C5a receptor 1 promotes autoimmunity, neutrophil dysfunction and injury in experimental anti-myeloperoxidase glomerulonephritis

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The prospects for complement-targeted therapy in ANCA-associated vasculitis have been enhanced by a recent clinical trial in which C5a receptor 1 (C5aR1) inhibition safely replaced glucocorticoids in induction treatment. C5aR1 primes neutrophils for activation by anti-neutrophil cytoplasmic antibody (ANCA) and is therefore required in models of glomerulonephritis induced by anti-myeloperoxidase antibody. Although humoral and cellular autoimmunity play essential roles in ANCA-associated vasculitis, a role for C5aR1 in these responses has not been described. Here, we use murine models to dissect the role of C5aR1 in the generation of anti-myeloperoxidase autoimmunity and the effector responses resulting in renal injury. The genetic absence or pharmacological inhibition of C5aR1 results in reduced autoimmunity to myeloperoxidase with an attenuated Th1 response, increased Foxp3$^+$ regulatory T cells and reduction in generation of myeloperoxidase-ANCA. These changes are mediated by C5aR1 on dendritic cells, which promotes activation, and thus myeloperoxidase autoimmunity and glomerulonephritis. We also use renal intravital microscopy to determine the effect of C5aR1 inhibition on ANCA induced neutrophil dysfunction. We found that myeloperoxidase-ANCA induce neutrophil retention and reactive oxygen species burst within glomerular capillaries. These pathological behaviors are abrogated by C5aR1 inhibition. Thus, C5aR1 inhibition ameliorates both autoimmunity and intra-renal neutrophil activation in ANCA-associated vasculitis.

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Anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV) is an autoimmune disease in which multiple immune participants contribute to the pathogenesis. A loss of self-tolerance results in ANCA production by B cells. These autoantibodies activate neutrophils, which induces their recruitment to glomeruli, degranulation, and extracellular trap formation.[1,2] This neutrophil activation results in both direct endothelial injury and extensive glomerular deposition of myeloperoxidase (MPO).[3,4] In mouse models, the response of MPO-specific effector T cells to glomerular MPO is a significant contributor to necrotizing glomerulonephritis associated with MPO autoimmunity.[5,6]

Complement plays an important role as a modulator and effector of immune responses. There are 3 activation pathways, the classical, lectin, and alternative, which converge to form C3 and C5 convertases. These result in the generation of multiple effector molecules, including the proinflammatory C5 fragment, C5a, which binds to C5a receptor 1 (C5aR1) and C5a receptor 2. The C5aR1 is expressed on myeloid cells, including neutrophils, mast cells, monocyte/macrophages, and dendritic cells (DCs).[7,8] The C5aR1 has been shown to be an important mediator of inflammation in murine models of autoimmunity.[9] In humans, therapeutic targeting of C5 has is a potent approach for treatment of diseases driven by dysregulation of complement, such as atypical hemolytic uremic syndrome.[10]

Previous preclinical studies have shown that complement amplified by the alternative pathway and signaling through C5aR1 plays a key role in the priming of neutrophils for activation by ANCA and in experimental glomerulonephritis induced by anti-MPO antibodies.[11–13] These data are supported by evidence of raised complement activation products in clinical samples from patients with active AAV.[14] The recently published phase II CLEAR study reported that the

oral C5aR1 antagonist CCX168 (Avacopan) was noninferior to standard-dose prednisolone as a component of induction therapy for AAV.[15] Complement inhibition, therefore, has the potential to be a paradigm changing therapy in AAV; a phase 3 clinical trial (NCT02994297) will further inform the validity of this approach.

Although therapeutic targeting of C5aR1 is in advanced clinical development, the importance of this receptor in the generation of the cellular and humoral anti-MPO autoimmunity that is required for this autoimmune disease has not been determined. Furthermore, how C5aR1 inhibition moderates ANCA-induced neutrophil dysfunction in the glomerulus *in vivo* has not been described. In these studies, we investigated the hypothesis that, independent of its effects on neutrophils, C5aR1 would promote autoreactivity to MPO and subsequent glomerulonephritis. In addition, we used multiphoton microscopy to define the effect of C5aR1 inhibition on neutrophil behavior in the glomerular microvasculature.

RESULTS

C5aR1 promotes cellular and humoral autoimmunity to MPO

To determine whether endogenous C5aR1 promotes autoimmune responses to MPO, anti-MPO autoimmunity was studied in C5aR1 intact (wild-type [WT]) and *C5aR1*$^{-/-}$ mice 10 days after immunization with native murine MPO (nmMPO) in Freund complete adjuvant (FCA). In *C5aR1*$^{-/-}$ mice, there was a reduction in the proliferation of antigen-restimulated lymphocytes (Figure 1a), and a reduction in the MPO-specific Th1 response (Figure 1b), the Th17 response was not affected (Figure 1c). This was accompanied by an increase in the proportion of CD4^{+} cells that were regulatory T cells (Tregs) (Figure 1d and e). Humoral immunity was assessed 28 days after initial immunization with nmMPO in FCA, followed by a boost dose of nmMPO in Freund incomplete adjuvant on day 7. MPO-ANCA titers were decreased in *C5aR1*$^{-/-}$ mice, largely related to a reduction in the IgG2b subclass. There were no significant differences between groups in other IgG subclasses (Figure 1f). Total serum IgG was measured in immunized mice and did not significantly differ between groups (Figure 1g). To assess whether inhibition, rather than genetic deletion, would affect adaptive immunity, we used the peptide inhibitor (Ac-Phe-[Orn-Pro-dCha-Trp-Arg], *PMX53*) to study the effect of C5aR1 inhibition on autoimmunity to MPO 10 days after immunization. Due to the drug's short life,[16] it was administered continuously through an osmotic infusion mini-pump. Similar to the findings with *C5aR1*$^{-/-}$ mice, T-cell proliferation (Figure 2a) and anti-MPO T-helper (Th)1 responses were reduced (Figure 2b), although there was no significant difference in the Th17 response or the proportion of CD4 cells with a Treg phenotype (Figure 2c and d).

C5aR1 regulates DC activation and subsequent T-cell response

C5aR1 has been reported to influence the generation of Th1, Th17, and Foxp3^{+} Tregs through signaling within both antigen-presenting cells (APCs) and T cells.[17–20] We investigated expression of costimulatory molecules and the cytokine profile of bone marrow (BM)-derived DCs (BMDCs) from *C5aR1*$^{-/-}$ and WT mice. DCs from WT mice exhibited higher expression of major histocompatibility complex-II, whereas CD40, CD80, and CD86 were similar between groups (Figure 3a–d). WT BMDCs secreted more tumor necrosis factor and interferon beta (Figure 3e and f), and whereas interleukin (IL)-12p70 was similar between groups, *C5aR1*$^{-/-}$ DCs secreted more anti-inflammatory IL-10 (Figure 3g and h). In addition, *C5aR1*$^{-/-}$ DCs were less efficient at internalizing antigen measured using ovalbumin conjugated to a fluorogenic substrate DQ-OVA (Figure 3i).

To determine whether the differences in DC activation and antigen uptake observed in *C5aR1*$^{-/-}$ DCs resulted in attenuated autoimmunity, we transferred recombinant murine MPO (rmMPO)-loaded BMDCs derived from either WT or *C5aR1*$^{-/-}$ mice into WT hosts to induce autoimmunity as previously described (Figure 4a).[21] Draining lymph node cells were collected 10 days after BMDC immunization for analysis of T-cell responses. Compared with WT DCs, transfer of *C5aR1*$^{-/-}$ DCs resulted in a reduced Th1 response (Figure 4b) and increased the proportion of Tregs without altering the Th17 response, mirroring results in mice globally deficient in the C5aR1 (Figure 4c and d).

To explore the additional possibility for a functional effect of a T-cell intrinsic C5aR1, we used *Foxp3* expressing green fluorescent protein (*Foxp3*^{GFP}) reporter mice with intact complement receptors or *Foxp3*^{GFP} mice deficient in both C5aR1 and C3aR (*C3aR*$^{-/-}$*C5aR1*$^{-/-}$*Foxp3*^{GFP}),[18] to isolate CD4^{+}Foxp3^{-} cells from naïve mice. These T cells were injected into *Rag1*$^{-/-}$ mice that were then immunized with nmMPO in FCA (Figure 4e). Ten days after immunization there was no difference in the generation of splenic interferon-γ^{+} or IL-17A^{+} and, although consistent with previous reports,[22] generation of *de novo* Foxp3^{+} Tregs was minimal, this did not differ between groups (Figure 4f). These findings indicate that in this experimental system, the absence of T-cell intrinsic C3aR/C5aR1 does not impact T-cell polarization.

C5aR1 on DCs induces T-cell–mediated anti-MPO glomerulonephritis

We have previously shown that when autoimmunity to MPO is induced in C57BL/6 mice by immunization, the humoral response generated is insufficient to cause glomerulonephritis. However, when glomerular neutrophil recruitment and MPO deposition are induced with low-dose sheep anti-mouse anti–glomerular basement membrane (GBM) globulin, mice develop MPO-specific T-cell–mediated necrotizing glomerulonephritis.[5,23] Importantly, previously published controls including the lack of injury in mice immunized with OVA and the induction of injury by MPO but not OVA-specific T-cell clones, confirm that glomerular injury is caused by MPO-specific effector T cells.[5,23,24] This model is dependent on glomerular neutrophil recruitment by anti-GBM globulin, and as glomerular neutrophil recruitment by immune complexes has been reported to be