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Introduction
Neuromyelitis optica (NMO) is an inflammatory demyelinating disease that primarily affects the spinal cord and optic nerves, leading to paralysis and visual impairment (1, 2). Serum autoantibodies against astrocyte water channel aquaporin-4 (AQP4), called NMO immunoglobulin G (NMO-IgG), are present in most NMO patients and are believed to be pathogenic (3–5). Human NMO lesions show marked cellular infiltration with eosinophils, neutrophils, and macrophages, loss of astrocyte AQP4 and glial fibrillary acidic protein, perivascular deposition of activated complement, and demyelination (6–9). Current NMO therapies include immunosuppression, plasma exchange and B cell depletion (10, 11).

Eosinophil infiltration is a prominent feature in NMO lesions but not in other demyelinating diseases including multiple sclerosis (2, 7). Eosinophils are also found in cerebrospinal fluid in NMO (9). They can damage cells by the release of toxins contained in intracellular granules, including eosinophilic granule major basic protein (MBPe), eosinophil cationic protein (ECP), eosinophil peroxidase, and eosinophil-derived neurotoxin (12). Eosinophil degranulation can be triggered by immunoglobulin binding to Fc receptors and by soluble effectors such as complement anaphylotoxins C3a and C5a, chemokines, and lipid mediators (13). Differentiation and maturation of eosinophils in bone marrow is under the control of transcription factor GATA-1, as well as IL-3, IL-5, and GM-CSF (12). Eosinophils are normally present in the gastrointestinal tract, as well as in the thymus, mammary gland, and uterus. Eosinophilia and exaggerated eosinophil responses occur in some infections, asthma, hypereosinophilic syndrome, eosinophilic gastrointestinal diseases, and certain tumors (14). Eosinophil-based therapies target eosinophil production or eosinophil-derived products, which broadly include glucocorticoids, myelosuppressive drugs, leukotriene antagonists and inhibitors, some second-generation antihistamines, cromoglicate, tyrosine kinase inhibitors, IFN-α, and anti–IL-5 antibodies (13, 14).

Here, we utilize mouse models of NMO, including ex vivo spinal cord slice cultures (15) and a new in vivo intracerebral infusion model, as well as cell cultures, to investigate the mechanisms of eosinophil-dependent NMO pathology. We report evidence of antibody-dependent cell-mediated cytotoxicity (ADCC) and complement-dependent cell-mediated cytotoxicity (CDCC), in which eosinophil toxins secreted upon degranulation injure target cells. We also show protection against eosinophil-dependent NMO pathology by small-molecule inhibitors of eosinophil degranulation, including 2 widely used second-generation antihistamines.

Results
Eosinophils produce NMO-IgG–dependent cytotoxicity. We performed in vitro experiments with murine eosinophils cultured from bone marrow. Cultures were treated with SCF and FLT3 ligand (FLT3-L) for 4 days to promote eosinophil progenitor cell growth, followed by IL-5 for 10 days to simulate eosinophil proliferation, and then GM-CSF for 24 hours before experiments to induce Fc receptor surface expression (16, 17). We produced a nearly pure suspension of eosinophils after 14 to 16 days in culture, showing cytoplasmic MBPe expression (Figure 1A).

The ability of eosinophils to produce NMO-IgG–dependent ADCC was studied after 3 hours’ incubation of AQP4-expressing CHO cell cultures with NMO-IgG and eosinophils. The percentage and density of live cells were quantified by live/dead (green/red) cell staining (Figure 1B). Substantial cell death was produced when NMO-IgG and eosinophils were present. Little cell death was seen in untreated (control) cells, in cells incubated with NMO-IgG or eosinophils alone, or in AQP4-null cells incubated with NMO-IgG and eosinophils. Also, little cell death was seen when NMO-IgG was replaced by a mutated NMO-IgG lacking ADCC effector
Eosinophils produce NMO-IgG–dependent ADCC and CDCC in cell cultures. (A) Culture and characterization of eosinophils from murine bone marrow. Culture method shown at the left and MBP expression shown in the micrograph. Scale bar: 20 μm. (B) ADCC. AQP4-expressing CHO cells were incubated for 3 hours with 20 μg/ml NMO-IgG plus 300,000 eosinophils. Live/dead (green/red) staining is shown with percentage and density of live cells given below the micrographs (SEM, n = 10). Controls included untreated cultures, NMO-IgG or eosinophils alone, AQmabADCC plus eosinophils, and NMO-IgG plus eosinophils in nontransfected (AQP4-null) cells. Scale bar: 100 μm. (C) CDCC. Cells were incubated for 60 minutes with submaximal (5 μg/ml) NMO-IgG, 1% hc, and 100,000 eosinophils, with controls indicated. Percentage of dead cells quantified as in B (SEM, n = 10). Scale bar: 100 μm. Experiments were replicated 3 times. Eos, eosinophils; MPPs, multipotent progenitors.

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(AQmab\textsuperscript{CDC}) indicated that the eosinophil-dependent pathology was complement dependent (C1q-binding dependent), implicating a CDCC mechanism. C5a is a complement anaphylotoxin that promotes eosinophil chemotaxis and degranulation (18). Figure 3B shows a marked loss of GFAP and AQP4 in slice culture incubated with eosinophils in the presence of C5a (in the absence of NMO-IgG), but not in culture incubated with eosinophils or C5a alone. We also found that the effect of eosinophils could be mimicked by the addition of purified human ECP, an eosinophil granule toxin, together with submaximal NMO-IgG and complement (Figure 3C).

Eosinophil-dependent NMO pathology in an in vivo mouse model of NMO. Prior passive-transfer mouse models of NMO involving intracerebral brain injection of NMO-IgG and complement produced NMO-like pathology, but without eosinophil infiltration (19, 20). After testing multiple variations of existing models, including eotaxin and IL-5 infusion together with NMO-IgG and complement administration, we found that human-like NMO pathology could be produced by 3-day continuous intracerebral infusion of NMO-IgG and hc (Figure 4A). Immunofluorescence showed lesions with astrocyte destruction, loss of AQP4, GFAP, and excitatory amino acid transporter 1 (EAAT1) immunoreactivity, as well as leukocyte infiltration and demyelination (Figure 4B). We did not observe lesions in identically treated \textit{Aqp4}\textsuperscript{−/−} mice. Within the lesion core, there was near-complete loss of AQP4 and myelin, perivascular deposition of activated complement, and prominent inflammation with eosinophil and neutrophil infiltration (Figure 4C); in the lesion periphery there was reduced AQP4 and myelin without complement deposition, and prominent perivascular infiltration of eosinophils and neutrophils. Figure 4D summarizes the extent of AQP4 loss, showing little pathology in \textit{Aqp4}\textsuperscript{−/−} mice or wild-type mice infused with NMO-IgG alone, or with control IgG with complement. Similar lesions were produced in mice infused continuously for 3 days with purified IgG from human NMO sera together with complement. Interestingly, after 1-day infusion with NMO-IgG and complement, the lesions contained many neutrophils but few eosinophils (Figure 4E).

Several types of studies were conducted to demonstrate eosinophil-dependent NMO pathology in vivo. In an initial set of
Figure 3
Eosinophils produce NMO-IgG–dependent pathology by a CDCC mechanism in ex vivo spinal cord slice cultures. (A) Spinal cord slice cultures prepared as in Figure 2 were incubated with submaximal NMO-IgG and hc as indicated (5 μg/ml NMO-IgG or AQmabCDC, 3 × 10⁶ eosinophils, 5% hc). The experiments were conducted in parallel with those in Figure 2A using the same control slice group (SEM; 8–11 slices per condition; *P < 0.001 versus the control group; 1-way ANOVA). Scale bar: 500 μm. (B) Eosinophil degranulation by recombinant human C5a produces NMO-like pathology (10⁷ eosinophils pretreated with 5 μg/ml cytochalasin B, 30 ng/ml C5a) (SEM; 5 slices per condition; *P < 0.01). Scale bar: 500 μm. (C) Eosinophil toxin ECP produces NMO-like pathology (5 μg/ml NMO-IgG, 5% hc, 300 ng/ml ECP) (SEM; 4 slices per condition; *P < 0.01). Scale bar: 500 μm. Experiments were replicated 3 times.
studies, lesions were compared in control mice receiving NMO-IgG and complement for 3 days with mice that were pretreated with anti–IL-5 to deplete eosinophils (21). Figure 5A summarizes peripheral eosinophil and neutrophil counts, showing the efficacy of the anti–IL-5 antibody. Figure 5B shows reduced lesion size and eosinophil infiltration in the anti-IL-5 antibody–treated mice. In a second set of studies, in order to isolate the effects of eosinophils versus neutrophils, we compared NMO lesions produced by NMO-IgG and complement in mice made neutropenic with anti-Ly6G antibody (20), without versus with anti–IL-5 antibody treatment. Treatment with anti-Ly6G antibody significantly reduced lesion size, which was further reduced by anti–IL-5 treatment (Figure 5C). Interestingly, in the neutropenic mice, the number of eosinophils in the lesion increased, particularly around blood vessels. In a third set of experiments, we tested the consequences of peripheral hypereosinophilia. Following infusion for 3 days with submaximal NMO-IgG and complement, lesions in transgenic hypereosinophilic (IL-5 Tg) mice showed marked eosinophil infiltration, with larger lesions compared with those in the control (normo-eosinophilic) mice (Figure 5D). Finally, studies in ΔdblGata1 mice, which lack eosinophils due to Gata1 promoter mutation, showed reduced lesion size (Figure 5E).

Eosinophil inhibitors reduce NMO-IgG–dependent pathology. We tested the possibility that compounds known to inhibit eosinophil degranulation would reduce NMO pathology. Figure 6A (left) shows in vitro inhibition of eosinophil degranulation, as assayed by EPO release, by cetirizine, a second-generation antihistamine, and IBMX, a phosphodiesterase inhibitor. The IC50...
for cetirizine inhibition was approximately 1 μM. Figure 6A (center) shows relatively lower potency of other eosinophil stabilizers including theophylline, ketotifen, cromolyn, and loratadine. Control studies showed that these compounds did not protect against CDC in AQP4-expressing cells produced by NMO-IgG and complement in the absence of eosinophils (Figure 6A, right). Figure 6B shows that cetirizine reduced eosinophil degranulation in AQP4-expressing cell cultures treated with NMO-IgG and eosinophils, with low EPO activity in control (eosinophil alone) measurements. We found protective effects of cetirizine, ketotifen, and IBMX for eosinophil-dependent NMO pathology in spinal cord slice cultures. Figure 6C shows significant protection in the eosinophil-dependent ADCC (top) and CDCC (bottom) models established in Figures 2 and 3.
Figure 6
Small-molecule eosinophil inhibitors reduce NMO-IgG–dependent cytotoxicity and NMO lesions. (A) Reduced EPO release following 30-minute incubation of eosinophils with 5 μM PAF and indicated inhibitors (representative of 3 sets of experiments). Inset shows dose response for cetirizine (at 5 μM PAF; n = 6) (left). Summary of EPO release data (SEM; n = 6) (center). Lack of compound effect on NMO-IgG–mediated CDC in AQP4-expressing CHO cells (SEM; n = 8) (right). (B) EPO release into media for AQP4-expressing CHO cells treated for 1 hour with 10^6 eosinophils without or with 20 μg/ml NMO-IgG. Where indicated, 5 μM cetirizine was present (SEM; n = 6; *P < 0.05). (C) Spinal cord slice cultures were exposed to NMO-IgG without complement (top, ADCC), or with complement (bottom, CDCC), and without or with eosinophils, as done in Figures 2 and 3. Where indicated, cetirizine (1 μM), ketotifen (50 μM), or IBMX (300 μM) were present during the 24-hour incubation. Scale bars: 500 μm. (D) Intracerebral infusion was done (as in Figure 5) without versus with cetirizine administration (10 mg/kg b.i.d., 1 day prior to and during 3-day infusion). Immunofluorescence staining is shown on the top and lesion scores are summarized on the bottom (SEM; n = 4–6; *P < 0.05). Scale bars: 2 mm; 50 μm. (E) Chemical structures of cetirizine and analogs (left). Summary of EPO release data (SEM; n = 6) (center). Summary of data from intracerebral infusion model (right) (each compound was given 10 mg/kg b.i.d.) (SEM; n = 3–7 for each group; *P < 0.05).
Cetirizine, which showed the greatest potency in vitro and ex vivo, was tested in mice in vivo. Mice were administered cetirizine (10 mg/kg b.i.d.) orally, as done previously in other disease models in mice (22), 1 day prior to and during the 3-day intracerebral infusion with NMO-IgG and complement. Figure 6D shows remarkably fewer eosinophils in lesions, with significantly reduced lesion severity.

Three first-generation antihistamine analogs of cetirizine, chlorphenamine, mepyramine, and hydroxyzine (Figure 6E, left) were also tested in this model. The analogs showed weak inhibition of eosinophil degranulation in vitro as assessed by EPO (Figure 6E, center), and no significant reduction of NMO lesion size in vivo (Figure 6E, right).

**Discussion**

The bulk of evidence supports an NMO pathogenic mechanism that involves NMO-IgG binding to AQP4 on astrocytes, which causes primary complement- and cell-dependent cytotoxicity and results in an inflammatory response involving cytokine release, blood-brain barrier disruption, and infiltration of granulocytes and macrophages (2, 4, 5). The consequent oligodendrocytic injury produces demyelination and neuronal death, resulting in neurological deficit. The supramolecular assembly of AQP4 in orthogonal arrays of particles is crucial for NMO pathogenesis, as AQP4 aggregates enhance NMO-IgG binding (23) and present NMO-IgG clusters for efficient C1q binding and consequent CDC (right), which involves binding of NMO-IgG to AQP4 on astrocytes, which causes eosinophil binding (involving Fcγ receptor) and degranulation. The released granule toxins damage astrocytes. Astrocyte damage is amplified when complement is present by CDC (right), which involves multiple mechanisms, including complement-dependent enhancement of eosinophil binding to AQP4-IgG and increased eosinophil accumulation and degranulation.

**Figure 7**

Proposed mechanism of eosinophil-dependent NMO pathogenesis. Eosinophils produce complement-independent and -dependent astrocyte damage. ADCC (left) involves binding of NMO-IgG to AQP4 on astrocytes, which causes eosinophil binding (involving Fcγ receptors) and degranulation. The released granule toxins damage astrocytes. Astrocyte damage is amplified when complement is present by CDC (right), which involves multiple mechanisms, including complement-dependent enhancement of eosinophil binding to AQP4-IgG and increased eosinophil accumulation and degranulation.
cyte cytotoxicity and NMO-like pathology in mouse models, but without marked inflammation or myelin loss (33). CDCC, which involves inflammatory cell activation by activated complement proteins, has been demonstrated to cause NMO pathology when NMO-IgG and complement are present, together with NK cells, neutrophils or macrophages (15, 33), and, as shown here, eosinophils. We thus conclude, as summarized in Figure 7, that NMO-IgG produces NMO pathology by a combination of CDC, CDCC, and ADCC mechanisms whose exact contributions may vary over time and remain to be quantified.

Eosinophil degranulation occurs in response to various stimuli including IgG and IgE, and proinflammatory mediators and cytokines such as PAF, FMLP, and complement (12). Proteins contained within eosinophil granules can exacerbate NMO pathology by multiple mechanisms: the granule toxins MBP, ECP, and eosinophil neurotoxins damage adjacent cells directly, and released cytokines and chemokines induce granulocyte and macrophage infiltration (13). Here, we found that even in the absence of NMO-IgG, the release of eosinophil granule contents caused by C5a or PAF can produce or exacerbate NMO lesions in spinal cord slice cultures.

Cetirizine (Zyrtec) and ketotifen (Zaditor) are clinically approved nonprescription drugs that are long-acting, second-generation histamine H1 receptor antagonists used in the treatment of atopic dermatitis, urticaria, and allergic rhinitis (34). Both drugs are reported to have multiple actions on eosinophils, including inhibition of eosinophil degranulation, adhesion to endothelial cells, migration in response to FMLP, C5a, and LTβ4 chemotaxin, and overall survival (13). The lack of protection by the first-generation cetirizine analogs chlorphenein, mepramine, and hydroxyzine indicates that the effects of cetirizine are histamine H1 receptor independent. Low concentrations of cetirizine increase lipid order in the exterior leaflet of the eosinophil plasma membranes, decrease membrane heterogeneity, and block PAF-induced changes in membrane fluidity (35). Cetirizine was originally designed for minimal brain-blood barrier penetration to reduce the sedative side effect of first-generation antihistamines. Notably, the blood-brain barrier is leaky in NMO lesions, suggesting effective penetration of cetirizine in these lesions. Pharmacokinetic studies showed a mean peak serum cetirizine concentration of 900 ng/ml (36), similar to its IC50 found here for the inhibition of eosinophil EPO release.

Though our data indicate the likely mechanisms by which eosinophil accumulation in NMO lesions produces NMO pathology, it is difficult to quantify the precise contribution of eosinophils in human NMO because of the limitations of available animal models of NMO. While cell cultures are useful for studying the mechanisms of NMO-IgG and AQP4-dependent cytotoxicity, the ex vivo and in vivo models of NMO can recapitulate the major features of human NMO pathology. An important advance here was the development of a continuous intracerebral infusion model that maintained within eosinophil granules can exacerbate NMO pathology by a combination of CDC, CDCC, and ADCC mechanisms whose exact contributions may vary over time and remain to be quantified.
nologies) were added to stimulate live cells and dead cells red. For assay of ADCC, cells were incubated for 3 hours at 37°C with 20 μg/ml NMO-IgG (or control IgG) and eosinophils at an effecter/target cell ratio of 30:1. For assay of CDC, cells were incubated for 60 minutes at 37°C with 10 μg/ml NMO-IgG and 2% IgG. In some experiments, test compounds were added for 30 minutes, followed 60 minutes later by complement and NMO-IgG. Cytotoxicity was measured by the AlamarBlue Assay (Life Technologies). Spinal cord slice culture model. On postnatal day 7 mouse pups were decapitated and the spinal cord was rapidly removed and placed in ice-cold HBSS. Transverse slices of cervical spinal cord of 300-μm thickness were cut using a vibratome (VT-1000S; Leica). Individual slices were placed on transparent, noncoated membrane inserts (Millipore-CM 0.4 μm pores, 30-mm diameter; Millipore) in 6-well (35-mm diameter) plates containing 1 ml culture medium, with a thin film of culture medium covering the slices. The culture medium, consisting of 50% MEM (Life Technologies), 25% HBSS, 25% horse serum (Life Technologies), 1% penicillin-streptomycin, 0.66% glucose, and 25 mM HEPES, was changed every 3 days. The slices were cultured in 5% CO2 at 37°C for 7 days. NMO-IgG and/or complement were added to both sides of the porous membrane, with 1 ml of medium added above the porous filter to fully immerse the slice. Eosinophils were added to the solution above the porous filter bathing the slice. In some experiments PAF (Sigma-Aldrich), ECP (Sigma-Aldrich), or recombinant human CSa, together with cytochalasin B (Sigma-Aldrich), were added directly to the slice medium for 24 hours. AQPF4- and GFAP-stained spinal cord slices were scored for lesion severity using the following scale: 0, intact slice with intact GFAP and AQPF4 staining; 1, intact slice with some astrocyte swelling (seen from GFAP stain) with weak AQPF4 staining; 2, at least 1 lesion with complete loss of GFAP and AQPF4 staining; 3, multiple lesions with loss of GFAP and AQPF4 staining in greater than 30% of area; and 4, extensive loss of GFAP and AQPF4 staining affecting greater than 80% of the slice area (15).

Intracerebral infusion model. Mice were implanted with an osmotic minipump (Alzet 1003D) with the needle positioned in the right lateral brain (from the bregma, anterior-posterior, -2 mm; medial-lateral, +1.5 mm; dorsal-ventral, 2.5 mm below the dura). NMO-IgG and/or IgG control were dissolved in PBS and delivered for 3 days at 3.3 μg per day and 16.7 μl per day, respectively. For the IL-5 Tg mice, submaximal human complement (3.4 μg/ml NMO-IgG or control IgG) and eosinophils at an effector/target cell ratio of 30:1 was added per well. EPO release from eosinophils was measured following the addition of PAF, which causes eosinophil degranulation (41). Substrate solution (100 μl) was added after 30 minutes, and the reaction stopped by 100 μl of 4 M sulfuric acid prior to the measurement of absorbence at 492 nm. In some experiments, eosinophil degranulation inhibitors (cetirizine, ketorizine, loratadine, cromolyn sodium, theophylline, IBMX) were added (separately) 30 minutes before PAF. EPO release was also measured after ADCC in which 250,000 cells in 100 μl were added to 96-well plates containing cultured CHO-AQPF4 cells or CHO-null cells. NMO-IgG or control IgG was added and cells were incubated at 37°C, 5% CO2 for 1 hour. Samples were centrifuged at 250 g for 15 minutes at 4°C. The supernatants (50 μl; triplicate) were dispensed into each well of a microplate, and 50 μl of substrate solution was added to each well.

Statistics. Where appropriate, differences were evaluated using a 2-tailed Student’s t test, a Mann-Whitney rank sum test, or a 1-way ANOVA rank test, as calculated using SigmaStat software, version 3.5 (Systat Software). Data are presented as the mean ± SEM. A P value of 0.05 or less was considered significant.

Study approval. All mouse procedures were approved by the University of California, San Francisco Committee on Animal Research and were conducted in accordance with the NIH guidelines for the care and use of animals.

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