A plasmid-encoded peptide from *Staphylococcus aureus* induces anti-myeloperoxidase nephritogenic autoimmunity

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Autoreactivity to myeloperoxidase (MPO) causes anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV), with rapidly progressive glomerulonephritis. Here, we show that a *Staphylococcus aureus* peptide, homologous to an immunodominant MPO T-cell epitope (MPO409-428), can induce anti-MPO autoimmunity. The peptide (6PGD391-410) is part of a plasmid-encoded 6-phosphogluconate dehydrogenase found in some *S. aureus* strains. It induces anti-MPO T-cell autoimmunity and MPO-ANCA in mice, whereas related sequences do not. Mice immunized with 6PGD391-410, or with *S. aureus* containing a plasmid expressing 6PGD391-410, develop glomerulonephritis when MPO is deposited in glomeruli. The peptide induces anti-MPO autoreactivity in the context of three MHC class II allomorphs. Furthermore, we show that 6PGD391-410 is immunogenic in humans, as healthy human and AAV patient sera contain anti-6PGD and anti-6PGD391-410 antibodies. Therefore, our results support the idea that bacterial plasmids might have a function in autoimmune disease.
Loss of tolerance to the neutrophil enzyme myeloperoxidase (MPO) leads to anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (MPO-AAV), an autoimmune disease that can affect multiple tissues but which often involves the kidney. In MPO-AAV, patients frequently develop rapidly progressive glomerulonephritis and are at risk of end-stage kidney failure. The other major autoantigen known to be clinically relevant in AAV is the neutrophil serine protease, proteinase-3 (PR3). MPO-AAV and PR3-AAV, while having some differences, share similar pathogenic features. In MPO-AAV, tissue injury is induced not only by autoantibodies binding to target tissues such as the kidney, but by anti-MPO autoantibodies (MPO-ANCA) that bind to and activate neutrophils causing glomerular neutrophil recruitment, degranulation, and NETosis. These activated neutrophils are not only themselves responsible for significant tissue injury and damage, they also deposit MPO in and around glomerular capillaries. Thus, MPO accumulating in glomeruli may function as an antigenic target for MPO-specific effector CD4+ and CD8+ T cells that induce a further wave of cell-mediated injury.

Although it is unclear how tolerance to neutrophil cytoplasmic antigens MPO and proteinase-3 (PR3) is lost and how disease is triggered, like many autoimmune diseases, both genetic and environmental factors are probably important. In particular, infection has been implicated both in clinical studies, and in vitro and in vivo experimental work. Nasal carriage of *Staphylococcus aureus* is associated with an increase in relapse of disease in granulomatosis with polyangiitis, characterized by loss of tolerance to PR3 (PR3-AAV). Less is known about *S. aureus* colonization of people with MPO-AAV. While chronic nasal carriage is uncommon in those with microscopic polyangiitis and renal limited vasculitis, usually associated with MPO-ANCA, nasal colonization does occur and case reports implicate *S. aureus* in the development of this condition. There are several mechanisms by which infections might influence AAV; superantigens have been hypothesized to have a function, and pathogen-associated molecular patterns stimulate antigen presentation, B cells, and prime neutrophils. The release of autoantigens (including PR3 and MPO) by neutrophils at sites of infection might also affect the maintenance of tolerance. A further potential consequence of the uptake of neutrophil-derived autoantigens by antigen-presenting cells at sites of inflammation with innate immune system activation could be the development of molecular mimicry. As molecular mimicry can lead to T-cell receptor (TCR) cross-reactivity, a microbial mimotope presented as a peptide by MHC Class II might activate TCRs that also recognize PR3 or MPO-derived epitopes presented by MHCII.

Some evidence supports the involvement of molecular mimicry in the loss of tolerance to neutrophil antigens in AAV. The complementary PR3 autoantigenic sequence, implicated in loss of tolerance to PR3, shares homology with bacterial peptides, including some from *S. aureus*. Another target neutrophil antigen, lysosomal antigen membrane protein-2 (LAMP-2), shares sequence homology with the bacterial adhesin FimH, with FimH immunization of rats inducing anti-LAMP-2 autoantibodies and glomerulonephritis. However, it is not known whether molecular mimicry has any function in loss of tolerance to MPO and the resultant development of MPO-AAV.

Here, we demonstrate that molecular mimicry mechanistically contributes to the loss of tolerance to MPO in AAV. We evaluate whether microbial-derived peptides, including those from *S. aureus*, with sequence homology to the immunodominant MPO CD4+ T-cell epitope can induce the expansion of naive CD4+ T cells that recognize MPO, with the subsequent development of cross-reactive anti-MPO autoimmunity leading to glomerulonephritis and AAV. We identify a *S. aureus* peptide, 6-phosphogluconate dehydrogenase (6PGD)391–410 derived from a plasmid-encoded protein that induces cellular and humoral anti-MPO autoimmunity and experimental anti-MPO glomerulonephritis. Thus, molecular mimicry mediated by a bacterial plasmid capable of horizontal transmission represents a potential mechanism of loss of tolerance in autoimmune disease.

**Results**

**Highly homologous peptides do not induce autoreactivity.** To determine if autoreactivity to the immunodominant MPO CD4+ T-cell epitope, mouse MPO409–428, could be induced by microbial peptides, we performed a protein BLAST (blastp) search using the core 11-mer sequence of the equivalent human MPO peptide, 441RLYQEARKIVG451 (mouse MPO peptide sequence and numbering: 415KLYQEARKIVG425). Sequences from the Animalia kingdom (taxid:33208) and microbes not known to colonize humans were excluded. Based on the search results, we selected the four most homologous sequences (Supplementary Table 1) and because we have demonstrated previously that a 20-mer peptide induces stronger immunoreactivity to MPO (concordant with MHCII molecules having an open binding groove), we synthesized 20-mers based on the four identified sequences. For example, for the *Aspergillus fumigatus* HEAT repeat protein331–441 (383RYQYEARKIIF441) the synthesized 20-mer was 823ISALPQRWYQYEARKIIEFAA444. To determine whether these sequences could induce anti-MPO autoimmunity we immunized C57BL/6 mice with individual 20mers and measured T-cell reactivity to either the immunizing peptide, MPO409–428, or recombinant mouse (rm)MPO using interferon-γ (IFN-γ) and interleukin (IL)-17A ELISPOTs and [3H]-T proliferation assays. While some homologous sequences induced reactivity to themselves, none induced reactivity to MPO409–428 or whole rmMPO (Fig. 1a–f), demonstrating that high sequence homology per se does not result in immunological cross-reactivity to MPO. A *S. aureus*-derived peptide induces anti-MPO autoimmunity. As *S. aureus* infections can precede the development of MPO-AAV, they are related to an overlapping form of vasculitis (PR3-AAV) and nasal colonization of *S. aureus* has been found in people with MPO-AAV. We identified a *S. aureus*-derived peptide with sequence homology to human MPO441–451 by protein BLAST. The highest scoring *S. aureus*-derived peptide containing the previously defined critical MPO441–451 T-cell epitope residues (Tyr443, Arg447, Ile449 and Val450) was 441RLYQYEARKIVG451. We synthesized 20-mers (Fig. 1a–f) from this sequence and measured T-cell reactivity using an I-Ab tetramer pre-plasmid pSJH101 from the clinically relevant *S. aureus* strain 428, and to rmMPO (Fig. 2a). MPO409–428-immunized mice served as a positive control. To determine if exposure to 6PGD397–408 induces in vivo expansion of MPO-specific T cells, we immunized mice with 6PGD397–408 as well as autoreactivity to both the immunodominant MPO CD4+ T-cell epitope, MPO409–428, and to rmMPO (Fig. 2a). MPO409–428-immunized mice served as a positive control. To determine if exposure to 6PGD397–408 then enumerated the peptide, 6–51 T-cell epitope, 6–51 (397TDYQEAALRDVVA408) was from 6-phosphogluconate dehydrogenase (6PGD), an enzyme of the pentose phosphate pathway, and was first described within the plasmid pSJH101 from the clinically relevant *S. aureus* strain JH132. To determine whether this 6PGD397–408 sequence induced autoreactivity to MPO, we immunized C57BL/6 mice with 6PGD391–410 (391YFKNIVTDYQEALRDVVATG410). Mice developed reactivity to 6PGD391–410 as well as autoreactivity to both the immunodominant MPO CD4+ T-cell epitope, MPO409–428, and to rmMPO (Fig. 2a). MPO409–428-immunized mice served as a positive control. To determine if exposure to 6PGD391–410 induces in vivo expansion of MPO-specific T cells, we immunized mice with 6PGD391–410 then enumerated the number of MPO-specific T cells using an I-Ab tetramer presenting the core mouse MPO T-cell epitope (415KLYQEARKIVG425). We compared the total numbers of MPOspecific T cells from naive mice, OVA323–335 immunized mice and...
MPO409–428 immunized mice using MPO1-Aβ tetramers. Cells were tetramer enriched using magnetic beads, then gated on live, CD4+, CD11b-, MPO1-Aβ tetramer+ cells. Compared with naïve mice and with mice immunized with OVA323–339, mice immunized with 6PGD391–410 exhibited a ~30-fold increase in MPO1-Aβ-specific CD4+ T cells (Fig. 2b). Thus, 6PGD391–410 induces expansion of MPO415–425-specific CD4+ cells and pro-inflammatory autoreactivity to MPO.

Serum from 6PGD391–410 immunized mice bound to fixed thioglycolate induced peritoneal neutrophils from C57BL/6 mice, in a perinuclear ANCA (pANCA) fashion (Fig. 3a) but not to MPO-deficient (Mpo−/−) mouse neutrophils, and to whole native mouse (nm)MPO by enzyme-linked immune sorbent assay (ELISA) (Fig. 3b), findings that meet the diagnostic criteria for MPO-ANCA positivity in humans33. Furthermore, purified serum IgG bound to the clinically relevant human linear B-cell epitope MPO447–459 (Fig. 3c)34. To demonstrate antibody cross-reactivity between 6PGD391–410 and MPO409–428, we performed an inhibition ELISA. Purified serum IgG from 6PGD391–410 immunized mice was pre-incubated with MPO409–428 then used to detect anti-MPO409–428 IgG by ELISA. Serum IgG from S. aureus 6PGD391–410 immunized mice pre-incubated with S. aureus 6PGD391–410 had lower antibody titers compared with serum IgG pre-incubated with blocking buffer only (Fig. 3d). Cross-reacting antibodies were functionally active, as serum IgG from 6PGD391–410 immunized mice induced reactive oxygen species production from LPS-primed bone marrow mouse neutrophils in vitro as detected by the conversion of dihydrorhodamine to rhodamine 123 (Fig. 3e). In vivo, passive transfer of this IgG fraction induced acute neutrophil glomerular recruitment in LPS-primed C57BL/6 mice, albeit at a low level (Fig. 3f). These data demonstrate that antibodies specific for S. aureus 6PGD391–410 cross-react with MPO409–428 and that the S. aureus-derived peptide induces both anti-MPO T-cell autoreactivity and biologically active MPO-ANCA.

To identify if the S. aureus-derived 6PGD protein is immunoreactive in healthy humans and in AAV patients, we measured IgG antibodies specific for the S. aureus pSJH101 6PGD protein by ELISA in sera from a Groningen cohort of healthy human subjects, 31 MPO-AAV patients and 30 PR3-AAV patients. We found detectable levels of S. aureus 6PGD-specific IgG in all three groups (Fig. 4a) implying that S. aureus pSJH101 6PGD is an immunogenic protein in humans. Furthermore, sera exhibited reactivity to the pSJH101 JH1 S. aureus 6PGD391–410 sequence by ELISA (Fig. 4b), demonstrating the immunogenicity of this sequence in humans. There were no significant differences in antibody titers between groups. To identify whether 6PGD391–410 can cross-react with anti-MPO antibodies in acute MPO-AAV, a Monash cohort of 15 patients with acute, active MPO-AAV was assessed (Supplementary Table 2). Purified IgG from these patients was assessed by inhibition ELISA by pre-incubulation with 6PGD391–410, then antibodies to human MPO435–454 (the homologous sequence to mouse MPO409–428) were examined by ELISA. Of the 15 patients, five showed a significant reduction in anti-human MPO435–454 titers after incubation with 6PGD391–410 (Fig. 4c).
**S. aureus clonal specificity for the 6PGD \(397-408\) mimotope.** This particular 6PGD \(397-408\) sequence is unique to the *Staphylococcus* genus. *S. aureus* makes up the majority of publicly available staphylococcal genomes and the 6PGD \(397-408\) sequence of interest predominates in a clinically relevant *S. aureus* genus. We assessed the multi-locus sequence type of 136 of the publicly available CC5 strains have been described in Asia, America, Australia, Africa, and Europe. There are 2544 publicly available CC5 *S. aureus* genomes, indicating that ~5% of sequenced CC5 strains contain the 6PGD \(397-408\) mimic sequence and found that 115 (85% of those typed) of them were CC5. *S. aureus* CC5 strains have been described in Asia, America, Australia, Africa, and Europe. These variant sequences most homologous to the pSJH101-derived 6PGD \(391-410\) sequence in *S. aureus* 143 sequenced variants most homologous to the pSJH101-derived 6PGD \(391-410\) (Fig. 6a). When we measured anti-MPO T-cell responses measured ex vivo to either OVA \(323-339\), MPO \(409-428\), 6PGD \(391-410\), or recombinant mouse MPO (rmMPO) using \[^{3}H\text{-thymidine proliferation assays (top row)}\), and ELISPOT for IFN-γ (middle row) or IL-17A (bottom row). Each dot represents one mouse; data are representative of two independent experiments. **b** In vivo expansion of MPO-specific CD4⁺ T cells. Cells from lymph nodes and spleen of C57BL/6 mice, either naive \((n = 4)\), immunized with OVA \(323-339\) \((n = 5)\), MPO \(409-428\) \((n = 6)\) or *S. aureus* pSJH101-derived 6PGD \(391-410\) \((n = 6)\). Results are expressed as number of MPO-I: A⁺ tetramer⁺ cells per mouse. Error bars represent the mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 by Kruskal-Wallis test. Source data are provided as a Source Data file.

**Immunization with 6PGD \(391-410\) leads to anti-MPO nephritis.** To determine if the loss of tolerance to MPO induced by *S. aureus* JH1-derived pSJH101 6PGD \(391-410\) could result in anti-MPO glomerulonephritis, we used our established model of T-cell-mediated anti-MPO glomerulonephritis. In this model, C57BL/6 mice immunized with MPO lose tolerance to MPO but do not develop ANCA of sufficient pathogenicity to induce glomerulonephritis. Therefore, MPO is deposited within the glomerulus via neutrophils transiently recruited by injection of low dose of heterologous anti-mouse basement membrane globulin. In this context, effector MPO-specific T cells recognize MPO peptides and mediate glomerular injury. MPO-immunized mice develop glomerulonephritis with pathological albuminuria and segmental glomerular necrosis. Using this protocol, mice immunized with the *S. aureus* JH1-derived pSJH101 6PGD \(391-410\)-immunized MPO-ANCA glomerulonephritis of similar severity to MPO-
immunized mice with elevated albuminuria, glomerular segmental necrosis, and inflammatory cell infiltrates (Fig. 7). Furthermore, the pSJH101 6PGD391–410 immunized mice developed MPO-ANCA and T-cell reactivity to rmMPO, detected by measuring dermal delayed type hypersensitivity to rmMPO. A further group of mice was immunized with the Variant 3 peptide of 6PGD391–410 (Supplementary Table 3), chosen because, of the four variants it was found most frequently in sequenced strains of *S. aureus*. As hypothesized, mice immunized with Variant 3 of 6PGD391–410 did not develop disease (Fig. 7), demonstrating the relative specificity of the JH1 pSJH101 6PGD391–410 sequence in nephritogenic anti-MPO autoimmunity.

**S. aureus JH1 with pSJH101 immunization leads to nephritis.** To address a specific role for the *S. aureus* pSJH101 plasmid-derived 6PGD391–410 sequence in anti-MPO autoimmunity and glomerulonephritis in the context of whole bacteria, we immunized mice with either heat-killed *S. aureus* JH1 strain containing the pSJH101 plasmid or heat-killed JH1 that had been cured of the pSJH101 plasmid (Supplementary Fig. 2a) and induced the same model of glomerulonephritis. Compared to mice immunized with cured heat-killed *S. aureus* JH1, mice immunized with *S. aureus* JH1 containing pSJH101 developed glomerulonephritis with pathological albuminuria, glomerular focal, and segmental necrosis and infiltrates of CD4+ T cells, CD8+ T cells and macrophages (Fig. 8). Mice immunized with *S. aureus* JH1 containing the pSJH101 plasmid also developed MPO-ANCA and MPO-specific secretion of IFN-γ and tumor necrosis factor (TNF) measured in supernatants of cultured splenocytes restimulated with rmMPO (Fig. 8). Therefore, the pSJH101 plasmid containing the cross-reactive *S. aureus* 6PGD sequence is required for anti-MPO cross-reactivity and disease.

**Plasmid and strain independent 6PGD induced anti-MPO immunity.** To determine if it is the specific 6PGD sequence that causes disease independent of other proteins encoded by pSJH101 and independent of the *S. aureus* strain, we cloned 6PGD containing the mimic 397TDYQELRDVQA408 sequence into the inducible vector pALC2073 (that does not otherwise express 6PGD) to create pALC2073-6PGD. We then transformed a common laboratory *S. aureus* strain (RN42209), that contains neither plasmids nor 6PGD 397TDYQELRDVQA408 with either pALC2073-6PGD or pALC2073 alone. Enhanced expression of 6PGD was confirmed after the induction of anhydroteracycline (Supplementary Fig. 2b). We immunized mice with heat-killed *S. aureus* RN4220 expressing 6PGD or heat-killed *S. aureus* RN4220 with pALC2073 alone, and disease was again triggered by low-
Here, we show that the core MPO T-cell epitope, MPO415 amino acids, de
dand HLA-DR15 mice (Supplementary Fig. 3a, b) and the critical
complementary Fig. 3c). To determine if the pSJH101-derived
ELISPOT for IFN-γ and TNF (Fig. 9). Mice
immunized with RN4220 with pALC2073 con-
trary to rmMPO (Fig.10a, b), supporting the notion that pSJH101
Mice immunized with S. aureus 6PGD391–454 homolog) in acute MPO-AAV. Serum IgG from patients with acute MPO-AAV (Fig.435–454 (the
MPO409–428 homolog) in acute MPO-AAV. Serum IgG from patients with acute MPO-AAV (Fig. 9) were pre-incubated with S. aureus pSJH101
6PGD391–410 then used to detect anti-hMPO435–454 IgG antibodies by ELISA. Values are quintuplicates. Error bars in a and b are mean ± s.d., in panel
c mean ± s.e.m. *P < 0.05, **P < 0.01, Mann–Whitney U-test. Source data are provided as a Source Data file.

6PGD391–410 sequence can be effectively presented and induce anti-MPO cross-reactivity by a variety of MHCII alleles.

**Discussion**

Although we know that a critical step in the development of autoimmune disease is the activation of pro-inflammatory T cells that react with self-antigens, the steps that precipitate the development and activation of these pathogenic T cells are still unclear. Recently, we have shown that peptide register is a key determinant of the phenotype of the autoreactive T-cell repertoire. While molecular mimicry is often flagged as a potential trigger for the activation of existing autoreactive pro-inflammatory T cells, fewer studies have formally demonstrated microbial-self-peptide cross-reactivity, which is often attributable to the lack of understanding of the self-antigen that precipitates disease. The current studies not only identify a mimotope peptide, pSJH101 6PGD391–410 that induces anti-MPO T and B-cell autoimmunity, they also highlight both the sensitivity of such mimicry, as very similar sequences to the mimic peptide were unable to induce cross-reactivity. Importantly our studies demonstrate the potential for mimicry to be induced by a plasmid-encoded microbial sequence, identifying a potential new role for bacterial plasmids in the pathogenesis of disease.

We and others have identified a “molecular hotspot” within MPO where an immunodominant T-cell epitope and a disease-associated antibody epitope overlap. PR3-AAA is classically associated with S.aureus, and reports also implicate S. aureus infections in MPO-AAV. However, despite the presence of neutrophil-derived MPO at sites of infection in a potentially “dangerous” immunological context, the links between the loss of tolerance to MPO and microbial-derived peptides are unclear. Using a standard and unbiased approach of searching microbial
In vitro restimulating antigens

**Fig. 5** Anti-MPO T-cell responses after immunization *S. aureus*-derived 6PGD391–410 sequences. C57BL/6 mice (*n* = 4 each group) were immunized with either **a** MPO409–428 (positive control), **b** pSJH101-derived 6PGD391–410 (YFKNIVTDYQDALRDVVATG), **c** *S. aureus* 6PGD391–410 Variant 1 (YFKNIVTDYQDALRDVVATG), **d** *S. aureus* 6PGD391–410 Variant 2 (YFKNIVTNYQDALRDVVATG), **e** *S. aureus* 6PGD391–410 Variant 3 (YFKNIVTYQDALRDVVATG), **f** *S. aureus* 6PGD391–410 Variant 4 (YFKNIVTNQDALRDVVATG). T-cell recall responses were measured ex vivo to either OVA323–339, the immunizing peptide, or MPO409–428 using [3H]-thymidine proliferation assays (top row), and ELISPOT IFN-γ (middle row) or IL-17A (bottom row). Each dot represents the response from an individual mouse, error bars represent the mean ± s.e.m. Data are representative of two independent experiments. *P* < 0.05, **P** < 0.01, ***P*** < 0.001 by Kruskal–Wallis test. Source data are provided as a Source Data file

proteomes in silico for peptide sequences with the highest sequence similarities to MPO441–451. We identified a number of microbial peptides from human pathogens, but experimentally these sequences did not induce anti-MPO cross-reactivity. However, when *S. aureus*-derived peptides sharing the critical amino acid residues were examined, we identified a plasmid-derived peptide that induces anti-MPO immunoreactivity in the context of several different MHCII molecules and that is immunogenic in humans.

This MPO mimotope, pSJH101 6PGD391–410, is overall less homologous than the other non-cross-reactive microbial-derived peptides tested, demonstrating that sequence similarity itself is not necessarily a predictor of molecular mimicry.\(^{41}\) Instead, specific structural determinants may be more of a contributory factor that leads to cross-reactivity.\(^{42}\) Our experiments, using similar 6PGD391–410 sequences from a range of *S. aureus* strains demonstrated that even single amino-acid substitutions were sufficient to abrogate anti-MPO cross-reactivity. For example, in Variant 1, a substitution from glutamic acid (E) to the smaller aspartic acid (D), and in Variant 2, a substitution from the negatively charged aspartic acid (D) to the uncharged asparagine (N), prevented the induction of anti-MPO cross-reactivity, highlighting the exquisite sensitivity of TCRs to specific peptide structures.

Using ex vivo restimulation assays, as well as MPO-I-A\(^{b}\) tetramers, we have demonstrated that pSJH101 6PGD391–410 can induce anti-MPO CD4\(^{+}\) T-cell cross-reactivity. Furthermore, in addition to cellular immunity, the 6PGD391–410 peptide also induces autoantibodies to whole nmMPO, to the disease-associated linear MPO peptide and to an overlapping linear MPO peptide. The 6PGD391–410 mimotope inhibited autoantibody binding to this peptide in mice via a solid phase competitive ELISA. 6PGD391–410 also inhibited binding to human MPO435–454 (equivalent to mouse MPO409–428) in 5/15 (33%) of humans with acute MPO-AAV. Collectively, these data confirm a functional interaction between these overlapping epitopes. Thus, the pSJH101 6PGD391–410 peptide cross reacts with an MPO T-cell epitope, but it is also likely to be relevant to these linked B-cell epitopes. While it is possible that antibodies to 6PGD391–410 serve as effectors, as for example in the seminal studies of Kaplan and Meyersian, and others for streptococcal antigens and acute rheumatic fever,\(^{43,44}\) we suggest that this type of direct reactivity at an effector level is less likely in MPO-AAV. Cross-reactivity at a B cell/B-cell receptor level is more likely to be relevant to the promotion of B-cell autoreactivity via binding of 6PGD391–410 to the B-cell receptor of potentially autoreactive B cells. This would promote autoreactive anti-MPO B-cell activation by autoreactive CD4\(^{+}\) T cells reacting to the same peptide. In this context, the relative affinities of 6PGD391–410 and MPO609–428 (in humans MPO435–454) to anti-MPO antibodies and whether 100% inhibition occurs, is unlikely to be of critical importance. Furthermore, 6PGD391–410 alone is unlikely to have a measurable effect on the
binding of MPO-ANCA to neutrophils by indirect immunofluorescence, as there are known to be multiple B-cell epitopes in active MPO-AAV.

There have been several studies of nasal carriage of S. aureus in people with PR3-AAV, due in part to sinonasal disease being common in PR3-AAV. However, the potential relationship between S. aureus and MPO-AAV has been largely unexplored, though colonization with S. aureus does occur in patients with this disease. Most S. aureus strains known to carry the nephritogenic 6PGD sequence belong to the CC5 clonal complex. In S. aureus carriers with established MPO-AAV, 11% of isolates were CC5 (healthy controls 5%, PR3-AAV 15%). CC5 is a globally distributed clonal complex of S. aureus found in both community and hospital settings.

It is not yet known in humans whether carriage or infection of S. aureus strains containing the cross-reactive 6PGD sequence promotes the induction of MPO-AAV or precipitates disease relapse. The conditions for 6PGD recognition to induce anti-MPO T-cell cross-reactivity may include S. aureus infection, intermittent colonization or chronic colonization. Furthermore, while nasal swabs are the most common way of screening for S. aureus, carriage also occurs on the skin, and in the throat, vagina, anus, and lower gastrointestinal tract. It is unlikely that the 6PGD mimotope is the sole factor that determines loss of tolerance to MPO, given the frequency of antibodies to the 6PGD protein and peptide, and the multiple genetic and environmental factors that contribute to the development of MPO-AAV.

Although our data do not conclusively prove a role for 6PGD, they suggest that exposure to certain S. aureus strains may be a precipitating factor in the loss of tolerance to MPO and the development of MPO-AAV. Our data also demonstrate that plasmids, acting as mobile genetic elements, may transfer a tendency to autoreactivity. The transfer of antibiotic resistance via plasmids is well known. However, the horizontal gene transfer of the cross-reactive 6PGD that we emulated by transforming S. aureus RN4220 with pALC2073-6PGD demonstrates that plasmids harboring cross-reactive peptide sequences can induce loss of tolerance. In conclusion, our findings identify pSJH101 6PGD mimotope peptide sequences as candidates that can induce loss of tolerance to MPO and experimental anti-MPO glomerulonephritis and MPO-AAV. This sequence is derived from a plasmid found in only some strains of S. aureus, implicating plasmid-derived antigens in the loss of tolerance to self-antigens.

Methods

Mice. C57BL/6 and BALB/c mice were obtained from the Monash Animal Research Platform, Clayton, Monash University. Mpo−/− mice and HLA-DR15 Tg mice were bred at the Monash Medical Center Animal Facility (MMCAF), Monash Medical Center, Clayton. Mice were housed in the SPF facilities at MMCAF and experiments were conducted in male mice aged 6–10 week. All animal studies were approved by the Monash University Animal Ethics Committee (Committee MMCB) and complied with the Australian code for the care and use of animals for scientific purposes (2013).

Human samples. Serum samples from AAV patients and healthy subjects (HS) were obtained from an existing collection of the ‘Groningen cohort of AAV’, and sera and plasma exchange effluent from Monash patients with acute MPO-AAV were obtained from the Monash Vasculitis Registry and Biobank. Institutional review board (IRB) approval was previously obtained from the Medical Ethics...
Photomicrographs glomeruli from glomeruli. Glomerular injury was measured by albuminuria, and by glomerular segmental necrosis on periodic acid-Schiff (PAS) stained kidney sections.

Fig. 7 Experimental anti-MPO glomerulonephritis in S. aureus pSJH101 6PGD391-410 immunized mice. C57BL/6 mice (n = 5 each group) were immunized with either OVA (negative control), MPO (positive control), S. aureus pSJH101 6PGD391-410 or the common S. aureus 6PGD391-410 variant, Variant 3. Low-dose heterologous anti-basement membrane globulin was injected intravenously to induce transient neutrophil recruitment and MPO deposition in glomeruli. Glomerular injury was measured by albuminuria, and by glomerular segmental necrosis on periodic acid-Schiff (PAS) stained kidney sections. Photomicrographs glomeruli from S. aureus pSJH101 6PGD391-410 or S. aureus Variant 3 6PGD391-410 immunized mice. Inflammatory cells within glomeruli were enumerated and expressed as cells per glomerular cross section (gcs). Anti-MPO autoreactivity determined by detection of anti-MPO IgG by ELISA and dermal delayed type hypersensitivity (DTH) swelling after recombinant mouse MPO intradermal challenge. Scale bar is 30 μm. Error bars represent the mean ± s.e.m. *P < 0.05, **P < 0.01, ***P < 0.001 by Kruskal-Wallis test. Source data are provided as a Source Data file.
Experimental anti-MPO glomerulonephritis

- **OVA immunized (negative control)**
- **MPO immunized (positive control)**

### Fig. 8

**Experimental anti-MPO glomerulonephritis in mice injected with *S. aureus* containing pSJH101**

- OVA (negative control, n = 4), MPO (positive control, n = 4), *S. aureus* JH1 with pSJH101 (n = 6) or cured *S. aureus* JH1 without pSJH101 (n = 6).

- OVA and MPO were emulsified in Freund’s complete adjuvant; *S. aureus* JH1 with or without pSJH101 were emulsified in Titermax. MPO was deposited in glomeruli using heterologous low-dose anti-basement membrane globulin. Glomerular injury was measured by albuminuria, and by glomerular segmental injury.

### Scale bar is 30 μm.

- Error bars represent the mean ± s.e.m.
- *P < 0.05, **P < 0.01, ***P < 0.001 by Mann-Whitney U-test.

### Source data are provided as a Source Data file.

#### Immediately following the Jun leucine zipper sequence. MPO-κAκA monomers were purified from baculovirus infected High Five insect cell supernatants through immobilized metal ion affinity (Ni Sepharose 6 Fast-Flow, GE Healthcare), size exclusion (S200 Superdex 16/600, GE Healthcare) and anion exchange (HiTrap Q, HP, GE Healthcare) chromatography. MPO-κAκA tetramers were assembled by the addition of Streptavidin-PE (BD Biosciences)54,55.

#### Plasmids and *Staphylococcus aureus* strains. The pSJH101 plasmid was found within a clinical isolate of *S. aureus* JH1 (also known as strain A8090)56. To cure the *S. aureus* JH1, cells were cultured with 0.004% SDS at 45 °C for 24 h57. To confirm the presence or absence of the pSJH101 plasmid containing 6PGD, PCR was performed on cell lysates using primers specific for: **cbl2**, forward primer 5′ GCAAGGTACCATGATAGAGTTATTATCCATTGC 3′, reverse primer 5′ ACCCCGTAAAATTTTGTTGAT 3′; **pSJH101**, forward primer 5′ CATTCAGCTTATGGAAGTGGTATGATGATGTAAGATAGGTGACAATAATTGTG 3′, reverse primer 5′ ACTCACCATTGTGGGGGACT 3′; and the pSJH101-derived 6PGD, which do not amplify the more common 6PGD (Variant 3) present in the chromosomal DNA of JH1: forward primer 5′ TCACTCATCTAACACGGGAAATG3′ and reverse primer 5′ ACCCCGTAAAATTTTGTTGAT 3′.

The 6PGD sequence (derived from pSJH101) was cloned into the tetracycline inducible pALC2073 plasmid58. *S. aureus* RN4220, which contains neither plasmids nor the 6PGD 3′TDYQEAARLDDVA sequence, was transformed by electroporation with either pALC2073 containing 6PGD or pALC2073 without 6PGD59,60. To confirm expression of 6PGD we performed PCR on cDNA from cultured *S. aureus* RN4220 containing pALC2073 with or without tetracycline, *S. aureus* RN4220 containing pALC2073 without 6PGD cultured with tetracycline. As a control for specificity, we performed PCR using chromosomal DNA of *S. aureus* RN4220. The primers we used were: forward primer 5′ TCACTCATCTAACACGGGAAATG3′ and reverse primer 5′ ACCCCGTAAAATTTTGTTGAT 3′ chromosomal DNA of chromosomal DNA of *S. aureus* RN4220.

The primers we used were: forward primer 5′ TCACTCATCTAACACGGGAAATG3′ and reverse primer 5′ ACCCCGTAAAATTTTGTTGAT 3′. For in silico multi-locus sequence typing (MLST), the software mlst was used to identify the sequence types (STs) after scanning the genomes of interest61, then STs were grouped into CC in which each ST in the CC shares at least six identical alleles of the seven loci with at least one other member of the group62.
Experimental anti-MPO glomerulonephritis

- OVA immunized (negative control)
- MPO immunized (positive control)

**Fig. 9** Experimental anti-MPO glomerulonephritis in mice injected with S. aureus RN4220 containing pALC2073. C57BL/6 mice were immunized with either OVA (negative control, n = 4), MPO (positive control, n = 3), S. aureus RN4220 containing pALC2073 without 6PGD (n = 6) or S. aureus RN4220 containing pALC2073 with or without 6PGD were emulsified in Freund’s complete adjuvant; S. aureus RN4220 containing pALC2073 with or without 6PGD were emulsified in Titermax. MPO was deposited in glomeruli using heterologous low-dose anti-basement membrane globulin. Renal injury was measured by albuminuria, and by glomerular segmental necrosis on periodic acid-Schiff (PAS) stained kidney sections. Photomicrographs depict glomeruli from mice immunized with either S. aureus RN4220 containing pALC2073 with 6PGD or RN4220 containing pALC2073 without 6PGD. Inflammatory cells within glomeruli were enumerated and expressed as cells per glomerular cross section (gcs). Anti-MPO autoreactivity determined by detection of anti-MPO IgG by ELISA and by measuring in vivo expansion of MPO-specific cells, mouse were first immunized with 10 µg of peptide emulsified in FCA subcutaneously at the base of the tail, then, 7 days later, the inguinal, axillary, brachial, cervical, mesenteric, and periarterial lymph nodes and spleen were harvested. Following, tetramer-based magnetic enrichment, cells were incubated with Live/Dead fixable Near IR Dead Cell Stain (Thermo Scientific) then stained with anti-mouse CD4-Pacific Blue (BioLegend, 100351, 1:400) and ‘dump’ antibodies anti-mouse CD11c (all BioLegend, 117311, 1:100), CD11b (101217, 1:100), F4/80 (123120, 1:100), CD8a (100723, 1:100), B220-Alexa Fluor 488 (103225, 1:100). The MPOI-A6 tetramer gate was set based on the CD4+ live lymphocyte population (see Supplementary Fig. 4 for gating strategy).

**Induction and assessment of T-cell responses.** Mice were immunized with 10 µg of peptide emulsified in Freund’s complete adjuvant (FCA) subcutaneously at the base of the tail. Ten days later, draining lymph node cells were isolated and cultured in [3H]-T proliferation assays and/or IFN-γ and IL-17A ELISPOTs. Lymph node cells were cultured in triplicate in supplemented RPMI media (10% vol/vol FCS, 2 mM l-glutamine, 100 U ml−1 penicillin, 0.1 mg ml−1 streptomycin, 50 µM 2-Mercaptoethanol) at 5 × 10⁵ cells per well in the presence or absence of peptide (10 µg ml⁻¹) or whole protein antigen (10 µg ml⁻¹) at 37 °C, 5% CO₂ for 72 h in proliferation assays and 18 h in ELISPOTs. In proliferation assays, [3H]-thymidine was added during the last 16 h of culture and results expressed as a stimulation index. For IFN-γ and IL-17A ELISPOTs (eBioscience, anti-IFN-γ antibodies 551216, 1:250 and 554410, 1:250; anti-IL-17A antibodies 555068, 1:1000 and 555067, 1:1000), spots were developed according to the manufacturer’s protocol and results expressed as the mean number of spots minus baseline (media alone). To determine the in vivo expansion of MPO-specific cells, mice were first immunized with 10 µg of peptide emulsified in FCA subcutaneously at the base of the tail, then, 7 days later, the inguinal, axillary, brachial, cervical, mesenteric, and periarterial lymph nodes and spleen were harvested. Following, tetramer-based magnetic enrichment, cells were incubated with Live/Dead fixable Near IR Dead Cell Stain (Thermo Scientific) then stained with anti-mouse CD4-Pacific Blue (BioLegend, 100351, 1:400) and ‘dump’ antibodies anti-mouse CD11c (all BioLegend, 117311, 1:100), CD11b (101217, 1:100), F4/80 (123120, 1:100), CD8a (100723, 1:100), B220-Alexa Fluor 488 (103225, 1:100). The MPOI-A6 tetramer gate was set based on the CD4+ live lymphocyte population (see Supplementary Fig. 4 for gating strategy).

**Induction and assessment of anti-MPO antibody responses.** C57BL/6 mice were immunized with 10 µg of either OVA, MPO, S. aureus pSH101-derived 6PGD, S. aureus Variant 1 6PGD, S. aureus Variant 2
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**Fig. 10** Anti-MPO T-cell responses in other *S. aureus* pSJH101 6PGD391-410 immunized mouse strains. a BALB/c (I-A<sup>a</sup>/I-E<sup>e</sup>) immunizing peptide, S. aureus pSJH101 6-PGD391-410. b HLA-DR15 transgenic (Tg, n = 6) and S. aureus pSJH101 6-PGD391-410. Then T-cell responses measured ex vivo to either OVA<sub>223-239</sub> (negative control), MPO<sub>409-428</sub> and recombinant mouse MPO using [3H]-thymidine proliferation assays (top row), and ELISOPT for IFN-γ (middle row) or IL-17A (bottom row). Each dot represents the response from an individual mouse, error bars represent the mean ± s.e.m. Data are representative of two independent experiments. *P < 0.05, **P < 0.01, ***P < 0.001 by Kruskal–Wallis test. Source data are provided as a Source Data file.

**ELISAs for anti-MPO and anti-6PGD antibodies.** Serum was collected from mice by cardiac puncture on day 28 and used either for the detection of anti-MPO IgG antibodies, anti-MPO<sub>447-459</sub> IgG antibodies by ELISA and inhibition ELISAs for the detection of anti-MPO<sub>409-428</sub> IgG antibodies. The anti-MPO IgG ELISA was performed on rmMPO coated, 2% casein/PBS blocked 96-well plates. Anti-MPO<sub>447-459</sub> IgG ELISA was performed on MPO<sub>409-428</sub> coated, 2% casein/PBS blocked 96-well plates. Serum (diluted 1:50 in PBS) or pooled IgG (100 μg ml<sup>−1</sup> in PBS) was incubated for 16 h at 4 °C, then anti-mouse IgG detected using a horseradish peroxidase (HRP) conjugated secondary antibody (Amersham, NA-931, 1:2000). For inhibition ELISA, serum IgG (10 μg ml<sup>−1</sup>) was pre-incubated with S. aureus pSJH101-derived 6PGD391-410 on a 96-well ELISA plate (coating concentration 10 μg ml<sup>−1</sup>), then transferred to an MPO<sub>409-428</sub> coated (10 μg ml<sup>−1</sup>) 96-well ELISA plate.

Human sera were tested for reactivity to 6PGD (HS n = 23, MPO-AAV n = 31 and PR3-AAV n = 30) and to S. aureus pSJH101 6PGD391-410 (HS n = 14, MPO-AAV n = 26) and PR3-AAV patients (n = 24) by ELISA. The HS groups were different between assays, and not all samples assayed for whole 6PGD were available for the S. aureus pSJH101 6PGD391-410 assay. ELISA plates (NUNC Maxisorp, Thermo Fisher Scientific, Breda, The Netherlands) were coated with 100 μl of 5 μg ml<sup>−1</sup> recombinant S. aureus pSJH101 6PGD or 10 μg ml<sup>−1</sup> S. aureus pSJH101 6-PGD391-410 peptide diluted in 0.1 M carbonate-bicarbonate buffer (pH 9.6) overnight. Plates were washed with PBS pH 7.4 with 0.05% TWEEN-20 and incubated for 1 h at room temperature (RT) with 200 μl 2% bovine serum albumin (BSA)/PBS per well to prevent non-specific binding. Next, plates were incubated with 100 μl per sample (1:50) in PBS 1% BSA, 0.05% TWEEN-20, 2 h at RT. After washing, plates were incubated with alkaline phosphatase goat anti-human IgG (Sigma, St. Louis, USA, A-5403; 1:1000) for 1 h at RT and p-nitrophenyl-phosphate disodium (Sigma) was used as a substrate. Absorbance was measured at 405 nm. For inhibition ELISA, IgG purified from sera or plasma exchange effluent (50 μg ml<sup>−1</sup>) was then pre-incubated with S. aureus pSJH101-derived 6PGD391-410 on a 96-well ELISA plate (coating concentration 10 μg ml<sup>−1</sup>), then transferred to a human MPO<sub>405-415</sub> coated (10 μg ml<sup>−1</sup>) 96-well ELISA plate.

**Induction of mouse anti-MPO glomerulonephritis.** C57BL/6 mice were immunized subcutaneously at the tail base with either 20 μg of OVA (control antigen), 20 μg of rmMPO, 100 μg of S. aureus pSJH101 6PGD391-410 100 μg of S. aureus Variant 3 6-PGD391-410, 10 mg of heat-killed S. aureus JH1, 10 mg of cured heat-killed S. aureus [H][1], 10 mg of heat-killed S. aureus RN4220 transformed with pALC2073 with 6PGD or 10 mg of heat-killed S. aureus RN2220 transformed with pALC2073 without 6PGD. Proteins and peptides were injected first emulsified in Freund’s Complete Adjuvant (FCA) (day 0), then 7 days later emulsified in Freund’s Incomplete Adjuvant (FIA) (day 7). S. aureus strains were emulsified in Titermax (Sigma-Aldrich) and injected on days 0 and 7. On day 16, MPO was deposited in glomeruli by recruiting neutrophils using a low dose of intravenously injected heterologous anti-mouse basement membrane antibodies<sup>9,38,65</sup>. Experiments ended on day 20. Albuminuria was determined by ELISA (Bethyl Laboratories, E90-134) on urine collected 24 h before the end of experiment. Seminal glomerular necrosis was assessed on formalin fixed, paraffin embedded, 3 μm thick, PAS-stained sections and defined as the accumulation of PAS-positive material with hydropcellularity.

**CD4<sup>+</sup> T cells, CD8<sup>+</sup> T cells, macrophages, and neutrophils were detected by immunoperoxidase staining frozen kidney sections. A minimum of 20 consecutively viewed glomeruli were assessed per animal. The primary mAbs used were clones GK1.5 (anti-mouse CD4; American Type Culture Collection, 20 μg ml<sup>−1</sup>), 53-67W (anti-CD8α; BioXcell, 10 μg ml<sup>−1</sup>), FA/11 (macrophages; anti-mouse CD68; from GL Koch, MRC Laboratory of Molecular Biology, Cambridge, United Kingdom, 10 μg ml<sup>−1</sup>), and RB6-8C5 (neutrophils, anti-Gr-1, 2.5 μg ml<sup>−1</sup>). MPO-specific delayed type hypersensitivity was measured by intradermal injection of 10 μg of rmMPO, diluted in PBS, into the left plantar footpad. The same volume of PBS was administered into the contralateral footpad. DTH was quantified 24 h later by measurement of the difference in footpad thickness. IFN-γ, TNF, IL-17A, and IL-6 in rmMPO stimulated spleocyte cultures was measured by cytometric bead array (BD Biosciences, 560485).

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability.** Source data for Figs. 1, 2, 3b–f, 4, 5b–c, 6–10, and Supplementary Figs. 1–3 are presented in the Source Data file. Other data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Author contributions**
J.D.O., P.H., A.Y.P. and A.R.K. designed the research and wrote the paper. J.D.O., J.-H.J., P.I.E., L.L.C., M.v.T., K.M.O’S., P.Y.G., and Y.Z. performed and analyzed experiments. K. L.L., H.H.R., and L.F. generated and provided analytical tools. K.T. analyzed data. C.A.S., A.R.K., J. Ryan, and L.R.S. provided samples from healthy humans and people with AAV. S.R.H., L.F., H.H.R. and J. Rossjohn provided intellectual input and technical support.

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