Rapid paper

Involvement of pentraxin-3 in anti-neutrophil cytoplasmic antibody production induced by aluminum salt adjuvant

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ABSTRACT

Objective. *Pentraxin 3 (PTX3) is a multifunctional soluble factor. PTX3 can be involved in the regulation of vasculitis and is expressed in the cytoplasm of neutrophils. As anti-neutrophil cytoplasmic antibody (ANCA) is recognised as a cause of vasculitis, we aimed to know the role of PTX3 in ANCA production in vivo.*

Methods. *To this end, we used aluminum salt (alum), which induces neutrophil extracellular traps, as an adjuvant for producing anti-myeloperoxidase-ANCA (MPO-ANCA). Specifically, we intraperitoneally injected alum and recombinant MPO (rMPO) into MPO-deficient mice and then measured the concentration of anti-MPO IgG in their blood. To show the involvement of extracellular PTX3 in this model, we assessed PTX3 protein content and host double-stranded DNA levels in the mice's peritoneal fluid after alum injection. In addition, we simultaneously administered recombinant PTX3, rMPO and alum to MPO-deficient mice to assess the function of PTX3 in producing anti-MPO IgG in vivo.*

Results. *Anti-MPO IgG was produced by the alum + rMPO immunisation model in MPO-deficient but not wild-type mice. Injection of alum induced extracellular PTX3 as well as double-stranded DNA and dead cells in MPO-deficient mice. Simultaneous injection of recombinant PTX3 with rMPO and alum attenuated the production of anti-MPO IgG in MPO-deficient mice.*

Conclusion. *Our current findings provide evidence that PTX3 attenuates the production of murine MPO-ANCA.*

Introduction

Pentraxin 3 (PTX3) is a multifunctional soluble factor (1) that is involved in the regulation of inflammation related to cell death (2) and in the development of several autoimmune diseases such as systemic lupus erythematosus, rheumatoid arthritis, and large- and small-vessel vasculitis (3-5). Anti-neutrophil cytoplasmic antibody (ANCA) is recognised as a cause of vasculitis (6) and myeloperoxidase (MPO) is a major antigen for ANCA in patients with vasculitis. Recent studies revealed that PTX3 is expressed in the cytoplasm of neutrophils, as well as MPO (6-8). Although the mechanisms through which MPO-ANCA participates in the priming and activation of neutrophils (9) and reacts with antigen on vessel walls (10) have been examined in detail, we have little evidence regarding how MPO-ANCA is produced *in vivo*.

Treatment with aluminum salt (alum) induces host cells to release free nucleic acid, which has adjuvant activity for autoantibody production (11). In addition, neutrophil extracellular traps (NETs), which contain free host double-stranded DNA (dsDNA), are involved in the development of MPO-ANCA-associated vasculitis (12). In fact, serum levels of PTX3 are higher in patients with vasculitis than in controls (13). Because recent studies have shown that both the cytoplasm of neutrophils and NETs contain PTX3 (8), we hypothesised that PTX3 has a role in MPO-ANCA production.

To examine the mechanism through which MPO-ANCA is produced *in vivo*, we injected alum into MPO-deficient mice and used this new murine model to reveal PTX3 as a regulator of MPO-ANCA production.

Material and methods

Mice and induction of aluminum-induced ANCA production

B6 background $MPO^{-/-}$ mice have been described elsewhere (7). We purchased

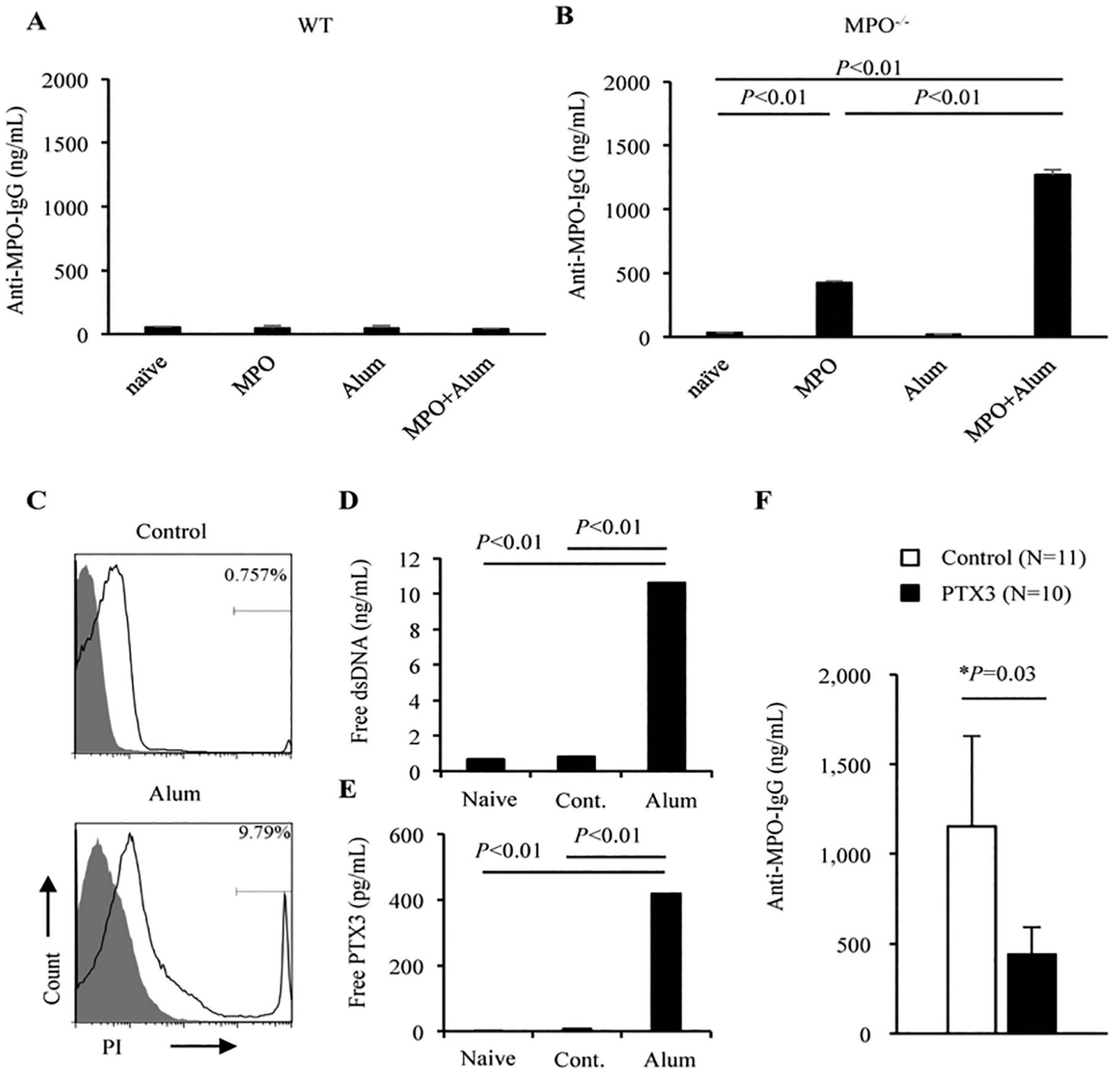

Fig. 1. Involvement of PTX3 in alum induced MPO-ANCA production model.
Recombinant MPO (rMPO; 10 μg) or vehicle was co-administered intraperitoneally with or without Alum into C57BL/6J background $MPO^{+/+}$ (WT) or $MPO^{-/-}$ mice on day 0. Serum concentrations of anti-MPO-IgG in (**A**) WT and (**B**) $MPO^{-/-}$ mice were measured by means of an ELISA at 14 days after immunisation. Data are shown as the mean value and S.E.M. of 3 to 8 mice. **C**. Cell-containing peritoneal fluid was collected from $MPO^{-/-}$ mice at 14 h after intraperitoneal administration of 4 mg alum. The percentage of dead cells (positive for propidium iodide [PI^+]) relative to total acquired cells was determined by flow cytometry. The concentrations of (**D**) cell-free double-stranded DNA (dsDNA) and (**E**) PTX3 in the samples of peritoneal fluids were determined also. **F**. Recombinant PTX3 (10 μg) or vehicle as control was co-administered with alum and MPO into $MPO^{-/-}$ mice. The concentration of anti-MPO IgG in consecutive samples was measured by means of an ELISA. Data are shown as the means ± SEM (n=11 [control] or 10 [rPTX3] mice).

C57BL/6J mice from Clea Japan. All mice were bred and housed in facilities that were specific pathogen free. Male and female mice (age, 12 to 16 weeks) were used in the current study. All experiments were performed in accordance with the guidelines and approval of the animal ethics committee of the University of Tsukuba Animal Research Center (approval no. 16-133). For immunisation, 10 μg of recombinant MPO (rMPO; catalogue no. 3667-MP-250, R&D Systems) in sterile saline and 4 mg of alum (catalogue no. 77161, Imject Alum, Thermo Scientific) were mixed at a volume ratio of 1:1 for 1 h at 4°C and then injected intraperitoneally into each mouse. The blood anti-MPO IgG concentration of immunised mice was examined by means of an enzyme-linked immunosorbent assay (ELISA) as previously described (7). To examine the involvement of PTX3 in this model, 15 μg of

recombinant mouse PTX3 (catalogue no. 2166-TS-025, R&D Systems) in 200 μl of sterile saline was co-administered with rMPO and alum, as described earlier.

Assessment of alum-induced cell death and soluble factors

Peritoneal lavage fluid (containing peritoneal cells) was collected from MPO$^{-/-}$ mice at 14 h after intraperitoneal injection of alum or sterile normal saline as a control. The proportion of dead cells was determined by using flow cytometry (FACScalibur, BD Biosciences) and FlowJo software (Tree Star) and was defined as the population of propidium iodide (PI)-positive cells among all acquired cells. Free dsDNA and PTX3 protein contents were measured by using Quanti-iT PicoGreen dsDNA Kit (catalogue no. P11496, Life Technologies) and PTX3 ELISA Kit (catalogue no. DY2166, R&D Systems), respectively.

Statistical analysis

Statistical significance between groups was determined by using the two-tailed Student's *t*-test, the Mann-Whitney U-test or Kruskal-Wallis one-way analysis with post-hoc Dunn's test to compare (GraphPad Prism). Statistical significance was set at $p<0.05$.

Results

Co-administration of recombinant MPO and aluminum salt induced MPO-ANCA production in MPO$^{-/-}$ mice

We immunised MPO$^{+/+}$ C57BL6/J (WT) and MPO$^{-/-}$ mice each with 10 μg of rMPO in the presence or absence of alum adjuvant. Whereas anti-MPO IgG titres did not differ among groups of WT mice (Fig. 1A), anti-MPO IgG specifically occurred in MPO$^{-/-}$ mice immunised with rMPO (Fig. 1B). Furthermore, antibody titres were higher in MPO$^{-/-}$ mice that received both alum and rMPO compared with rMPO alone.

Alum induced cell death and increased cell-free nucleic acid and PTX3 levels in the peritoneal lavage fluids of MPO$^{-/-}$ mice

In previous studies, it was shown that alum had adjuvant activity by release of free nucleic acid from dead host cells (11). Compared with control mice that received control, our alum-injected MPO$^{-/-}$ mice demonstrated a population clearly identified as dead cells (Fig. 1C) as well as considerable quantities of free dsDNA (Fig. 1D). In addition, PTX3 levels were markedly increased in MPO$^{-/-}$ mice after intraperitoneal injection of alum (Fig. 1E).

Co-administration of recombinant PTX3 (rPTX3) attenuated the production of MPO-ANCA in MPO$^{-/-}$ mice that received alum and rMPO

We next examined whether exogenous PTX3, a soluble factor thought to be in NETs and to promote the removal of dead cells (2), is involved in MPO-ANCA production. To this end, we measured the concentration of anti-MPO IgG in successive samples from MPO$^{-/-}$ mice immunised with MPO and alum in the presence or absence of rPTX3 (Fig. 1F). The anti-MPO IgG concentration was significantly lower in MPO$^{-/-}$ mice with co-administration of rPTX3 than in those that did not receive rPTX3.

Discussion

Neutrophil cytoplasmic proteins are involved in the development of ANCA-associated vasculitis (14). PTX3 is expressed in the cytoplasm of neutrophils, as is MPO, a representative granule protein and an antigen for ANCA (8). PTX3 might play important roles in immunologic regulation, neutrophil function, and ANCA-associated vasculitis.

WT and MPO$^{-/-}$ mice showed specificity of MPO-ANCA in this rMPO and alum immunisation model. Although we cannot clearly explain why WT mice did not produce MPO-ANCA, we suggest that alum is a weak adjuvant and therefore was unable to overcome the immunologic tolerance for MPO in WT (MPO$^{+/+}$) mice, whereas rMPO immunisation with intraperitoneal Freund's adjuvant (7) produced high titres of MPO-ANCA even in WT mice in our experience (data not shown).

We also cannot clearly explain why MPO injection without alum led to MPO-ANCA production in MPO$^{-/-}$ mice but not WT mice. However, recent evidence shows that neutrophil-derived MPO attenuates the development of autoimmunity by dampening effects on antigen-presenting cells (15), and MPO$^{-/-}$ mice show enhanced auto-immunity. Together, these previous findings (15) and our current results suggest that endogenous MPO hampers MPO-ANCA production in WT mice.

As our current results show, intraperitoneal injection of alum leads to increased levels of dead cells in the peritoneal cavity and effects on the activation of inflammatory dendritic cells (11). In addition, our results are consistent with previous reports regarding the release of free nucleic acids as NETs from host cells after treatment with alum adjuvant (11), but no previous data indicated that alum induces the release of PTX3 as well. Although we did not identify the sources of the endogenous PTX3 in the model we used in this study, PTX3 has been shown to associate with nuclear DNA or histones, to contribute to NET formation, to reside in NETs, and to regulate cytotoxicity (16). Therefore, it is reasonable to surmise that PTX3 attenuated MPO-ANCA production after rMPO and alum injection by interacting with components of NETs.

The immunological pathogenesis of MPO-ANCA-related vasculitis has some stepwise processes that involve several organ systems. As the first step, loss of tolerance to MPO results in anti-MPO autoimmunity in secondary lymphoid organs (15), and then, ANCA binds to primed (*e.g.* by infection) neutrophils and induces inflammatory changes in neutrophils. Finally, ANCA-activated neutrophils react with antigen on vessel walls and injure organs such as kidneys and lungs. In this study, we have focused on the first step by establishing anti-MPO autoimmunity with adjuvant stimulation. Therefore, these results do not explain how PTX3 is involved in every process to develop clinical phenotype of MPO-ANCA-related vasculitis.

ANCA-related vasculitis has a poor prognosis. Because ANCA against MPO causes vasculitis (6, 9, 10), the inhibition of ANCA production might be both therapeutically and prophylac-

tically effective. Current therapies for ANCA-related vasculitis have significant toxicities and side effects, mainly due to infectious complications. PTX3 negatively regulates acute and chronic inflammation and leads to favourable effects in host animals during infection (17). Together, these results suggest that PTX3 is a good molecular target for ANCA-related vasculitis.

Here we presented a new mouse model of MPO-ANCA production after immunisation with alum, and provided evidence for the regulatory role of PTX3 in this process. Our findings suggest that the administration of rPTX3 to attenuate the production of ANCA has potential therapeutic applications in ANCA-related vasculitis.

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