinine and serum urea (data not shown). We found that in vivo administration of PDL-1/Fc fusion protein resulted in a significant reduction in albuminuria and maintenance of normal renal function at Day 28, when compared to controls. A marked reduction in the number and severity of the glomerular abnormalities was observed, especially in the development of crescents. There was also a significant reduction in the severity of tubular damage in the interstitium, although interestingly there was no reduction in interstitial inflammation. However, this was very mild in the positive control group, which may account for this finding.

A significant reduction in the numbers of glomerular macrophages, total T cells, CD4+ T cells, CD8+ T cells and PD1+ cells was also observed. As described in our previous studies, a transient increase in CD4+ T cells precedes a glomerular influx of CD8+ T cells and macrophages [12]. As a result, most of the glomerular T cells at
Although there was a reduction in the severity of the deposits of IgG on the GBM, suggesting PDL-1/Fc downstream cellular signalling events, diminishing cellular proliferation and/or cytokine production [29, 30]. Thus, in vivo administration of PDL-1/Fc fusion protein has an inhibitory effect on cell-mediated immunity in EAG.

We observed that in vivo administration of PDL-1/Fc fusion protein had no effect on the level of circulating anti-α3(IV)NC1 antibodies at any dilution or at any time point during the disease. In addition, no reduction in the levels of circulating IgG1 or IgG2a anti-α3(IV)NC1 autoantibody was observed (data not shown), suggesting that stimulating PD-1 on T cells had no direct effect on the capability of Th-1 or Th-2 cells to stimulate antibody production. Likewise, no reduction was observed on the intensity of deposits of IgG on the GBM, suggesting PDL-1/Fc therapy had no apparent effect on humoral immunity, even although there was a reduction in the severity of the disease. However, this could be because both circulating and deposited antibodies are well established by Day 18 (the start of treatment) in our model of EAG, and there may have been insufficient time to see any inhibitory effect of PDL-1/Fc on humoral immunity by Day 28. We also analysed the immune response in the rat to the human Fc component of the fusion protein (data not shown). Although an elevated rat anti-human IgG response was detected, it did not seem to affect the function of the PDL-1/Fc fusion protein, in that this was still effective in treating established disease.

In conclusion, we have demonstrated for the first time that administration of a PDL-1/Fc fusion protein is capable of stimulating the PD-1/PDL-1 T-cell co-inhibitory pathway in the context of renal autoimmunity in vivo and is effective in treating disease in a well-characterized animal model of autoimmune glomerulonephritis. This observation confirms the importance of the PD-1/PDL-1 T-cell co-inhibitory pathway in autoimmune renal disease and suggests that strategies designed to stimulate this pathway may provide a novel approach to the treatment of human glomerulonephritis and other autoimmune diseases.

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