

# Basophil activation through ASGM1 stimulation triggers PAF release and anaphylaxis-like shock in mice

Yan Yang<sup>1,2</sup>, Daling Li<sup>1,3</sup>, Foad Katirai<sup>1,4</sup>, Bin Zhang<sup>1</sup>, Yong Xu<sup>1</sup>,  
Ping Xiong<sup>1</sup>, Feili Gong<sup>1</sup> and Fang Zheng<sup>1</sup>

<sup>1</sup> Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

<sup>2</sup> Laboratory of Infection and Immunity, Wuhan Institute of Virology, Chinese Academy of Sciences, Wuhan, China

<sup>3</sup> Department of Anesthesiology, Wuhan Central Hospital, Wuhan, China

<sup>4</sup> Clinical Medical School, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China

Basophils have been shown to contribute to anaphylaxis through either an IgE–FcεRI-dependent pathway or an IgG–FcγR pathway. However, it remains largely unclear whether basophils can be activated to promote anaphylaxis via a non-FcR pathway as well. The glycolipid receptor ASGM1 (Asialoganglioside ganglioside gangliosylceramide), which has an exposed GalNAcβ1–4Gal moiety and serves as a receptor for pathogen associated molecular patterns such as flagellin, was recently found to be expressed on basophils. Here, we demonstrate that stimulation of basophils with anti-ASGM1 antibodies promotes platelet-activating factor (PAF) secretion in vitro and in vivo. Moreover, we found that ASGM1 stimulation triggers basophil- and PAF-dependent anaphylactic shock in pertussis toxin (PTX)-pretreated mice. Thus, ASGM1 has a crucial role in basophil activation and basophil-mediated anaphylaxis-like shock in mice, especially when the vascular permeability is increased by PTX treatment. Our findings describe a novel anaphylaxis-associated pathway that is antigen-, antibody-, and FcR-independent.

**Keywords:** Anaphylaxis · ASGM1 · Basophil · PAF



Additional supporting information may be found in the online version of this article at the publisher's web-site

## Introduction

Anaphylaxis is a serious life-threatening allergic reaction characterized by rapid onset and potentially fatal outcomes [1]. The major manifestations of anaphylaxis include bronchoconstriction, pulmonary hypertension, systemic hypotension, and vascular leakage. A classical IgE- and FcεRI-dependent pathway has long been recognized to be associated with anaphylaxis. Binding of

antigens to IgE and high-affinity cross-linking with FcεRI trigger the release of histamines from mast cells, resulting in anaphylaxis. Recently, various human and animal studies have indicated that IgG- and FcγR-involving pathways also mediate anaphylaxis in an IgE-independent fashion [2–4]. IgG antibodies, FcγRIIIA and FcγRIV, macrophages and neutrophils, as well as platelet-activating factor (PAF) are the main players in IgG-induced passive and active systemic anaphylaxis, in which IgE antibodies, FcεRI, mast cells, and histamine play a minor role [5]. It has been reported that in the absence of a specific allergen and antibodies, treatment with two injections of the cytokine IL-33 can trigger a third pathway for anaphylaxis in mice [6]. Kojima

**Correspondence:** Dr. Fang Zheng  
e-mail: zhengfangtj@hust.edu.cn

et al. also reported that in mice the anti-CD200R3 antibody Ba91 induced systemic and local anaphylaxis via an IgE- and IgG1-independent pathway that involved CD200R3 receptor activation [7]. Currently, it is largely unclear whether and how non-FcR receptors activate immune cells and trigger anaphylaxis.

Basophils represent less than 1% of peripheral blood leukocytes. They are thought to contribute to anaphylaxis by releasing histamine and PAF. It has long been recognized that antigen binding to IgE and high-affinity cross-linking with FcεRI trigger the release of histamines from basophils, resulting in anaphylactic shock. Recently, compelling evidence supports that basophils are dispensable for IgE-mediated anaphylactic shock but are responsible for a fatal outcome in IgG1-mediated anaphylaxis in mice through the release of PAF rather than histamine [2]. Serum PAF levels were directly correlated with the severity of anaphylaxis [8].

Asialoganglioside ganglioside GM1, ASGM1) is a glycolipid receptor with an exposed GalNAcβ1-4Gal moiety that serves as a receptor for pathogen associated molecular pattern such as flagellin from a large number of pathogens [9–11]. ASGM1 also acts as a coreceptor for TLR5 [12–16]. Anti-ASGM1 antibodies can mimic the interaction of flagellin with ASGM1 [17] and have long been used in mice for in vivo depletion of NK cells, which express high levels of ASGM1 [18–21]. Recent studies have revealed that, in addition to NK cells, basophils express ASGM1 whereas anti-ASGM1 antibody (αASGM1) treatment targets basophils and abolishes IgE-mediated chronic cutaneous allergic inflammation regardless of the presence or absence of NK cells in mice [22]. Although these findings have suggested the crucial role of basophil in allergic responses and the depleting effect of αASGM1 on basophil, it remains largely unclear whether and how ASGM1-mediated activation of basophil contributes to the development of anaphylactic shock.

In this study, we found that ASGM1 stimulation using anti-ASGM1 antibodies activates basophils to produce and secrete PAF in vitro and in vivo. Moreover, we observed that ASGM1 stimulation triggers anaphylactic shock in pertussis toxin (PTX)-pretreated mice. The basophil and PAF were identified as the pivotal mediators in anti-ASGM1-induced anaphylactic shock. Interestingly, PTX was shown to augment the susceptibility of mice to PAF-mediated anaphylaxis via enhancing vascular permeability and upregulating FcεRI expression on basophils. Thus, this study indicates a critical role for ASGM1 in basophil activation and PAF-mediated anaphylaxis-like shock in mice.

## Results

### Anti-ASGM1 antibody induces basophil activation and PAF release in vitro

The effects of ASGM1-mediated stimulation on basophil were detected by treating basophils derived from bone marrow (BMBs) cultured in vitro with αASGM1. BMBs (DX5<sup>+</sup>FcεRI<sup>+</sup>c-kit<sup>+</sup>) were obtained with more than 90% purification (Fig. 1A) and expressed

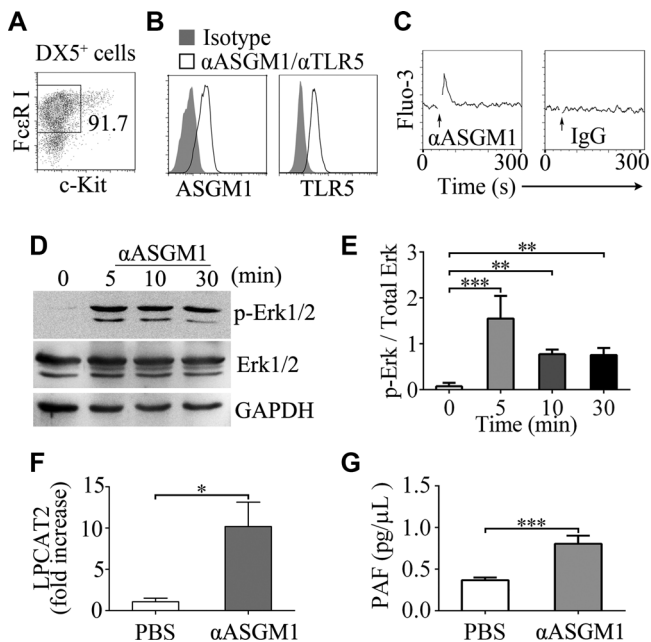


**Figure 1.** Basophil activation through ASGM1 stimulation in vitro. (A) Basophil purification. DX5<sup>+</sup> cells were purified from mice bone marrow cells that had been cultured for 10 days with IL-3 using a MACS column and magnetically labeled DX5-specific antibody. Next, the purity of DX5<sup>+</sup> bone marrow-derived basophils (BMBs) was determined by flow cytometry using FcεRI- and c-Kit-specific antibodies. The mean value of the percentages of FcεRI<sup>+</sup>c-Kit<sup>+</sup> cells in the three independent experiments was 91.7%. (B) ASGM1 (left) and TLR5 (right) are both expressed on BMBs. The expression of ASGM1 and TLR5 on the cell surface of DX5<sup>+</sup>FcεRI<sup>+</sup>c-Kit<sup>+</sup> BMBs was determined by flow cytometry using specific antibodies and isotype controls. (C) Calcium mobilization in ASGM1-stimulated BMBs. Fluo-3-AM (5 μM)-loaded BMBs were assessed for calcium influx by flow cytometry. Changes in fluorescence of Fluo-3/Ca<sup>2+</sup> were determined after cell treatment with αASGM1 (10 μg/mL) or control-IgG (IgG) (10 μg/mL) at the time point that the arrows indicate. (D–E) Activation of ERK1/2 MAPK in ASGM1-stimulated BMBs. αASGM1 (10 μg/mL)-treated BMBs (1 × 10<sup>6</sup> cells) were lysed and phosphorylated ERK1/2, total ERK1/2, and control GAPDH levels were detected by Western blotting using specific antibodies at a dilution of 1:1000. (D) A blot that is representative of three independent experiments is shown. (E) The band densities were assessed using scanning densitometry. Data are shown as mean + SEM of three samples. (F) Increased mRNA expression of Lysophosphatidylcholine acetyltransferase 2 (LPCAT2) in ASGM1-stimulated BMBs. LPCAT2 mRNA levels were assessed in BMBs using real-time PCR after treatment with αASGM1 (10 μg/mL) or PBS. Data from real-time PCR were normalized to GAPDH mRNA levels and are presented as fold increase vs. untreated BMBs. Data are shown as mean + SEM of five samples. (G) Increased secretion of platelet-activating factor (PAF) by ASGM1-stimulated BMBs. The supernatant of BMBs that had been treated with αASGM1 (10 μg/mL) or PBS for 30 min was collected and PAF levels were measured using LC/MS. Data are shown as mean + SEM of six samples. (A–C) Data shown are representative of three independent experiments. (E–G) Data shown are pooled and calculated from three independent experiments. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001 in two-tailed Student's *t*-test.

high level of ASGM1 and TLR5 (Fig. 1B). αASGM1 treatment induced an immediate and a rapid transient rise of [Ca<sup>2+</sup>]<sub>i</sub> which then returned to baseline values within 150 s (Fig. 1C) and strong ERK1/2 phosphorylation within 5 min (Fig. 1D and E). In addition, αASGM1 treatment upregulated LysoPAFAT/LPCAT2 (a PAF biosynthetic enzyme) mRNA expression (Fig. 1F) and PAF release (Fig. 1G) within 30 min. These results suggest that stimulation

with  $\alpha$ ASGM1 can activate basophil and promote the synthesis and release of PAF.

### Anti-ASGM1 antibody triggers PAF-mediated shock in PTX-pretreated mice

The *in vivo* effects of ASGM1-mediated stimulation were assayed by injection of  $\alpha$ ASGM1 into mice. The procedure is schemed (Fig. 2A). C57BL/6 mice were intraperitoneally (i.p.) injected with PTX (200 ng) or PBS on day 0 and 2, then were i.v. injected with  $\alpha$ ASGM1 (35  $\mu$ g) on day 7 (Fig. 2A).  $\alpha$ ASGM1 treatment had no significant effects on body temperature and activity in PBS-pretreated mice (Fig. 2B). However,  $\alpha$ ASGM1 treatment resulted in anaphylaxis-like shocks in PTX-pretreated mice, with salient features of rapid drops in body temperature, altered mouse behavior, to such an extent that death occurred within 60 min (Fig. 2B). In contrast, neither isotype IgG treatment nor PTX treatment again affected the body temperature, activity, and survival of the PTX-pretreated mice (Fig. 2B), which indicated that the anaphylaxis-like shock is not antibody dependent and PTX actions are biological in nature and do not act as allergen. Anti-NK1.1 antibody PK136 treatment that activates complement *in vivo*, also had no significant effect on the PTX-pretreated mice (Fig. 2B), which suggest complement activation was not involved in the above anaphylaxis-like shock.

PAF and histamine are two major mediators of human and murine anaphylaxis, either of which can directly cause shock if injected into rodents in sufficient quantities. In this study, pretreatment with the PAF antagonist CV-3988 almost completely prevented the anaphylaxis-like shock triggered by  $\alpha$ ASGM1 in PTX-pretreated mice (Fig. 2C), however pretreatment with triprolidine, a histamine H1 receptor antagonist, had no significant effect on the shock (Fig. 2D). Fifteen minutes after  $\alpha$ ASGM1 injection, mRNA levels of LysoPAFAT/LPCAT2 in the pulmonary tissue were significantly upregulated (Fig. 2E). PTX pretreatment had no significant effect on the mRNA levels of LysoPAFAT/LPCAT2 (Fig. 2E). These results suggest that  $\alpha$ ASGM1 triggers PAF, rather than histamine, mediated shock in PTX-pretreated mice.

### ASGM1 expressed on basophil contributes to PAF-mediated shock

ASGM1 expression on some immune cells was detected. It was found that high levels of ASGM1 expressed on NK cells, basophils, and a subpopulation of monocytes and macrophages whereas very low levels of ASGM1 were expressed on mast cells. No expression of ASGM1 was detected on neutrophils (Fig. 3A). Further, specific antibodies or reagents were used for depleting ASGM1<sup>+</sup> cell populations *in vivo*. Only MAR-1 pretreatment and Ba103 pretreatment, both for basophil depletion, abrogated  $\alpha$ ASGM1-induced temperature drops in PTX-pretreated mice (Fig. 3B). Treatment of mice with either gadolinium chloride (GdCl<sub>3</sub>) for monocytes/macrophages depletion or PK136 for NK cells and NKT

cells elimination had no significant effects on drops in body temperature (Fig. 3B). MAR-1 pretreatment did not change the count of intact or degranulated mast cells (Fig. 3C and D). These observations indicate that ASGM1-expressing basophil contributes to the PAF-mediated shock triggered by anti-ASGM1 antibodies in PTX-pretreated mice.

### Increased vascular permeability and susceptibility to PAF-induced anaphylaxis in PTX-treated mice

The effects of PTX on murine endothelial cells were measured *in vitro*. PTX treatment resulted in the retraction of the SVEC4–10 murine endothelial cells. The cell–cell contact was also lost with diminished stress fibers detected (Fig. 4A). In contrast, PTX plus PAF treatment induced more severe retracement of cells and disruption of cell–cell contacts, even cell losses (Fig. 4A). Not only PTX or PAF treatment alone reduced the average size of cells significantly, but also PTX plus PAF minimized the size of the cells (Fig. 4B). *In vivo*, mice treated with PTX displayed substantially increased Evans Blue leakage in the lungs, heart, and esophagus (Fig. 4C). The results indicate that PTX itself or cooperating with PAF acts on endothelial cells to promote vascular permeability.

Next, the cooperation of PTX and PAF was detected *in vivo*. PAF at a dose of 100 ng triggered slight temperature drops with no significant activity changes and no death in mice (Fig. 4D). Although PTX treatment alone did not induce shock, injection of 100 ng PAF in PTX-pretreated mice resulted in severe hypothermia, lower activity, and higher mortality rates (Fig. 4D), suggesting that PTX can increase the susceptibility of mice to PAF-induced anaphylaxis.

### Fc $\epsilon$ RI expression on basophil and levels of serum IgE are upregulated in PTX-pretreated mice

The effects of PTX on basophil *in vivo* were further assayed. PTX pretreatment for 7 days not only significantly increased the frequency of basophil in the spleen (Fig. 5A), but also upregulated the expression of high-affinity IgE receptor (Fc $\epsilon$ RI) on splenic basophil (Fig. 5A). Meanwhile, in the bone marrow, the percentage of basophil was not changed but Fc $\epsilon$ RI level on them was increased by PTX pretreatment (Fig. 5B). Moreover, elevated levels of serum IgE were also detected in PTX pretreated mice (Fig. 5C), while serum IgG1 and IgG2 levels were not influenced (Fig. 5D and E).

## Discussion

In 2011, Nishikado et al. firstly reported that the entire population of basophil in mice homogeneously expresses high levels of ASGM1 and that *in vivo* administration of anti-ASGM1 readily ablates most of basophils, in addition to NK cells, in a variety of mouse strains [22]. At the same time, we used  $\alpha$ ASGM1 to investigate the effects of NK cells on EAE development *in vivo*.

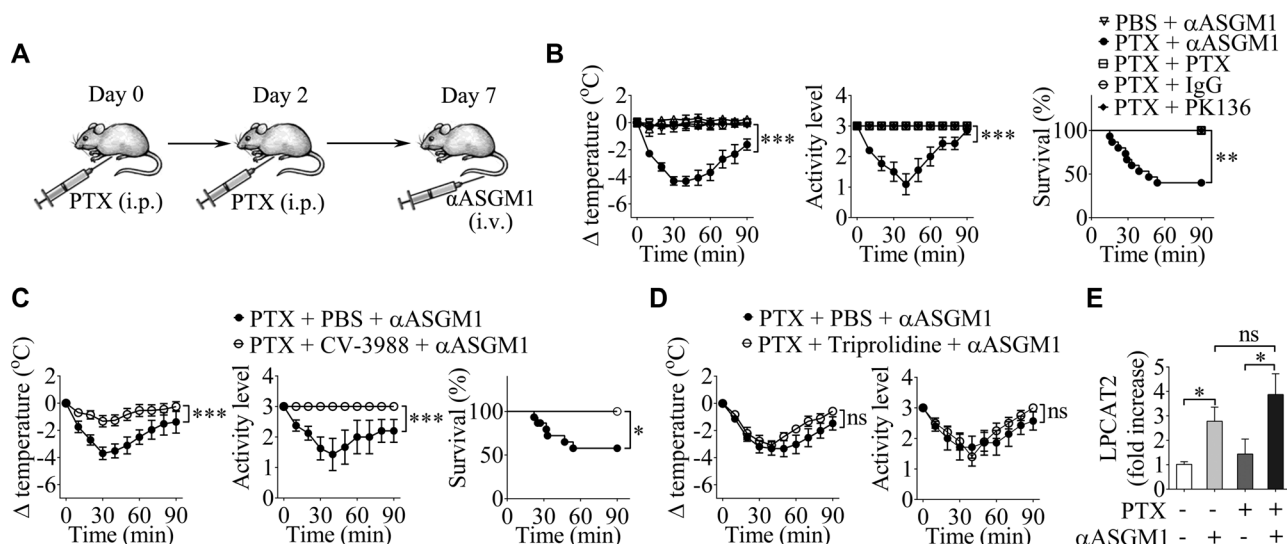


**Figure 2.** ASGM1 stimulation induces manifestations of anaphylactic shock in PTX-pretreated mice. (A) Scheme of PTX and  $\alpha$ ASGM1 administration. C57BL/6 mice were i.p. injected with PTX (200 ng in 200  $\mu\text{L}$  PBS) on days 0 and 2, then were i.v. injected with  $\alpha$ ASGM1 (35  $\mu\text{g}$  in 200  $\mu\text{L}$  PBS) or other reagents on day 7. (B) Manifestations of anaphylactic shock following injection of  $\alpha$ ASGM1. C57BL/6 mice pretreated with PTX or PBS were intravenously (i.v.) injected with  $\alpha$ ASGM1 (35  $\mu\text{g}$ ), rabbit isotype IgG (35  $\mu\text{g}$ ), PTX (200 ng), or the NK-cell depleting antibody PK136 (200  $\mu\text{g}$ ) in 200  $\mu\text{L}$  PBS on day 7. Rectal temperature (left), levels of activity (middle), and survival rate (right) were recorded. (C) PAF antagonist ameliorates  $\alpha$ ASGM1-induced anaphylactic shock. The PAF antagonist CV-3988 (80  $\mu\text{g}$  in 200  $\mu\text{L}$  PBS) or PBS were injected i.v. in mice 10 min before  $\alpha$ ASGM1 injection. (D) Histamine antagonist has no effect on  $\alpha$ ASGM1-induced anaphylactic shock. Mice were injected i.p. with the histamine antagonist triprolidine (200  $\mu\text{g}$  in 200  $\mu\text{L}$  PBS) or PBS 30 min before the  $\alpha$ ASGM1 injection. (E) LPCAT2 mRNA expression is upregulated in the lungs of  $\alpha$ ASGM1-injected mice. Total RNA was extracted from the lungs of PTX-pretreated or nonpretreated mice 15 min after  $\alpha$ ASGM1 injection. LPCAT2 mRNA levels were examined by real-time PCR (RT-PCR). Data from real-time PCR were normalized to GAPDH and presented as fold increase vs. uninjected mice. Data are shown as mean  $\pm$  SEM of five mice per group (two tailed Student's t-test). (B–D) Data shown as mean  $\pm$  SEM (temperature and activity rates) or as a survival curve (survival rates) of initially (B and C) 15 or (D) 10 mice per group and are pooled from three experiments. \* $p$  < 0.05, \*\* $p$  < 0.01, \*\*\* $p$  < 0.001. ns: no significance, two way ANOVA test was used for temperature and activity data, Log-rank (Mantel–Cox) test for survival data.

We performed i.p. injection of anti-ASGM1 antibodies to deplete NK cells on day 7, following the active induction of EAE according to the general protocol (the mice were firstly treated with PTX for two consecutive days and then actively immunized with MOG<sub>35–55</sub> and CFA). Unexpectedly,  $\alpha$ ASGM1 treatment resulted in anaphylaxis-like shock in mice with salient features of respiratory distress, rapid drop in body temperature and high mortality within 180 min (Supporting Information Fig. 1A). Interestingly, anti-ASGM1 antibodies induced anaphylaxis-like shock only in PTX-pretreated mice but not in myelin oligodendrocyte glycoprotein (MOG)- and CFA-pretreated or PBS-pretreated mice (Supporting Information Fig. 1A and B). Notably, i.v. injection of  $\alpha$ ASGM1 induced decrease of body temperature and altered animal behavior, to such an extent that death occurred within 60 min in PTX-pretreated mice group just as illustrated in Figure 2, more rapidly than i.p. injection and similar in induction speed to the typical experimental anaphylaxis. In this study, we clearly demonstrate that  $\alpha$ ASGM1 activates basophil to synthesize and release PAF in vitro and in vivo, as well as induces shock in PTX-pretreated mice.

Although basophil was first described more than 100 years ago, their precise biological functions are still unclear. Because basophil expressed the high-affinity IgE receptor (Fc $\epsilon$ RI) and release histamine in response to Fc $\epsilon$ RI-mediated stimulation, they have long been considered to contribute to the IgE-mediated anaphylactic shock [23]. However, recent compelling evidence shows that

basophils are dispensable for IgE-mediated anaphylaxis but shown to be responsible for a fatal outcome in IgG1-mediated anaphylaxis in mice through the release of PAF rather than histamine [2]. In our findings, although the shock triggered by  $\alpha$ ASGM1 involves activation of basophil and PAF release, it is allergen and antibody receptor independent. Here  $\alpha$ ASGM1 mimics the interaction of flagellin with ASGM1 [17].  $\alpha$ ASGM1 acts rather as an agonist of basophil, but not as a blocker or the ligand of Fc $\gamma$ R on the basophil.

In the present study, in untreated mice, ASGM1 mediated stimulation can activate basophil to release PAF, but has no significant effects on the general manifestations, such as body temperature and activity and so on. While in PTX-pretreated mice, the same ASGM1-mediated stimulation induces lethal shock. It suggests that PTX treatment increases the response of mice to  $\alpha$ ASGM1 mediated stimulation. PTX, a noncovalently linked heterohexameric protein, is the major toxic secretion of *Bordetella pertussis*, which can increase vascular permeability and open up the blood–brain barrier to inflammatory leukocytes [24]. It has been reported that PTX increases the susceptibility to anaphylaxis in other experimental conditions. Mice for EAE induction without PTX need a fivefold dosage of self-peptide to induce anaphylaxis as compared to EAE mice treated with PTX [25, 26]. Anti-very late appearing antigen-4 antibody induced anaphylaxis is correlated with high levels of mortality in PTX-injected EAE mice, but not in PTX-untreated mice [27]. Here, we show that PTX can increase the vascular permeability, which is consistent with

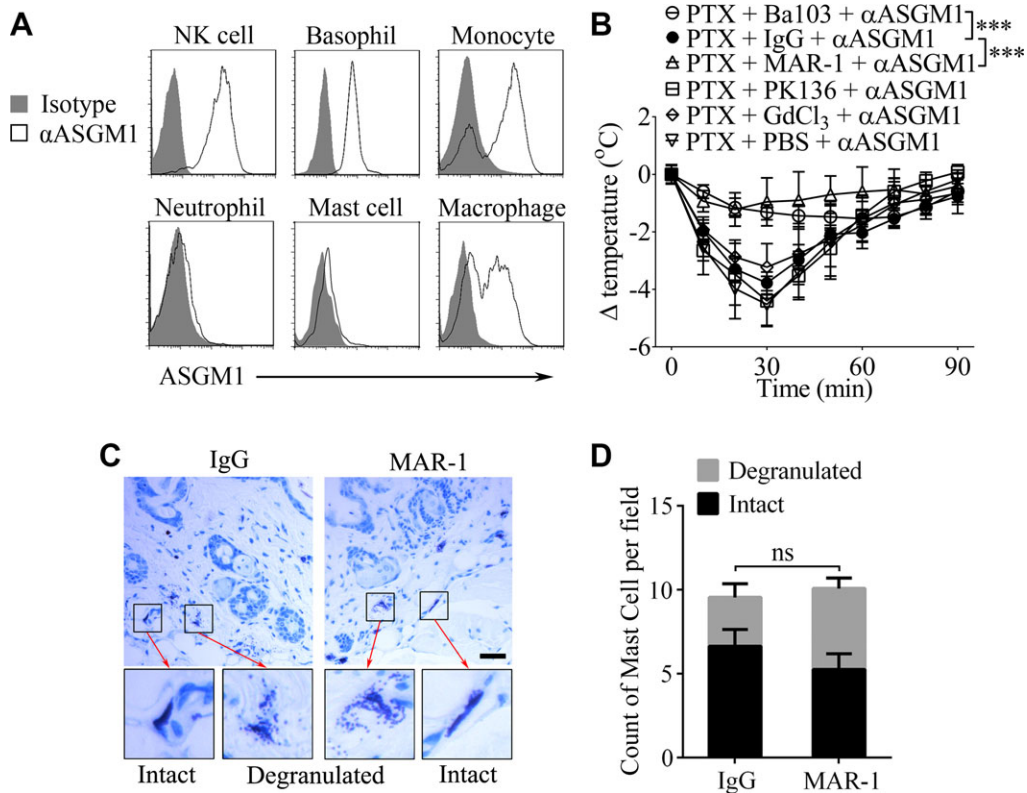


**Figure 3.** Basophils are indispensable for  $\alpha$ ASGM1-induced anaphylaxis. (A) ASGM1 expression profile in innate immune cells. ASGM1 expression was assessed by flow cytometry in following splenic cell populations: NK cells (gated on CD3<sup>+</sup>, NK1.1<sup>+</sup>); basophils (gated on c-Kit<sup>+</sup>, DX5<sup>+</sup>, Fc $\epsilon$ RI<sup>+</sup>); monocytes (gated on CD11b<sup>+</sup>, Gr1<sup>+</sup>); macrophage (gated on F4/80<sup>+</sup>); neutrophils (gated on CD11b<sup>+</sup>, Gr1<sup>high</sup>). In addition, mast cells (gated on c-Kit<sup>+</sup>, Fc $\epsilon$ RI<sup>+</sup>) obtained from the peritoneal cavity were analyzed. Data are representative of results obtained from six mice. (B) Dynamics of body temperature following depletion of ASGM1-expressing cells. Three days before injection of PTX-pretreated mice with  $\alpha$ ASGM1, basophils were deleted through the daily administration of MAR-1 twice (5  $\mu$ g in each time) or the daily treatment with Ba103 once (10  $\mu$ g). Alternatively, one day before injection of PTX-pretreated mice with  $\alpha$ ASGM1, NK, and NKT cells were deleted using a single PK136 (200  $\mu$ g) injection, or monocytes and macrophages were deleted following GdCl<sub>3</sub> (1 mg) administration. IgG (200  $\mu$ g) or PBS (200  $\mu$ L) was administered in the control groups one day before  $\alpha$ ASGM1 injection. The rectal temperature was measured after  $\alpha$ ASGM1 injection. Data are shown as mean  $\pm$  SEM of 10 mice per group pooled from three experiments performed are shown. \*\*\* $p$  < 0.001, two way ANOVA. (C) Mast cells staining with Toluidine blue. Paraffin sections at 5  $\mu$ m of skin samples from MAR-1- or control IgG-treated mice were stained with 0.1% Toluidine blue at pH 2.3 for 3 min. Scale bar, 25  $\mu$ m. (D) The number of mast cells per fields was determined under a light microscope at a 400 $\times$  magnification. Data are shown as mean  $\pm$  SEM of 14 fields assessed, ns: no significance (two tailed Student's  $t$ -test).

previous findings that lack sphingosine-1-phosphate in plasma or PI3K-C2 $\alpha$  knockdown in endothelial cells or treatment with IL-4/IL-13 or IL-33 exhibit higher vascular permeability that result in susceptibility to anaphylaxis [28–32]. Furthermore, our results reveal that PTX can increase Fc $\epsilon$ RI expression on basophil and elevate serum IgE. It has been reported that IgE can bind Fc $\epsilon$ RI to prolong basophil survival whereas the exposure of basophil to IgE leads to the upregulated expression of Fc $\epsilon$ RI $\alpha$  [33, 34]. IgE-dependent enhancement of mast cell Fc $\epsilon$ RI expression results in enhancement of IgE-dependent mast cell mediator release [33]. Thus, our current findings support the notion that PTX promotes anaphylactic reactions triggered by the activation of basophil, no matter it is IgE-dependent or IgE-independent.

Our data indicated that an individual with increased vascular permeability is more likely to be susceptible to anaphylaxis when exposed to even low dosage of PAF. It may help to explain types of allergic reactions such as exercise-induced anaphylaxis

that have hitherto been without a known allergen, but conclusive evidence for this theory is still far away. Accordingly, endothelial cells must play an important role in anaphylactic responses. Some situations such as oxidative stress, trauma, infection, and inflammation can damage endothelium to increase the vascular permeability directly [35]. Ideally, vascular permeability and dysfunction of endothelial cells might be valuable tools for future evaluation in understanding susceptibility to anaphylaxis. As one of the bacterial components, flagellin is a ligand to ASGM1 [11]. Basophil activation and anaphylactic shock may be triggered upon bacterial infection. As shown in Supporting Information Figure 2A, purified recombinant flagellin treatment promoted ERK1/2 phosphorylation in basophils in dose dependent in vitro. As illustrated in Supporting Information Fig. 2B, i.v. injection of purified recombinant flagellin induced a moderate decrease in rectal temperature in PTX-pretreated mice as compared with control mice. Targeting endothelium and the activated receptors on the

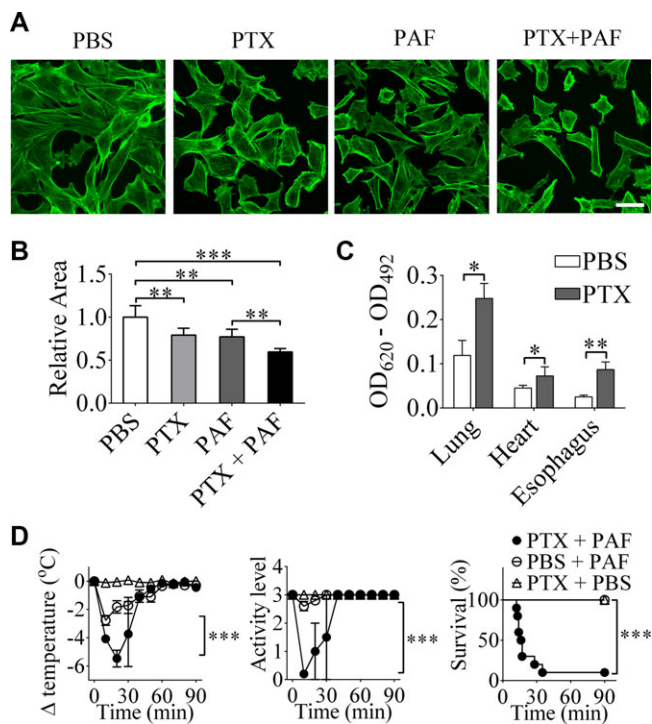


**Figure 4.** PTX increases vascular permeability and susceptibility to PAF-mediated anaphylaxis. (A and B) Murine endothelial cell monolayers are disturbed by PTX, PAF, or their combination. The SVEC4–10 murine endothelial cells were incubated with 100 ng/mL PTX or PBS for 24 h and then incubated with 100 ng/mL PAF or PBS for 30 min. (A) PTX- and PAF-induced morphological changes were assessed by microscopy after staining F-actin with 50  $\mu$ g/mL of FITC-Phalloidin. Data shown are representative of five independent experiments. Scale bar, 5  $\mu$ m. (B) Mean area of SVEC4–10 cells was calculated using ImageJ 1.47 software in each group and normalized with mean area of PBS group as relative area. Data are shown as mean  $\pm$  SEM of 25 assessed fields. (C) PTX increases vascular permeability in mice. Mice with or without PTX pretreatment were i.v. injected with 100  $\mu$ L of 1% Evans blue dye. Thirty minutes later, the mice were perfused with saline to remove the intravascular Evans blue dye. Whole lung, heart, and esophageal tissues were excised and the extravasated dye was extracted from the organs and measured by spectrophotometry. Data are shown as mean  $\pm$  SEM of five mice. (D) PTX promotes manifestations associated with PAF-mediated anaphylactic shock. Mice were i.v. injected with 100 ng PAF or PBS 7 days after the PTX or PBS pretreatment. Then the dynamic of rectal temperature, activity level, and survival rate was recorded. Temperature and activity data are shown as mean  $\pm$  SEM from 10 mice in each group. Survival data are shown as survival curve. (B and C) Data shown are pooled from four independent experiments and were evaluated using two-tailed Student's *t*-test. (D) Data shown are pooled from two independent experiments. Temperature and activity data were evaluated using two-way ANOVA. Survival data were evaluated using Long rank (Mantel Cox) test. \**p* < 0.05, \*\**p* < 0.01, \*\*\**p* < 0.001.

basophil might well be a way to find a treatment method for anaphylaxis.

In conclusion, our results show that ASGM1-mediated stimulation can trigger basophil activation and PAF-mediated shock in PTX-pretreated mice, which provide new insights in understanding the mechanisms of anaphylactic shock. Further studies may facilitate the potential development of targeting ASGM1 and endothelial cells as therapeutic intervention for anaphylactic shock.

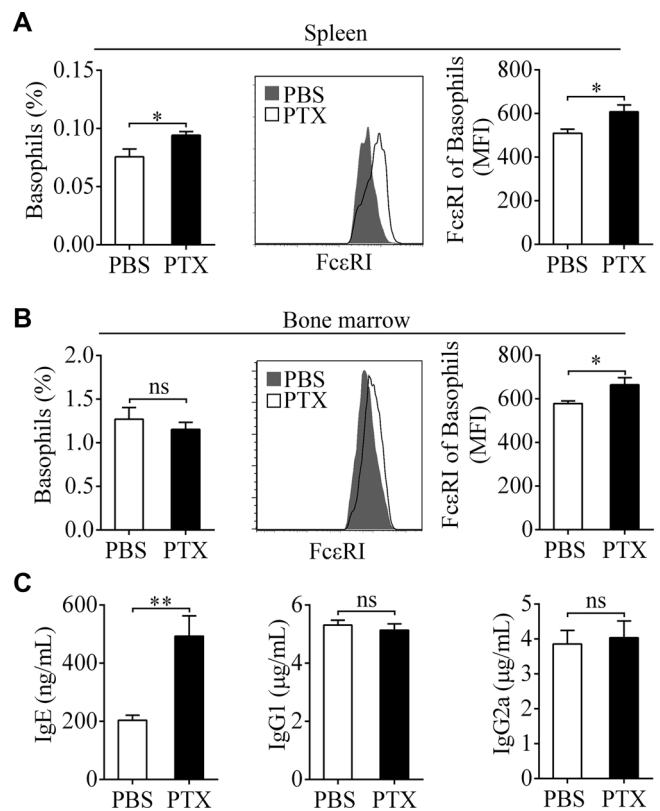


**Figure 5.** Influence of PTX on basophils and serum antibody levels in vivo. C57BL/6 mice were treated with PTX (200 ng) on day 0 and 2 and the measurements were performed on day 7. (A and B) The percentages of basophil and FcεRI expression in the spleen and bone marrow. Spleen and bone marrow cells were separated and stained with specific antibodies for analysis with flow cytometry. Basophils were gated on DX5<sup>+</sup>, FcεRI<sup>+</sup> cells (left). Expression of FcεRI on basophils (middle and right) was analyzed. MFI: mean fluorescence intensity. (C) Antibody levels in the sera of PTX-pretreated mice. The sera of mice were collected and total IgE (left), IgG1 (middle), and IgG2a (right) were measured using ELISA. (A–C) Data are shown as mean  $\pm$  SEM of (A) 15, (B) 5, or (C) 10 mice per group and are pooled from three independent experiments. \**p* < 0.05, \*\**p* < 0.01, ns no significance, two tailed Student's *t*-test.

## Materials and methods

### Cell cultures

Bone marrow from the femurs and tibias of adult female C57BL/6 mice was flushed with RPMI 1640 medium using a Syringe with a 0.45 mm diameter needle. Clusters within the marrow suspension were disintegrated via pipetting. Bone marrow-derived basophils (BMBs) were derived from bone marrow cultures at a density of  $1 \times 10^6$  cells/mL and medium was changed every 3 days for 10 days in RPMI-1640 medium supplemented with 10% FBS and 20 ng/mL IL-3 (Peprotech). For purification of BMBs, DX5<sup>+</sup> cell were separated by a MACS (Miltenyi Biotec). SVEC4–10 cells were maintained in DMEM supplemented with 10% fetal bovine serum. All cells were cultured in a humidified 5% CO<sub>2</sub> incubator at 37°C.

## Mice

Female C57BL/6 mice (6–8 weeks) were purchased from SLAC Laboratory Animal Co. Ltd. (Shanghai, China). All mice were maintained in our specific pathogen-free facilities. All animal studies were performed in accordance with the Guidelines and permission of Tongji Medical College Animal Care and Use Committee.

## Antibodies and reagents

$\alpha$ ASGM1, a polyclonal rabbit IgG antibody (Wako), PTX (Sigma-Aldrich), PAF (Sigma-Aldrich) was purchased.

## Intracellular calcium measurement

Purified BMBs were loaded with a final concentration of 5  $\mu$ M Fluo-3-AM (Beyotime) for 45 min at 37°C and changes in dye fluorescence after stimulation with  $\alpha$ ASGM1 (10  $\mu$ g/mL) were analyzed using a LSR II flow cytometer. Kinetic changes of Fluo-3 fluorescence were then plotted versus time using FlowJo software [36, 37].

## Western blotting

Fifty micrograms of cellular protein were subjected to electrophoresis on a 12% SDS–PAGE and transferred onto PVDF membranes (Hybond). Specific antibodies to Phospho-ERK1/2 (Thr202/Tyr204), ERK1/2 (Cell Signaling), GAPDH (Santa Cruz) were used to detect the indicated proteins. Quantitative assessment of band densities was performed by scanning densitometry using ImageJ 1.47 (NIH, USA).

## Real-time quantitative PCR

Total RNA was extracted using Trizol reagents (Invitrogen). The first-strand was synthesized with First-Strand cDNA Synthesis SuperMix kit. Real-time PCR was performed using SYBR Green Real-time PCR Master Mix (TOYOBO) and ABI PRISM 7000 sequence detection system. The primer sequences used in PCR were as follows: GAPDH (sense, 5'-TTCACCACCATGGAGAAGGC-3' and anti-sense, 5'-GGCATGGACTGTGGTCATGA-3'), acetyl-CoA: 1-O-alkyl-*sn*-glycero-3-phosphocholine acetyltransferase (LysoPAFAT)/lysophosphatidylcholine acyltransferase 2 (LPCAT2); (sense, 5'-GTCCAGCAGACTACGATCAGTG-3' and anti-sense 5'-CTTATTGGATGGGTCAGCTTTC-3'). The relative expression levels for the target gene were normalized to GAPDH, calculated using the  $2^{-\Delta\Delta Ct}$  method and presented as fold increases versus control.

## Measurement of PAF

Purified BMBs ( $1 \times 10^5$  in 100  $\mu$ L) were incubated with  $\alpha$ ASGM1 (10  $\mu$ g/mL) at 37°C. Culture supernatants were collected after

30 min and mixed with an equal volume of 20% acetic acid immediately. Purification of PAF-containing lipids was performed by the method of Bligh and Dyer with a mixture of chloroform-methanol-water (1:1:0.9) [8, 38]. The concentration of PAF was measured using the 6460 Triple Quadrupole Liquid Chromatography/Mass Spectrometry (6460 QQQ LC/MS; Agilent Technologies) system. Chromatographic separation was performed on a HPLC Symmetry<sup>®</sup> C18 column (4.6 mm  $\times$  250 mm, 5  $\mu$ m) (Waters Corporation, USA) at 46°C with a flow rate of 1 mL/min. Mobile phases A and B are water and acetonitrile/formic acid (100:0.1), respectively. The detection was under the negative electrospray ionization and selected reaction monitoring mode. Fragment ions 568.1–59  $m/z$  were monitored for PAF [39]. Data were processed using Masshunter Qualitative Analysis software (Agilent Technologies).

## Observation of the shock

The rectal temperature was measured with a Digital Thermocouple Thermometer (Model BAT-12) before tests and then every 10 min for a maximum of 120 min. At the same time, mouse activity was assessed and quantitated on a 4-point scale as described by Strait et al. [3].

## In vivo blocking and depletion

Eighty micrograms of PAF receptor antagonist CV-3988 (Enzo life science) was i.v. injected 10 min before the  $\alpha$ ASGM1 injection. Two hundred micrograms of histamine H1 receptor antagonist triprolidine (Sigma-Aldrich) were i.p. injected 30 min before the  $\alpha$ ASGM1 injection. For basophil depletion, mice were given twice-daily i.p. injections of 5  $\mu$ g of anti-mouse-Fc $\epsilon$ RI (MAR-1; eBioscience) or Anti-CD200R3 (Ba103; Hycult biotech) on day 4, 5, and 6 [40, 41]. For NK-cell or/and NKT-cell depletion, mice were injected i.p. with 200  $\mu$ g of anti-NK1.1 (PK136) on day 6. For monocyte/macrophage function inhibition, mice were given i.p. injection of 1 mg gadolinium chloride (GdCl<sub>3</sub>; Sigma-Aldrich) on day 6 [3].

## Mast cell staining

Skin samples were fixed in 4% paraformaldehyde and paraffin sections at 5  $\mu$ m were stained with 0.1% Toluidine blue (Sigma) at pH 2.3. The number of mast cells per view at 40  $\times$  objective lenses was determined under a light microscope. A “degranulated” mast cell was defined as a cell showing extrusion of 10% cell granules [42].

## Flow cytometry

FITC-anti-mouse-CD3e (145–2C11), PE-anti-mouse-CD49b (DX5), allophycocyanin-anti-mouse-Fc $\epsilon$ RI (MAR-1), PE/Cy7-anti-

mouse-c-Kit (CD117; 2B8), PerCP/Cy5.5-anti-mouse-NK1.1 (PK136), and Alexa Fluor 488-asialo GM1 (Polyclonal) were purchased from eBioscience. PerCP/Cy5.5-anti-mouse-CD11b (M1/70), allophycocyanin-anti-mouse-Gr1 (RB6–8C5), and allophycocyanin-F4/80 (BM8) were purchased from BioLegend. Cells that from spleens or cultures were stained with fluorescence-conjugated antibodies in staining buffer (PBS, 1% FBS) and analyzed either on a LSR II or a FACSCalibur flow cytometers (Becton Dickinson). Data were acquired using FACSDiva and analyzed using FlowJo software (TreeStar).

### Measurement of vascular leakage

PTX-pretreated or untreated mice were i.v. injected with 100  $\mu$ L of a 1% solution of Evans blue dye (Sigma-Aldrich) in saline. Thirty minutes later, the mice were perfused with saline in the right ventricle to remove the intravascular Evans blue dye. The whole lung, heart, and esophagus tissues were excised and extravasated dye was extracted in 1 mL of formamide at 55°C overnight. Evans blue content was measured by spectrophotometry and determined as absorbent density at 620 nm wavelength ( $OD_{620}$ ) minus absorbent density at 492 nm wavelength ( $OD_{492}$ ) of the formamide extract [29, 30].

### Confocal fluorescence microscopy

SVEC4–10 cells were grown as a monolayer on sterile coverslips. After incubation with 200 ng/mL PTX for 24 h, or 100 ng/mL PAF for 30 min cells, or both together, the cells were fixed, permeabilized and incubated with 50  $\mu$ g/mL of FITC-phalloidin (Sigma-Aldrich, St Louis, MO, USA) for 45 min at 37°C to label actin filaments and stained with 0.1  $\mu$ g/mL of DAPI (Sigma-Aldrich, St Louis, MO, USA) for 2 min at room temperature. Cells were examined with confocal laser scanning biological microscope (FV-500, Olympus Inc., Japan) with a 40 $\times$  objective lens and images were acquired using Fluoview software [43]. Mean area of cells was calculated using ImageJ 1.47 (NIH, USA) and normalized with mean area of PBS group as relative area [44].

### Enzyme-linked immunosorbent assays

Blood samples were collected for serum preparation. For measurement of serum IgE levels, mouse IgE, IgG1, and IgG2a Ready-SET-Go ELISA kit (eBioscience) was used. The detection range limits of IgE, IgG1, and IgG2a measurement kits are 4–250 ng/mL, 3.13–200 ng/mL, and 3.90–250 ng/mL, respectively.

### Statistical analysis

Experimental data were expressed as means  $\pm$  SEM or means  $\pm$  SEM. The statistical significance of differences was determined

using a two-tailed unpaired Student *t* test or a two-way ANOVA. Survival data were analyzed by using a Mental–Cox test. Values of *p* < 0.05 were considered significant.

**Acknowledgments:** We thank Dr. Min Fang and Dr. Zheng Tan (Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology) for their discussion about the project. This work was supported by grants from the National Natural Sciences Foundation of China to Fang Zheng (No. 81072466) and Major State Basic Research Development Program of China to Feili Gong (973 Program) (No. 2013CB530505).

**Conflict of interest:** The authors declare no financial or commercial conflict of interest.

### References

- Korhonen, H., Fisslthaler, B., Moers, A., Wirth, A., Habermehl, D., Wieland, T., Schutz, G. et al., Anaphylactic shock depends on endothelial Gq/G11. *J. Exp. Med.* 2009. **206**: 411–420.
- Tsujimura, Y., Obata, K., Mukai, K., Shindou, H., Yoshida, M., Nishikado, H., Kawano, Y. et al., Basophils play a pivotal role in immunoglobulin-G-mediated but not immunoglobulin-E-mediated systemic anaphylaxis. *Immunity* 2008. **28**: 581–589.
- Strait, R. T., Morris, S. C., Yang, M., Qu, X.-W. and Finkelman, F. D., Pathways of anaphylaxis in the mouse. *J. Allergy Clin. Immunol.* 2002. **109**: 658–668.
- Jiao, D., Liu, Y., Lu, X., Pan, Q., Zheng, J., Liu, B., Liu, Y. et al., Characteristics of anaphylaxis-inducing IgG immune complexes triggering murine passive systemic anaphylaxis. *Allergy* 2013. **68**: 236–245.
- Jonsson, F., Mancardi, D. A., Kita, Y., Karasuyama, H., Iannascoli, B., Van Rooijen, N., Shimizu, T. et al., Mouse and human neutrophils induce anaphylaxis. *J. Clin. Invest.* 2011. **121**: 1484–1496.
- Komai-Koma, M., Brombacher, F., Pushparaj, P. N., Arendse, B., McSharry, C., Alexander, J., Chaudhuri, R. et al., Interleukin-33 amplifies IgE synthesis and triggers mast cell degranulation via interleukin-4 in naive mice. *Allergy* 2012. **67**: 1118–1126.
- Kojima, T., Obata, K., Mukai, K., Sato, S., Takai, T., Minegishi, Y. and Karasuyama, H., Mast cells and basophils are selectively activated in vitro and in vivo through CD200R3 in an IgE-independent manner. *J. Immunol.* 2007. **179**: 7093–7100.
- Vadas, P., Gold, M., Perelman, B., Liss, G. M., Lack, G., Blyth, T., Simons, F. E. et al., Platelet-activating factor, PAF acetylhydrolase, and severe anaphylaxis. *N. Engl. J. Med.* 2008. **358**: 28–35.
- Janshoff, A., Steinem, C., Sieber, M., el Baya, A., Schmidt, M. A. and Galla, H. J., Quartz crystal microbalance investigation of the interaction of bacterial toxins with ganglioside containing solid supported membranes. *Eur. Biophys. J.* 1997. **26**: 261–270.
- Soong, G., Reddy, B., Sokol, S., Adamo, R. and Prince, A., TLR2 is mobilized into an apical lipid raft receptor complex to signal infection in airway epithelial cells. *J. Clin. Invest.* 2004. **113**: 1482–1489.

- 11 Krivan, H. C., Roberts, D. D. and Ginsburg, V., Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids. *Proc. Natl. Acad. Sci. USA* 1988. **85**: 6157–6161.
- 12 Moore, M. L., Chi, M. H., Goleniewska, K., Durbin, J. E. and Peebles, R. S., Jr., Differential regulation of GM1 and asialo-GM1 expression by T cells and natural killer (NK) cells in respiratory syncytial virus infection. *Viral Immunol.* 2008. **21**: 327–339.
- 13 McNamara, N., Khong, A., McKemy, D., Caterina, M., Boyer, J., Julius, D. and Basbaum, C., ATP transduces signals from ASGM1, a glycolipid that functions as a bacterial receptor. *Proc. Natl. Acad. Sci. USA* 2001. **98**: 9086–9091.
- 14 Ratner, A. J., Bryan, R., Weber, A., Nguyen, S., Barnes, D., Pitt, A., Gelber, S. et al., Cystic fibrosis pathogens activate Ca<sup>2+</sup>-dependent mitogen-activated protein kinase signaling pathways in airway epithelial cells. *J. Biol. Chem.* 2001. **276**: 19267–19275.
- 15 McNamara, N., Gallup, M., Sucher, A., Maltseva, I., McKemy, D. and Basbaum, C., AsialoGM1 and TLR5 cooperate in flagellin-induced nucleotide signaling to activate Erk1/2. *Am. J. Respir. Cell Mol. Biol.* 2006. **34**: 653–660.
- 16 de Bentzmann, S., Roger, P., Dupuit, F., Bajolet-Laudinat, O., Fuchey, C., Plotkowski, M. C. and Puchelle, E., Asialo GM1 is a receptor for *Pseudomonas aeruginosa* adherence to regenerating respiratory epithelial cells. *Infect. Immun.* 1996. **64**: 1582–1588.
- 17 Du, J. W., Zhang, F., Capo-Aponte, J. E., Tachado, S. D., Zhang, J., Yu, F. S., Sack, R. A. et al., AsialoGM1-mediated IL-8 release by human corneal epithelial cells requires coexpression of TLR5. *Invest. Ophthalmol. Vis. Sci.* 2006. **47**: 4810–4818.
- 18 Kasai, M., Iwamori, M., Nagai, Y., Okumura, K. and Tada, T., A glycolipid on the surface of mouse natural killer cells. *Eur. J. Immunol.* 1980. **10**: 175–180.
- 19 Bukowski, J. F., Woda, B. A., Habu, S., Okumura, K. and Welsh, R. M., Natural killer cell depletion enhances virus synthesis and virus-induced hepatitis in vivo. *J. Immunol.* 1983. **131**: 1531–1538.
- 20 Warfield, K. L., Perkins, J. G., Swenson, D. L., Deal, E. M., Bosio, C. M., Aman, M. J., Yokoyama, W. M. et al., Role of natural killer cells in innate protection against lethal ebola virus infection. *J. Exp. Med.* 2004. **200**: 169–179.
- 21 Yamaji, O., Nagaishi, T., Totsuka, T., Onizawa, M., Suzuki, M., Tsuge, N., Hasegawa, A. et al., The development of colitogenic CD4(+) T cells is regulated by IL-7 in collaboration with NK cell function in a murine model of colitis. *J. Immunol.* 2012. **188**: 2524–2536.
- 22 Nishikado, H., Mukai, K., Kawano, Y., Minegishi, Y. and Karasuyama, H., NK cell-depleting anti-asialo GM1 antibody exhibits a lethal off-target effect on basophils in vivo. *J. Immunol.* 2011. **186**: 5766–5771.
- 23 Karasuyama, H., Obata, K., Wada, T., Tsujimura, Y. and Mukai, K., Newly appreciated roles for basophils in allergy and protective immunity. *Allergy* 2011. **66**: 1133–1141.
- 24 Lando, Z. and Ben-Nun, A., Experimental autoimmune encephalomyelitis mediated by T-cell line. II. Specific requirements and the role of pertussis vaccine for the in vitro activation of the cells and induction of disease. *Clin. Immunol. Immunopathol.* 1984. **30**: 290–303.
- 25 Musio, S., Pedotti, P., Mantegazza, R., Ohtsu, H., Boon, L., Steinman, L., Galli, S. J. and Pedotti, R., Anaphylaxis to a self-peptide in the absence of mast cells or histamine. *Lab. Invest.* 2009. **89**: 398–405.
- 26 Pedotti, R., Mitchell, D., Wedemeyer, J., Karpuij, M., Chabas, D., Hattab, E. M., Tsai, M. et al., An unexpected version of horror autotoxicus: anaphylactic shock to a self-peptide. *Nat. Immunol.* 2001. **2**: 216–222.
- 27 Ji, N., Rao, N., Guentzel, N. M., Arulanandam, B. P. and Forsthuber, T. G., Anaphylaxis and mortality induced by treatment of mice with anti-VLA-4 antibody and pertussis toxin. *J. Immunol.* 2011. **186**: 2750–2756.
- 28 Strait, R. T., Morris, S. C., Smiley, K., Urban, J. F., Jr. and Finkelman, F. D., IL-4 exacerbates anaphylaxis. *J. Immunol.* 2003. **170**: 3835–3842.
- 29 Camerer, E., Regard, J. B., Cornelissen, I., Srinivasan, Y., Duong, D. N., Palmer, D., Pham, T. H. et al., Sphingosine-1-phosphate in the plasma compartment regulates basal and inflammation-induced vascular leak in mice. *J. Clin. Invest.* 2009. **119**: 1871–1879.
- 30 Yoshioka, K., Yoshida, K., Cui, H., Wakayama, T., Takuwa, N., Okamoto, Y., Du, W. et al., Endothelial PI3K-C2alpha, a class II PI3K, has an essential role in angiogenesis and vascular barrier function. *Nat. Med.* 2012. **18**: 1560–1569.
- 31 Choi, Y. S., Choi, H. J., Min, J. K., Pyun, B. J., Maeng, Y. S., Park, H., Kim, J. et al., Interleukin-33 induces angiogenesis and vascular permeability through ST2/TRAF6-mediated endothelial nitric oxide production. *Blood* 2009. **114**: 3117–3126.
- 32 Khodoun, M., Strait, R., Orekov, T., Hogan, S., Karasuyama, H., Herbert, D. R., Kohl, J. and Finkelman, F. D., Peanuts can contribute to anaphylactic shock by activating complement. *J. Allergy Clin. Immunol.* 2009. **123**: 342–351.
- 33 Yamaguchi, M., Lantz, C. S., Oettgen, H. C., Katona, I. M., Fleming, T., Miyajima, I., Kinet, J. P. and Galli, S. J., IgE enhances mouse mast cell Fc(epsilon)RI expression in vitro and in vivo: evidence for a novel amplification mechanism in IgE-dependent reactions. *J. Exp. Med.* 1997. **185**: 663–672.
- 34 Saini, S. S., Klion, A. D., Holland, S. M., Hamilton, R. G., Bochner, B. S. and Macglashan, D. W., Jr., The relationship between serum IgE and surface levels of FcepsilonRI on human leukocytes in various diseases: correlation of expression with FcepsilonRI on basophils but not on monocytes or eosinophils. *J. Allergy Clin. Immunol.* 2000. **106**: 514–520.
- 35 Park, J. H., Okayama, N., Gute, D., Krsmanovic, A., Battarbee, H. and Alexander, J. S., Hypoxia/aglycemia increases endothelial permeability: role of second messengers and cytoskeleton. *Am. J. Physiol.* 1999. **277**: C1066–C1074.
- 36 Chang, H., Yanachkov, I. B., Michelson, A. D., Li, Y., Barnard, M. R., Wright, G. E. and Frelinger, A. L., 3rd, Agonist and antagonist effects of diadenosine tetraphosphate, a platelet dense granule constituent, on platelet P2Y1, P2Y12 and P2X1 receptors. *Thromb. Res.* 2010. **125**: 159–165.
- 37 Schepers, E., Glorieux, G., Dhondt, A., Leybaert, L. and Vanholder, R., Flow cytometric calcium flux assay: evaluation of cytoplasmic calcium kinetics in whole blood leukocytes. *J. Immunol. Methods* 2009. **348**: 74–82.
- 38 Bligh, E. and Dyer, W. J., A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.* 1959. **37**: 911–917.
- 39 Kita, Y., Takahashi, T., Uozumi, N. and Shimizu, T., A multiplex quantitation method for eicosanoids and platelet-activating factor using column-switching reversed-phase liquid chromatography-tandem mass spectrometry. *Anal. Biochem.* 2005. **342**: 134–143.
- 40 Yoshimoto, T., Yasuda, K., Tanaka, H., Nakahira, M., Imai, Y., Fujimori, Y. and Nakanishi, K., Basophils contribute to T(H)2-IgE responses in vivo via IL-4 production and presentation of peptide-MHC class II complexes to CD4+ T cells. *Nat. Immunol.* 2009. **10**: 706–712.
- 41 Obata, K., Mukai, K., Tsujimura, Y., Ishiwata, K., Kawano, Y., Minegishi, Y., Watanabe, N. and Karasuyama, H., Basophils are essential initiators of a novel type of chronic allergic inflammation. *Blood* 2007. **110**: 913–920.
- 42 Ujike, A., Ishikawa, Y., Ono, M., Yuasa, T., Yoshino, T., Fukumoto, M., Ravetch, J. V. and Takai, T., Modulation of immunoglobulin (Ig) E-mediated systemic anaphylaxis by low-affinity Fc receptors for IgG. *J. Exp. Med.* 1999. **189**: 1573–1579.

- 43 Li, P., Zheng, G., Yang, Y., Zhang, C., Xiong, P., Xu, Y., Fang, M. et al., Granzyme B is recovered by natural killer cells via clathrin-dependent endocytosis. *Cell Mol. Life Sci.* 2010. **67**: 3197–3208.
- 44 Dandapani, S. V., Sugimoto, H., Matthews, B. D., Kolb, R. J., Sinha, S., Gerszten, R. E., Zhou, J. et al., Alpha-actinin-4 is required for normal podocyte adhesion. *J. Biol. Chem.* 2007. **282**: 467–477.

**Abbreviations:** ASGM1: Asialoganglioside gangliotetraosylceramide ·  $\alpha$ ASGM1: anti-ASGM1 antibody · BMB: bone marrow-derived basophil · LPCAT2: lysophosphatidylcholine acyltransferase 2 · LysoPAFAT: acetyl-CoA:1-O-alkyl-sn-glycero-3-phosphocholine (LysoPAF) acetyltransferase · MOG: myelin oligodendrocyte glycoprotein · PAF: platelet-activating factor · PTX: pertussis toxin

**Full correspondence:** Dr. Fang Zheng, Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology, 1022 Room, 2nd Teaching building, 13# Hangkong Road, Wuhan 430030, China  
Fax: +86-27-83693500  
e-mail: zhengfangtj@hust.edu.cn

Received: 4/10/2013  
Revised: 20/3/2014  
Accepted: 23/4/2014  
Accepted article online: 28/4/2014

# European Journal of Immunology

**Supporting Information  
for**

**DOI 10.1002/eji.201344144**

Yan Yang, Daling Li, Foad Katirai, Bin Zhang, Yong Xu, Ping Xiong, Feili Gong  
and Fang Zheng

**Basophil activation through ASGM1 stimulation triggers PAF release and  
anaphylaxis-like shock in mice**

## Supporting Information

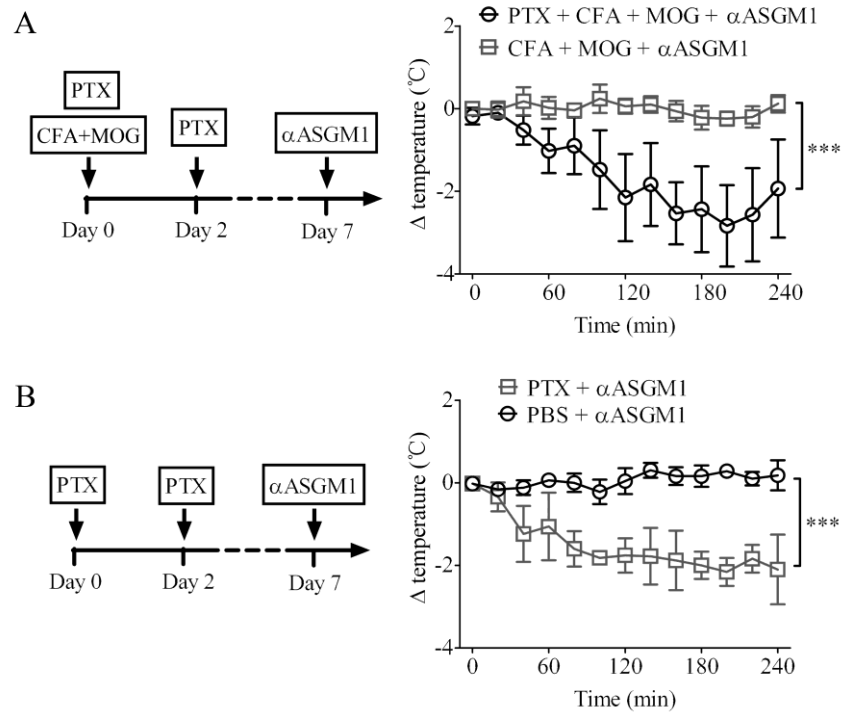


Figure 1. PTX and anti-ASGM1 antibody, but not MOG and CFA, accounted for anaphylaxis-like shock. **(A)** C57BL/6 mice were immunized with MOG<sub>35-55</sub> and CFA on day 0 and treated with PTX on day 0 and 2, and control mice were under the immunization without the treatment of PTX. Anti-ASGM1 antibody was intraperitoneally injected in mice on day 7. **(B)** Mice treated with PTX or PBS were injected intraperitoneally with anti-ASGM1 antibody. Changes in rectal temperature with time were shown. (A,B) Data are shown as mean  $\pm$  SEM from 5 mice in each group. \*\*\* $P < 0.001$ , two way ANOVA. Data are representative of three independent experiments.

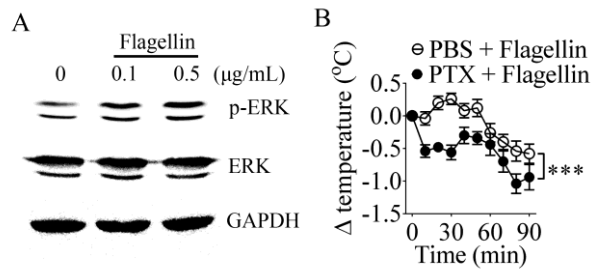


Figure 2. Flagellin treatment increased the phosphorylation of ERK in basophils and decreased the body temperature of PTX-pretreated mice. (A) BMBs were treated with flagellin from *S. typhimurium* (Invivogen) in indicated concentration for 10 min. Cells were lysed and indicated proteins were detected with specific primary antibodies at dilution of 1:1000 by western blotting. Data are representative of three independent experiments. (B) C57BL/6 mice were treated with PTX (200ng) on days 0 and 2, and intravenously injected with 1 µg flagellin on day 7. Changes in rectal temperature were shown. Data are shown as mean  $\pm$  SEM from 5 mice in each group. \*\*\* $P < 0.001$ , two way ANOVA. Data are representative of three independent experiments.

**Basophil activation through ASGM1 stimulation triggers PAF release and anaphylaxis-like shock in mice**

Yan Yang, Daling Li, Foad Katirai, Bin Zhang, Yong Xu, Ping Xiong, Feili Gong and Fang Zheng

Correspondence: Dr. Fang Zheng, Department of Immunology, Tongji Medical College, Huazhong University of Science and Technology. 1022 Room, 2nd Teaching building, 13# Hangkong Road, Wuhan 430030, China

---

|                  |                            |                 |
|------------------|----------------------------|-----------------|
| Review Timeline: | Submission date:           | 4 October 2013  |
|                  | First Editorial decision:  | 5 November 2013 |
|                  | First Revision received:   | 11 January 2014 |
|                  | Second Editorial decision: | 30 January 2014 |
|                  | Second Revision received:  | 20 March 2014   |
|                  | Accepted:                  | 23 April 2014   |

---

Handling Executive Committee member: Prof. Rikard Holmdahl

Please note that the correspondence below does not include the standard editorial instructions regarding preparation and submission of revised manuscripts, only the scientific revisions requested and addressed.

**First Editorial Decision – 5 November 2013**

Dear Dr. Zheng,

Manuscript ID eji.201344144 entitled "ASGM1-mediated stimulation triggers anaphylaxis-like shock via activating basophils to release PAF in mice" which you submitted to the European Journal of Immunology has been reviewed. The comments of the referees are included at the bottom of this letter.

A revised version of your manuscript that takes into account the comments of the referees will be reconsidered for publication.

You should also pay close attention to the editorial comments included below. \*In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.\*

Furthermore, the Executive Editor feels that it is incorrect to show only representative examples of the data. The editor feels you should show and calculate all data to demonstrate the true variation.

Please note that submitting a revision of your manuscript does not guarantee eventual acceptance, and that your revision will be re-reviewed by the referees before a decision is rendered.

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology and we look forward to receiving your revision.

Yours sincerely,  
Karen Chu

On behalf of Prof. Rikard Holmdahl

Dr. Karen Chu  
Editorial Office  
European Journal of Immunology  
e-mail: [ejied@wiley.com](mailto:ejied@wiley.com)  
[www.eji-journal.eu](http://www.eji-journal.eu)

\*\*\*\*\*

Reviewer: 1

### Comments to the Author

The authors report that the ASGM1 receptor on murine basophils can activate basophils to produce PAF and thereby mediate anaphylaxis. However, this anaphylactic response required treatment of mice with pertussis toxin to increase vascular leakage. This finding is based on an unexpected observation when the authors studied the role of basophils in the EAE model where they depleted basophils with anti-ASGM1 and induced EAE with PTX+MOG peptide. The present study follows up on this observation and provides new insights regarding the potential mechanism of this response. The study is well performed but would benefit from addressing the following points

Specific comments

1. The authors should mention in the introduction that also anti-CD200R3 induces an IgE- and IgG1-independent anaphylaxis in mice (Kojima et al. J Immunol 179:7093).
2. Figure 1. Could the authors also show quantification of released PAF in the supernatant in addition to the RT-PCR data?
3. Figure 3. As MAR-1 can also modulate the activity of mast cells it would be more convincing to include either mast cell deficient mice in which this type of anaphylaxis should still occur and/or basophil-deficient mice in which the response should be ablated.
4. Figure 5. The link between PTX treatment, basophilia, FcεRI upregulation and IgE production is not worked out very well. PTX has many effects in vivo including blockade of chemokine receptors which might explain basophil-accumulation in the blood. Are basophils also increased in other organs including bone marrow? Does PTX directly act on basophils to promote upregulation of FcεRI? Are other antibody isotypes also increased in PTX treated mice or is it selective for IgE?
5. Please specify in the Materials and Methods that anti-ASGM1 is a polyclonal rabbit antibody.

Reviewer: 2

Comments to the Author

Yang et al have investigated activation of basophils via non-FcR pathway and claimed that ASGM1, a glycolipid receptor involved in activation and development of basophils was responsible from anaphylaxis-like shock in mice, with increased vascular permeability in response to PTX. Although this is a novel subject, the work is well planned and shows clear, nice data, it is not presented well due to a number of grammar and typographic errors. I have some comments, which may improve the manuscript.

Comments:

1. In the introduction part, what is the relation of IL-33 with this study? If related, it should be better defined. (page 3, line 36, 37)
2. In introduction page 8, Line 50 Figure 4C must be 4D.
3. It is mentioned that ASGM1 is a co-receptor for TLR5, which is known to be responsible from flagellin recognition. Did the authors investigate effects of TLR5-triggering by flagellin? Is TLR5 expressed in mice basophils?

## Peer review correspondence

4. Authors demonstrate roles of ASGM-1 together with pertussis toxin. PTX is known to increase permeability, however, in general it is known to be an immune-inhibitory substance. Authors should also consider investigating roles of other bacterial toxins with similar effects on permeability together with ASGM-1. What is the authors opinion about cholera toxin?
5. Compare to ASGM1 (Figure 2A) response the flagellin is completely different. Authors should discuss this discrepancy. Also, Flagellin is claimed to activate basophils, but this data is presented in the supplementary figures, it might be better to move this data into Figure 1.
6. Results should be more definitive, it is very difficult to follow. For example Figure-2 related results should be edited as 2A, B, C, D. Fig 2D should be discuss in last order not first for better understanding. Last result session, Figure 5 contains only for lines, even figure has many data to discuss.
7. Some abbreviations lack definitions, like i.p., MOG, CFA, anti-VLA-4 as seen in discussion. It may be good to define these, for better understanding of the manuscript.
8. In methods, generally needs definitely more explanation, for example totally how many mice were studied in the experiments?  
Why was female mice specifically selected?  
Completely, lack isolation of bone-marrow cells, and purification of basophils. The procedures should be added to the methods.  
Give all details (Company, State, etc..) for the substances and antibodies used in the study. For example page 14, line 14 (Peprotech, ??.) or same page line 44 (Beyotime ??)  
What is the sensitivity and specificity for IgE?
9. Can the measurement of rectal temperature and determination of mouse activities reveal shock status of mice perfectly?
10. On page 16 and line 49, what is a FACSCalibur machine? It should better be written as “..... and analysed either on a LSR II or a FACSCalibur flow cytometry running ..... software. What was the software used for data acquisition?
11. What is OD620 minus OD492 on page 17 line 14? Determination of evans blue content should be better defined. By which method? What do these numbers mean,..etc.
12. On page 22, line 46, how was the mice treated with PTX? Why was the dose 200ng selected? For all doses used, were dose-response experiments converted? It is better to define volumes of the doses injected to mice.
13. On figure 1A, filled and solid lines should be defined in the legend. Is the filled one an isotype control? It is claimed that purity of basophils were 90%, did the authors characterize the contaminating cells in remaining 10%?
14. What does “general performance” mean on page 11, line 31?
15. Is it possible to analyse band strengths in Western Blots so that objective comparison in between different time points or doses can be made?
16. In supplement figure 1B CFA + aAGM1 condition is missing should be presented.

17. Although authors evaluated statistical significance by unpaired T test, it is better to investigate by paired-T test or non-parametric Wilcoxon test, according to sample numbers. Same cells or animals under different stimulations and time points were investigated, which requires paired tests for proper statistical analysis.

18. In general text definitely needs intensive editing.

Reviewer: 3

### Comments to the Author

The authors describe a basophil d-dependent model of anaphylaxis in mice. Anti-ASGM1 antibodies administered in PTX-pretreated mice induce anaphylaxis through PAF release from basophils. The authors show in vitro and in vivo data that both PTX and PAF can alter the integrity of endothelial monolayers that PTX increases the susceptibility of the mice to PAF-mediated anaphylaxis. They then propose that this is the mechanism of anaphylaxis in their animal model.

There are a few issues that need to be addressed:

1. The model used for these experiments is not well described. It is not clear to me how much of the anti-ASGM-1 antibody was administered, how much PTX and what was the timing of the administrations.
2. The supplemental data are only discussed in the "Discussion" section. They should be inserted at the appropriate area in the "Results" section.
3. The experiments are well performed and the conclusions are very clear and well supported. However, I am not convinced about the biological significance of the observation. The authors propose that flagellin may use this system to induce anaphylaxis, but what is the evidence that flagellin does that and what does it mean physiologically? Inclusion of more data on the ability of flagellin to induce a similar anaphylactic-type reaction would be helpful.
4. It is not clear to me what do the data shown in Figure 5 contribute to the point the authors try to make with this manuscript. The authors state in the abstract: "PTX augments the susceptibility of mice to PAF-mediated shock by enhancing the vascular permeability and upregulating FcεRI expression on basophils". What is the evidence that the results they describe are FcεRI dependent?
5. On page 7, lines 9-15 the authors state that "complement activation was not involved in the anaphylaxis-like shock. I am not convinced that this is an appropriate conclusion. The data presented in Fig 2C only indicate that complement activation following PTX treatment is not sufficient to cause anaphylaxis.

### First Revision – authors' response –11 January 2014

To Reviewer 1

1. The authors should mention in the introduction that also anti-CD200R3 induces an IgE- and IgG1-independent anaphylaxis in mice (Kojima et al. J Immunol 179:7093).

Answer: The paper (Kojima et al. J Immunol 179:7093) has been cited (ref. 7) and mentioned in the introduction of revised manuscript.

2. Figure 1. Could the authors also show quantification of released PAF in the supernatant in addition to the RT-PCR data?

Answer: The quantification of released PAF has been performed using the 6460 Triple Quadrupole Liquid Chromatography/Mass Spectrometry system. The data are illustrated in the revised figure 1G and described in the revised main text. Anti-ASGM1 antibody stimulation increased the release of PAF from basophils in vitro. Because the blood extraction from shocked mouse is almost impossible, released PAF data in vivo were not shown.

3. Figure 3. As MAR-1 can also modulate the activity of mast cells it would be more convincing to include either mast cell deficient mice in which this type of anaphylaxis should still occur and/or basophil-deficient mice in which the response should be ablated.

Answer: We absolutely support the reviewer. We tried our best to purchase the mast cells or basophils deficient mice in the previous and present study, however unfortunately we could not obtain them so far. In the revised manuscript, we detected the modulation of MAR-1 on the activity of mast cells (fig 3C and 3D), and found that the numbers of intact and degranulated mast cells were not significantly affected. In addition, we used Ba103, another basophils depletion antibody, and observed the similar result with MAR-1 (fig 3E). In the future, we will continuously look for mast cell-deficient mice and basophils-deficient mice.

4. Figure 5. The link between PTX treatment, basophilia, FcεRI upregulation and IgE production is not worked out very well. PTX has many effects in vivo including blockade of chemokine receptors which might explain basophil-accumulation in the blood. Are basophils also increased in other organs including bone marrow? Does PTX directly act on basophils to promote upregulation of FcεRI? Are other antibody isotypes also increased in PTX treated mice or is it selective for IgE?

Answer: As shown in revised fig 5B, PTX treatment did not increase basophils in bone marrow, but promoted upregulation of FcεRI on basophils. As illustrated in revised fig 5C, IgG1 and IgG2 level were

not significantly affected in PTX treated mice. It has been reported that IgE can bind FcεRI to prolong basophil survival whereas the exposure of basophils to IgE leads to the upregulated expression of FcεRIα (J Exp Med 1997. 185: 663-672. J Allergy Clin Immunol 2000. 106: 514-520). We conclude that PTX not only increase the vascular permeability, but also prime basophils. We will carry out further study next.

5. Please specify in the Materials and Methods that anti-ASGM1 is a polyclonal rabbit antibody.

Answer: "ASGM1 is a polyclonal rabbit IgG antibody" has been added in the Materials and Methods.

To Reviewer 2

1. In the introduction part, what is the relation of IL-33 with this study? If related, it should be better defined. (page 3, line 36, 37)

Answer: In the present study, IL-33 is not related with our results. In the introduction part, we would like to show there are some reports about non-antibody-antibody receptor dependent pathways for anaphylaxis. IL33 pathway is one of them.

2. In introduction page 8, Line 50 Figure 4C must be 4D.

Answer: Yes, Figure 4C should be 4D. We have re-edited intensively all of the figures number in the revised manuscript.

3. It is mentioned that ASGM1 is a co-receptor for TLR5, which is known to be responsible from flagellin recognition. Did the authors investigate effects of TLR5-triggering by flagellin? Is TLR5 expressed in mice basophils?

Answer: Both of ASGM1 and TLR5 recognize flagellin. Mice basophils express TLR5, as shown in revised fig 1B. Although we did not investigate the downstream signal molecules of TLR5-triggering by flagellin in vitro, such as TRAF6, NK-κB and so on, we detect the effects of flagellin in vivo, as illustrated in supporting information fig 2 right panel. We conclude that the change of body temperature is the overlap of both signals from ASGM1 and TLR5 triggering by flagellin.

4. Authors demonstrate roles of ASGM-1 together with pertussis toxin. PTX is known to increase permeability, however, in general it is known to be an immune-inhibitory substance. Authors should also consider investigating roles of other bacterial toxins with similar effects on permeability together with ASGM-1. What is the authors opinion about cholera toxin?

## Peer review correspondence

Answer: We absolutely support the reviewer. We will try cholera toxin in the next study. As we discussed in Discussion part, the materials or toxins, which increase the permeability of vessels will increase the sensitivity to PAF. In the present study, the anaphylaxis like shock is not induced by immune response, so we do not discuss the immune-inhibitory role of PTX.

5. Compare to ASGM1 (Figure 2A) response the flagellin is completely different. Authors should discuss this discrepancy. Also, Flagellin is claimed to activate basophils, but this data is presented in the supplementary figures, it might be better to move this data into Figure 1.

Answer: Flagellin has the similar effects with that of anti-ASGM1 antibody, such as inducing the phosphorylation of ERK in vitro and body temperature decrease in vivo. The bioactivities of flagellin are more than anti-ASGM1 antibody, because flagellin can bind ASGM1, TLR5 and other receptors and produce multiple effects. We have discussed it in Discussion part. Because most of data are from anti-ASGM1 antibody treatment and this antibody is an artificial reagent not a nature molecule, we will research the effects of some nature molecules such as flagellin on the activation of basophils and anaphylaxis in the next project. In the present study, we put the data of flagellin in the supplementary figures to display the physiological significance of non-antibody-antibody receptor pathway in natural condition.

6. Results should be more definitive, it is very difficult to follow. For example Figure-2 related results should be edited as 2A, B, C, D. Fig 2D should be discuss in last order not first for better understanding. Last result session, Figure 5 contains only for lines, even figure has many data to discuss.

Answer: All of figures including Fig 2 and Fig 5 have been re-edited carefully. We added the labels or keys in the revised figures to let the results more definitive. Meanwhile, the interpretation of data and figure legends have been re-edited in the revised manuscript.

7. Some abbreviations lack definitions, like i.p., MOG, CFA, anti-VLA-4 as seen in discussion. It may be good to define these, for better understanding of the manuscript.

Answer: All abbreviations have been given full name or definition in the revised manuscript.

8. In methods, generally needs definitely more explanation, for example totally how many mice were studied in the experiments?

Answer: The number of mice studied in the experiments has been defined in the figure legends in the revised manuscript.

## Peer review correspondence

Why was female mice specifically selected?

Answer: Female mice were selected to induce EAE according to nature protocol. Because in the present study, anaphylaxis-like shock was observed in EAE induced mice, we used female mice.

Completely, lack isolation of bone-marrow cells, and purification of basophils. The procedures should be added to the methods.

Answer: The procedures about isolation of bone marrow cells and purification of basophils have been added to the Materials and methods part.

Give all details (Company, State, etc..) for the substances and antibodies used in the study. For example page 14, line 14 (Peprotech, ??.) or same page line 44 (Beyotime ??)

Answer: The details for the substances and antibodies used in the study have been given in the revised manuscript following EJI standards.

What is the sensitivity and specificity for IgE?

Answer: In the present study, we detected the total IgE level in the serum. We did not characterize their sensitivity and specificity.

9. Can the measurement of rectal temperature and determination of mouse activities reveal shock status of mice perfectly?

Answer: The best parameter displaying shock status is blood pressure, however, it is very difficult to be measured. So far, the rectal temperature and mouse activities are still most popular parameters to reveal shock status or systemic anaphylaxis. (Kojima et al. J Immunol 179:7093)

10. On page 16 and line 49, what is a FACSCalibur machine? It should better be written as “..... and analysed either on a LSR II or a FACSCalibur flow cytometry running ..... software. What was the software used for data acquisition?

Answer: It has been changed to “Cells that from spleens or cultures were stained with fluorescence-conjugated antibodies in staining buffer (PBS, 1% FBS) and analyzed either on a LSR II or a FACSCalibur flow cytometers (Becton Dickinson). Data were acquired using FACSDiva software and analyzed using FlowJo software (TreeStar).”

11. What is OD620 minus OD492 on page 17 line 14? Determination of evans blue content should be better defined. By which method? What do these numbers mean,..etc.

Answer: It has been changed to "The whole lung, heart and esophagus tissues were excised and extravasated dye was extracted in 1 ml of formamide at 55 °C overnight. Evans blue content was measured by spectrophotometry and determined as absorbent density at 620nm wavelength (OD620) minus absorbent density at 492nm wavelength (OD492) of the formamide extract."

12. On page 22, line 46, how was the mice treated with PTX? Why was the dose 200ng selected? For all doses used, were dose-response experiments converted? It is better to define volumes of the doses injected to mice.

Answer: The scheme indicating PTX treatment has been added as fig 2A. In brief, C57BL/6 mice were intraperitoneal cavity injected (i.p.) with PTX (200ng/volume) on day 0 and day 2. 200ng of PTX was selected to induce EAE actively via preliminary experiment. As mentioned above and in the manuscript, the anaphylaxis-like shock was observed when we carried out the study about EAE. In this study, via preliminary experiments, we found that 200ng indeed was appropriate dose. All of doses used in the experiments were determined by preliminary experiments. The volumes of the doses were added in the revised figure legends.

13. On figure 1A, filled and solid lines should be defined in the legend. Is the filled one an isotype control? It is claimed that purity of basophils were 90%, did the authors characterize the contaminating cells in remaining 10%?

Answer: All lines have been defined in the figures and figure legends. The filled one is an isotype control. We did not characterize the contaminating cells in remaining 10% excluding basophils. The phenotype of the contaminating cells should be DX5<sup>+</sup>FcγRI-c-kit<sup>-</sup> or DX5<sup>+</sup>FcγRI-c-kit<sup>+</sup>.

14. What does "general performance" mean on page 11, line 31?

Answer: Here "general performance" means body temperature, activities et al outward performance. We have changed it to "outward manifestation".

15. Is it possible to analyse band strengths in Western Blots so that objective comparison in between different time points or doses can be made?

Answer: Yes, we have analyzed bands strengths using grayscale scanning. The data have been added in the fig 1E.

16. In supplement figure 1B CFA + aAGM1 condition is missing should be presented.

Answer: Because supplement figure 1A has illustrated that CFA+MOG+αASGM1 condition could not elicit shock, CFA+αASGM1 condition is not necessary to be presented in supplement figure 1B.

17. Although authors evaluated statistical significance by unpaired T test, it is better to investigate by paired-T test or non-parametric Wilcoxon test, according to sample numbers. Same cells or animals under different stimulations and time points were investigated, which requires paired tests for proper statistical analysis.

Answer: We absolutely support the reviewer. Full statistical analysis has been performed and detailed in each figure legend in revised manuscript. Proper statistical analysis is selected according to the stimulation and time points.

18. In general text definitely needs intensive editing.

Answer: The text has been intensively edited according to the comments of editors and reviewers.

To Reviewer 3

1. The model used for these experiments is not well described. It is not clear to me how much of the anti-ASGM-1 antibody was administered, how much PTX and what was the timing of the administrations.

Answer: The scheme of the model used in the study was added in the fig 2A. The dosage of anti-ASGM1 antibody (35 μg) and PTX (200ng) was described in the figure legends.

2. The supplemental data are only discussed in the “Discussion” section. They should be inserted at the appropriate area in the “Results” section.

Answer: I worry about putting the supplemental data in the “Results” section will distribute the topic of the study. The supplemental figure 1 illustrates that the cooperation of PTX and anti-ASGM1 antibody on anaphylaxis-like shock in the presented study was found unexpectedly when we focused on EAE research. It is actually a part of background. Supplemental figure 2 shows the effects of flagellin on the basophils activation and anaphylaxis like shock in mice. We put the data to display the physiological significance of non-antibody-antibody receptor pathway in natural condition. Because most of data in the present study are from anti-ASGM1 antibody treatment and this antibody is an artificial reagent not a

nature molecule, we will research the effects of some nature molecules such as flagellin on the activation of basophils and anaphylaxis in the next project.

3. The experiments are well performed and the conclusions are very clear and well supported. However, I am not convinced about the biological significance of the observation. The authors propose that flagellin may use this system to induce anaphylaxis, but what is the evidence that flagellin does that and what does it mean physiologically? Inclusion of more data on the ability of flagellin to induce a similar anaphylactic-type reaction would be helpful.

Answer: We absolutely support the reviewer. More data on the ability of flagellin to induce a similar anaphylactic-type reaction would be helpful. We will try it in the following research. We propose that flagellin-ASGM1-basophils-PAF pathway would be involved in some infection shocks.

4. It is not clear to me what do the data shown in Figure 5 contribute to the point the authors try to make with this manuscript. The authors state in the abstract: "PTX augments the susceptibility of mice to PAF-mediated shock by enhancing the vascular permeability and upregulating FcεRI expression on basophils". What is the evidence that the results they describe are FcεRI dependent?

Answer: It has been reported that IgE can bind FcεRI to prolong basophil survival whereas the exposure of basophils to IgE leads to the upregulated expression of FcεRIα (J Exp Med 1997. 185: 663-672. J Allergy Clin Immunol 2000. 106: 514-520). We tried to use this evidence to support the roles of PTX in this anaphylactic-like shock. The results we described in the manuscript are not FcεRI dependent, but the expression of FcεRI is important for the survival and activation of basophils.

5. On page 7, lines 9-15 the authors state that "complement activation was not involved in the anaphylaxis-like shock. I am not convinced that this is an appropriate conclusion. The data presented in Fig 2C only indicate that complement activation following PTX treatment is not sufficient to cause anaphylaxis.

Answer: Antibody PK136 can activate complement and delete NK cells at the same time. As shown in revised fig 2B and fig 3B, PK136 treatment did not change the body temperatures and activities of mice, so we concluded that complement activation was not involved in the anaphylaxis-like shock.

### **Second Editorial Decision – 30 January 2014**

Dear Dr. Zheng,

Thank you for submitting your revised manuscript ID eji.201344144.R1 entitled "ASGM1-mediated stimulation triggers anaphylaxis-like shock via activating basophils to release PAF in mice" to the European Journal of Immunology. Your manuscript has been re-reviewed and the comments of the referees are included at the bottom of this letter.

Although the referees have recommended publication, some revisions to your manuscript have been requested. Therefore, I invite you to respond to the comments of referees 2 and 3 and revise your manuscript accordingly. \*\*We also strongly encourage you to have your revised manuscript proofread by a native English speaker before re-submission to improve the language.\*\*

You should also pay close attention to the editorial comments included below. \*In particular, please edit your figure legends to follow Journal standards as outlined in the editorial comments. Failure to do this will result in delays in the re-review process.\*

If the revision of the paper is expected to take more than three months, please inform the editorial office. Revisions taking longer than six months may be assessed by new referees to ensure the relevance and timeliness of the data.

Once again, thank you for submitting your manuscript to European Journal of Immunology. We look forward to receiving your revision.

Yours sincerely,  
Karen Chu

on behalf of Dr. Rikard Holmdahl

Dr. Karen Chu  
Editorial Office  
European Journal of Immunology  
e-mail: ejied@wiley.com

[www.eji-journal.eu](http://www.eji-journal.eu)

\*\*\*\*\*

Reviewer: 1

Comments to the Author

The authors addressed my questions and improved the quality of the manuscript

Reviewer: 2

Comments to the Author

After answering the required questions manuscript is enhanced, become more clear and publishable.

1. "sensitivity and specificity for IgE measurement" means the detection range limits of IgE measurement kits, please give this detail regarding IgE measurement.

2. Still there are some typographic errors, text may be spell-checked once more to clear these errors.

a. Last line of abstract, instead of suggest use suggesting

b. In the results part "[Ca<sup>2+</sup>]<sub>i</sub>", is this correctly written?

c. Page 14: line 14; After aASGM1 injection 15 minutes, .... should be corrected as Fifteen minutes after aASGM1 injection,.....

d. Several times, basophil / basophils are written erroneously singular or plural, which should be corrected. Example: basophils depletion should be basophil depletion.

e. Page 15 line 5, "GdCl<sub>3</sub>" definition of this abbreviation should be here instead of being in the methods part.

f. Page 18, line 8, basophiles should be corrected as basophils.

g. Page 18 line 9, anit-ASGM1 should be corrected as anti-ASGM1

h. Page 22, line 12, (Invitrogen,) should be corrected as (Invitrogen)

i. Page 22, line 15, Primer sequences used in PCR was should be corrected as were.

j. Page 25, line 3 data were or data was ?

k. Page 25, line 10: correct as spectrophotometry, absorbent, also line 11 as absorbent.

Reviewer: 3

Comments to the Author

I believe that in general the authors have replied well to the reviewers questions.

I still would like to see the supplemental data discussed also in the results section and would like to see a more clear discussion of the fact that the increased FcεRI expression on basophils and increased IgE in the serum is not the mechanism of the presented results.

### **Second Revision – authors' response –20 March 2014**

To review 2:

1. "sensitivity and specificity for IgE measurement" means the detection range limits of IgE measurement kits, please give this detail regarding IgE measurement.

Answer: The detection range limits of IgE measurement kits are 4 ng/mL-250 ng/mL. This detail is added in the "methods".

2. Still there are some typographic errors, text may be spell-checked once more to clear these errors.

a. Last line of abstract, instead of suggest use suggesting

Answer: The abstract has been revised.

b. In the results part "[Ca<sup>2+</sup>]<sub>i</sub>", is this correctly written?

Answer: [Ca<sup>2+</sup>]<sub>i</sub> is correctly written.

c. Page 14: line 14; After aASGM1 injection 15 minutes, .... should be corrected as Fifteen minutes after aASGM1 injection,.....

Answer: It has been corrected as "Fifteen minutes after aASGM1 injection....."

d. Several times, basophil / basophils are written erroneously singular or plural, which should be corrected. Example: basophils depletion should be basophil depletion.

Answer: They have been corrected.

e. Page 15 line 5, "GdCl<sub>3</sub>" definition of this abbreviation should be here instead of being in the methods part.

Answer: "GdCl<sub>3</sub>" has been defined in the "results".

f. Page 18, line 8, basophiles should be corrected as basophils.

Answer: It has been corrected as "basophils".

g. Page 18 line 9, anit-ASGM1 should be corrected as anti-ASGM1

Answer: It has been corrected as "anti-ASGM1".

## Peer review correspondence

h. Page 22, line 12, (Invitrogen,) should be corrected as (Invitrogen)

Answer: The comma has been removed.

i. Page 22, line 15, Primer sequences used in PCR was should be corrected as were.

Answer: "was" has been corrected as "were".

j. Page 25, line 3 data were or data was ?

Answer: It should be "data were".

k. Page 25, line 10: correct as spectrophotometry, absorbent, also line 11 as absorbent.

Answer: They have been corrected as "spectrophotometry" and "absorbent".

To review 3:

I still would like to see the supplemental data discussed also in the results section and would like to see a more clear discussion of the fact that the increased FcεRI expression on basophils and increased IgE in the serum is not the mechanism of the presented results.

Answer: The supplemental data are help to understand the findings of the study. They are not the critical results, so we do not put them in the "results" section.

It has been reported that IgE can bind FcεRI to prolong basophil survival whereas the exposure of basophils to IgE leads to the upregulated expression of FcεRIα (Yamaguchi M et al. JEM.1997.17:663; Saini SS et al. J Allergy Clin Immunol.2000.106: 514). IgE-dependent enhancement of mast cell FcγRI expression results in enhancement of IgE-dependent mast cell mediator release (Yamaguchi M et al. JEM.1997.17:663). Thus, we conclude that the increased FcγRI expression and IgE enhance the sensitivity of basophil activation, no matter it is IgE-dependent or IgE-independent.

### **Third Editorial Decision – 1 April 2014**

Dear Dr. Zheng,

It is a pleasure to provisionally accept your manuscript entitled "Basophil activation through ASGM1 stimulation triggers PAF release and anaphylaxis-like shock in mice" for publication in the European Journal of Immunology. For final acceptance, please follow the instructions below and return the requested items as soon as possible as we cannot process your manuscript further until all items listed below are dealt with.

Please note that EJI articles are now published online a few days after final acceptance (see Accepted Articles: [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1521-4141/accepted](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1521-4141/accepted)). The files used for the Accepted Articles are the final files and information supplied by you in Manuscript Central. You should therefore check that all the information (including author names) is correct as changes will NOT be permitted until the proofs stage.

## Peer review correspondence

We look forward to hearing from you and thank you for submitting your manuscript to the European Journal of Immunology.

Yours sincerely,  
Laura Soto Vazquez

on behalf of  
Dr. Rikard Holmdahl

Editorial Office  
European Journal of Immunology  
e-mail: [ejied@wiley.com](mailto:ejied@wiley.com)  
[www.eji-journal.eu](http://www.eji-journal.eu)