BRIEF COMMUNICATION

Myeloperoxidase Peptide–Based Nasal Tolerance in Experimental ANCA–Associated GN


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ABSTRACT

Less toxic treatment options for patients with myeloperoxidase (MPO)-ANCA–associated GN are needed. Using an established murine model of focal necrotizing GN mediated by autoimmunity to MPO (autoimmune anti–MPO GN), we assessed the capacity for nasal tolerance induced by nasal insufflation of the immunodominant nephritogenic MPO peptide (MPO409–428) to attenuate this disease. Compared with mice that received an irrelevant immunodominant ovalbumin (OVA) peptide, OVA323–339, mice that received MPO409–428 were protected from the development of humoral and cell-mediated autoimmunity to full-length MPO and the development of GN. In mice with established anti–MPO autoimmunity, nasal insufflation of MPO409–428 as a therapeutic attenuated anti–MPO GN. To investigate the nature of this induced tolerance, we isolated CD4+ T cells from the upper airway draining lymph nodes of both OVA323–339- and MPO409–428-tolerized mice. Adoptive transfer of CD4+ T cells from MPO409–428- but not OVA323–339-tolerized mice to animals with established anti–MPO autoimmunity attenuated the subsequent development of GN, confirming that the immunosuppression induced by these T cells is antigen specific. Ex vivo studies showed that nasal tolerance to MPO is mediated by both conventional and induced T regulatory cells. The strong homology between the pathogenic human MPO B cell epitope recognized by ANCA in patients with acute vasculitis and the nephritogenic murine T cell MPO epitope emphasizes the clinical relevance of this study.


ANCA–associated GN is the most common form of crescentic GN. Among patients with ANCA–associated GN, most have ANCA that reacts to myeloperoxidase (MPO). MPO, the major constituent of the azurophilic granules in neutrophils, is a heme-containing enzyme that plays a key role in the generation of injurious oxidant products. These products are potent antimicrobial agents1 but can also mediate tissue injury in GN.2

Cyclophosphamide is still the standard of care for patients with MPO–ANCA–associated GN.3 This drug has adverse side effects, including severe and life-threatening infections, sterility, acute myeloid leukemia, bladder cancer, and hemorrhagic cystitis.4–6 New, more targeted, less toxic therapies are needed. Experimental animal models have provided a depth of understanding of the pathogenesis of MPO–ANCA–associated GN. This disease begins with the loss of tolerance and development of autoimmunity to MPO. In 2012, the dominant disease–inducing murine MPO epitope, MPO409–428, was defined7 and shown to have high homology with the immunodominant human MPO B cell epitope recognized by sera from patients with active MPO–ANCA–associated GN.8

Discovery of an immunodominant nephritogenic MPO peptide offers the opportunity for the development of peptide–based immunomodulatory therapies that may provide antigen–specific immunosuppression capable of restoring immune homeostasis with antigenic specificity.9 Mucosal tolerance is a phenomenon, whereby peripheral tolerance is maintained by administering the antigen of interest through the oral or nasal/respiratory routes.10–12 The induction of tolerance by mucosal administration of autoantigens or immunodominant/immunogenic peptides has been reported in various experimental models of autoimmune diseases, including encephalomyelitis,13,14 myasthenia gravis,15,16 uveitis,16,17 diabetes,18–20 arthritis,21–23 and autoimmune GN.24,25 In several of
these studies, nasal administration of the immunodominant peptide was more effective in inducing tolerance than oral peptide administration.\textsuperscript{22,23,25,26} Despite considerable research in this area, the mechanisms involved in the induction of mucosal tolerance are uncertain.

In these studies, we examined the effects of nasal administration of the mouse immunodominant MPO T cell peptide (mouse sequence numbering MPO\textsuperscript{409–428}; mapped to the heavy-chain region of the MPO molecule) in the prevention and treatment of GN in experimental autoimmune anti–MPO GN.\textsuperscript{8} Induction of mucosal tolerance is dependent on the dose of antigen administered. In some experimental models of autoimmune diseases, administration of high-dose peptide is more effective to treat established disease by favoring the induction of clonal anergy or deletion. Low-dose therapy prevents the development of autoimmunity by generating a subset of regulatory T cells (Tregs) independent on the dose of antigen administered. In some experimental models of autoimmune diseases, administration of high-dose peptide is more effective to treat established disease by favoring the induction of clonal anergy or deletion. Low-dose therapy prevents the development of autoimmunity by generating a subset of regulatory T cells (Tregs) independent on the dose of antigen administered. In some experimental models of autoimmune diseases, administration of high-dose peptide is more effective to treat established disease by favoring the induction of clonal anergy or deletion. Low-dose therapy prevents the development of autoimmunity by generating a subset of regulatory T cells (Tregs) independent on the dose of antigen administered. In some experimental models of autoimmune diseases, administration of high-dose peptide is more effective to treat established disease by favoring the induction of clonal anergy or deletion. Low-dose therapy prevents the development of autoimmunity by generating a subset of regulatory T cells (Tregs) independent on the dose of antigen administered.

To determine the optimal dosage of MPO\textsuperscript{409–428} in nasal tolerance induction in experimental autoimmune anti–MPO GN, we administered different dosages of MPO\textsuperscript{409–428} (cumulative dose of 3, 30, or 150 \(\mu\)g divided over three consecutive daily administrations; days –3, –2, and –1) by nasal insufflation before the induction of anti–MPO T cell autoimmunity by subcutaneous MPO immunization (day 0). Nasal insufflation of 150 \(\mu\)g MPO\textsuperscript{409–428} effectively limited the development of anti–MPO T cell autoimmunity compared with the effect of lower dosages of MPO\textsuperscript{409–428} (cumulative dose of 3 or 30 \(\mu\)g), which were assessed by the frequency of MPO\textsuperscript{409–428} or whole-MPO–specific IFN–\(\gamma\)– and IL-17A–producing cells in nasal insufflation of either MPO\textsuperscript{409–428} or the irrelevant immunodominant ovalbumin (OVA) peptide OVA\textsuperscript{323–339} (days –3, –2, and –1). Tolerized mice were immunized with MPO\textsuperscript{409–428} emulsified in Freund’s complete adjuvant, and immune response was boosted with MPO\textsuperscript{409–428} emulsified in Freund’s incomplete adjuvant (days 0 and 7, respectively). GN was triggered by intravenous injection of a subnephritogenic dose of sheep anti–mouse glomerular basement membrane (GBM) antibody for the purpose of recruiting neutrophils to the glomeruli to deposit MPO (days 14 and 15). Immune responses and renal injury assessed 4 days later before any adaptive immune response were directed against the sheep anti–mouse GBM antibody. Anti-MPO responses were measured by isolating draining LN cells and culturing these cells with whole MPO \textit{in vitro}. Compared with mice that received OVA\textsuperscript{323–339} nasal insufflation of mice with MPO\textsuperscript{409–428} reduced anti–MPO proliferative responses of cells from MPO\textsuperscript{409–428}–immunized mice (Figure 1A) as well as the frequency of IFN–\(\gamma\)– and IL-17A–producing cells (Figure 1, B and C). Furthermore, nasal insufflation of MPO\textsuperscript{409–428} decreased circulating MPO-ANCA IgG titers (Figure 1D). Collectively, these results indicate that nasal administration of

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\caption{Nasal insufflation of MPO\textsuperscript{409–428} prevents the development of anti-MPO GN. LN cells draining MPO immunization sites were restimulated with whole-MPO protein \textit{in vitro}. (A) Mice that received MPO\textsuperscript{409–428} nasally (n=8) had reduced MPO–specific T cell proliferation with reduced frequency of (B) IFN–\(\gamma\)– and (C) IL-17A–producing cells compared with OVA\textsuperscript{323–339} (n=8). (D) Circulating MPO-ANCA titers were diminished in MPO\textsuperscript{409–428}–tolerized mice and similar to naïve C57BL/6 mice (dotted line). Nasal insufflation of MPO\textsuperscript{409–428} resulted in a marked reduction in (E) albuminuria and (F and G) glomerular segmental necrosis and (H) fewer infiltrating glomerular CD4\textsuperscript{+} T cells, macrophages, and neutrophils. *\textit{P}<0.05; **\textit{P}<0.01; ***\textit{P}<0.001. gcs, Glomerular cross-section; M\(\Phi\), macrophages; OD, optical density; PMN, polymorphonuclear leukocytes.}
\end{figure}
MPO \textsubscript{409–428} has the capacity to suppress the development of anti-MPO autoimmunity. The reduced systemic anti-MPO autoimmunity in MPO \textsubscript{409–428} nasally insufflated mice limited the development of GN with reduced proteinuria compared with mice receiving OVA\textsubscript{323–339} (Figure 1E). This was associated with fewer glomeruli developing segmental glomerular necrosis (Figure 1, F and G) and a similar reduction in the accumulation of effector leukocytes (CD4\textsuperscript{+} T cells, macrophages, and neutrophils) in glomeruli of mice nasally insufflated with MPO\textsubscript{409–428} compared with control OVA\textsubscript{323–339}–t lerized mice (Figure 1H).

To determine the efficacy of administering MPO\textsubscript{409–428} nasally as a potential therapeutic in experimental anti-MPO GN, mice with established MPO autoimmunity were nasally insufflated with OVA\textsubscript{323–339} or MPO\textsubscript{409–428} (days 10–12), and the severity of GN was assessed on day 20. Compared with nasal insufflation of OVA\textsubscript{323–339}, MPO\textsubscript{409–428} treatment attenuated GN, with fewer glomeruli exhibiting segmental necrosis (Figure 2, A and B) and fewer intraglomerular CD4\textsuperscript{+} T cells and macrophages (Figure 2, C and D). No difference in glomerular neutrophils was observed between groups (Figure 2E). Functional renal injury as measured by proteinuria paralleled the attenuated histologic injury observed in MPO\textsubscript{409–428}–treated mice with anti-MPO GN (Figure 2F). Collectively, these results show that nasal insufflation of MPO\textsubscript{409–428} can prevent the development of anti-MPO autoimmunity and attenuate anti-MPO GN, even when administered after induction of active anti-MPO autoimmunity.

The mechanisms involved in the development of mucosal tolerance to autoantigens are not well understood. It has been previously shown that nasal tolerance induces antigen–specific Tregs in a model of delayed type hypersensitivity responses to OVA as a foreign antigen and that CD4\textsuperscript{+} T cell transfer from OVA–tolerized mice can prevent the development of delayed type hypersensitivity to OVA in recipient mice.27 To define mechanisms by which nasal administration of MPO\textsubscript{409–428} may prevent anti-

**Figure 2.** Nasal insufflation of MPO\textsubscript{409–428} in mice with established MPO autoimmunity (day 14) is therapeutic in the development of anti-MPO GN. (A and B) Nasal insufflation of MPO\textsubscript{409–428}–attenuated GN (n=8) assessed histologically by the percentage of segmental glomerular necrosis and (C) infiltration of glomerular CD4\textsuperscript{+} T cells and (D) macrophages compared with OVA\textsubscript{323–339}–t lerized mice (n=8). (E) No difference in numbers of glomerular neutrophils between groups. (F) Renal function as measured by proteinuria was attenuated in mice nasally tolerized with MPO\textsubscript{409–428}. gcs, Glomerular cross-section. *P<0.05; **P<0.01; ***P<0.001.
MPO autoimmunity and attenuate anti-MPO GN, we assessed Treg function in an *ex vivo* coculture system using Foxp3-GFP mice. Anti-MPO Teffs were generated by immunizing Foxp3-GFP mice subcutaneously with MPO. Draining LNs from MPO immunization sites were harvested 10 days later, and CD4⁺Foxp3⁻ Teff sorted cells were isolated. These anti-MPO CD4⁺ Teffs were cocultured with either CD4⁺Foxp3⁺ or CD4⁺Foxp3⁻ cells isolated from upper respiratory tract draining LNs from Foxp3-GFP mice that received either MPO409–428 or OVA323–339 intranasally (150-µg cumulative dose). Cocultured cells were incubated with whole MPO. MPO409–428-tolerized CD4⁺ T cells, regardless of Foxp3 expression, significantly reduced anti-MPO Teff cell proliferation, whereas OVA323–339–tolerized CD4⁺Foxp3⁺ or CD4⁺Foxp3⁻ cells did not (Figure 3, A and B). Enhanced CD4⁺ (Foxp3⁺ and Foxp3⁻) immunoregulatory cell function observed from mice nasally insufflated with MPO409–428 was also associated with increased production of IL-10 compared with CD4⁺ cells from OVA323–339-tolerized mice after MPO recall challenge (Figure 3, C and D). Characterization of CD4⁺Foxp3⁺ and CD4⁺Foxp3⁻ cells from upper respiratory draining LNs of MPO409–428-tolerized mice for Treg cell surface markers showed increased expression of inducible T-cell co-stimulator compared with upper respiratory draining LN cells from naïve mice (Figure 3E). Interestingly, among the MPO409–428 nasally tolerized mice, only the CD4⁺Foxp3⁻ population of upper respiratory draining LN cell expression of cytotoxic T-lymphocyte-associated protein 4 and Folate R4 was significantly increased compared with CD4⁺ cells from naïve mice, an increase not observed in CD4⁺Foxp3⁺ cells (Figure 3, F and G). No difference in glucocorticoid-induced tumour-necrosis-factor-receptor expression was observed between groups (Figure 3H).

Given that nasal insufflation of MPO409–428 enhances the regulatory function of CD4⁺ cells and also, generates both conventional Tregs (CD4⁺Foxp3⁺) and Type 1 regulatory (CD4⁺Foxp3⁻)
CD4+ cells were adoptively transferred to mice with established anti-MPO autoimmunity (day 10) to determine whether they can functionally suppress the development of anti-MPO GN. MPO-immunized mice that did not receive tolerized MPO409–428 CD4+ T cells were used as controls. Transfer of MPO409–428–tolerized CD4+ T cells reduced the percentage of glomeruli with segmental glomerular necrosis (Figure 4, A and B) and proteinuria (Figure 4C). This was associated with decreased numbers of glomerular CD4+ T cells and macrophages (Figure 4, D and E). No difference in glomerular neutrophils was observed between groups (Figure 4F). To ascertain if the therapeutic nature of adoptively transferring nasally tolerized CD4+ T cells is antigen specific, OVA323–339–tolerized CD4+ T cells were adoptively transferred to mice with established anti-MPO autoimmunity. MPO-immunized mice that did not receive OVA323–339–tolerized CD4+ T cells served as controls. OVA323–339–tolerized CD4+ T cells did not attenuate GN, and disease severity was similar to control mice that did not receive cells (Supplemental Figure 2).

In conclusion, this study reveals that donor Foxp3-GFP mice were isolated by magnetic cell sorting (CD4+ T cells) and CD4+Foxp3+T cells compared with CD4+ T cells from naive C57BL/6 mice. (H) No difference in expression of GITR was observed between groups. *P<0.05; **P<0.01. CTLA4, cytotoxic T-lymphocyte-associated protein 4; GITR, glucocorticoid-induced tumour-necrosis-factor-receptor; ICOS, inducible T-cell co-stimulator.

Assessment of Systemic Autoimmune Responses to MPO
For in vitro measurement of MPO–specific cell proliferation, draining LNs from experimental mice were harvested, and single-cell suspensions were prepared. Cells were seeded at 5×10^5 cells per well. IFN-γ and IL-17A production was assessed by ELISPOT (Mouse IFN-γ ELISPOT Kit and Mouse IL-17A ELISPOT Kit; BD Biosciences), with draining LN cells restimulated with 5 μg/ml either MPO409–428 or recombinant MPO for 18 hours. IFN-γ- and IL-17A–producing cells were enumerated with an automated ELISPOT reader system. For the T cell proliferation assay, cells were restimulated with 10 μg/ml MPO409–428 or recombinant MPO and incubated for 72 hours. During the last 18 hours, 0.5 μCi [3H]-thymidine (PerkinElmer) was added. Cells were harvested, and [3H]-thymidine incorporation was measured as previously described. Measurement of circulating MPO-ANCA titers in sera was measured by ELISA as previously described.

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**CONCISE METHODS**

**Mice and Experimental Design**
Eight- to twelve-week-old male C57BL/6 (wild type) and Foxp3-GFP mice (from Alexander Rudensky, University of Washington, Seattle, WA) were bred and housed in specific pathogen–free conditions at Monash Medical Centre Animal Facilities, Monash University. Studies were approved by the Monash University Animal Ethics Committee in accordance with the Australian National Health and Medical Research Council animal experimentation guidelines.

Peptides MPO409–428 (PRWNGEKLY-QEARKIVGAMV) and the control irrelevant peptide MPO409–428 (ISQAVHAHAINEAGR) were synthesized at Mimotopes Australia. To determine the optimal dose of intranasal MPO409–428, three groups of mice (n=4) were given MPO409–428 in 20 μl saline nasally on 3 consecutive days (days −3, −2, and −1) with total cumulative doses of 3, 30, and 150 μg. Control mice received 20 μl saline nasally (n=4). On day 0, mice were immunized with 25 μg MPO409–428 in Freund’s Complete Adjuvant subcutaneously at the base of tail, and they were killed on day 10. Draining LNs from MPO immunization sites were aseptically removed for analysis of systemic immunity.

For prevention of experimental anti-MPO GN, 150 μg MPO409–428 or OVA323–339 in 20 μl saline was administered nasally for 3 consecutive days. Mice were then immunized on day 0 with 100 μg MPO409–428 in Freund’s Complete Adjuvant subcutaneously at the base of tail, and on day 7, they were immunized with 100 μg MPO409–428 in Freund’s Incomplete Adjuvant subcutaneously at the neck. On days 14 and 15, GN was triggered by administration of 1 mg sheep anti-mouse GBM globulin intravenously. Mice were humanely culled on day 18. To treat mice with established anti-MPO autoimmunity, nasal administration of a cumulative dose of 150 μg MPO409–428 or OVA323–339 began 14 days post-MPO409–428 immunization. GN was triggered by sheep anti-mouse GBM globulin on days 17 and 18, and mice were culled on day 22.

For adoptive transfer of tolerized CD4+ T cells to mice with established anti-MPO autoimmunity, donor Foxp3-GFP mice were nasally insufflated with MPO409–428 or OVA323–339 as above. Four days after initial administration of MPO409–428, upper airway draining LNs were collected, single-cell suspensions were obtained, and CD4+ T cells were isolated by magnetic cell sorting (CD4+ T Cell Isolation Kit; Miltenyi Biotec, North Ryde, Australia); 5×10^6 MPO409–428–tolerized CD4+ T cells or OVA323–339–tolerized CD4+ T cells were adoptively transferred to MPO-immunized mice on day 10, and control MPO–immunized mice received no cells. GN was triggered and culled as described above.
Assessment of Renal Injury

Mice were housed individually in cages for urine collection over the final 24 hours of the experiment. Proteinuria was measured by Bradford’s method using Bradford’s reagent and calculated from the 24-hour urine volume and the urinary protein concentration (expressed as milligrams per 24 hours). The proportions of glomerular segmental necrosis were determined by evaluating, using coded slides, a minimum of 30 glomerular cross-sections per mouse on periodic acid–Schiff reagent–stained kidneys. For assessment of glomerular macrophages, neutrophils, and CD4+ T cell infiltrations, frozen periodate lysine paraformaldehyde–fixed kidneys were cut at 6 μm and stained using a three-layer immunoperoxidase technique. The primary mAbs used were FA/11 (anti-CD68) for macrophages, RB6–8C5 (anti-Gr-1) for neutrophils, and GK1.5 (anti–CD4+ T cells) for CD4+ T cells. A minimum of 30 glomeruli per mouse was assessed, and results are expressed as cells per glomerular cross-section.

Statistical Analyses

Data are given as means ± SEMs. An unpaired t test was used for statistical analysis, and one-way ANOVA and Tukey’s post hoc test were used for multiple group comparisons (GraphPad Prism; GraphPad Software Inc., San Diego, CA). Differences were considered to be statistically significant at P<0.05.

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DISCLOSURES

None.

REFERENCES


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