

Asparaginase immune complexes induce Fc- γ RIII-dependent hypersensitivity in naive mice

Sanjay Rathod,^{*,†} Manda Ramsey,^{*,†} Danielle DiGiorgio,^{*,†} Roberto Berrios,^{*,†} Fred D. Finkelman,^{‡,§} and Christian A. Fernandez^{*,†,1}

^{*}Center for Pharmacogenetics and [†]Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; [‡]Division of Immunology, Allergy, and Rheumatology, Department of Internal Medicine, College of Medicine, University of Cincinnati, Cincinnati, Ohio, USA; and [§]Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio, USA

ABSTRACT: Asparaginase (ASNase) is an important drug for the treatment of leukemias. However, hypersensitivity to ASNase can increase the risk of leukemia relapse. Two mechanisms of ASNase hypersensitivity have been identified in mice. The existence of a pathway involving anti-ASNase IgG and Fc- γ receptor III (Fc- γ RIII) implies that IgG and ASNase immune complexes (ICs) could directly induce hypersensitivity. The aim of this study was to detect ASNase ICs in mice after hypersensitivity reactions and determine their role in hypersensitivity. Protein G beads were used to detect plasma ASNase ICs by flow cytometry. Anti-ASNase IgG was purified from the plasma of sensitized mice, and ASNase ICs were prepared *ex vivo* at various ratios of ASNase to anti-ASNase IgG. The levels of ASNase ICs detected after hypersensitivity reactions correlated with reaction severity ($R^2 = 0.796$; $P = 0.0005$). ASNase ICs prepared *ex vivo* required high levels of anti-ASNase IgG for formation, and binding to naive and sensitized immune cells depended on soluble anti-ASNase IgG, antigen:antibody ratio, and Fc- γ RIII. Similarly, basophil activation by ASNase ICs depended on the antigen:antibody ratio and Fc- γ RIII. Consistent with the *ex vivo* results, naive mice receiving ASNase ICs developed hypersensitivity reactions. Our data demonstrate that ASNase ICs can directly contribute to the onset and severity of ASNase hypersensitivity.—Rathod, S., Ramsey, M., DiGiorgio, D., Berrios, R., Finkelman, F. D., Fernandez, C. A. Asparaginase immune complexes induce Fc- γ RIII-dependent hypersensitivity in naive mice. FASEB J. 33, 000–000 (2019). www.fasebj.org

KEY WORDS: antidrug antibodies • leukemia • basophil activation test • anaphylaxis • immunogenicity

Asparaginase (ASNase) is an essential component of multidrug chemotherapy used in the treatment of leukemias and lymphomas. It is a nonhuman enzyme derived from bacterial sources that prevents the proliferation of leukemic cells with low asparagine synthetase levels by depleting the nonessential amino acid asparagine (1). However, patients with pediatric acute lymphoblastic leukemia (ALL) treated with ASNase can develop hypersensitivity reactions and anti-ASNase antibodies that limit ASNase levels and increase the risk of leukemia relapse (2–8). Furthermore, clinical studies have demonstrated that hypersensitivity reactions to native *Escherichia coli* ASNase correlate with high anti-ASNase

IgG levels (4, 9). Nevertheless, many patients with anti-ASNase IgG antibodies never develop clinical ASNase hypersensitivity but have decreased ASNase levels caused by antibody-mediated neutralization and accelerated clearance. The presence of anti-ASNase IgG antibodies in the absence of hypersensitivity is commonly referred to as silent hypersensitivity and is considered a major risk factor for ALL relapse (10).

ASNase-induced hypersensitivity during ALL treatment is most common upon ASNase reexposure (4), suggesting that patients are likely sensitized to the agent when they first receive it during induction therapy. The sensitization mechanism therefore likely involves ASNase epitope presentation to T cells, T-cell activation, and B-cell proliferation and differentiation into antibody-secreting plasma cells. In accordance with the development of a humoral immune response, pharmacogenomic studies of the ASNase immune response have identified human leukocyte antigen-*DRB1* (11) and nuclear factor of activated T cells 2 (12) variants that are associated with increased risk of ASNase hypersensitivity.

Consistent with the features of clinical ASNase hypersensitivity, 2 immunologic mechanisms of hypersensitivity

ABBREVIATIONS: ALL, acute lymphoblastic leukemia; ASNase, asparaginase; AUC, area under curve; Fc- ϵ RI, Fc- ϵ receptor I; Fc- γ R, Fc- γ receptor; IC, immune complex; mMCP-1, mouse mast cell protease 1; PAF, platelet activating factor; PEG, polyethylene glycol

¹ Correspondence: Department of Pharmaceutical Sciences, Center for Pharmacogenetics, School of Pharmacy, University of Pittsburgh, 335 Sutherland Dr., Pittsburgh, PA 15261, USA. E-mail: chf63@pitt.edu

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have been described involving ASNase-specific antibodies (13). The classic pathway is mediated by Fc- ϵ receptor I (Fc- ϵ RI) and antigen-specific IgE antibody. In this pathway, free antigens and allergens in circulation bind to cell-associated, antigen-specific IgE, leading to the release of the mediators of hypersensitivity, such as histamines (14). In contrast, the alternative pathway involves the formation of antigen- and IgG antigen-specific antibody immune complexes (ICs), which can bind to Fc- γ RIII-expressing immune cells (15). Nevertheless, if antigen-specific antibody levels are low, insufficient ICs will bind to Fc- γ RIII and no hypersensitivity reaction will be induced because of low Fc- γ RIII affinity for ICs (16). Similar to the classic pathway of anaphylaxis, activation of immune cells by ICs results in the release of hypersensitivity mediators, such as platelet activating factor (PAF), and the onset of anaphylaxis (14, 17).

Recent studies of murine ASNase hypersensitivity demonstrate that the classic and alternative pathways of anaphylaxis simultaneously contribute to hypersensitivity reactions (18, 19). However, no study has reported detecting the presence of ASNase ICs after hypersensitivity reactions or investigating whether they can directly contribute to the severity and onset of ASNase hypersensitivity. We hypothesized that ASNase ICs form after ASNase reexposure in sensitized mice and that ASNase ICs can directly induce hypersensitivity reactions *via* Fc- γ RIII when administered to ASNase-naïve mice. Herein, we describe methods for detecting plasma ASNase ICs after hypersensitivity reactions and demonstrate that ASNase ICs prepared at high ratios of anti-ASNase IgG to ASNase bind and activate immune cells, whereas ASNase ICs prepared at lower ratios do not. Moreover, we show that ASNase ICs are sufficient to induce hypersensitivity reactions in naïve mice. Taken together, our study indicates that ASNase ICs can induce hypersensitivity reactions in mice and require high anti-ASNase IgG antibody levels to mediate these reactions.

MATERIALS AND METHODS

Mice

Female C57BL/6J mice weighing 18–22 g (6–8 wk old) were obtained from The Jackson Laboratory (Bar Harbor, ME, USA) and housed at the University of Pittsburgh (Pittsburgh, PA, USA) animal facility. All experiments with mice were reviewed and conducted under a protocol approved by the University of Pittsburgh Institutional Animal Care and Use Committee.

Reagents

L-ASNase produced from *E. coli* was obtained from BioVendor Laboratory Medicine (Asheville, NC, USA). Aluminum hydroxide Inject Alum Adjuvant and PBS (both from Thermo Fisher Scientific, Waltham, MA, USA) were used for mouse sensitization with ASNase. Streptavidin-Coated Magnetic Beads (Spherotech, Lake Forest, IL, USA), EZ-Link Sulfo-NHS-LC-Biotinylation Kit, IgG Elution Buffer, and Tween 20 (all from Thermo Fisher Scientific) were used to purify anti-ASNase IgG. ASNase was labeled with Alexa Fluor 647 (Alexa Fluor 647

Protein Labeling Kit; Thermo Fisher Scientific) and polyethylene glycol (PEG) 6000 (Thermo Fisher Scientific) was used for preparation and purification of ASNase ICs. Fixation and Permeabilization Buffer Set (Thermo Fisher Scientific), New Zealand-origin heat-inactivated horse serum (Thermo Fisher Scientific), red blood cell lysis solution (10 $\times$) (Miltenyi Biotec, Bergisch Gladbach, Germany), and EDTA (MilliporeSigma, Burlington, MA, USA) were used.

ASNase sensitization and detection of anaphylaxis

Female mice were injected twice intraperitoneally 14 d apart with 10 μ g of *E. coli* ASNase formulated with 1 mg of aluminum hydroxide adjuvant (18). Nonsensitized naïve control mice received adjuvant with PBS only. ASNase hypersensitivity was induced in sensitized mice by challenging them with a 100- μ g i.v. dose of *E. coli* ASNase 10 d after the second immunization. Hypersensitivity was detected by monitoring decreases in rectal temperature using a digital thermometer (BAT-12; Physitemp Instruments, Clifton, NJ, USA). Rectal temperature measurements were performed immediately before (time 0) and following the ASNase challenge at different time points for 2 h. The area under the temperature *vs.* time curve was calculated using the trapezoidal rule, and lower area under curve (AUC) values indicated more severe reactions. Differences in the severity of ASNase hypersensitivity reactions among groups were determined by comparing AUC values. Prechallenge (*i.e.*, 1 d before challenge) and after challenge (*i.e.*, 2 h after) blood samples were collected by cardiac puncture. Results from naïve or sensitized mice were represented as green or red data points, respectively.

Anti-ASNase IgG antibody purification and characterization by ELISA and Western blotting

Purification of anti-ASNase IgG and confirmation by ELISA

E. coli ASNase (1 mg) was biotinylated per manufacturer's instructions (EZ-Link Sulfo-NHS-LC-Biotinylation Kit). One hundred microliters of streptavidin-coated magnetic beads (Spherotech) and 0.2 μ g of biotinylated ASNase were combined and incubated for 90 min while shaking at room temperature. The beads were washed and blocked with 100 μ l of phosphate buffered saline with 0.05% Tween 20 and 5% bovine serum albumin. Sensitized mouse plasma (100 μ l) was diluted with PBS and added to streptavidin beads while shaking at room temperature for 15 min. After discarding the supernatant, 100 μ l of IgG elution buffer (pH 3; Thermo Fisher Scientific) was added and a magnetic column (Miltenyi Biotec) was used to separate the beads from the eluate. 10 M Tris (pH 8) neutralization buffer was added to the eluate for pH adjustment prior to lyophilization. Pooled eluate samples were lyophilized overnight and reconstituted with 100 μ l of sterile water. Murine anti-ASNase IgG concentrations were determined using a Bovine Serum Albumin Kit (Thermo Fisher Scientific), and confirmation of anti-ASNase IgG antibody activity was demonstrated by ELISA (18, 19).

Western blotting

Forty-five micrograms of *E. coli* ASNase was separated on SDS-PAGE gels and transferred to a PVDF membrane using a Trans-Blot Semi-Dry Transfer Cell (Bio-Rad, Hercules, CA, USA). After transfer, the membrane was blocked using 5% milk and incubated with purified anti-ASNase IgG or with control mouse IgG overnight at 4°C on a shaker. Mouse horseradish peroxidase-conjugated anti-IgG secondary antibody (1:10,000) was used for detection. Specific bands were visualized with ECL

substrate (Thermo Fisher Scientific) and exposed onto films with an AX 700LE film processor (Alphatek, Salem, WI, USA).

Mouse mast cell protease 1 ELISA

Mouse mast cell protease 1 (mMCP-1) concentrations were measured from plasma samples using a commercially available ELISA kit according to the manufacturer's instructions (Thermo Fisher Scientific).

ASNase activity determination

The ASNase activity in plasma samples was determined by monitoring the enzymatically coupled oxidation of NADH to NAD⁺ in a 96-well format, as previously described in Fernandez *et al.* (20).

Detection of ASNase ICs

ASNase ICs were detected in plasma collected after 2 h of ASNase challenge. Twenty-five microliters of plasma was precipitated with an equal volume of 8% PEG (MilliporeSigma) and incubated with protein G-coated polystyrene particles 6–8 μ m in size for 60 min at 4°C on a shaker. FITC-labeled goat anti-mouse IgG Fc antibody (Thermo Fisher Scientific) was incubated for 30 min at 4°C with the beads and the washed beads were resuspended in 250 μ l of running buffer (Miltenyi Biotec) for acquisition by flow cytometry. Naive mouse plasma, as well as protein G beads alone, were used as a negative control.

Ex vivo formation of ASNase anti-ASNase IgG ICs

Preparation

ASNase ICs were prepared *ex vivo* using ASNase labeled with Alexa Fluor 647 at 8 μ g/ml and purified mouse or commercially available rabbit polyclonal anti-ASNase IgG at various weight ratios of ASNase to anti-ASNase IgG antibody (1:1, 1:2.5, 1:5, 1:10, or 1:20). For studies using sensitized mouse plasma for ASNase IC preparation instead of purified anti-ASNase IgG, 5 μ l of sensitized plasma was added to ASNase at 8 μ g/ml. All samples were incubated for 1 h at room temperature (25°C) before isolating the ASNase ICs by PEG precipitation. An equal volume of 8% PEG was added to samples and incubated overnight at 4°C, as previously described in refs. 21 and 22. Precipitated ASNase ICs were pelleted by centrifugation at 14,000 rpm for 30 min at 4°C, washed in 4% PEG, and resuspended in PBS. To confirm that ASNase or anti-ASNase IgG was not precipitated by 4% PEG, free fluorescent ASNase or anti-ASNase IgG was treated with PEG as described for sensitized mouse plasma. ASNase was detected by flow cytometry using anti-ASNase IgG-coated protein G beads. Free anti-ASNase IgG antibodies were detected by flow cytometry using protein G beads to capture IgG antibodies and fluorescent ASNase for staining. Non-PEG-precipitated free fluorescent ASNase and anti-ASNase IgG were used as positive controls.

Detection of ASNase ICs by flow cytometry

After PEG precipitation, pellets were resuspended in PBS and incubated with 6–8- μ m diameter protein G polystyrene particles for 60 min at 4°C. The beads were washed with PBST and resuspended in 250 μ l of running buffer for acquisition by flow cytometry.

Detection of ASNase ICs by gel retardation assay

E. coli ASNase (1 μ g), purified anti-ASNase IgG (10 μ g), and ASNase ICs prepared at a 1:10 weight ratio were separated by PAGE. The separated protein samples were stained for 2 h at room temperature with GelCode Blue Stain Reagent (Thermo Fisher Scientific) and de-stained with water. The ASNase IC band was excised, dissociated, and separated by PAGE.

Ex vivo analysis of ASNase IC binding to immune cells

Peripheral blood cells were collected from naive and sensitized mice (*i.e.*, 1 d before challenge). Samples were stained with ASNase ICs that were prepared with fluorescent ASNase and immune cell marker panels for 30 min in the dark at 4°C. Cells were lysed, washed, and acquired by flow cytometry to determine the binding of ASNase ICs on different populations of immune cells. To determine Fc- γ R expression, nucleated blood cell samples were stained with anti-Fc- γ RIII (CD16- α) and immune cell marker panels, as described in Supplemental Data.

Effect of ASNase ICs binding to immune cells after Fc- γ R and Fc- ϵ RI blocking

Blood from sensitized mice (*i.e.*, 1 d before challenge) was incubated with EM-95 (Zelig Eshhar, Weizmann Institute, Rehovot, Israel), 2.4G2 (American Type Culture Collection; Rockville, MD, USA), both (1 μ g/ml), or medium (as a negative control) for 1 h. Samples were stained with ASNase ICs, CD45, CD49b, and IgE for 30 min in the dark at 4°C, erythrocyte lysed, washed, and acquired by flow cytometry to determine ASNase IC binding on leukocytes (CD45) or basophils in the presence and absence of blocking antibodies. To determine the extent of ASNase IC binding that is independent of soluble anti-ASNase IgG antibody, samples were first washed and erythrocyte lysed to remove soluble antibodies before staining with fluorochrome-labeled ASNase, as previously described in Rathod *et al.* (19).

Murine basophil activation

Basophil activation was performed as previously described in refs. 19, 23, and 24 with some modification. Briefly, 100 μ l of blood from ASNa-naive or -sensitized mice was diluted with Roswell Park Memorial Institute medium at 1:1 and incubated for 15 min at 37°C before addition of 100 μ l of Roswell Park Memorial Institute medium containing 500 ng of EM-95 (anti-IgE mAb), 500 ng of 2.4G2 (anti-Fc- γ RIIB/III mAb), 1 IU of ASNase, ASNase ICs (prepared at a weight ratio of 1:10 of ASNase to anti-ASNase IgG), or medium (as a negative control). The cells were blocked with 15% horse serum in PBS for 30 min on ice, washed, and stained with anti-IgE, anti-CD49b, anti-CD200R1 (basophil), and anti-CD200R3. The cells were then lysed, washed, and acquired by flow cytometry. The percent change of CD200R1 and CD200R3 expression were calculated as previously described in Rathod *et al.* (19).

Murine model of ASNase IC hypersensitivity

One microgram of *E. coli* ASNase and 10 μ g of purified mouse anti-ASNase IgG were mixed at a 1:10 weight ratio at room temperature for 1 h. ASNase-naive plasma was mixed with ASNase as a negative control. Naive C57BL/6J female mice received 1 μ g of *E. coli* ASNase, ASNase ICs prepared at a

1:10 = weight ratio of ASNase to anti-ASNase IgG, or 150 μ l of PBS as a negative control. To investigate whether mice developed anaphylaxis as a result of ASNase IC administration, rectal temperature measurements were performed immediately before (time 0) and at different time points for 2 h after the challenge.

Flow cytometric analysis

Detailed description of the flow-based methods is found in the Supplemental Data.

Statistical analysis

All data were plotted and analyzed by using Prism 7 statistical software (GraphPad Software, La Jolla, CA, USA). Statistical analysis was performed with a 2-tailed Student's *t* test between 2 groups, or 2-way ANOVA for multiple groups, followed by Tukey's *post hoc* test. For correlation analysis, the Spearman rank correlation test was performed to measure association. A value of $P < 0.05$ was considered significant. Unless stated otherwise, data are represented as means $\pm$ SD.

RESULTS

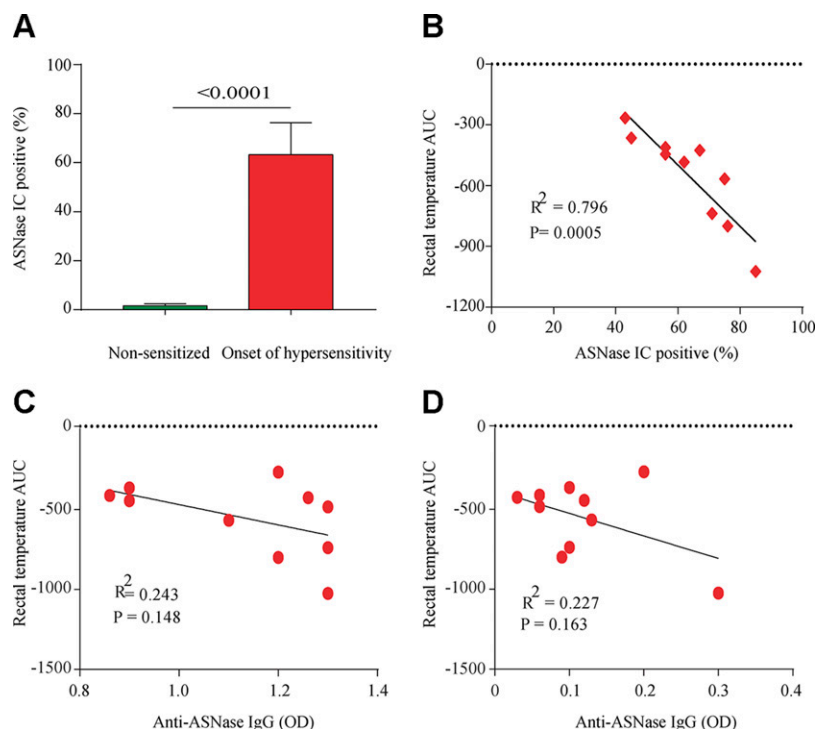
ASNase ICs are detected after ASNase hypersensitivity in mice

Previously, we demonstrated that ASNase-specific recognition and hypersensitivity in mice are Fc- γ RIII-dependent, suggesting that soluble anti-ASNase antibodies are required for both to occur (19). In agreement with our previous findings, we found that the binding of ASNase to leukocytes, including B cells and

basophils, is significantly decreased after removing soluble antibodies (Supplemental Fig. S1A–C). Moreover, ASNase binding can be restored by the addition of sensitized mouse plasma containing anti-ASNase IgG (Supplemental Fig. S1C). In contrast, the residual binding of ASNase to basophils after removing soluble antibodies is likely caused by cell-associated anti-ASNase IgE (Supplemental Fig. S1C) and, therefore, is blocked by the anti-IgE mAb, EM-95 (Supplemental Fig. S1D). Similar results are observed with free ASNase in purified peripheral blood mononuclear cells and splenocytes (Supplemental Fig. S2A, B), indicating that IC formation is dependent on soluble anti-ASNase IgG and form after intravenous ASNase administration.

We sought to determine whether ASNase ICs were present in plasma samples collected after the onset of ASNase hypersensitivity in mice, where ASNase hypersensitivity was confirmed by detecting the onset of hypothermia and measuring mMCP-1 levels after challenging mice with ASNase (Supplemental Fig. S3A, B). Plasma ASNase ICs were indeed detected in sensitized mice 2 h after the ASNase challenge by precipitating ICs with 4% PEG and binding them with protein G-coated beads. The beads were then stained with mouse anti-IgG Fc-FITC secondary antibody and analyzed by flow cytometry (Fig. 1A and Supplemental Fig. S4). Because our previous studies indicated that the severity of ASNase hypersensitivity is dose dependent (18), we determined whether the concentration of ASNase ICs correlates with the severity of ASNase hypersensitivity, as indicated by hypothermia (reported as rectal temperature AUC; Fig. 1B). Our results show that levels of ASNase ICs negatively correlate with rectal temperature AUC (Fig. 1B; $R^2 = 0.796$; $P = 5 \times 10^{-4}$), which suggests that high levels of ASNase ICs lead to more

Figure 1. ASNase ICs are detected after an ASNase hypersensitivity reaction, and their levels correlate with the severity of the reaction. *A*) ASNase ICs were detected in plasma samples collected 2 h after the onset of an ASNase hypersensitivity reaction. *B*) The percentage of ASNase ICs positively correlates with the severity of ASNase hypersensitivity, as quantified by rectal temperature AUC. *C*, *D*) No correlation was detected between anti-ASNase IgG antibody levels measured before (*C*) or after (*D*) the onset of ASNase hypersensitivity. OD, optical density.



severe ASNase hypersensitivity. In contrast, no significant correlation was found between the severity of hypersensitivity and anti-ASNase IgG level measured either before (Fig. 1C) or after (Fig. 1D) the onset of ASNase hypersensitivity reactions.

ASNase ICs can be prepared *ex vivo* and their binding to immune cells is Fc- γ R-dependent

ASNase ICs were prepared *ex vivo* using fluorescent ASNase and either rabbit anti-ASNase IgG, purified mouse anti-ASNase IgG, or plasma from ASNase-sensitized mice. Anti-ASNase IgG was purified from mouse plasma using biotinylated ASNase and streptavidin-coated polystyrene beads. Purified mouse anti-ASNase antibody was characterized by ELISA and Western blot (Supplemental Fig. S5A, B). ASNase ICs prepared from all sources of anti-ASNase IgG show similar binding to protein G beads (Fig. 2A). The optimal weight ratio of

ASNase to murine anti-ASNase IgG for IC formation was experimentally evaluated by determining the concentration of anti-ASNase or ASNase, at which further addition did not increase the detection of ASNase ICs (Supplemental Fig. S6A, B). Our results suggest that high levels of anti-ASNase IgG are required to form saturated ICs (*i.e.*, at or above a weight ratio of ASNase to anti-ASNase IgG of 1:10, which is equivalent to a 1:2 molar ratio). Our observation that little to no ASNase or antibody remains after PEG precipitation supports the conclusion that the ASNase fluorescence detected *via* our method is from ICs rather than free ASNase (Supplemental Fig. S7A–F). The presence of ASNase and anti-ASNase IgG within the ICs was confirmed by flow cytometry and PAGE analysis of the excised IC band (Supplemental Fig. S5C–E).

Soluble anti-ASNase IgG is required to form an ASNase IC, but no other ASNase-specific mechanism is required to mediate the binding and recognition of

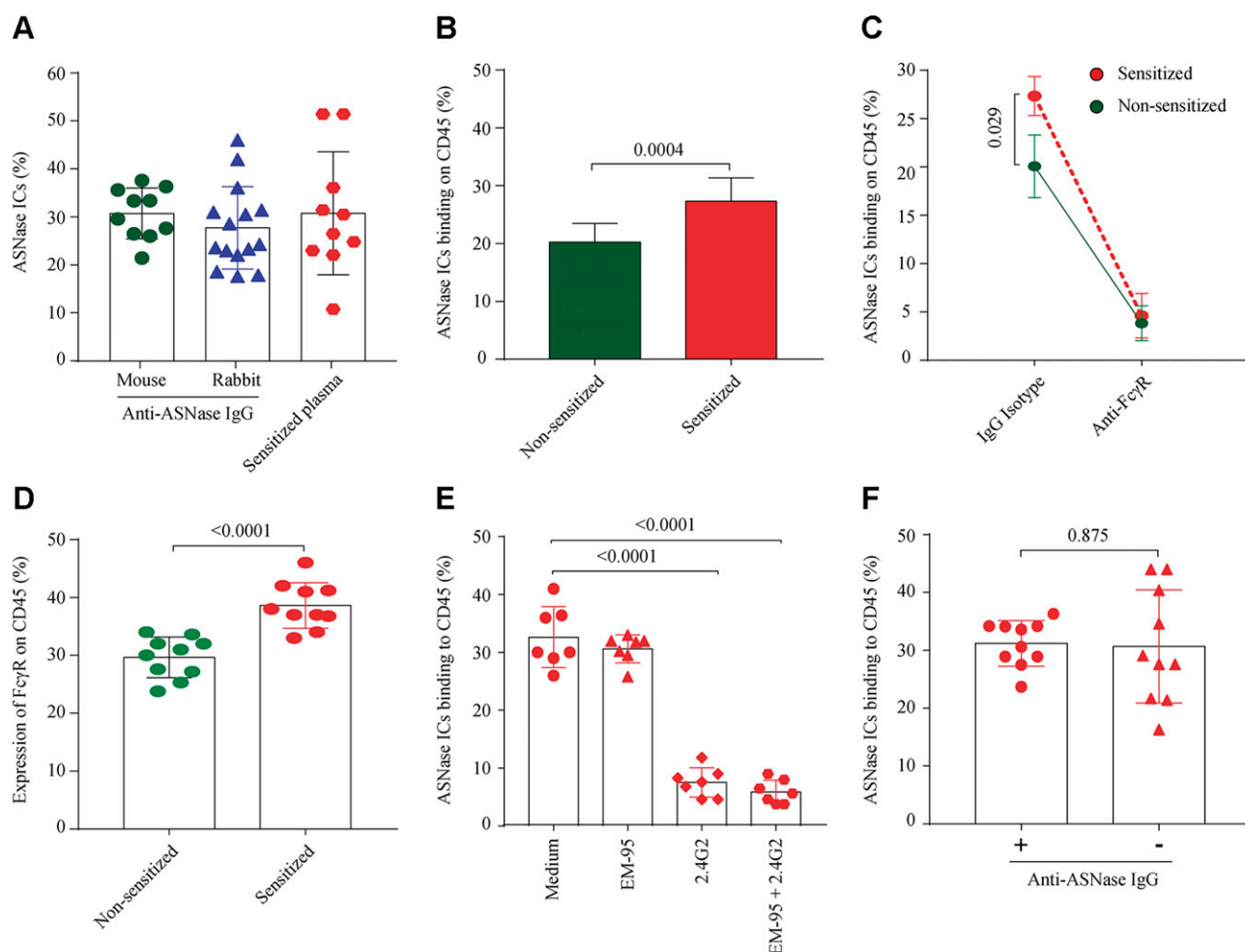


Figure 2. ASNase IC binding to immune cells is Fc- γ RIII-dependent. *A*) ASNase ICs were prepared *ex vivo* using either commercially available mouse or rabbit anti-ASNase IgG, or ASNase-sensitized mouse plasma that contained anti-ASNase IgG and detected by flow cytometry. *B*) ASNase ICs prepared *ex vivo* at an ASNase to anti-ASNase IgG weight ratio of 1:10 show strong binding to naive and ASNase-sensitized total leukocytes (CD45⁺). *C*) Blocking Fc- γ RIII using an mAb inhibits ASNase IC binding to naive and ASNase-sensitized leukocytes. *D*) The Fc- γ RIII expression of leukocytes was determined by flow cytometry and indicates that naive mice have lower Fc- γ RIII expression than ASNase-sensitized mice. *E*, *F*) Blocking anti-ASNase IgE with anti-IgE mAb (EM-95) (*E*), or removing soluble anti-ASNase IgG through lysis and washing (*F*) shows that ASNase IC binding to leukocytes is not dependent on IgE and Fc- ϵ RI or soluble anti-ASNase IgG.

ASNase ICs by immune cells expressing Fc- γ RIII. We therefore investigated which immune cells bound ASNase ICs and whether there were any differences between ASNase-naive and -sensitized mice. We observed significantly higher ASNase IC binding to sensitized leukocytes relative to naive leukocytes (Fig. 2B; $P = 0.0004$). We confirmed that the IC binding was Fc- γ R-dependent among both naive and sensitized leukocytes (Fig. 2C), and we speculated that the difference in ICs binding between naive and sensitized leukocytes is caused by the higher Fc- γ R expression measured in sensitized leukocytes relative to naive leukocytes (Fig. 2D; $P < 0.0001$). We also confirmed that none of the ASNase ICs binding to sensitized leukocytes is dependent on anti-ASNase IgE (Fig. 2E) or soluble anti-ASNase IgG (Fig. 2F). The residual binding of ICs to sensitized leukocytes after 2.4G2 anti-Fc- γ RII β /RIII mAb $\pm$ EM-95 anti-IgE mAb treatment (Fig. 2E) may represent nonspecific binding or incomplete Fc- γ R blocking.

ASNase IC binding to immune cells requires high anti-ASNase IgG levels

Many patients with leukemia receiving ASNase develop anti-ASNase IgG antibodies but not ASNase hypersensitivity (*i.e.*, silent hypersensitivity) (4). We hypothesized that this can occur if antibody levels are low and are not sufficient to form ICs capable of binding to the Fc- γ R. To test this hypothesis, we prepared ASNase ICs at a weight ratio of ASNase to anti-ASNase IgG of 1:1, 1:2.5, 1:5, 1:10, or 1:20 and measured IC binding to naive leukocytes. We observed that IC binding to CD45⁺ leukocytes is dependent on high anti-ASNase IgG ratios (Fig. 3A). We further analyzed ASNase IC binding to sensitized and naive macrophages, neutrophils, basophils, B cells, and T cells at a 1:1, 1:10, or 1:20 weight ratio (Fig. 3B–F). We found that the ASNase ICs prepared at a 1:10 weight ratio had higher binding to all sensitized immune cells as compared with both lower and higher antibody ratios (Fig. 3B–D; $P < 0.05$). Binding of ICs to neutrophils and basophils from naive mice was also highest when ICs were prepared at a 1:10 ASNase to anti-ASNase IgG weight ratio, although IC binding to naive macrophages was similar for ICs generated at 1:10 and 1:20 ratios (Fig. 3B–D). Furthermore, ASNase ICs prepared at a 1:10 weight ratio showed significantly higher binding to sensitized than to naive macrophages, neutrophils, and basophils, whereas no difference between sensitized and naive B cells was observed (Fig. 3B–E). As expected, the magnitude of ASNase IC binding in both naive and sensitized mice was higher for immune cells with higher Fc- γ R expression (Fig. 3G). The magnitude of binding correlated with Fc- γ R expression of macrophages, neutrophils, and basophils (Fig. 3H–J).

ASNase ICs activate basophils in an Fc- γ RIII-dependent manner and induce anaphylaxis in naive mice

Basophil activation tests were performed to determine whether activation by ASNase ICs is dependent on the

ratio of antigen to antibody used to prepare ASNase ICs. Blood samples from sensitized and naive mice were stimulated with medium (negative control), 2.4G2 mAb (IgG-mediated activation), EM-95 mAb (IgE-mediated activation), ASNase, or ASNase ICs prepared at a 1:1, 1:10, or 1:20 ratio. The positive controls, EM-95 and 2.4G2, activate basophils by stimulating Fc- ϵ RI or Fc- γ Rs, respectively, with basophil activation respectively detected by the up-regulation of CD200R1 or the down-regulation of CD200R3 (Supplemental Fig. S8A–D). As previously described in Rathod *et al.* (19), samples from naive mice showed no ASNase-induced basophil activation, whereas sensitized mice stimulated with ASNase showed both a down-regulation of CD200R3 and an up-regulation of CD200R1 relative to naive mice (Fig. 4A, B). Consistent with the ASNase IC binding data, we found that ASNase ICs prepared at a 1:10 and a 1:20 weight ratio down-regulated CD200R3 in both naive and sensitized mice (Fig. 4B), without affecting CD200R1 expression (Fig. 4A), confirming that ASNase ICs only activate basophils *via* the Fc- γ R and that anti-ASNase IgE is not playing a role in the binding and activation of sensitized murine basophils by ICs. Consistent with the higher Fc- γ RIII expression among sensitized basophils (Fig. 3G) and the increased binding of ASNase ICs to sensitized *vs.* naive basophils (Fig. 3D), the down-regulation of CD200R3 was greater in sensitized *vs.* naive basophils for ASNase IC prepared at a 1:10 ratio (Fig. 4B). In contrast, ASNase ICs prepared at a 1:1 weight ratio, which showed low binding to macrophages, neutrophils, basophils, and B cells (Fig. 3B–E), did not activate basophils (Fig. 4B). ASNase ICs prepared at a weight ratio of 1:20 activated basophils similar to those prepared at 1:10 (Fig. 4B), supporting that high antibody levels are required to form ASNase ICs capable of binding and activating immune cells in an Fc- γ R-dependent manner. Based on the immune cell binding and basophil activation results, we speculated that ASNase ICs can induce ASNase hypersensitivity in naive mice without requiring soluble anti-ASNase IgG, cell-associated IgE, or any previous exposure to ASNase. Naive mice were therefore challenged with ASNase ICs prepared at a 1:10 ratio, ASNase, or vehicle control. Our results indicate that naive mice dosed with ASNase ICs developed ASNase hypersensitivity relative to both controls (Fig. 5A; $P < 0.002$). Consistent with reactions mediated by IgG and Fc- γ R, we detected no increase in mMCP-1 levels relative to sensitized mice challenged with ASNase or naive controls (Fig. 5B). Furthermore, no anti-ASNase IgG or ASNase activity (*i.e.*, drug levels) was detected in mice after receiving ASNase ICs (Fig. 5C, D). These studies confirm that ASNase ICs are sufficient to induce ASNase hypersensitivity and play a role in ASNase hypersensitivity in mice.

DISCUSSION

ASNase is an essential component of optimal ALL treatment. However, antibody responses to this chemotherapeutic agent worsen treatment outcome by

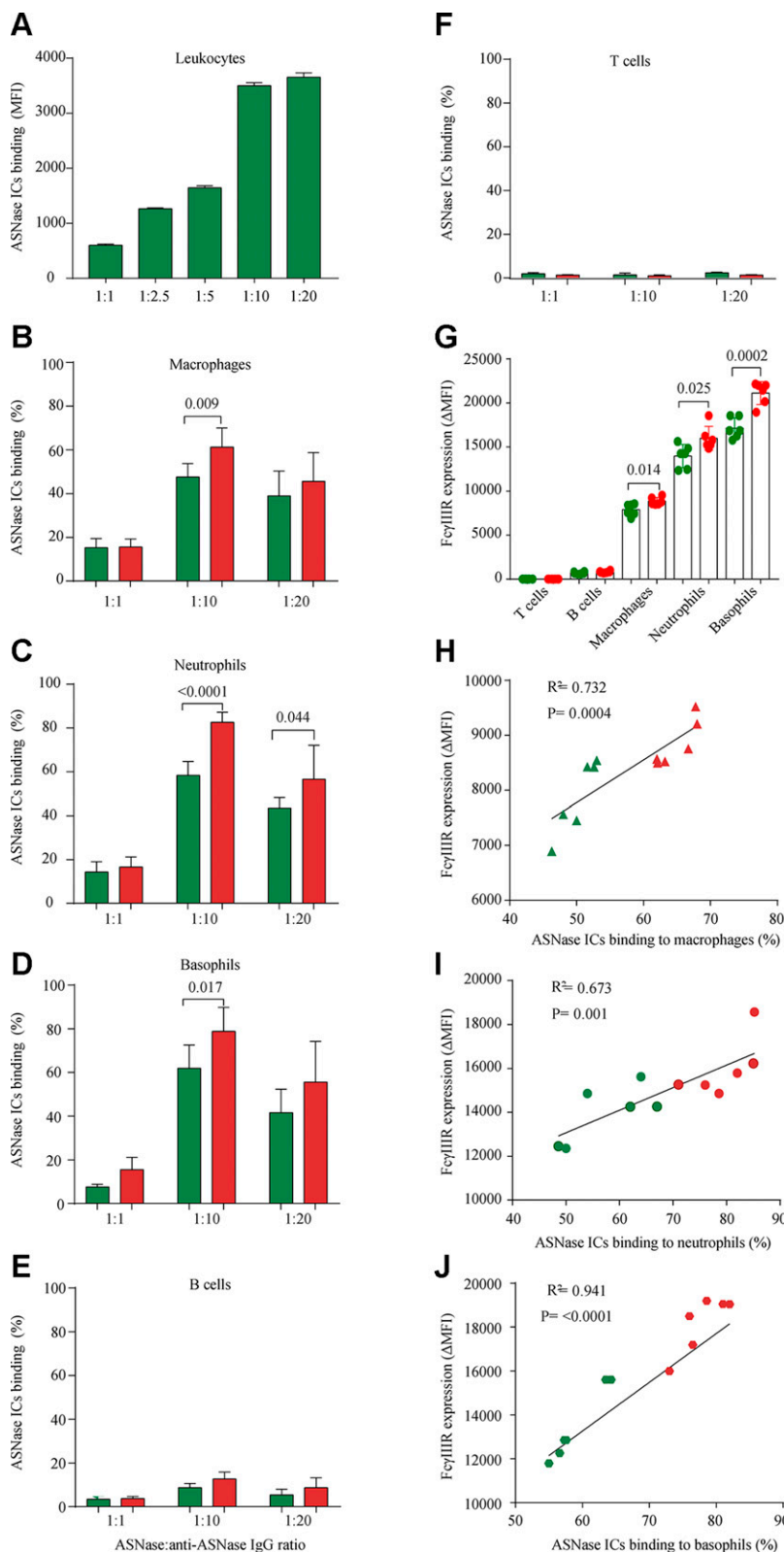


Figure 3. Binding of ASNase ICs to immune cells requires high anti-ASNase IgG antibody levels and correlates with Fc- γ RIII expression. *A*) *Ex vivo* ASNase ICs were prepared at weight ratios of ASNase to anti-ASNase IgG of 1:1, 1:2.5, 1:5, 1:10, and 1:20 and their binding to total leukocytes (CD45⁺) was determined. *B–F*) ASNase IC binding to naive and sensitized macrophages (*B*), neutrophils (*C*), basophils (*D*), B cells (*E*), and T cells (*F*) was determined at a 1:1, 1:10, and 1:20 weight ratio of ASNase to anti-ASNase IgG ($n = 15$). *G*) The Fc- γ RIII expression of ASNase-naive and -sensitized macrophages, neutrophils, basophils, B cells, and T cells was determined, and found to positively correlate with the ASNase ICs binding of macrophages, neutrophils, and basophils (*H–J*). MFI, mean fluorescence intensity.

decreasing ASNase concentration (25). To successfully prevent or mitigate the effect of ASNase hypersensitivity on treatment outcome, the mechanisms leading to hypersensitivity reactions require elucidation. To understand the underlying cause of clinical ASNase hypersensitivity, our group developed the first murine

model of immediate ASNase hypersensitivity and discovered 2 concurrent mechanisms of hypersensitivity in mice; these involve soluble ASNase binding to mast cell and basophil-associated IgE anti-ASNase antibody, and IgG anti-ASNase antibody and ASNase IC binding to Fc- γ RIII (19). This study attempted to provide evidence for

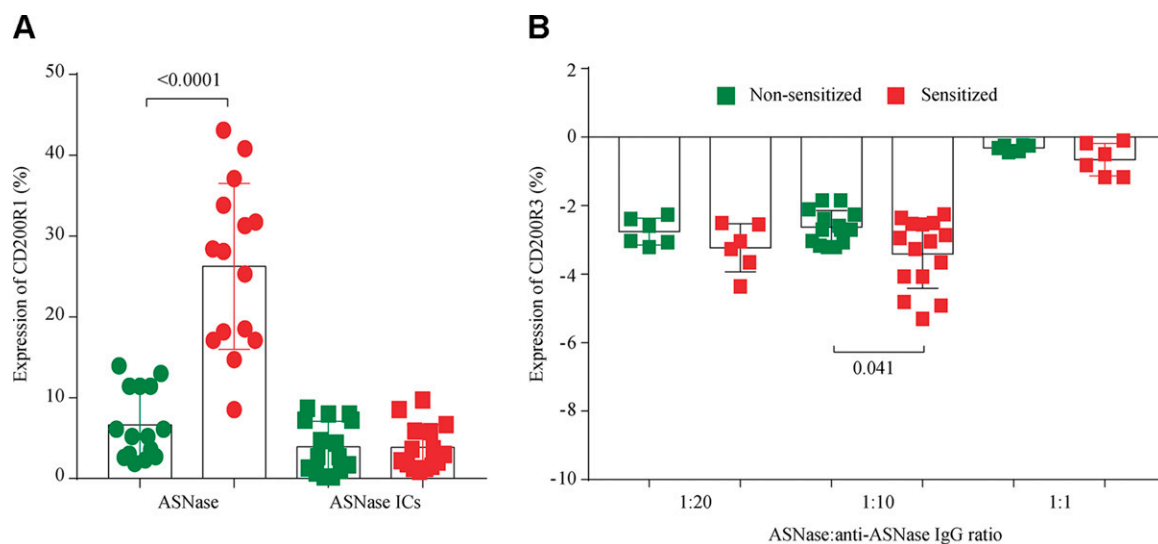


Figure 4. ASNase ICs activate naive and sensitized basophils in an Fc- γ R-dependent manner. *A*) ASNase exposure induces ASNase-sensitized basophils to up-regulate CD200R1 and down-regulate CD200R3 comparably with their respective modification by anti-IgE mAb (EM-95) and anti-Fc- γ R1B/III mAb (2.4G2), respectively, which were used as positive controls. In contrast, ASNase ICs do not affect CD200R1 expression, suggesting that they activate basophils through an Fc- γ R-dependent mechanism. *B*) The down-regulation of CD200R3 by ASNase ICs only occurs at higher weight ratios of ASNase to anti-ASNase IgG antibody.

the importance of the second mechanism by demonstrating the presence and role of ASNase ICs during ASNase hypersensitivity. Our results demonstrate the presence of ASNase ICs in ASNase hypersensitivity and show that these can directly bind to and activate naive and ASNase-sensitized immune cells. Moreover, we found that the role of these ICs during ASNase hypersensitivity depends on anti-ASNase IgG levels because ASNase ICs formed at lower ratios of antibody to ASNase are not capable of binding to immune cells expressing Fc- γ R1B/III. These observations help explain why patients with pediatric ALL can develop neutralizing anti-ASNase IgG antibodies during therapy without clinical hypersensitivity (*i.e.*, silent hypersensitivity) (4, 9). That is, if anti-ASNase IgG antibody or immune cell Fc- γ R expression levels are low, the probability of silent hypersensitivity is increased.

The alternative pathway of anaphylaxis requires soluble antigen-specific IgG antibodies to form ICs capable of binding to the Fc- γ R1B/III of immune cells, with hypersensitivity mediated predominantly by PAF. We therefore hypothesized that ASNase ICs prepared *ex vivo* could induce hypersensitivity in naive mice. In agreement with this hypothesis, we found that ASNase IC binding to naive immune cells is dependent on soluble anti-ASNase IgG and Fc- γ R1B/III (Fig. 2C, D and Supplemental Fig. S1B–D) and correlates with Fc- γ R1B/III expression (Fig. 3H–J). Furthermore, ASNase ICs prepared *ex vivo* induce hypersensitivity in naive mice with little or no increase in mMCP-1 levels (Fig. 5B), suggesting that mucosal mast cells are not playing a major role in ASNase IC-mediated hypersensitivity (26). These results, along with previous experiments using a PAF receptor antagonist during ASNase hypersensitivity (19), demonstrate that ASNase hypersensitivity is, in part, Fc- γ R1B/III-dependent and that ASNase ICs form from soluble anti-ASNase IgG antibodies after drug

administration with hypersensitivity mediated by PAF. Our studies also indicate that the severity of ASNase hypersensitivity, as determined by the area under the temperature *vs.* time curve, correlates with the challenge dose of ASNase (18), ASNase IC levels (Fig. 1B), and mMCP-1 concentrations (18, 19) (observations that support the contribution of both hypersensitivity pathways to ASNase hypersensitivity).

ASNase silent hypersensitivity during ALL treatment is a major complication because of their effect on outcome (6–8, 27, 28). The strong correlation between clinical ASNase hypersensitivity and high anti-ASNase antibody levels suggests that silent hypersensitivity occurs because of low antibody levels that are sufficient to neutralize considerable ASNase but insufficient to form ICs capable of inducing clinical hypersensitivity (4, 6–8, 27, 28). To assess the mechanism of silent hypersensitivity, we purified anti-ASNase IgG antibodies from sensitized mice and prepared ASNase ICs *ex vivo*. These ASNase ICs had similar properties to ASNase ICs isolated from plasma samples (Fig. 2A) and likewise bound to cells expressing Fc- γ R1B/III (Fig. 3B–D). Our studies using purified anti-ASNase IgG demonstrate that ASNase ICs formed at high ratios of antigen to antibody can bind and activate basophils (Fig. 4B), whereas ICs formed at lower ratios cannot, presumably because of the lower binding affinity of these ICs for Fc- γ R1B/III. These results are consistent with clinical silent hypersensitivity correlating with lower antibody levels (4).

No previous study, to our knowledge, has detected ASNase ICs in plasma samples. We detected these ICs in plasma by PEG precipitation (21, 22, 29), which separates ICs from free antibody and antigen by precipitating ICs and leaving any free ASNase and soluble anti-ASNase IgG in solution (Supplemental Fig. S7C–F). Using this technique, we found no involvement of

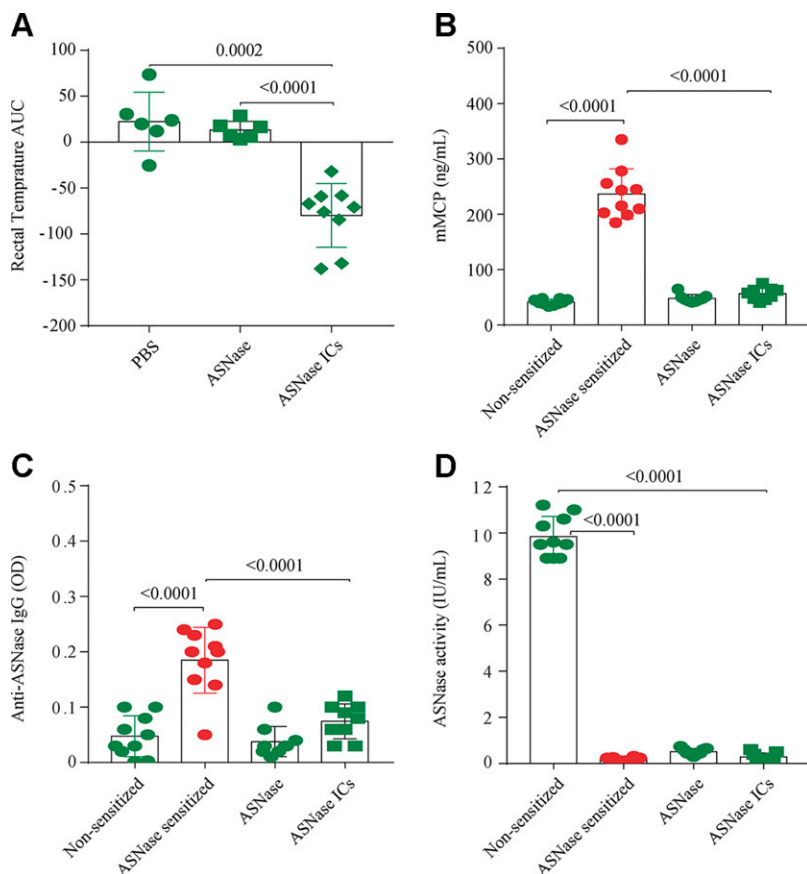


Figure 5. ASNase ICs prepared *ex vivo* can trigger anaphylaxis in ASNase-naive mice. *A*) Naive C57BL/6 mice received intravenous vehicle control (PBS), ASNase (1 μ g), or ASNase ICs prepared *ex vivo* at a 1:10 ratio of ASNase (1 μ g) to anti-ASNase IgG (10 μ g). The core body temperatures of mice were monitored for the development of hypothermia and the onset of hypersensitivity. *B*, *C*) The data indicate that mMCP-1 (*B*) and anti-ASNase IgG (*C*) levels were lower in naive mice receiving ASNase ICs compared with ASNase-sensitized mice challenged with ASNase. *D*) Low ASNase activity levels were detected in naive mice receiving ASNase ICs and ASNase-sensitized mice challenged with ASNase. OD, optical density.

anti-ASNase IgE in ASNase IC binding to basophils or activation (Figs. 2E and 4A, respectively). Similarly, ASNase IC binding to cells expressing Fc- γ RIII did not depend on soluble anti-ASNase IgG (Fig. 2F), supporting the view that the IgG binding, basophil activation, and clinical hypersensitivity we detected were mediated solely by ICs.

In summary, we have shown that ASNase ICs are detected in mice after the onset of ASNase hypersensitivity and that these ICs can bind and activate immune cells in an Fc- γ RIII-dependent manner. Furthermore, ASNase ICs prepared *ex vivo* are sufficient for inducing ASNase hypersensitivity in naive mice. Taken together, our data explicitly indicate that anti-ASNase antibodies can mediate hypersensitivity by forming ICs. However, when IgG anti-ASNase antibody levels are low, ASNase ICs formed will not have sufficient affinity for Fc- γ RIII to activate immune cells, leading to silent hypersensitivity. Although it remains unknown whether these results apply to clinical ASNase hypersensitivity, detecting ASNase ICs after the onset of ASNase hypersensitivity in clinical samples would support the involvement of the alternative pathway of anaphylaxis in humans. **[F]**

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AUTHOR CONTRIBUTIONS

S. Rathod, F. D. Finkelman, and C. A. Fernandez conceived and designed research; S. Rathod, M. Ramsey, D. DiGiorgio, and R. Berrios performed research; and S. Rathod and C. A. Fernandez analyzed the data and wrote the manuscript.

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Asparaginase immune complexes induce FcγRIII-dependent hypersensitivity in naïve mice

Sanjay Rathod,¹ Manda Ramsey,¹ Danielle DiGiorgio,¹ Roberto Berrios,¹ Fred D. Finkelman,² and Christian A. Fernandez¹

¹Center for Pharmacogenetics and Department of Pharmaceutical Sciences, University of Pittsburgh, Pittsburgh, PA 15261, USA

²Department of Internal Medicine, Division of Immunology, Allergy and Rheumatology, University of Cincinnati College of Medicine, Cincinnati, OH 45267; and the Division of Immunobiology, Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, USA.

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Corresponding Author:

Christian A. Fernandez

Center for Pharmacogenetics

Department of Pharmaceutical Sciences

School of Pharmacy

335 Sutherland Drive

Pittsburgh, PA 15261

412-383-8108 phone

chf63@pitt.edu

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Supplementary Methods

Flow cytometry analysis

Freshly collected blood cells in EDTA were stained with the following anti-mouse conjugated antibody fluorophores and clones were used for this study: anti-CD3-eFluor450 (clone: 17A2), anti-B220-PerCP (clone: RA3-6B2), anti-CD11b-PE-Cy7 (clone: M1/70), anti-CD11c-FITC (clone: N418), anti-I-A^b (MHC class-II)-Pacific Blue (clone: AF6-120.1), anti-Ly6c-APC-Cy7 (clone: HK1.4), anti-F4/80-PE-eFluor 610 (clone: BMB), anti-Ly6G (Gr-1)-FITC (clone: RB6-8C5), anti-FcyRIII-PerCP (clone: #002), anti-IgE-PE (clone: RME-1), anti-CD49b-FITC (clone: DX5), and anti-CD200R-APC (clone: OX110) were all from ThermoFisher Scientific, Waltham, MA, USA except for anti-CD45-VioGreen (clone: REA737) and anti-CD200R3-VioBlue (Clone: REA128) from (Milteny Biotec, Germany). After staining, blood cells were suspended in running buffer (Milteny Biotec, Germany) and acquired on a MACSQuant Analyzers Flow cytometer (Milteny Biotec, Germany). Gates were determined using unstained and single-stained samples obtained from the blood. Compensation and FMO controls used for final gating and analysis. Results were analyzed using FlowJo_V10 (TreeStar, USA).

Preparation of PBMCs and splenocytes: Peripheral blood was collected at a 1:10 volume/volume ratio of anti-coagulant (0.5 M EDTA) to whole blood and was immediately mixed well. ASNase binding to peripheral blood cells in the absence of anti-ASNase IgG antibody was determined. PBMCs and splenocytes were isolated from naïve and sensitized mice by centrifugation over a ficoll gradient.

Immune cell populations were defined as follows: All phenotypes were gated from leukocytes (CD45⁺), B cells (B220⁺CD3⁻CD11b⁻CD11c⁻), T cells (CD3⁺B220⁻CD11b⁻CD11c⁻), macrophages (⁺MHC-II⁺F4/80⁺), monocytes (MHC-II⁻Ly6c⁺Ly6G⁻), neutrophils (MHC-II⁻Ly6c⁻/⁺Ly6G⁺) and basophils (CD49b⁺IgE⁺), see supplementary Figure 9 for details and flow cytometry gating strategy. Fluorochrome -labeled ASNase ICs was used to determine IC binding to immune cell populations.

Supplementary Figures Legends

Supplemental Figure 1. ASNase binding to the immune cells of sensitized mice is IgG and IgE-dependent. (A-B) ASNase binding to total leukocytes (CD45⁺), B cells, and T cells in the presence and absence of anti-ASNase IgG antibodies demonstrates that binding is dependent on

soluble anti-ASNase IgG (n=5). (C) ASNase binding to leukocytes and basophils is restored after the addition of soluble anti-ASNase IgG (n=15). (D) ASNase binding to sensitized basophils in the absence of anti-ASNase IgG is dependent on cell-associated anti-ASNase IgE and is blocked by anti-IgE mAb (EM-95; n=8 per group).

Supplemental Figure 2. ASNase binding to PBMCs and splenocytes is dependent on soluble anti-ASNase IgG antibodies. Blood and spleen samples were collected from naïve and sensitized mice. PBMCs and splenocytes were isolated by ficoll density gradient centrifugation. (A) ASNase binding to total leukocytes (CD45⁺), B cells, and T cells from PBMCs and (B) splenocytes of sensitized or naïve mice were determined (n=5 in each group), confirming that ASNase binding to immune cells is dependent on soluble anti-ASNase antibodies and IC formation.

Supplemental Figure 3. ASNase-sensitized mice develop a hypersensitivity reaction after an ASNase challenge. (A) Sensitized mice experienced a drop in rectal temperature that is quantified by estimating the AUC of the rectal temperature versus time curve. (B) Sensitized mice develop elevated levels of mMCP-1 after challenge compared to non-sensitized mice (n=10 per group).

Supplemental Figure 4. Schematic representation of ASNase ICs detection by flow cytometry. ASNase ICs were detected from sensitized mice after challenge and the onset of hypersensitivity by flow cytometry. Plasma samples were precipitated with an equal volume of 8% PEG (final concentration, 4% PEG) and then incubated with protein G beads for 60 minutes at 4° on shaker. The protein G beads were stained with fluorochrome-labeled goat anti-mouse IgG Fc antibody and analyzed by flow cytometry. Naïve mouse plasma and protein G beads were used as a negative control.

Supplemental Figure 5. Characterization of purified anti-ASNase IgG and ASNase ICs. (A) Purified mouse anti-ASNase IgG antibody was confirmed by anti-ASNase IgG ELISA, as described previously (18, 19), and (B) by western blotting, where *E.coli* ASNase was stained with purified anti-ASNase IgG and murine anti-IgG HRP secondary antibody. ASNase ICs prepared at a 1:10 weight ratio of ASNase to anti-ASNase IgG were detected by flow cytometry (C). ASNase (34kDa), anti-ASNase IgG (heavy chain 55kDa; light chain 28 kDa), and ASNase

ICs (~100 kDa) were separated by PAGE (D). The ASNase IC band was excised and separated by PAGE to demonstrate that it is comprised of ASNase and anti-ASNase IgG antibody (E).

Supplemental Figure 6. Determining the optimal ratio of ASNase to anti-ASNase IgG for the preparation of ASNase ICs. (A) Labeled ASNase (80 ng) was incubated with different concentrations of purified murine anti-ASNase IgG (10 to 2000 ng) and analyzed by flow cytometry for IC formation (B) Similarly, purified anti-ASNase IgG antibody (800 ng) was incubated with different concentrations of labeled ASNase (0.8 to 1600 ng) and analyzed by flow cytometry for the ASNase IC formation. The results indicate that ASNase ICs formation is saturated at a weight ratio of 1:10 (ASNase to anti-ASNase IgG).

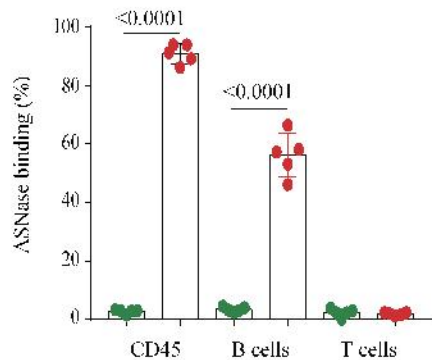
Supplemental Figure 7. 4% PEG precipitation separates free ASNase and anti-ASNase IgG from ASNase/anti-ASNase IgG ICs, allowing for detection of ASNase ICs by flow cytometry. (A) The ability of 4% PEG precipitation to separate fluorescent ASNase from ASNase/anti-ASNase IgG ICs was determined using anti-ASNase antibody-coated protein G beads and flow cytometry to detect ASNase-associated fluorescence on these beads. (B) Removal of anti-ASNase IgG by 4% PEG precipitation was determined using protein G beads to capture IgG antibodies in the precipitate, followed by staining with fluorescent ASNase. Non-precipitated (No 4% PEG) samples were used as positive controls for each experiment. 4% PEG does not precipitate (C) free ASNase, (D) rabbit anti-ASNase IgG, or (E) purified mouse anti-ASNase IgG from naïve plasma. (F) Nor does the 4% PEG addition precipitate anti-ASNase IgG that is not part of an IC from ASNase sensitized plasma.

Supplemental Figure 8. Basophil activation test. (A) anti-IgE mAb (EM-95) upregulates CD200R1 expression following FcεRI stimulation. (B) Anti-FcγRIIB/III mAb (2.4G2), downregulates CD200R3 following FcγR stimulation. (C) Anti-FcγRIIB/III mAb (2.4G2) does not effect CD200R1 basophil expression. (D) Anti-IgE mAb (EM-95) does not downregulate CD200R3 basophil expression.

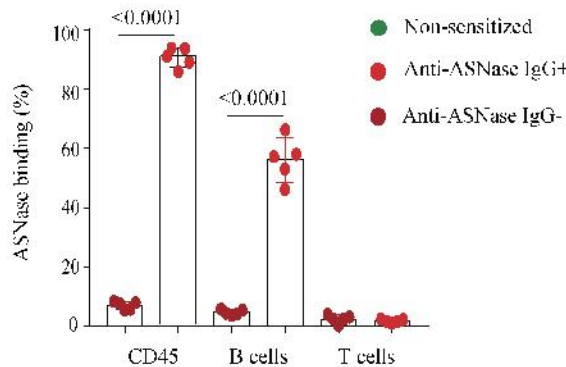
Supplemental Figure 9. Gating strategies. The gating strategy for immunophenotypes are shown. Live peripheral blood cells were gated based on FSC-A/SSC-A, exclusion of doublets (FSC-H/FSC-A) and gate singlets. Total leukocytes were identified by gating CD45⁺ populations; from total leukocytes, all other populations were gated. T and B cells were gated based on CD3 vs. B220 expression, and the CD11b⁺CD11c⁺ population was separated into B and

CD3⁺ T cells. For neutrophils and macrophage, CD45 populations were gated as MHC-II⁺ and F4/80⁺MHC-II⁻ populations. Neutrophils were gated as MHC-II⁻Ly6c^{-/+}Ly6G⁺ and macrophages as MHC-II⁺F4/80⁺. Basophils were gated as CD49⁺IgE⁺ cells from CD45 populations. Single color and FMO controls were used for setting.

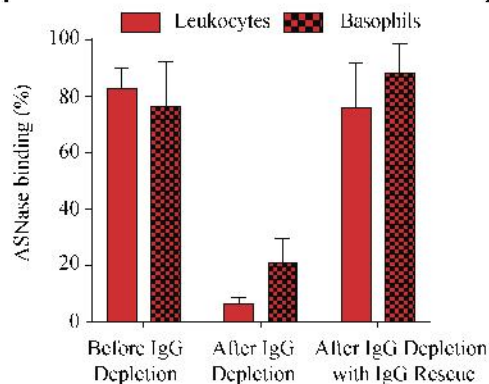
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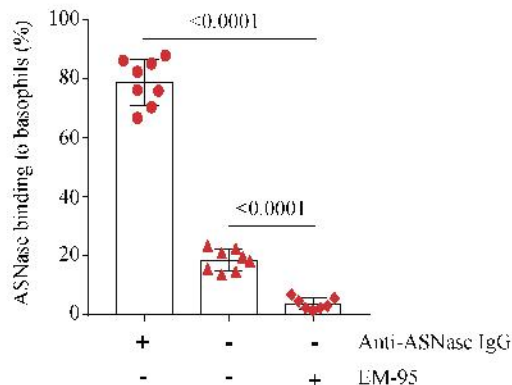
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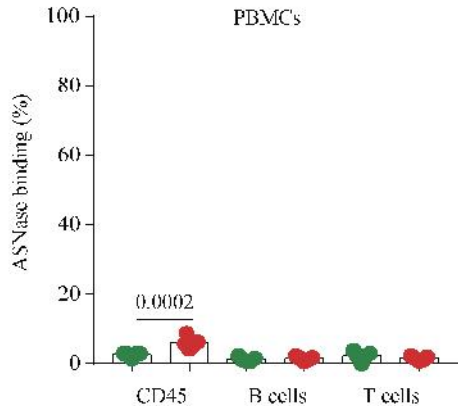
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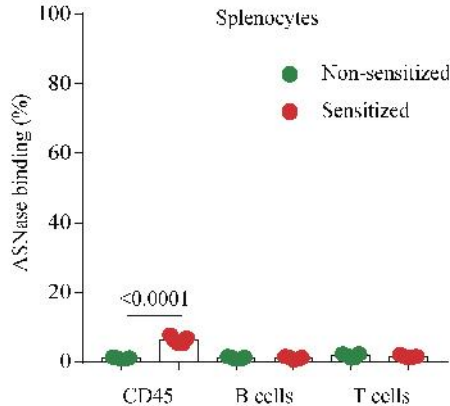
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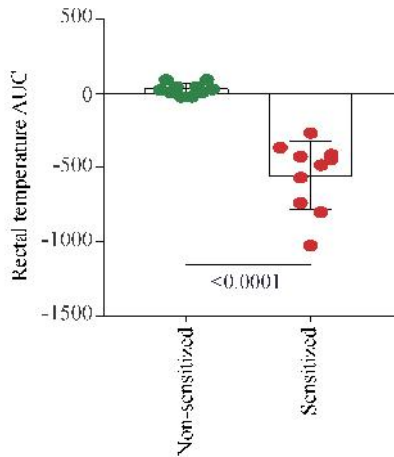
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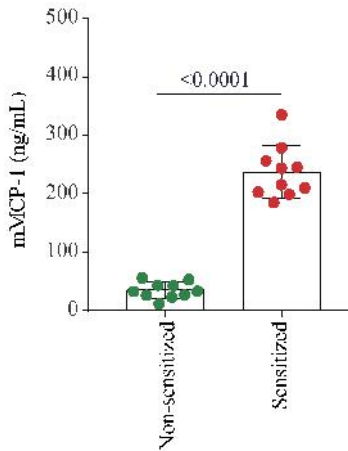
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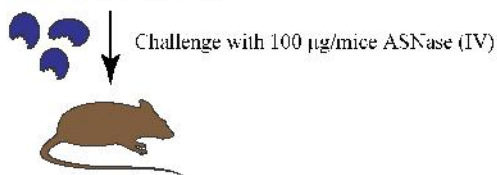
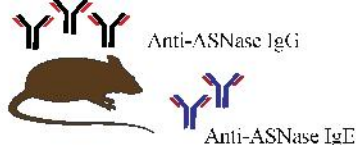


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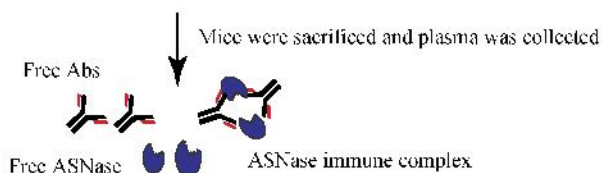


B.

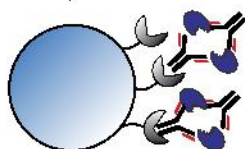
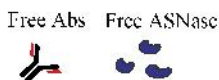




Mice developed ASNase-induced hypersensitivity condition



PEG precipitation

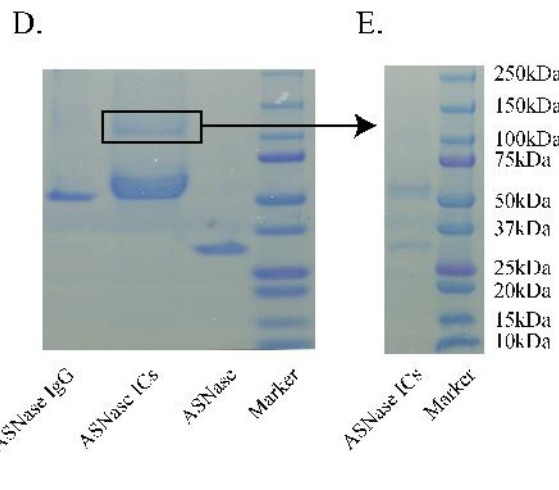
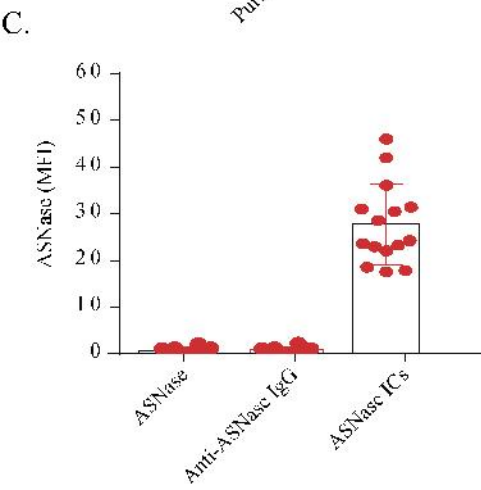
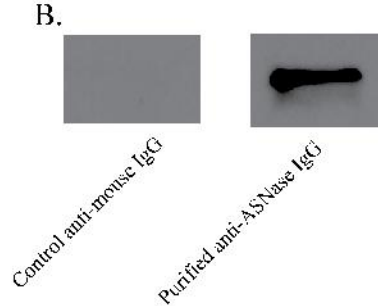
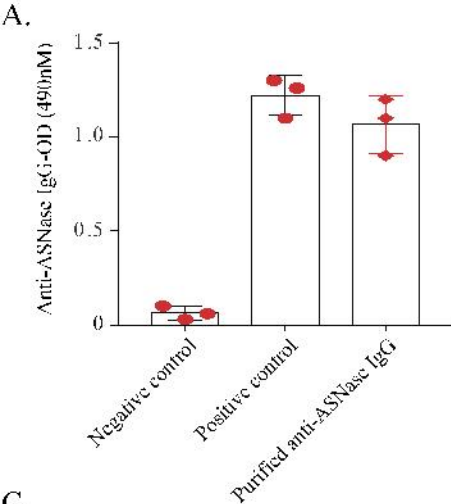


Goat anti-mouse Fc secondary Ab

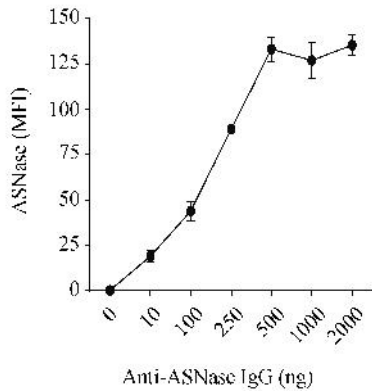


Protein G Beads (6-8 µm in Size)

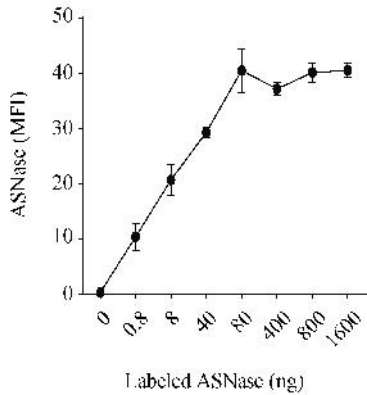
Detected by flow cytometry



A.

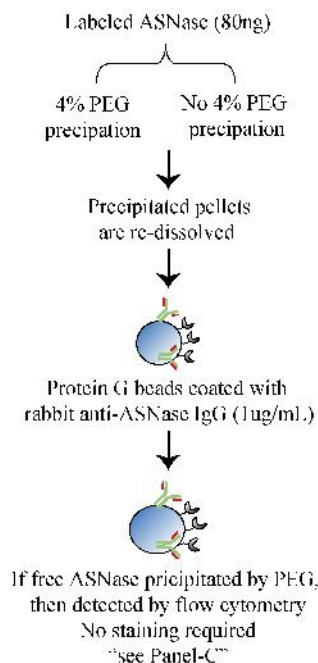


B.



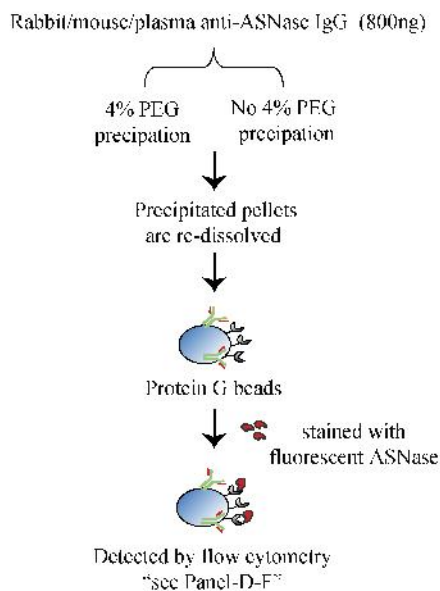
A.

Method used for detection of free ASNase

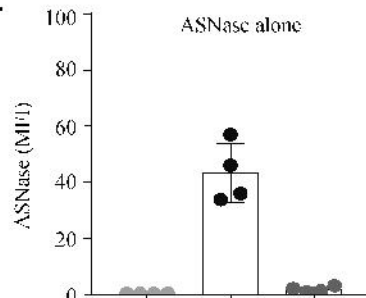


B.

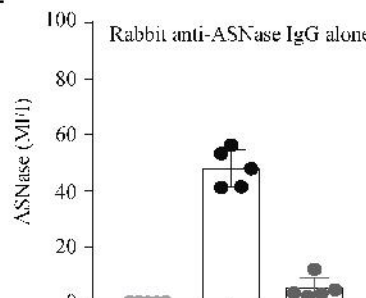
Method used for detection of free anti-ASNase IgG



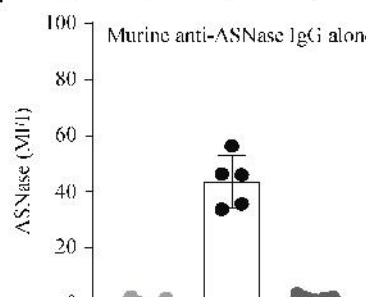
C.



D.



E.



F.

