A plasmid-encoded peptide from Staphylococcus aureus induces anti-myeloperoxidase (anti-MPO) nephritogenic autoimmunity

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- Introduction/Background Information
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ANCA-associated Vasculitis (AAV)

- Anti-neutrophil cytoplasmatic antibodies (ANCA)

- Systemic vasculitis affecting small vessels and accompanied by presence of ANCA in serum

- Disease entities include
  - Microspcopic polyangiits (MPA)
  - Granulomatosis with polyangiitis (GPA)
  - Eosinophilic granulomatosis with polyangiitis (EGPA)
  - Drug-induced ANCA-associated vasculitis

Hallmarks

MPA

- Histological
  - necrotizing vasculitis
  - granulomatous inflammation is absent

- Clinical representation
  - Symptoms due to renal failure
  - Fever
  - joint pain

GPA

- Histological presentation
  - necrotizing vasculitis
  - granulomatous inflammation

- Clinical representation
  - Rhinorhea
  - Hemoptysis
  - Fever
  - symptoms due to renal failure

Anti-neutrophil cytoplasmatic antibodies (ANCAs)

p-ANCA

- Perinuclear staining
- Myeloperoxidase (MPO) →MPO-ANCA
- Typically positive in MPA and 50% of EGPA

c-ANCA

- Diffuse staining in the cytoplasm
- Proteinase 3 (PR3) → PR3-ANCA
- Typically positive in GPA

Pathogenesis

- ANCAs induce excessive activation of neutrophil granulocytes

  → release inflammatory cytokines, reactive oxygen species and lytic enzymes

  → formation of neutrophil extracellular traps (NETs)

- Disordered regulation of NETs contributes to ANCA production

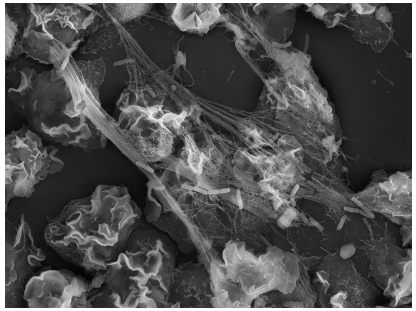
Sangaletti, S. et al. Neutrophil extracellular traps mediate transfer of cytoplasmic neutrophil antigens to myeloid dendritic cells toward ANCA induction and associated autoimmunity. *Blood* **120**, 3007–3018

Neutrophil extracellular traps (NETs)

- extracellular meshes composed of histones, DNA and neutrophil granular proteins

- nonphagocytic mechanisms of neutrophil-mediated bacterial killing

  → entrap bacteria and serve as scaffold to promote high local concentrations of anti-microbial components



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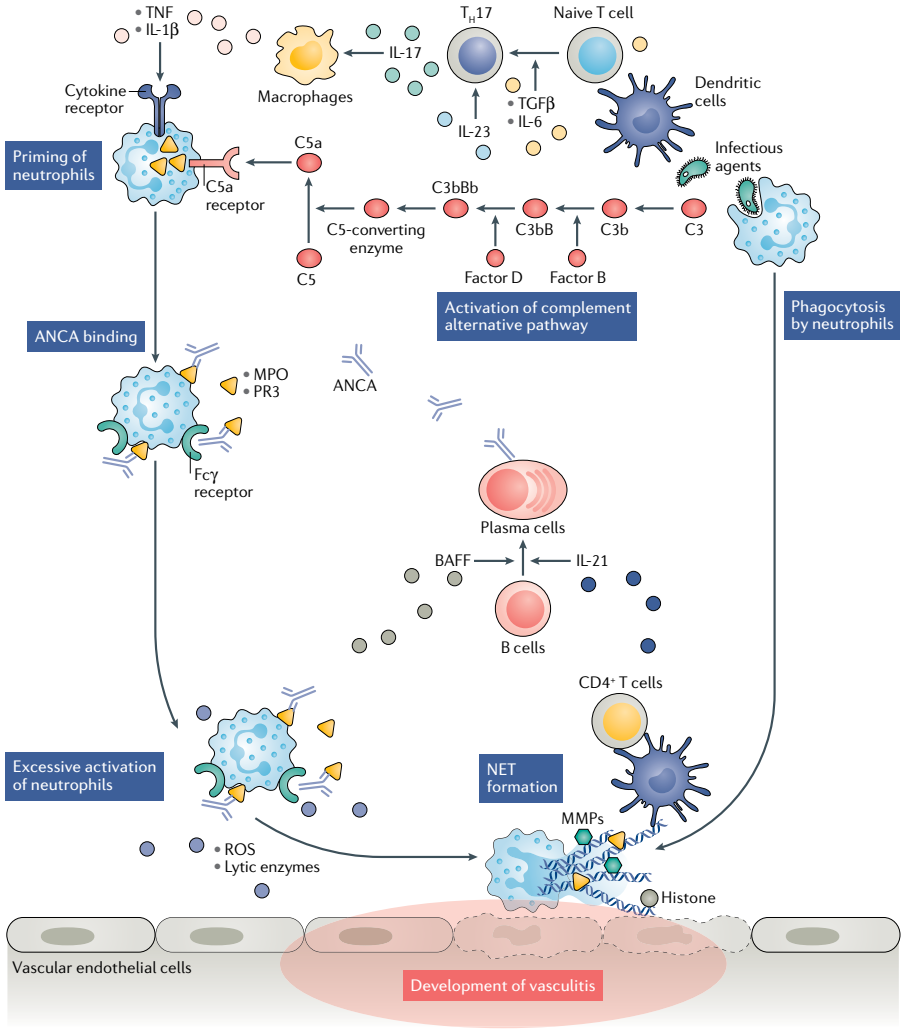


1) Priming of neutrophils
   - macrophage activation
   - phagocytosis by neutrophils

2) NETs formation
   - release of granules
   - antigen presentation
   - ANCA production

3) Excessive activation of neutrophils

Nakazawa D, Masuda S, Tomaru U, Ishizu A. Pathogenesis and therapeutic interventions for ANCA-associated vasculitis. Nat Rev Rheumatol. 2019;15(2):91–101.

ANCA Production

- Three essential steps

  1) Modification of antigenicity by mixture of chromatin fibers and with contents release from neutrophilic granules and binding to DNA during NETs formation

  2) Incomplete degradation of NETs and distuption of tolerogenicity towards MPO and PR3

  3) Antigen presentation and continuous stimulation of neutrophils

Materials and Methods

- Synthesis of peptides with homology to MPO
- Generation of MPO:I-A^b tetramers
- Plasmids and Staphylococcus aureus strains
- Induction and assessment of T-cell responses
- Induction and assessment of anti-MPO antibody responses
- ELISAs for anti-MPO and anti-6PGD antibodies
- Induction of mouse anti-MPO glomerulonephritis.

Peptides

- All peptides were synthesized at > 95% purity
- Immunizations to induce $CD4^+$ T-cell responses were performed with 20-mers containing the core 11 amino acids → additional amino acids on either side enhance immunoreactivity

- $MPO_{409-428}$, $OVA_{323-339}$
  Treponema vincentii hypothetical $protein_{167-186}$
  Aspergillus fumigatus HEAT repeat $protein_{825-844}$
  Helicobacter pylori RNA polymerase factor $sigma\text{-}54_{163-182}$
  Bacteroides sp. chloramphenicol O- $acetyltransferase_{104-123}$
  S. aureus pSJH101-derived $6PGD_{391-410}$, Variants 1-4

MPO:I-A^b tetramers

- MHCII monomers were produced in High Five insect cells
- DNA encoding the I-A^b α- and β-chains and the mouse $MPO_{415-428}$ fused to the N-terminus of the β-chain
  → cloned into plasmidic vector and propagated in Sf9 insect cells
- Fos and Jun leucine zippers on the C-terminal ends to promote correct heterodimeric pairing
- BirA ligase recognition sequence for biotinylation and poly-histidine tag for purification

- → MPO:I-A^b monomers were purified from baculovirus infected High Five insect cell supernatants

- immobilized metal ion affinity, size exclusion and anion exchange chromatography

- MPO:I-A^b tetramers were assembled by the addition of Streptavidin-PE

Plasmids and S.aureus strains

- The pSJH101 plasmid was found within a clinical isolate of S. aureus JH1
- 0.004% SDS at 45 ˚C for 24 h
- confirm the presence or absence of the pSJH101 plasmid containing 6PGD PCR was performed on cell lysates
- 6PGD sequence (derived from pSJH101) was cloned into the tetracycline inducible pALC2073 plasmid
- S. aureus RN4220, which contains neither plasmids nor the 6PGD sequence, was transformed with either pALC2073 containing 6PGD or pALC2073 without 6PGD

Induction and assessment of T-cell response

- Mice were immunized with 10 μg s.c. of peptide emulsified in Freund's complete adjuvant (FCA)

- After 10 days draining lymph node cells were isolated
  → cultured in [^{3}H]-Thymidine proliferation assays
  IFN-γ and IL-17A ELISPOTs
- cultured in triplicate in supplemented RPMI media at 5 × 105 cells per well in the presence or absence of peptide or whole protein at 37 ˚C, 5% CO2

- 72 h in proliferation assays and 18 h in ELISPOTs

In vivo expansion of MPO-specific cells

- Mice were immunized with 10 μg of peptide
  → 7 days later lymph nodes and spleen were harvested

- cells were incubated with Live/Dead fixable Near IR Dead Cell Stain, anti- mouse CD4-Pacific Blue and “dump” antibodies (anti- mouse CD11c, CD11b, CD8a, B220-Alexa Fluor 488)
- The MPO:I-Ab tetramer^{+} gate was set based on the CD4+ live lymphocyte population

Induction and assessment of anti-MPO antibody responses

- C57BL/6 mice were immunized with 10 μg of either $OVA_{323-339}$, $MPO_{409-428}$, S. aureus pSJH101- derived $6PGD_{391-410}$ and Variants 1-4 on day 0,boosted on days 7 and 14
- Serum was collected from mice by cardiac puncture on day 28
- Thioglycolate induced peritoneal neutrophils were obtained from either $Mpo^{+/+}$ or $Mpo^{-/-}$ C57BL/6 mice – pANCA staining
- Pooled serum IgG was incubated with slides for 1 h, detected using a chicken anti-mouse Alexa Fluor 488 secondary antibody DAPI was used as a nuclear stain

ELISAs for anti-MPO and anti-6PGD antibodies

- cardiac puncture on day 28 for serum collection
- detection of anti-MPO IgG antibodies, anti-$MPO_{447-459}$ IgG antibodies by ELISA and inhibition ELISAs for the detection of anti-$MPO_{409-428}$ IgG antibodies
- For inhibition ELISA, serum IgG (10 μg ml−1) was pre-incubated with S. aureus pSJH101-derived $6PGD_{391-410}$
- transferred to an $MPO_{409-428}$ coated

- Human sera tested seperately (healthy, MPO-AAV, PR3-AAV)

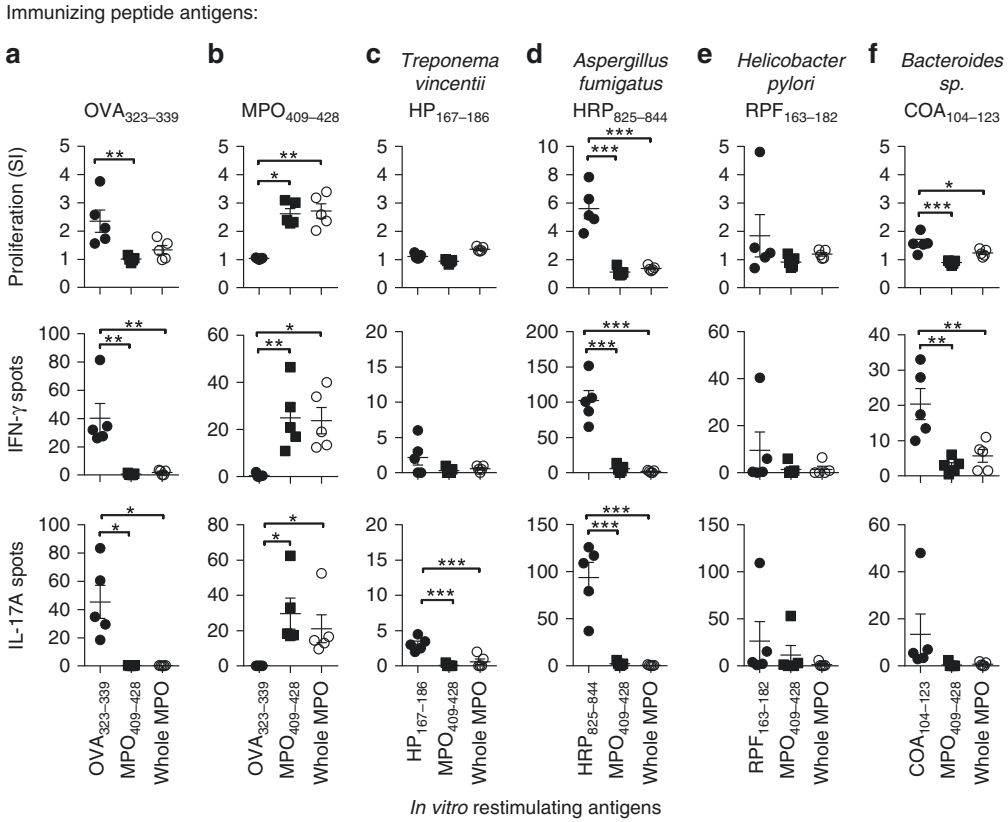
Induction of mouse anti-MPO glomerulonephritis

- C57BL/6 mice were immunized subcutaneously
- Proteins and peptides were injected on day 0 and 7 days later
- S. aureus strains were emulsified in Titermax and injected on days 0 and 7.
- Day 16 → MPO was deposited in glomeruli by injection of anti-mouse basement membrane antibodies
- Albuminuria, segmental glomerular necrosis, immune cell infiltration
- IFN-γ, TNF, IL-17A, and IL-6 in rmMPO stimulated splenocyte cultures was measured by cytometric bead array

Results

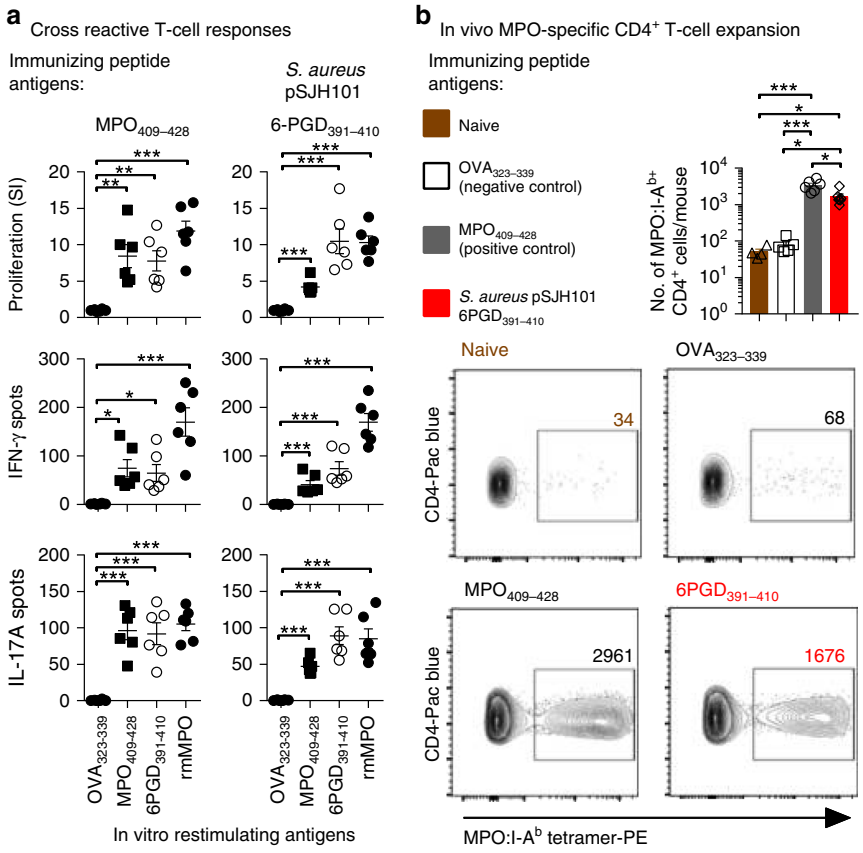
- Highly homologous peptides do not induce autoreactivity
- A S. aureus-derived peptide induces anti-MPO autoimmunity
- S. aureus clonal specificity for the 6PGD397–408 mimotope
- Immunization with 6PGD391–410 leads to anti-MPO nephritis
- S. aureus JH1 with pSJH101 immunization leads to nephritis
- Plasmid and strain independent 6PGD induced anti-MPO immunity
- MHCII promiscuous induction of anti-MPO cross-reactivity

Microbe-derived peptides with closest sequence homology to $MPO_{409–428}$ do not induce cross-reactivity to MPO



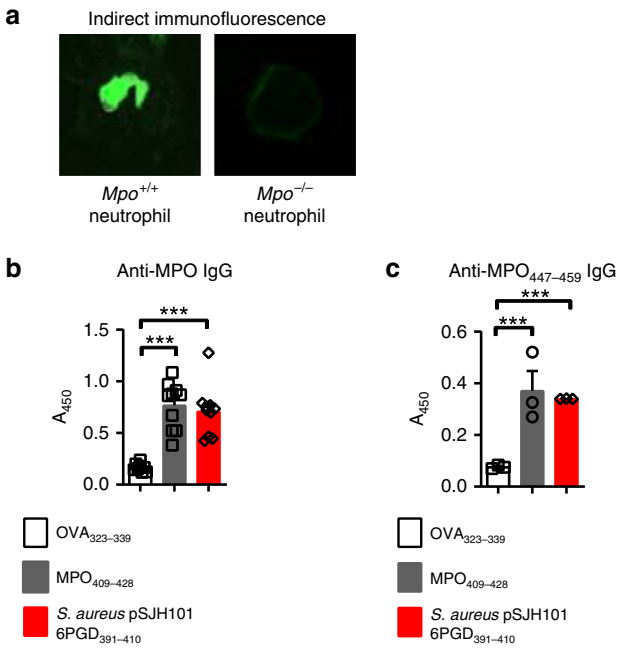
High sequence homology per se does not result in immunological cross-reactivity to MPO

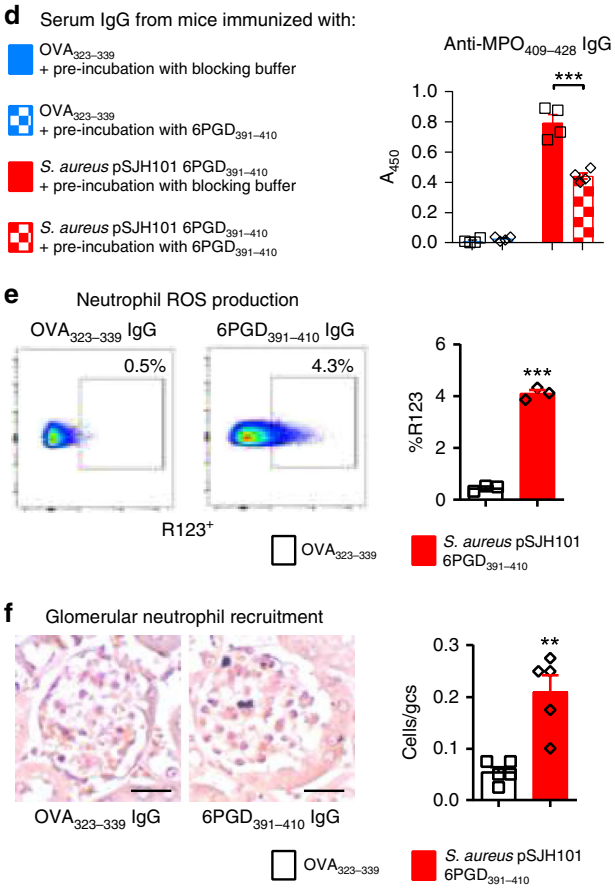
Immunization with S. aureus pSJH101-derived $6PGD_{391–410}$ induces anti-MPO T-cell responses.



$6PGD_{391–410}$ induces pro- inflammatory autoreactivity to MPO and expansion of $MPO_{415–425}$-specific CD4+ cells

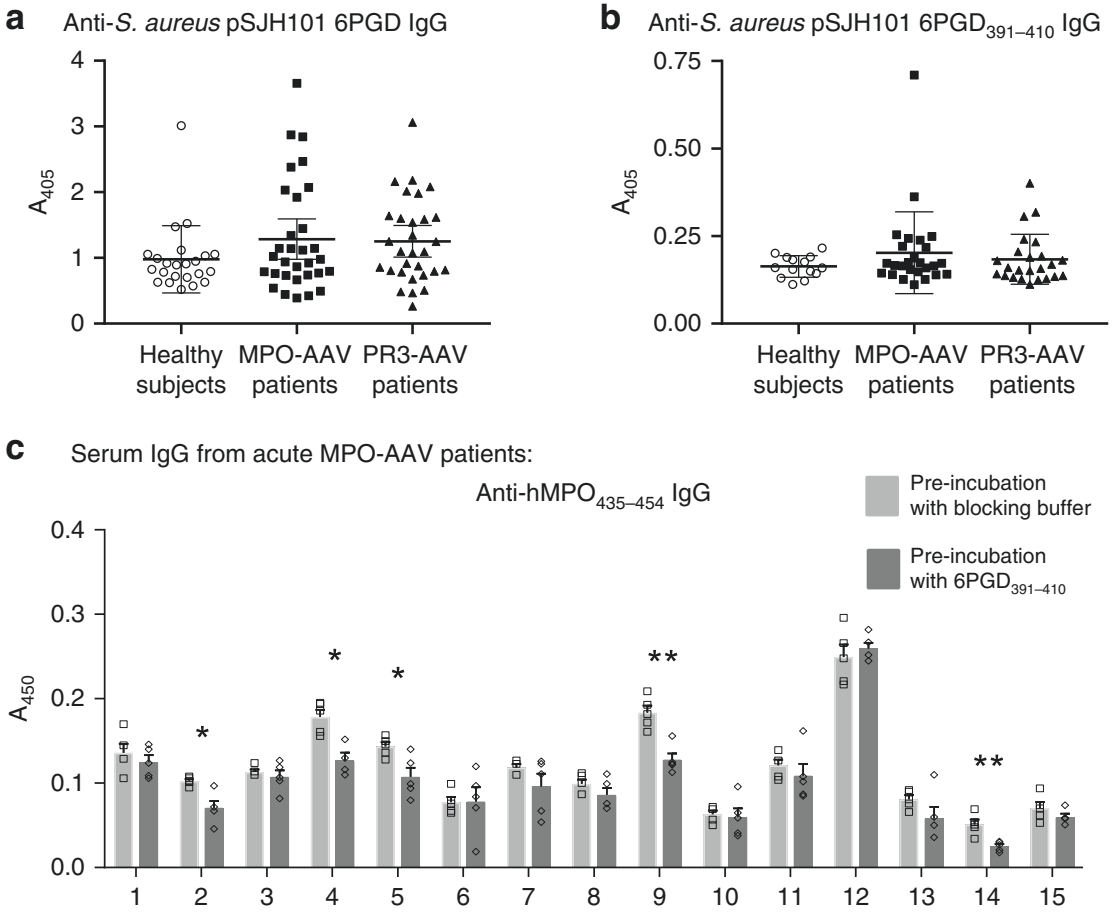
MPO-ANCA production in S. aureus pSJH101 6PGD$_{391–410}$ immunized mice





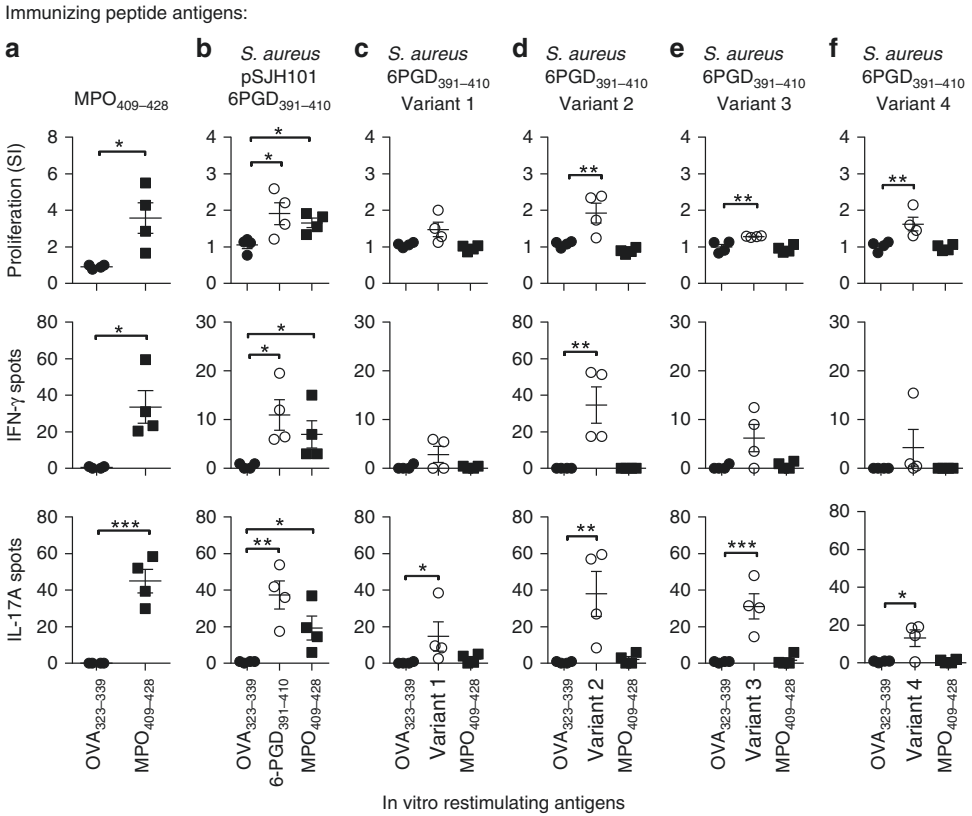
6PGD$_{391–410}$ mimotope inhibited autoantibody binding to MPO in mice.

Humoral responses to 6PGD and S. aureus pSJH101 $6PGD_{391–410}$ in humans



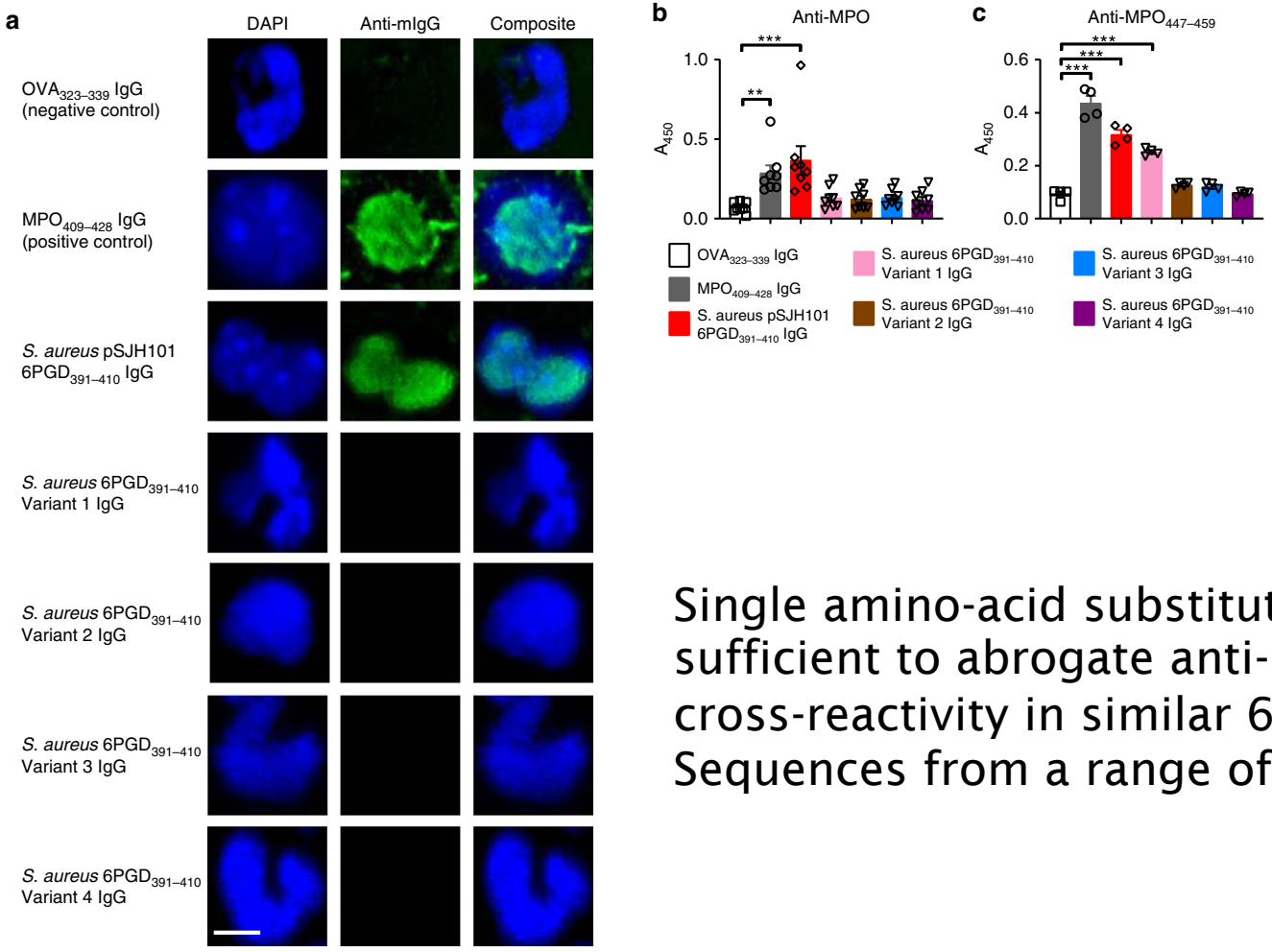
$6PGD_{391–410}$ inhibits binding to human $MPO_{435–454}$ (equivalent to mouse $MPO_{409–428}$) in 5/15 (33%) of humans with acute MPO-AAV

Anti-MPO T-cell responses after immunization with S. aureus-derived $6PGD_{391–410}$ sequences



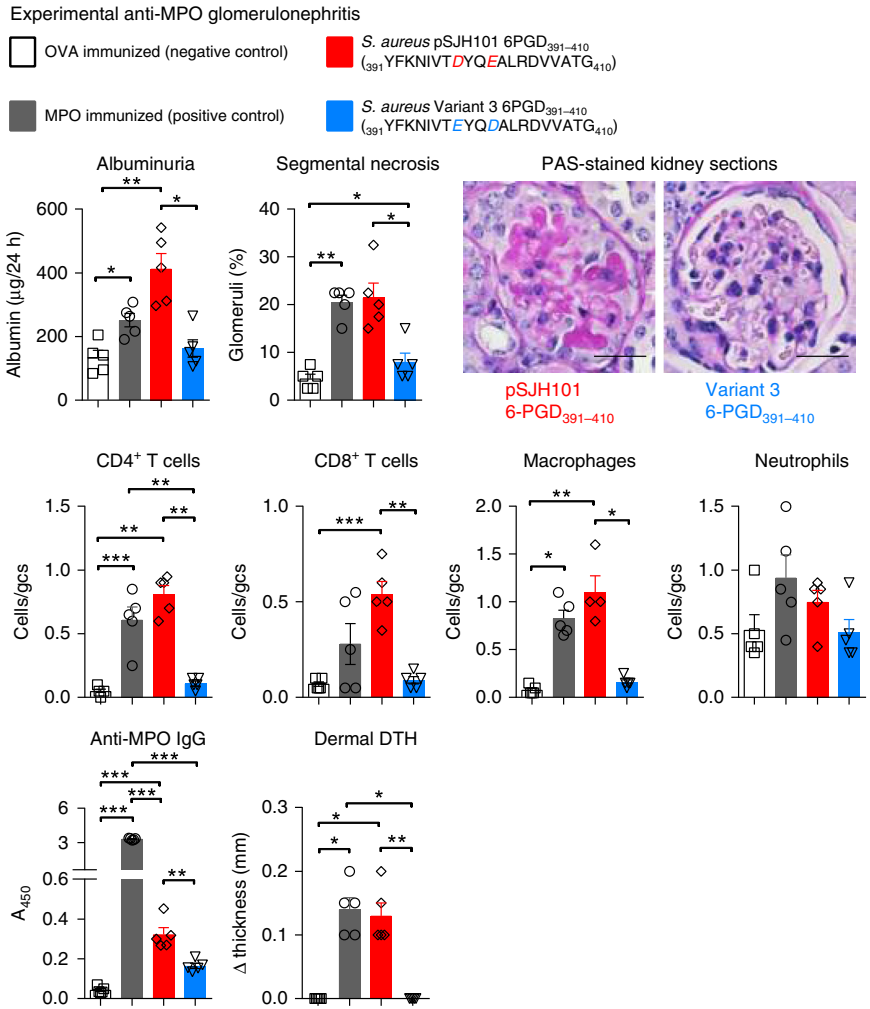
Single amino-acid sequence changes in the 6PGD peptide are sufficient to abrogate anti-MPO immunity

MPO-ANCA after immunization with S. aureus-derived $6PGD_{391–410}$ sequences

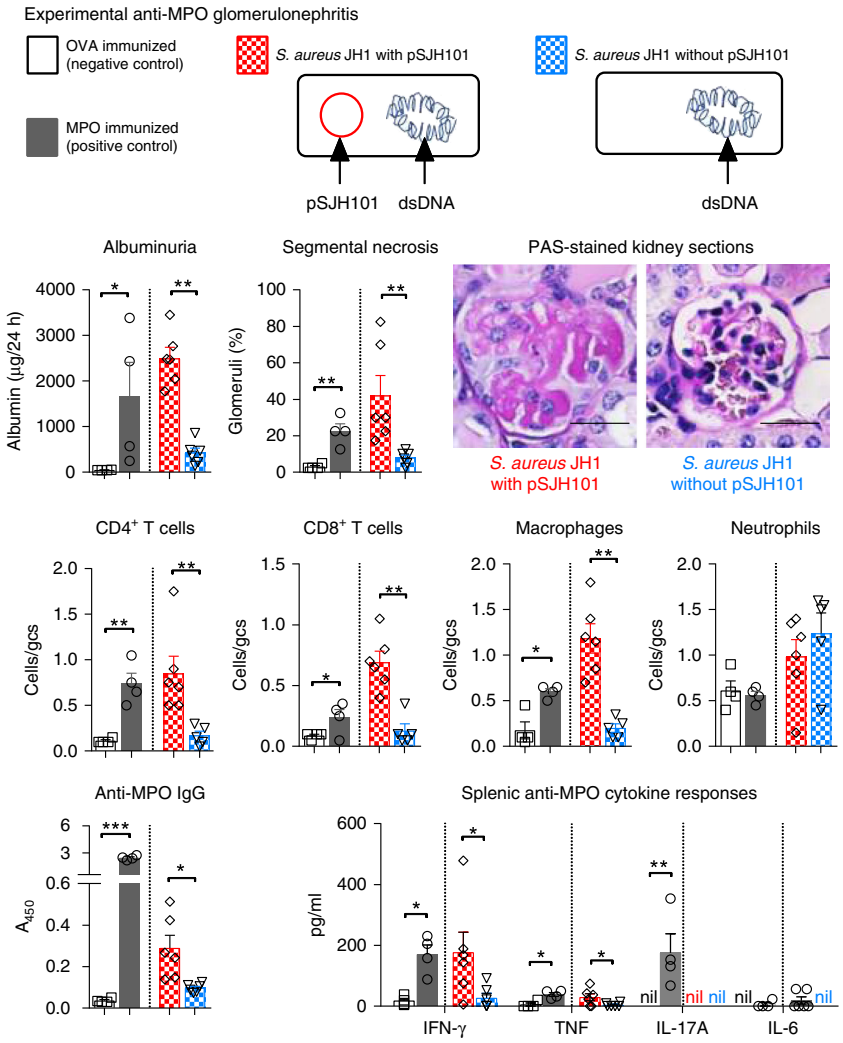


Single amino-acid substitutions are sufficient to abrogate anti-MPO cross-reactivity in similar $6PGD_{391–410}$ Sequences from a range of S. aureus strains

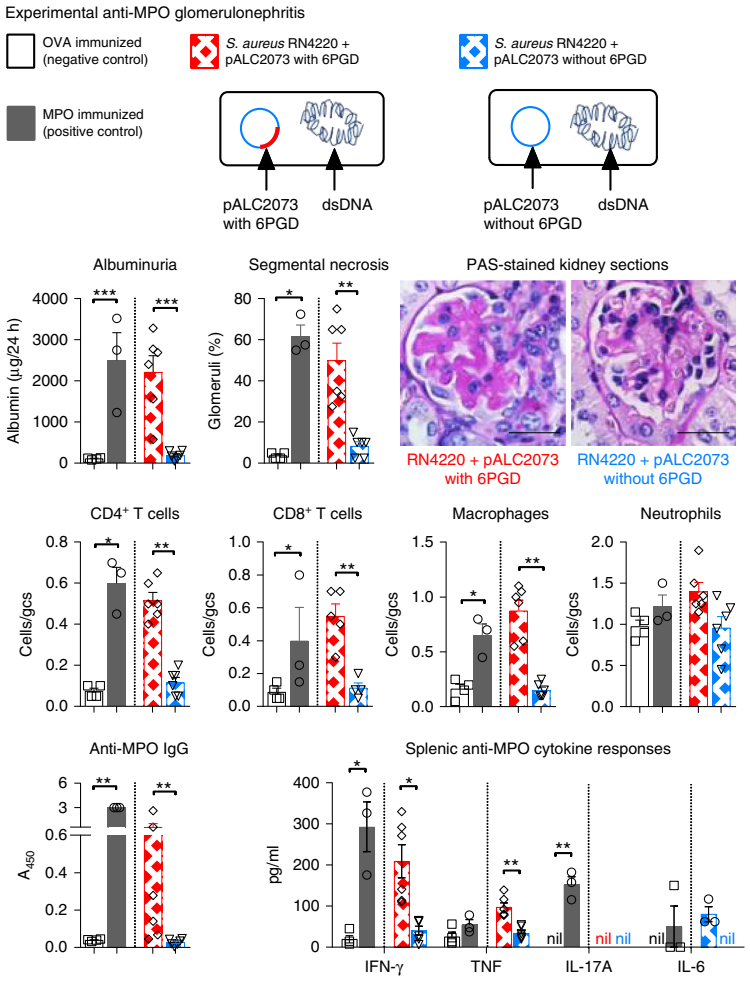
Experimental anti-MPO glomerulonephritis in S. aureus pSJH101 6PGD$_{391–410}$ immunized mice



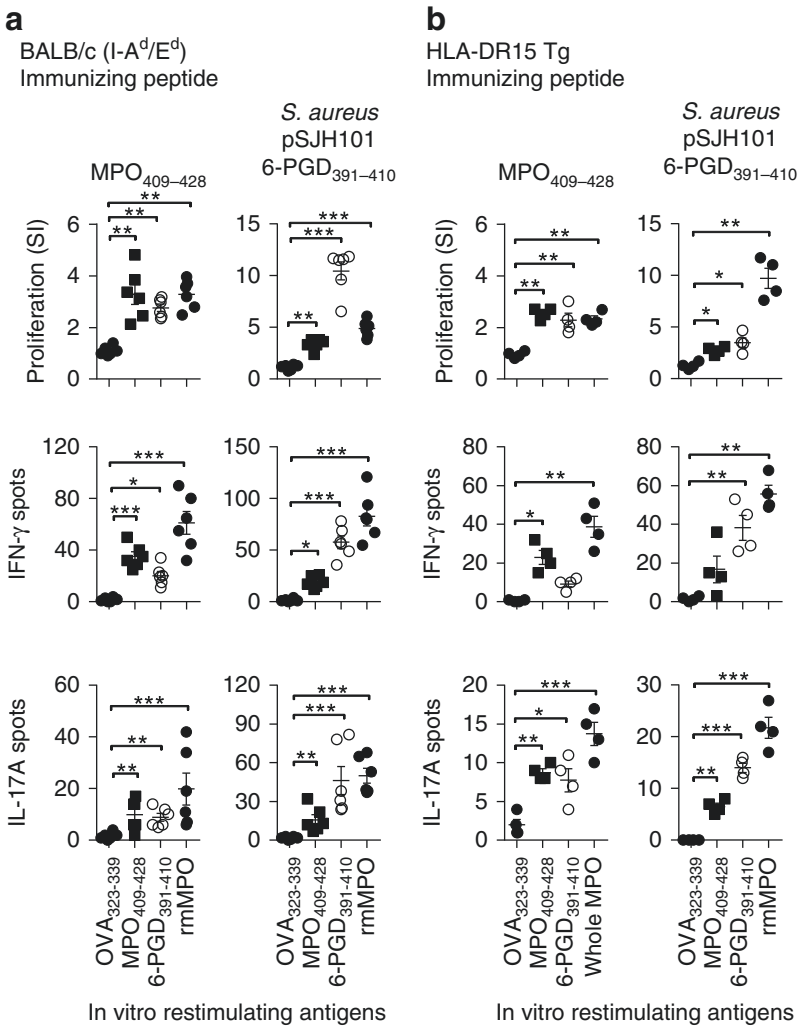
Experimental anti-MPO glomerulonephritis in mice injected with S. aureus containing pSJH101 6PGD$_{391–410}$



Experimental anti-MPO glomerulonephritis in mice injected with S. aureus RN4220 containing pALC2073



Anti-MPO T-cell responses in other S. aureus pSJH101 $6PGD_{391–410}$ immunized mouse strains



Conclusion

- PR3-AAV is classically associated with S.aureus and reports also implicate S. aureus infections in MPO-AAV
- links between the loss of tolerance to MPO and microbial-derived peptides are unclear
- S. aureus-derived peptides with sequence similarities to $MPO_{441-451}$ identified
  → plasmid- derived peptide (6PGD) that induces anti-MPO immunoreactivity

- → structural determinants rather than sequence similarity as predictor of molecular mimicry

- Unlikely that $6PGD_{391-410}$ mimotope is the sole factor that determines loss of tolerance to MPO
- frequency of antibodies to the 6PGD protein and peptide, multiple genetic and environmental factors that contribute to the development of MPO-AAV.

- $6PGD_{391-410}$ exposure may be a precipitating factor in the loss of tolerance to MPO and the development of MPO-AAV.
- Plasmids may transfer a tendency to autoreactivity (S. aureus RN4220 with pALC2073-6PGD)

- pSJH101 6PGD391–410 as an MPO cross-reactive mimotope peptide

- Induce loss of tolerance to MPO and experimental anti-MPO glomerulonephritis and MPO-AAV

- Derived from a plasmid found in certain strains of S. aureus
  → implicating role of plasmid-derived antigens in the loss of tolerance to self-antigens.

Thank you for your attention