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HLA-DR15-specific inhibition attenuates autoreactivity to the Goodpasture antigen

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ABSTRACT

Goodpasture's disease manifests as rapidly progressive glomerulonephritis. Current immunosuppressive treatments do not specifically target the pathological immune response and have significant side effects. Like most autoimmune diseases, the strongest genetic association is with the HLA alleles. Inheritance of HLA-DR15 confers susceptibility, and structure-function studies have shown that HLA-DR15 plays a causative role in activating autoreactive pro-inflammatory T cells. Thus, specific inhibition of HLA-DR15 would provide a targeted therapeutic approach. We hypothesised that PV-267, an HLA-DR15-specific inhibitor, would effectively block HLA-DR15 presentation of the dominant epitope, attenuate the activation of autoreactive T cells, and limit disease. Using humanised HLA-DR15 transgenic mice, $\alpha3_{135\text{-}145}$-specific, pro-inflammatory T cell recall responses were measured using IFN-γ and IL-17A ELISPOTs and by proliferation assay. To determine if PV-267 could limit disease, experimental autoimmune anti-GBM glomerulonephritis was induced in HLA-DR15 transgenic mice (on an *Fcgr2b*$^{-/-}$ background), and functional and histological disease endpoints were measured. PV-267 effectively inhibited $\alpha3_{135\text{-}145}$-specific immune responses and disease development. Mice treated prior to immunization with $\alpha3_{135\text{-}145}$ had reduced $\alpha3_{135\text{-}145}$-specific recall responses, and limited disease by albuminuria, histological glomerular injury, IgG deposition, and inflammatory cell infiltrates. PV-267 treatment commencing after the onset of active anti-α3(IV)NC1 autoimmunity attenuated functional and histological renal injury. When treatment was administered after disease was established, PV-267 limited the severity of histological injury. In conclusion, HLA-DR15 inhibition attenuates α3(IV)NC1-specific pro-inflammatory responses and could be used as an adjunct therapy for anti-GBM disease.

1. Introduction

Anti-glomerular basement membrane (GBM) disease, also known as Goodpasture's disease, results from autoimmunity against the non-collagenous domain of the α3 chain of type IV collagen, α3(IV)NC1, present in the GBM [1]. This disease manifests as rapidly progressive glomerulonephritis, with glomerular crescent formation and linear staining of glomerular antibody deposits [2]. Patients also develop pulmonary haemorrhage. Both humoral and cell mediated effectors contribute to the disease pathogenesis, with pathogenic anti- α3(IV)NC1 antibodies and autoreactive $CD4^+$ T cells found in patients with anti-GBM disease and in experimental animal models of disease [3–8]. Anti-GBM disease is strongly associated with the MHC class II allele HLA-DRB1*15:01 (HLA-DR15, previously known as HLA-DR2b), with an average odds ratio of 8.5 [9]. $CD4^+$ T cells reactive to the immunodominant $CD4^+$ T cell epitope, $\alpha3_{135\text{-}145}$ ($_{135}$GWISLWKGFSF$_{145}$), are expanded in HLA-DR15$^+$ humans and induce disease in HLA-DR15 transgenic mice (HLA-DR15$^+$, lacking mouse MHC class II), demonstrating the important contribution of HLA-DR15-mediated $CD4^+$ T cell responses in this disease [10].

Treatments for autoimmune disease have remained largely unchanged for many years, with few advances that provide better options for patients. Current treatment for anti-GBM disease involves high dose corticosteroids, cyclophosphamide, and acute plasmapheresis to

Abbreviations: GBM, glomerular basement membrane; HLA, human leukocyte antigen; α3(IV)NC1, non-collagenous domain of the alpha 3 chain of type IV collagen

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remove autoantibodies [2]. These toxic immunosuppressants often have severe side effects, including those that compromise protective immune function, risking life-threatening infections. There is a need for better treatments, in this and in other autoimmune kidney diseases.

Most autoimmune diseases have genetic associations with HLA alleles, although their mechanistic contribution to autoimmunity is unclear [11]. In anti-GBM disease, however, a mechanism by which HLA polymorphisms influence disease risk has been defined. HLA-DR15 increases disease susceptibility by presenting $\alpha3_{135\text{-}145}$ in a conformation that activates pro-inflammatory T cells; in contrast, the negatively associated HLA-DR1 confers protection by presenting $\alpha3_{135\text{-}145}$ in a conformation that activates regulatory T cells [8]. Thus, blocking the ability of HLA-DR15 to present $\alpha3_{135\text{-}145}$ could specifically inhibit pro-inflammatory $\alpha3_{135\text{-}145}$-specific responses while allowing protective immunity to be effected by the other unaffected HLA class II allomorphs found in each human (HLA-DQ, HLA-DP, and if not homozygous for DR15, the other HLA-DR). PV-267 is one such HLA-DR15-specific inhibitor, a small molecule that binds with high affinity and specificity to HLA-DR15 [12]. Thus, we hypothesize that HLA-DR15 inhibition with PV-267 will block the activation of $\alpha3_{135\text{-}145}$-specific T cells and attenuate experimental autoimmune anti-GBM disease. We tested these hypotheses using humanised HLA-DR15 transgenic mice.

2. Material and methods

2.1. Peptides and PV-267

The $\alpha3_{135\text{-}145}$ peptide (GWISLWKGFSF) and $OVA_{323\text{-}339}$ peptide (ISQAVHAAHAEINEAGR) were synthesised to at least 95% purity (Mimotopes, Clayton, Australia). The PV-267 peptide [Ac–V(Chg)R (Tic)F–NH2] was designed and synthesised by Provid Pharmaceuticals (Monmouth Junction, NJ, USA). PV-267 was dissolved in 0.1 M sodium phosphate buffer (pH 7.4, with 0.02% Tween 80).

2.2. Mice

HLA-DR15 transgenic mice (mouse MHC class $II^{-/-}$, HLA-DRA1*01:01 transgenic, HLA-DRB1*15:01 transgenic, *Fcgr2b*$^{+/+}$ or *Fcgr2b*$^{-/-}$) were generated as previously described [10]. Mice deficient in *Fcgr2b* have increased susceptibility for glomerulonephritis and are used in these experiments to measure the effect of PV-267 on limiting disease. Mice 6–10 weeks of age, male and female, were used in experiments and kept in specific pathogen-free conditions at Monash Medical Centre Animal Facilities; animal experiments were approved by the Monash University Animal Ethics Committee.

2.3. Immune responses to $\alpha3_{135\text{-}145}$ after PV-267 treatment

DR15^{+}*Fcgr2b*$^{+/+}$ mice were immunized subcutaneously with 10 μg of $\alpha3_{135\text{-}145}$ emulsified in Freund's complete adjuvant (Sigma-Aldrich, Sydney, Australia) on day 0. Mice were administered vehicle (0.1 M sodium phosphate buffer) or PV-267 at 30 mg/kg intraperitoneally every day from day −1 to day 10. At day 10 mice were culled and draining lymph nodes were harvested. Recall responses were measured by lymphocyte proliferation using [^{3}H]-thymidine incorporation, and IFN-γ and IL-17A ELISPOTs (BD Biosciences, North Ryde, Australia) as previously described [10].

2.4. Experimental autoimmune anti-GBM glomerulonephritis; prevention and treatment with PV-267

DR15^{+}*Fcgr2b*$^{-/-}$ mice were immunized subcutaneously with 100 μg $\alpha3_{135\text{-}145}$, emulsified in Freund's complete adjuvant for the first injection and subsequently in Freund's incomplete adjuvant (Sigma-Aldrich), subcutaneously on days 0, 7, and 14 [10]. These mice develop autoimmunity by day 21, with increased albuminuria at day 28, and by day 42 have significant functional and histological renal injury. In a prevention model, mice were administered vehicle or PV-267 at 30 mg/kg intraperitoneally every second day from day −1. To attenuate disease development, mice were administered vehicle or PV-267 at 50 mg/kg daily from day 21. In treating established disease, mice were administered vehicle or PV-267 at 50 mg/kg daily from day 28. Experiments ended at day 42. Animals were randomly allocated to either a vehicle control or PV-267 treatment group.

2.5. Assessment of renal injury

Urinary albumin was measured by ELISA (Bethyl Laboratories, Montgomery, TX, USA) following the manufacturer's protocol. Urine creatinine was measured using an autoanalyzer (Monash Health, Clayton, Australia).

2.6. Assessment of histological injury and inflammatory cell infiltrates

Glomerular segmental necrosis and crescents were assessed by periodic acid-Schiff (PAS) staining of formalin-fixed, paraffin-embedded (FFPE) kidney sections. Segmental necrosis was scored based on regions of glomeruli with hypocellularity and PAS-positive staining, and crescents were defined as two or more layers of cells lining the Bowman's capsule. Fibrin deposition was examined by immunoperoxidase staining of FFPE kidney sections using a rabbit anti-mouse fibrinogen antibody (R-4025) and DAB substrate (Sigma-Aldrich). Inflammatory cell infiltrates were assessed by immunoperoxidase staining of periodate-lysine-paraformaldehyde (PLP)-fixed, frozen kidney sections using DAB substrate and primary mAbs to detect macrophages (FA/11; anti-mouse CD68), neutrophils (RB6-8C5; anti-mouse Gr1), and $CD4^{+}$ T cells (GK1.5; anti-mouse CD4). Anti-GBM IgG deposition was assessed by immunofluorescent staining of snap-frozen kidney sections with FITC-conjugated rabbit anti-mouse IgG (Thermo Fisher Scientific, Waltham, MA, USA). A minimum of 30 glomeruli were assessed per animal.

2.7. Flow cytometry analysis of $\alpha3_{135\text{-}145}$-specific T cells

One kidney from each mouse was harvested and digested with 0.125% (w/v) collagenase D (Roche Diagnostics, Indianapolis, IN, USA) and 0.1% (w/v) DNase I (Roche). Kidney cells were enriched for $CD45^{+}$ cells using CD45 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany), then incubated with anti-mouse CD16/CD32 (BD Biosciences) to block Fc binding sites. To each kidney sample, 10 μl of AccuCount particles (Spherotech, Lake Forest, IL, USA) were added. The cells were then stained with Pacific Blue-labelled anti-mouse CD4 (BD Biosciences), APC-eFluor780-labelled anti-mouse CD8a (eBioscience, Scoresby, Australia), PE-labelled $\alpha3_{135\text{-}145}$-DR15-tetramer [8], AF488-labelled anti-mouse/human CD11b (BioLegend, San Diego, CA, USA), AF488-labelled anti-mouse CD11c (Biolegend), AF488-labelled anti-mouse F4/80 (Biolegend), AF488-labelled anti-mouse/human CD45R/B220 (Biolegend), and Fixable Viability Dye eFluor 520 (Thermo Fisher). Analysis was performed on a FACSCanto II (BD Biosciences) and FlowJo software (BD).

2.8. Renal HLA-DR expression

HLA-DR expression was assessed by immunofluorescent staining of snap-frozen kidney sections from DR15^{+}*Fcgr2b*$^{-/-}$ mice using PE-labelled mouse anti-human HLA-DR antibody (clone L243; Biolegend).

2.9. Statistical analyses

Mann-Whitney test was used for comparisons between two groups.

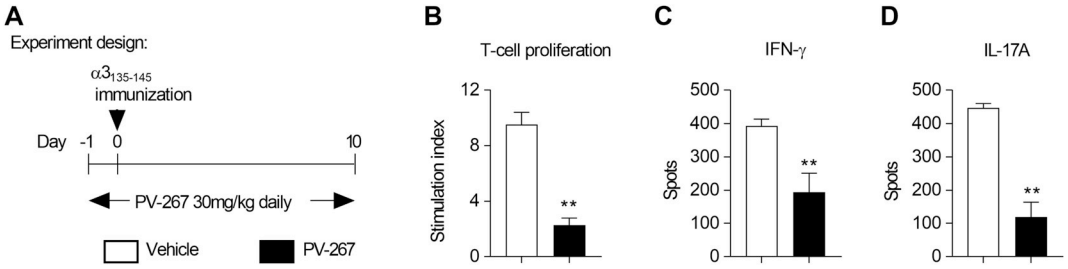

Fig. 1. HLA-DR15 inhibition attenuates inflammatory $\alpha3_{135\text{-}145}$-specific immune responses. **A**: DR15^{+}*Fcgr2b*$^{+/+}$ mice were immunized with $\alpha3_{135\text{-}145}$ and administered PV-267 or vehicle daily, (n = 5 per group), then lymph node cells were cultured and re-stimulated with $OVA_{323\text{-}339}$ or $\alpha3_{135\text{-}145}$. Immune responses were determined by proliferation measured by **B**: [^{3}H]-thymidine incorporation, and **C**: IFN-γ and **D**: IL-17A production measured by ELISPOT. Results are represented as the mean ± SEM. $^{**}P < 0.01$ by Mann-Whitney test.

3. Results

3.1. Blocking HLA-DR15 inhibits $\alpha3_{135\text{-}145}$-specific immune responses

To determine whether PV-267 could limit the activation of $\alpha3_{135\text{-}145}$-specific T cells, HLA-DR15 transgenic (DR15^{+}*Fcgr2b*$^{+/+}$) mice were administered either vehicle or PV-267 (30 mg/kg, intraperitoneally) daily from one day prior to $\alpha3_{135\text{-}145}$ immunization (Fig. 1A). Ten days after $\alpha3_{135\text{-}145}$ immunization, $\alpha3_{135\text{-}145}$-specific responses were measured on cells from draining lymph nodes by proliferation using [^{3}H]-thymidine incorporation, and IFN-γ and IL-17A ELISPOTs after *ex vivo* stimulation with $\alpha3_{135\text{-}145}$ or an irrelevant peptide ($OVA_{323\text{-}339}$). Stimulation with $OVA_{323\text{-}339}$ did not produce any response. Cells from mice that received PV-267 were less reactive to $\alpha3_{135\text{-}145}$, with approximately 75% reduction in proliferation and IL-17A spots, and 50% fewer cells producing IFN-γ (Fig. 1B–D). These results demonstrate that the inhibition of HLA-DR15 with PV-267 prior to $\alpha3_{135\text{-}145}$ immunization attenuates $\alpha3_{135\text{-}145}$-specific T cell responses.

3.2. HLA-DR15 inhibition protects mice from developing experimental autoimmune anti-GBM glomerulonephritis

To determine if PV-267 inhibited disease, we tested it in experimental autoimmune anti-GBM glomerulonephritis [8,10]. DR15^{+}*Fcgr2b*$^{-/-}$ mice received either vehicle or PV-267 (30 mg/kg) starting from one day before $\alpha3_{135\text{-}145}$ immunization (Fig. 2A). Compared to vehicle treated controls, mice that received PV-267 developed markedly reduced disease. Albuminuria as a measure of renal injury was reduced, and histological glomerular injury was markedly attenuated, with only minimal segmental necrosis and crescent formation (Fig. 2B–D). Glomerular IgG deposition was also reduced (Fig. 2E). Glomerular fibrin deposition, a feature of local T cell-mediated delayed type hypersensitivity [13], was markedly lower (Fig. 2F); and there were fewer CD4^{+} T cells, macrophages and neutrophils within glomeruli (Fig. 2G–I). These results indicate the near absence of disease in mice given PV-267, suggesting that inhibition of HLA-DR15 is effective at halting the development of experimental autoimmune anti-GBM glomerulonephritis.

3.3. HLA-DR15 inhibition after established autoimmunity attenuates disease development in mice

To test the ability of PV-267 to block disease development after autoimmunity is established, DR15^{+}*Fcgr2b*$^{-/-}$ mice were administered PV-267 from day 21 using the same experimental model of disease (Fig. 3A). Renal injury was significantly reduced in treated mice with no observable histological glomerular injury (Fig. 3B–D). Treated mice also had no detectable IgG deposition in glomeruli (Fig. 3E). Glomerular fibrin was absent in the PV-267 treated group, and inflammatory cell infiltrates were reduced, with fewer macrophages and neutrophils (Fig. 3F–I). Although there was no difference in the numbers of infiltrating CD4^{+} T cells when measured immunohistologically (Fig. 3G), flow cytometry analyses of whole kidney digests revealed fewer intrarenal $\alpha3_{135\text{-}145}$-specific CD4^{+} T cells in mice given PV-267 (Supplementary Fig. 1). These results demonstrate that inhibiting HLA-DR15 antigen presentation during active autoimmunity can block the development of disease.

3.4. HLA-DR15 inhibition following established renal injury attenuates disease severity in mice

To assess the efficacy of PV-267 treatment after the development of functional renal injury, mice were administered PV-267 from day 28. By this time point DR15^{+}*Fcgr2b*$^{-/-}$ mice had developed increased albuminuria and were then randomised into either the vehicle control group or the PV-267 treatment group (Fig. 4A and B). In this model, PV-267 treatment limited histological glomerular injury as evidenced by a decrease in segmental glomerular necrosis (Fig. 4C). However, albuminuria, glomerular IgG deposition and inflammatory cell infiltrates were unaffected (Fig. 4D–H). These results suggest that PV-267 is effective, at least, in limiting disease severity.

3.5. HLA-DR shows periglomerular expression in mice

To examine the cells that PV-267 may affect within the kidney, tissue samples from DR15^{+}*Fcgr2b*$^{-/-}$ diseased mice were stained for HLA-DR. The staining was observed to be in a periglomerular pattern (Supplementary Fig. 2), demonstrating that HLA-DR is expressed on renal interstitial cells that can be acted on by PV-267.

4. Discussion

Specifically inhibiting HLA allomorphs in autoimmune renal disease may offer a more targeted approach than current broadly immunosuppressive treatments. In the current studies, we explored the possibility of using a small molecule inhibitor, PV-267, to block HLA-DR15 mediated antigen presentation in an HLA transgenic mouse model of autoimmune anti-GBM glomerulonephritis. PV-267 effectively suppressed inflammatory responses to $\alpha3_{135\text{-}145}$ (Fig. 1) and protected~~ing~~ mice from developing disease (Fig. 2). Furthermore, even after autoimmunity against α3(IV)NC1 was induced PV-267 treatment prevented the development of renal injury (Fig. 3). When treatment was initiated after functional injury was established, PV-267 limited the severity of histological disease (Fig. 4). However, immune cell infiltration was not attenuated, suggesting that while PV-267 may not be able to reduce the numbers of immune cells that have been recruited to the kidney, it can reduce the pathogenicity of the immune cells that are in the kidney.

HLA-peptide complexes are responsible for antigen presentation and function in immune responses. In addition to its expression in the tubulointerstitium, HLA-DR is expressed on intrinsic glomerular cells,

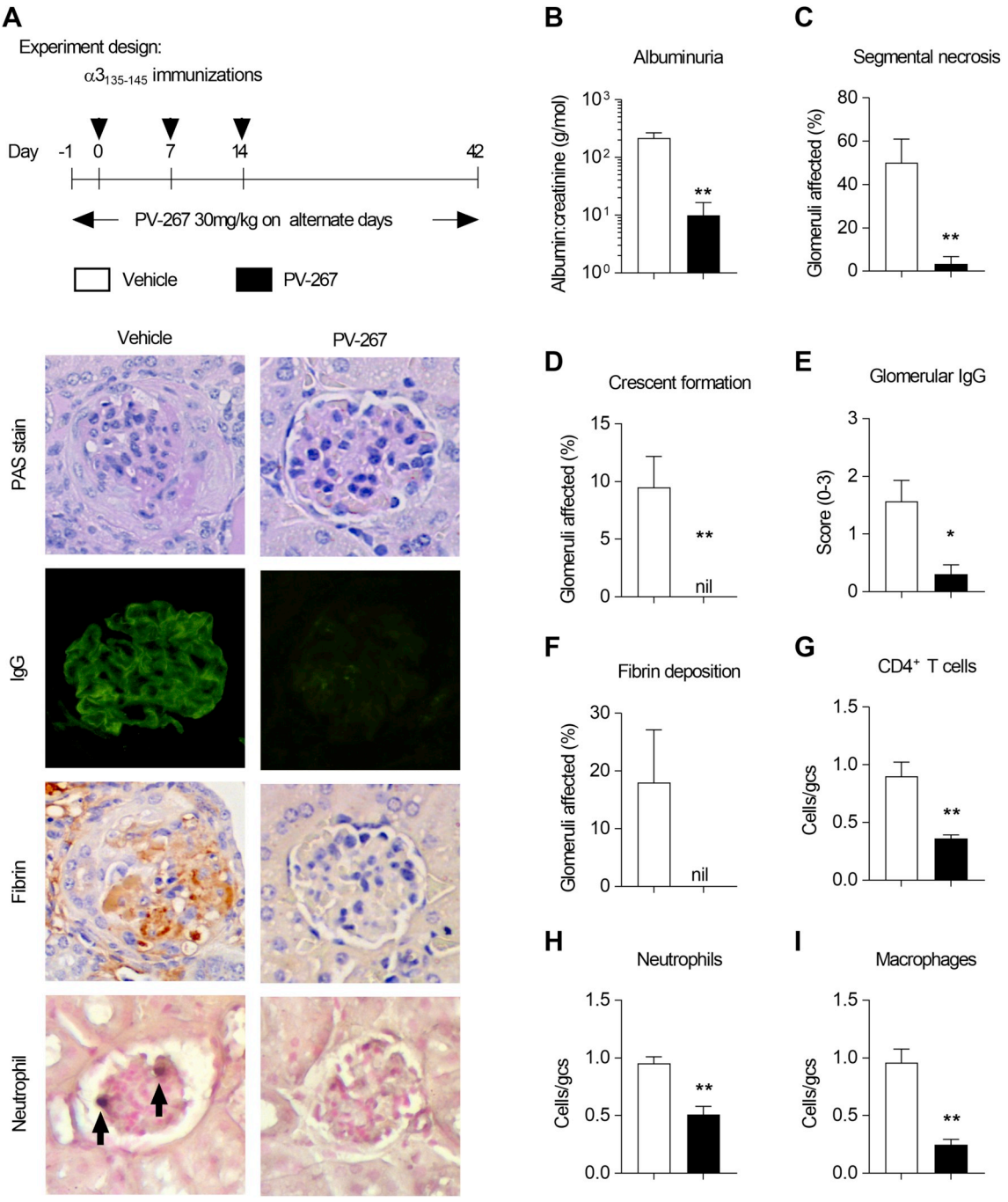

Fig. 2. HLA-DR15 inhibition protects DR15^{+}*Fcgr2b*$^{-/-}$ mice from experimental autoimmune anti-GBM glomerulonephritis. **A**: DR15^{+}*Fcgr2b*$^{-/-}$ mice immunized with $\alpha3_{135\text{-}145}$ were administered PV-267 ($n = 6$) or vehicle ($n = 5$) on alternate days in a prevention model. Disease was assessed by **B**: urinary albumin, **C**: segmental glomerular necrosis, **D**: crescent formation, **E**: glomerular IgG deposits, **F**: glomerular fibrin, and **G**: and infiltrating CD4^{+} T cells, **H**: neutrophils, **I**: macrophages. Photomicrographs (400x) show glomeruli from mice that received vehicle or PV-267, showing crescent formation by PAS stain, immunofluorescent staining of linear IgG deposits, and immunoperoxidase staining of fibrin deposition and inflammatory cell infiltrates with neutrophils (indicated by the arrows). IgG deposition is scored semiquantitatively; inflammatory cell infiltrates are expressed as cells per glomerular cross section (gcs). Results are represented as the mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$ by Mann-Whitney test.

including endothelial cells in normal human kidneys, while in mice, MHC II is present on interstitial renal mononuclear phagocytes but not the glomerular endothelium [14]. Here, immunofluorescent staining for HLA-DR in diseased DR15^{+}*Fcgr2b*$^{-/-}$ mice showed similar periglomerular staining in the interstitium, and recent data demonstrates that MHC class II-expressing monocytes within glomerular capillaries present antigens to effector T cells [15]. These findings imply that PV-267 in the current studies may be acting not just on HLA-DR15 in secondary lymphoid organs, but also on HLA-DR15 expressed on cells within the kidney and its microvasculature, preventing recognition of α3(IV)NC1-derived peptides by effector CD4^{+} T cells.

In the context of other studies of MHC inhibition in autoimmunity, disruption of the MHC-peptide loading machinery by Cathepsin S inhibition has been shown to attenuate experimental lupus nephritis [16]. The efficacy of selective MHC inhibition by direct binding to the peptide binding cleft has been demonstrated in autoimmune diabetes, where blocking NOD mouse I–A^{g7} delayed disease onset in mice, and in an open-label clinical trial, specific inhibition of HLA-DQ8 decreased

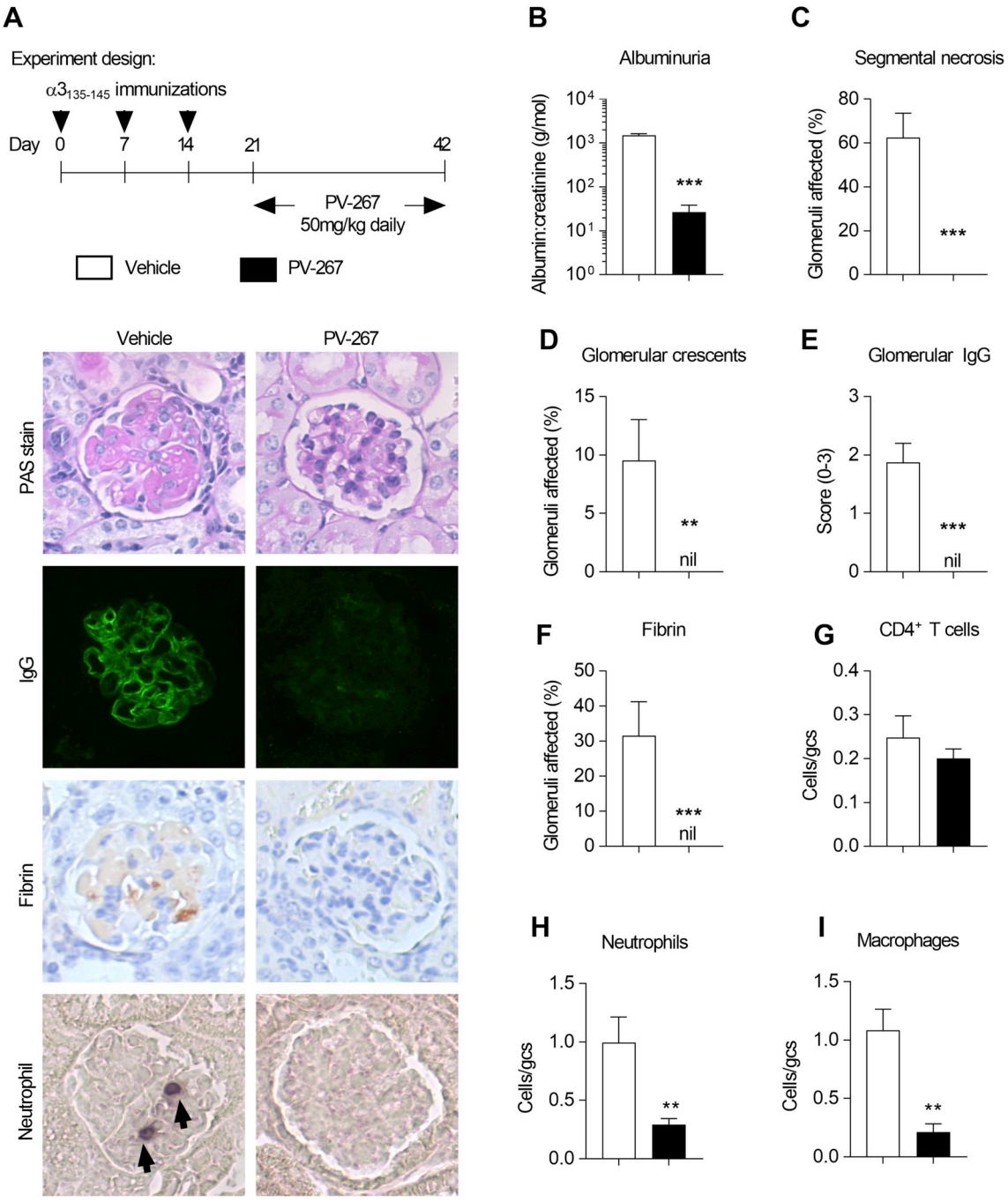

Fig. 3. HLA-DR15 inhibition, after inducing autoimmunity with $\alpha 3_{135\text{-}145}$ immunizations, attenuates disease development in DR15^{+}*Fcgr2b*$^{-/-}$ mice. **A**: DR15^{+}*Fcgr2b*$^{-/-}$ mice immunized with $\alpha 3_{135\text{-}145}$ were administered PV-267 ($n = 8$) or vehicle ($n = 7$) daily from day 21. **B**: Disease was assessed by urinary albumin, **C**: segmental glomerular necrosis, **D**: crescent formation, **E**: glomerular IgG deposits, **F**: glomerular fibrin, and recruitment of **G**: CD4^{+} T cells, **H**: neutrophils, **I**: macrophages. Photomicrographs (400x) show glomeruli from mice that received vehicle or PV-267, showing segmental necrosis by PAS stain, immunofluorescent staining of linear IgG deposits, and immunoperoxidase staining of fibrin deposition and inflammatory cell infiltrates with neutrophils (indicated by arrows). IgG deposition is scored semiquantitatively; inflammatory cell infiltrates are expressed as cells per glomerular cross section (gcs). Results are represented as the mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ by Mann-Whitney test.

inflammatory T cell responses in patients [17]. Previous research has established that PV-267 does not impair T cell responses from other MHC II molecules, nor cause non-specific activation of human PBMCs [12]. When administered therapeutically, recovery times were improved in a mouse model of experimental autoimmune encephalomyelitis, underscoring its potential as a treatment for multiple sclerosis [12]. PV-267 is well tolerated, even at high doses in mice [12]. Similar attempts in the past aimed to produce altered peptide ligands which would modify the T cell response to become suppressive, however in some instances patients had adverse reactions; it was suggested that a peptide antagonistic for one particular TCR may have been agonistic for another [18], so designing a peptide to inhibit rather than modify the direction of the T cell response may be more efficacious in humans. Here, we have demonstrated that inhibiting the T cell response by blocking presentation of the immunodominant CD4^{+} T cell epitope is an effective means of attenuating the autoimmune response and resultant disease.

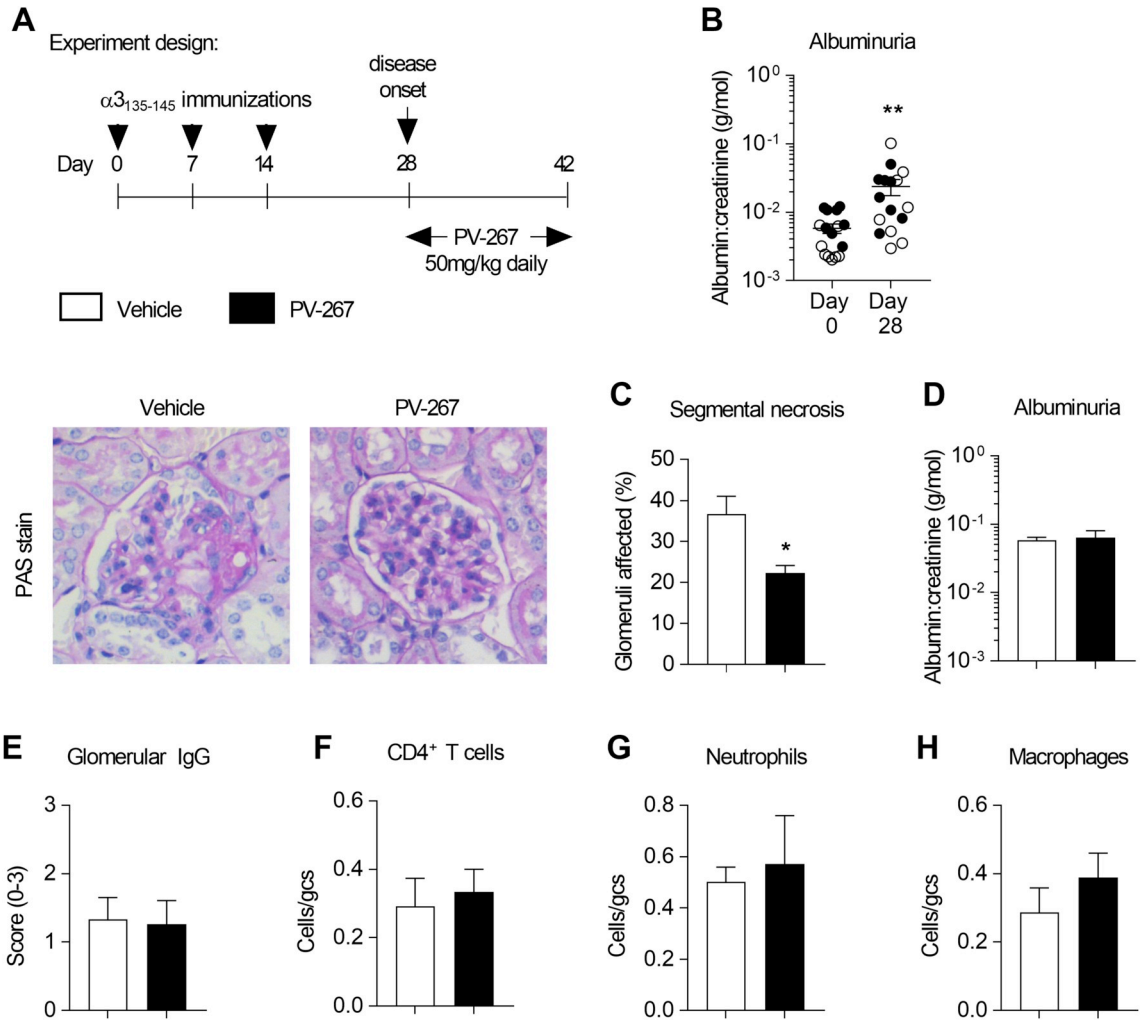

Fig. 4. HLA-DR15 inhibition attenuates disease severity in DR15^{+}*Fcgr2b*$^{-/-}$ mice. **A,B**: DR15^{+}*Fcgr2b*$^{-/-}$ mice immunized with $\alpha3_{135\text{-}145}$ were treated with PV-267 ($n = 6$) or vehicle ($n = 7$) daily from day 28 after increased albuminuria was established. At day 42, disease was assessed by **C**: segmental glomerular necrosis, **D**: urinary albumin, **E**: glomerular IgG deposits and glomerular recruitment of **F**: CD4^{+} T cells, **G**: neutrophils, **H**: macrophages. Photomicrographs (400x) show glomerular necrosis by PAS stain. IgG deposition is scored semiquantitatively; inflammatory cell infiltrates are expressed as cells per glomerular cross section (gcs). Results are represented as the mean ± SEM. *$P < 0.05$, **$P < 0.01$ by Mann-Whitney test.

5. Conclusion

This study has shown that specific HLA-DR15 inhibition by PV-267 can attenuate renal injury, demonstrating antigen-specific therapeutic potential. If used as an adjunct treatment for anti-GBM disease, PV-267 may be able to provide a less toxic alternative and reduce adverse outcomes, for example by allowing lower doses of current therapies to be used. Furthermore, these results support the use of selective HLA class II inhibition in other autoimmune diseases, where specific HLA allomorphs confer an increased risk of disease.

Author contributions

M.H., G.L.O., N.B.R., C.R.S., Y.S., S.R.H., A.R.K. and J.D.O. designed the experiments; M.H., P.E. and J.D.O. performed the experiments and collected the data; M.H., A.R.K. and J.D.O. drafted and revised the paper; all authors approved the final version of the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jaut.2019.05.004.

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