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Running Title: The lung retains antigen during anaphylaxis

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ABSTRACT

Mouse models of active systemic anaphylaxis rely predominantly on IgG antibodies forming IgG-allergen immune complexes that induce IgG receptor-expressing neutrophils and monocytes/macrophages to release potent mediators, leading to systemic effects. Whether anaphylaxis initiates locally or systemically remains unknown. Here we aimed at identifying the anatomical location of IgG-allergen immune complexes during anaphylaxis. Active systemic anaphylaxis was induced following immunization with BSA and intravenous challenge with fluorescently-labelled BSA. Antigen retention across different organs was examined using whole body fluorescence imaging, comparing immunized and naïve animals. Various mouse models and *in vivo* deletion strategies were employed to determine the contribution of IgG receptors, complement component C1q, myeloid cell types and anaphylaxis mediators. We found that following challenge, antigen diffused systemically, but specifically accumulated in the lungs of mice sensitized to that antigen, where it formed large antibody-dependent aggregates in the vasculature. Antigen retention in the lungs did not rely on IgG receptors, C1q, neutrophils or macrophages. IgG2a-mediated, but neither IgG1- nor IgG2b- mediated, passive systemic anaphylaxis led to antigen retention in the lung. Neutrophils and monocytes significantly accumulated in the lungs after challenge and captured high amounts of antigen, which lead to downmodulation of surface IgG receptors and triggered their activation. Thus, within minutes of systemic injection in sensitized mice, antigen formed aggregates in the lung and liver vasculature, but accumulated specifically and dose-dependently in the lung. Neutrophils and monocytes recruited to the lung captured antigen and became activated. However, antigen aggregation in the lung vasculature was not necessary for anaphylaxis induction.

KEY POINTS

- Active systemic anaphylaxis in mice shows antigen retention specifically in the lung
- Antigen is predominantly captured by neutrophils and monocytes
- Neutrophils and monocytes activate locally in the lung during anaphylaxis

KEY WORDS

Anaphylaxis; IgG; immune complexes; lungs; neutrophil; monocyte; macrophage; FcγR; Platelet-activating Factor; Histamine.

ABBREVIATIONS USED

Alum: Aluminum hydroxide

CFA: Complete Freund's adjuvant

IFA: Incomplete Freund's adjuvant

FcγR: IgG Fc receptor

PAF: Platelet-activating Factor

WT: Wild-Type

PSA: Passive Systemic Anaphylaxis

ASA: Active Systemic Anaphylaxis

BSA: Bovine Serum Albumin

OVA: Ovalbumin

mAb: Monoclonal Antibody

PBS: Phosphate Buffered Saline

SEM: Standard Error of the Mean

NETs: Neutrophil-extracellular traps

MPO: Myeloperoxidase

Gfi-1: Growth factor independence 1

C1q: Complement component 1q

TNP: Trinitrophenyl

INTRODUCTION

Anaphylaxis is a severe, systemic allergic reaction with increasing worldwide incidence (1, 2). Reactions usually appear rapidly (within minutes to hours) and principally affect the vasculature and the lungs, i.e., intense vasodilation and bronchoconstriction, which can lead to hypotension, hypothermia, tachycardia, respiratory distress and eventually lung or heart failure. Anaphylaxis is classically attributed to an IgE-mediated reaction involving mast cell activation and release of histamine and tryptase (3), but increasing numbers of reports describe non-IgE-mediated anaphylaxis (reviewed in (4-6)). Our recent clinical study on human drug-induced anaphylaxis (mechanisms reviewed in (7)) demonstrated the existence of an IgG-mediated reaction driven by neutrophil activation via their IgG receptors (FcγR), which contributes to severe anaphylaxis in patients; including patients lacking biomarkers of IgE-dependent anaphylaxis (8). This IgG-mediated reaction was associated with the release of platelet-activating factor, elastase and neutrophil-extracellular traps (NETs). Serum levels of platelet-activating factor (PAF) have been found to directly correlate with anaphylaxis severity (9), whereas histamine and mast cell tryptase levels do not reflect the severity of reactions (10, 11).

Experimental models of anaphylaxis can be induced following systemic antigen/allergen challenge in mice previously immunized against that antigen/allergen (called active systemic anaphylaxis, ASA). These models largely entail IgG-driven reactions, triggered by IgG-immune complexes forming in circulation and subsequent activation of FcγR-expressing myeloid cells. Indeed, for the most part, mouse ASA models proceed independently of IgE antibodies and their receptors (FcεR) (12, 13). Depending on the experimental anaphylaxis model used, several FcγR-expressing cell

types have been proposed to drive the anaphylactic response, including neutrophils, monocytes/macrophages, basophils and mast cells (reviewed in (3, 4, 14)). PAF release by neutrophils and monocytes/macrophages, or histamine release by basophils and mast cells, can cause anaphylactic symptoms. In these models, no overt symptoms of anaphylaxis are observed when mice are treated with both PAF and histamine receptor antagonists (13, 15, 16). We and others recently demonstrated that FcγR-expressing platelets could also be major players in anaphylaxis induction and severity by releasing serotonin (17, 18) (reviewed in (19)). Thus IgG-mediated anaphylaxis can rely on neutrophils, monocytes/macrophages, basophils and mast cells in humans and wild-type mice, but also on platelets in humans.

In mice, four IgG subclasses exist– IgG1, IgG2a/c, IgG2b, IgG3 – without structural or functional equivalence to human IgG subclasses. We have shown that mouse IgG1, IgG2a/c and IgG2b are all capable of triggering anaphylaxis in mice; almost exclusively via mouse FcγRIII (20). This reaction can be inhibited by mouse FcγRIIB in the case of IgG1 and IgG2b, but not IgG2a. Models of anaphylaxis relying on immunization of mice result in polyclonal IgG responses comprised mostly of IgG1; while IgG2a/c and IgG2b are provoked only by certain adjuvants and/or genetic backgrounds e.g., alum immunization induces IgG1 but not IgG2 antibodies in C57BL/6 mice (13).

In both humans and mice, IgG-dependent anaphylaxis is considered to occur chiefly when high amounts of antigen/allergen are present in circulation, thereby enabling the formation of a critical amount of circulating IgG-immune complexes; as seen in drug-induced anaphylaxis in humans (8) or in a mouse model of food-induced anaphylaxis, for example (21). This notion has nevertheless been challenged by titrating down the quantities of IgG antibodies and antigen: using cocktails of monoclonal IgG binding

different epitopes on a model allergen (OVA), merely tens of micrograms of IgG and micrograms of antigen were sufficient to induce severe IgG-mediated anaphylaxis in mice (22). This study also demonstrated that IgG-mediated anaphylaxis could potentiate IgE-mediated anaphylaxis, in line with our recent results on human cases of severe drug-induced anaphylaxis (8). Anaphylaxis can arise in humans after encounter with inhaled, ingested or injected antigens/allergens (including intravenous, intradermal, subcutaneous, intramuscular routes), and by virtually any route of challenge in sensitized mice, and entail cutaneous, respiratory, oral, gastrointestinal and/or cardiac symptoms. Whether a particular anatomical location favors the initiation of systemic anaphylaxis remains unknown. In the case of IgG-dependent anaphylaxis, the circulatory system and highly vascularized tissues are prime candidates for the site of anaphylaxis initiation because IgG-antigen/allergen immune complexes must be formed prior to interacting with FcγR-expressing cells.

Here, we sought to interrogate the anatomical location of IgG-dependent anaphylaxis initiation. We postulated that this would correspond to a site with high antigen density after injection and evidence of antigen capture by local FcγR-expressing myeloid cells, and their activation. We characterized antigen retention sites *in vivo* in mice and identified the myeloid cell populations responsible for immune complex capture, requirements for FcγR expression and reliance on IgG subclasses.

MATERIAL & METHODS

Mice

C57BL/6J mice, myeloperoxidase-deficient (MPO^{KO}) mice, and $CX3CR1^{GFP}$ knock-in mice that express EGFP in monocytes, dendritic cells, NK cells, and brain microglia(23) were purchased from Charles River, BALB/cJRj mice from Janvier, and used for experiments after maintaining the mice for at least 1 week in SPF conditions after arrival in Institut Pasteur's animal facility. $Fc\gamma R^{null}$ mice (18), NOTAM mice (24), $C1q^{KO}$ mice on the C57BL/6 background (generated by Marina Botto, London, UK) (25) and $Gfi\text{-}1^{KO}$ mice on the C57BL/6 background (18, 24-26) were described previously (26). $Fc\gamma R^{null}$ $C1q^{KO}$ mice were generated by intercrossing of $Fc\gamma R^{null}$ mice and $C1q^{KO}$ mice on a mixed C57BL/6NTac x 129S6/SvEvTac (generated by Regeneron Pharmaceuticals; VG598 mice) (27). Mice were bred at Institut Pasteur and used for experiments at 7-11 weeks of age. All animal care and experimentation were conducted in compliance with the guidelines and specific approval of the Animal Ethics committee CETEA (Institut Pasteur, Paris, France) registered under #170043, and by the French Ministry of Research.

Reagents

PBS-liposomes and Clodronate-liposomes were purchased from Liposoma (The Netherlands). BSA was from Miltenyi Biotec. OVA, Freund's adjuvant, Alum, luminol sodium salt, ABT-491 and cetirizine were from Sigma-Aldrich. Anti-Ly6G antigen-specific antibody clone NIMP-R14, IgG1 anti-TNP (TIB-191) provided by D. Voehringer (Universitätsklinikum, Erlangen, Germany), IgG2a anti-TNP (Hy1.2) provided by Shozo Izui (University of Geneva, Geneva, Switzerland), and IgG2b anti-TNP (GORK) provided by B. Heyman (Uppsala Universitet, Uppsala, Sweden) were reported previously (20) and purified from hybridoma supernatant on Protein G affinity columns. BSA or OVA were conjugated to VivoTag680 fluorochrome using the VivoTag 680XL Protein Labeling Kit (Perkin Elmer). Antibodies against CD31 (MEC 13.3), Ly6G (1A8), CD11b (M1/70), MHC class II (M5/114), Siglec F (REA798), CD45 (30F11), Ly6C (AL21), CD16/32 (2.4G2) were purchased from eBioscience, Becton Dickinson, Miltenyi Biotec or Biolegend, anti-myeloperoxidase (MPO; AF3667) from R&D Systems.

Active Systemic Anaphylaxis

Mice were injected intraperitoneally on day 0 with 200µg BSA in complete Freund's adjuvant (CFA) or Alum, as indicated, and boosted intraperitoneally on day 14 and day

21 with 200µg BSA in incomplete Freund's adjuvant (IFA) or Alum, as indicated. Control mice were immunized with OVA instead of BSA following the same protocol. BSA-specific IgG1 and IgG2a/b/c antibodies in serum were titered by ELISA on day 30, as described (13). Mice with comparable antibody titers were challenged i.v. with 500 µg BSA (unless otherwise specified) or 500 µg OVA as control 10 days after the last immunization. Central temperature was monitored using a digital thermometer with rectal probe (YSI), and time of death was recorded. Absolute counts and proportions of granulocytes in peripheral mouse blood were determined using an ABC Vet automatic blood analyzer (Horiba ABX).

If indicated, 300µg/mouse anti–Ly6G (NIMP-R14) or corresponding rat IgG2b isotype control mAbs, or 300µL /mouse PBS- or clodronate-liposomes were injected intravenously 24 hours before challenge. In the case of double depletion of neutrophils and monocytes/macrophages, 300µg/mouse anti–Ly6G (NIMP-R14) were injected intravenously 29 hours before challenge, followed 5 hours later by 300µL/mouse clodronate-liposomes intravenously. PAF-R antagonist ABT-491 (25µg/mouse) or H1-receptor antagonist cetirizine DiHCl (300µg/mouse) diluted in 0.9% NaCl were injected intravenously or intraperitoneally, respectively, 15min prior to challenge.

Passive Systemic Anaphylaxis

Mouse IgG1, IgG2a or IgG2b anti-TNP antibodies were administered intravenously at a dose of 4 mg, if not otherwise indicated, in 200 µL 0.9% NaCl, followed by an intravenous challenge with 200 µg of the antigen (TNP-BSA) in 0.9% NaCl 16 hours later. Lung and liver tissues were collected 15 min after challenge for further analysis by microscopy.

Whole body and tissue fluorescence imaging

Depilated and anesthetized mice were challenged i.v. with 500 µg BSA (composed of 50 µg BSA-VT680 + 450 µg unlabeled BSA to avoid instrument saturation) and its anatomical distribution was analyzed on an IVIS 100 (Caliper Life Sciences) using 1 sec exposition time, small binning and 675 – 720 nm filter band. Whole body imaging was performed from 1 min to 30 min post-challenge, then mice were perfused with 10 ml of PBS and tissues (lung, liver, salivary gland, thymus, spleen, kidney, heart, stomach) were collected for ex vivo imaging using the same acquisition settings. Average radiant

efficiency (photons/seconds/cm^2/surface radiance) of indicated regions of interest were calculated using Living Image software (Perkin Elmer).

Whole body bioluminescence imaging of myeloperoxidase activity

Luminol has been proposed as a reporter for myeloperoxidase activity in vivo (28). Mice were injected i.p. with 15 mg/mouse luminol before image acquisition on an IVIS 100 (Caliper Life Sciences) using 3 min exposure time and medium binning. For ex vivo imaging on explanted tissue/organs, mice were perfused with 10 ml of PBS, tissues were collected and imaged 20 min after luminol (Sigma Aldrich) injection and 30 min post challenge. Total photon flux of each region of interest was calculated using Living Image software.

Immunofluorescence

Fifteen minutes post challenge mice were perfused with 10 ml of PBS, lung and liver tissues were collected and fixed in 1 % paraformaldehyde overnight at 4°C. Tissue dehydration was performed in successive sucrose gradient baths (10%, 20% and 30% for 2h each) then tissues were embedded in OCT compound. Eight µm-thick sections were permeabilized with PBS containing 0.1% Saponin and stained with DAPI, anti-CD31 antibody (1/500) and/or anti-MPO antibody (4 µg/mL) for 2h at room temperature and revealed with FITC-coupled goat anti-rat Fab fragments (Jackson laboratories). Slides were washed 3 times and mounted with coverslips using Prolong Gold antifade reagent (Invitrogen) with DAPI. Samples were imaged by using a confocal microscope Leica SP5 and analyzed using ImageJ software.

Flow cytometry

BSA was fluorescently labeled with FITC. Lung cells were freshly isolated using Lung Dissociation kit and gentleMACS dissociator (Miltenyi Biotec). Cells were stained with the following antibodies for 30 min at 4°C: Ly6G (1A8), CD11b (M1/70), MHC class II (M5/114), SiglecF (REA798), CD45 (30F11), Ly6C (AL21), CD16/32 (2.4G2). Data were acquired using MACSQuant (Miltenyi Biotec) flow cytometer and analyzed by FlowJo software. Dead cells, labeled with propidium iodide were excluded from the analysis. Immune cell populations were identified as follows: neutrophils ($CD45^+/CD11b^+/Ly6G^+$), alveolar macrophages ($CD45^+/CD11b^{mid}/SiglecF^{high}$),

eosinophils ($CD45^+/CD11b^+/SiglecF^+$), $Ly6C^+$ monocytes ($CD45^+/CD11b^+/ SiglecF^-/ Ly6G^-/ MHCII^-/Ly6C^+$), $Ly6C^-$ monocytes ($CD45^+/CD11b^+/ SiglecF^-/Ly6G^-/MHCII^-/Ly6C^-$), $CD11b^+$ dendritic cells ($CD45^+/CD11b^+/SiglecF^-/Ly6G^-/MHCII^{mid}/Ly6C^{+/-}$) and interstitial macrophages ($CD45^+/CD11b^+/SiglecF^-/Ly6G^-/MHCII^{high}/Ly6C^-$), B cells ($CD45^+/CD11b^-/MHCII^{high}$).

Fluorescence microscopy on fresh lung explant

OVA-sensitized (CFA+IFA) $CX3CR1^{GFP}$ mice were injected i.v. with 5 µg anti-Ly6G-BV421 antibody. Challenge with 500 µg of OVA-VT680 was performed i.v. 5 min later. Five minutes after challenge mice were euthanized and the lungs perfused with 10 ml PBS, explanted and immediately sliced into 300 µm slices using a vibratome (Leica). Samples were glued onto a petri dish filled with 37°C RPMI medium (minus phenol red) using Vetbond (3M Co., Maplewood, MN). Two-photon excitation fluorescence imaging was performed on a Zeiss LSM 710 NLO microscope equipped with a W Plan-Apochromat 20× numerical aperture (NA) 1.0 differential interference contrast (DIC) M27 75-mm water immersion objective. Excitation was produced at 940 nm by a tunable pulsed laser (Chameleon; Coherent, Santa Clara, CA). SHG was epicollected by a dedicated nondescanned detector (NDD) coupled with a 427- ± 20-nm bandpass filter, green fluorescent protein (GFP) by a 500-to-550-nm NDD, rhodamine B or Alexa Fluor 568 by a 565-to-610-nm NDD, and Alexa Fluor 633 by a 660-to-730-nm NDD. Z-stacks were rendered using a maximum-intensity projection.

Statistical analyses

Statistical analyses were performed with GraphPad Prism version 9.0 (GraphPad Software Inc.). Fluorescence data between two groups were compared with a Mann-Whitney two-tailed test (Fig.1B, 2A-B, 3A, 3D, Supp.1C, Supp.3B, Supp.4A-B). Fluorescence data between more than two groups were compared with a two-way ANOVA (Fig.1D, 3B, 3E, 4C-D, 5C, Supp.1D, Supp.3C (top), Supp.3D, Supp.4C). Survival data between two groups were compared using the Log-rank (Mantel-Cox) test (Supp.3C bottom). Time course responses were compared with an unpaired t-test, two-tailed (Fig.5B).

RESULTS

We hypothesized that the anatomical location of IgG-mediated anaphylaxis initiation would correspond to a site with high antigen density immediately after antigen injection. Antigen accumulation would favor local generation of IgG antibody-antigen immune complexes, and subsequent complement activation and/or myeloid cell activation through their FcγRs, leading to clinical signs of anaphylaxis. We therefore took an approach to track the antigen during a mouse model of ASA: we labeled a model antigen, BSA, with a near-infrared fluorochrome compatible with non-invasive whole-body imaging (VivoTag680; VT680). C57BL/6J mice were immunized with BSA or control antigen OVA in Freund's adjuvant (CFA/IFA). Five minutes after challenge with BSA-VT680, we observed an accumulation of fluorescence signal in the liver area using whole body imaging, and in the lung area specifically in BSA-sensitized mice (Fig.1A; Supp.Fig.S1A). Quantification of antigen accumulation in explanted tissues 30 min after challenge demonstrated a 120-fold increase of fluorescence in the lungs of BSA-sensitized mice, but only a ~2-fold increase in the liver, spleen or thymus compared to naive animals (Fig.1B; Supp.Fig.S1B). No increase in antigen-associated fluorescence was detected in the salivary glands or in the stomach, whereas the kidneys and heart showed an inverse ~2-fold reduction in fluorescence in sensitized compared to naive animals (Fig.1B; Supp.Fig.S1C).

Immunofluorescence assessment of frozen sections of lung and liver from sensitized mice collected 15 min post-challenge demonstrated the presence of large extracellular aggregates of antigen in the lung alveolar capillaries and liver sinusoids. These aggregates were multifocally distributed exclusively in vascular spaces. Such aggregates were not seen in the lungs of naive mice injected with BSA-VT680, and barely detectable in the liver (Fig.1C). In sensitized mice, the accumulation of antigen in the

lung was dose-dependent, whereas the degree to which antigen was detected in the liver did not change with varying antigen dose (Fig.1D), nor in other tissues (Supp.Fig.1D). Of note, challenge with a low dose (10μg) of antigen resulted in minimal antigen accumulation in the lung 30 min after challenge (Fig.1D) and did not significantly reduce central body temperature (Fig.1E), a hallmark of anaphylaxis in the mouse. Higher antigen doses (i.e., 50 and 500μg) induced significant antigen accumulation in the lung and >5°C drop of body temperature, with up to 50% mortality in mice challenged with 500μg antigen (Fig.1D-E). Altogether, these experiments indicate that antigen accumulates specifically and dose-dependently in the lung during experimental active systemic anaphylaxis, with massive antigen aggregates observed in the lung vasculature.

We next investigated if antigen was associating with specific immune cell populations in the lung. We examined cell populations reported to be involved in experimental anaphylaxis, i.e., neutrophils, macrophages (alveolar and interstitial), $Ly6C^{lo}$ and $Ly6C^{hi}$ monocytes, but also FcγR-expressing eosinophils, $CD11b^{+}$ dendritic cells (DCs) and B cells. To ensure similar proportions of immune cells and total circulating antibody between experimental and control groups, mice were either immunized with BSA and challenged with FITC-labeled BSA (experimental group, leading to BSA-immune complex formation and anaphylaxis) or immunized with OVA and challenged with FITC-labeled BSA (control group, no immune complexes and no anaphylaxis). Among all the cell types examined, the numbers of $Ly6G^{+}$ neutrophils, $Ly6C^{hi}$ monocytes and $Ly6C^{lo}$ monocytes significantly increased in the lung during anaphylaxis (Fig.2A, gating information in Supp.Fig.2A). >95% of neutrophils and $Ly6C^{hi}$ monocytes were antigen-positive, along with the vast majority of interstitial macrophages, eosinophils and alveolar macrophages, and 20% of B cells (Fig.2B). Neutrophils, interstitial macrophages and $Ly6C^{hi}$ monocytes exhibited the highest amount

337 of antigen-associated fluorescence on a per cell basis (Supp.Fig.2B-C) As a proportion of
338 the antigen-capturing cells, however, neutrophils and Ly6C^{hi} monocytes represented
339 >20% and >10%, respectively, whereas Ly6C^{lo} monocytes were barely detectable. ~40%
340 of all antigen-capturing cells were alveolar macrophages and interstitial macrophages
341 (Fig.2C).

342 To further investigate the anatomical distribution of antigen aggregates and
343 antigen-capturing cells, we performed 2-photon microscopy analysis of lung explants of
344 OVA-sensitized CX3CR1^{GFP} mice that express enhanced GFP (EGFP) in monocytes,
345 dendritic cells and a minor subset of NK cells, DCs or macrophages (29, 30) (but neither
346 neutrophils, eosinophils, B cells, T cells nor alveolar macrophages) (23), and which were
347 injected with BrilliantViolet421-labelled anti-Ly6G to visualize neutrophils in another
348 channel. As expected, labeled OVA antigen (OVA-VT680) could be readily imaged in
349 the lung explants of OVA-sensitized mice, but not in non-sensitized mice, taken 5 min
350 after OVA challenge. We observed accumulation of GFP^{+} cells and neutrophils in the
351 lungs of immunized and challenged mice, with evidence of antigen-neutrophil-GFP^{+} cell
352 foci and antigen capture by neutrophils (Fig.2D) and GFP+ cells (Supp.Fig.3A). The
353 velocity of neutrophils, but not of CX3CR1+ cells, was also significantly increased within
354 the lung tissue (Supp.Fig.3B) suggestive of persistent neutrophil recruitment and
355 activation. These experiments indicate that neutrophils and inflammatory monocytes
356 infiltrate the lung and take up antigen during anaphylaxis and represent, together with
357 tissue-resident monocytes/macrophages, grossly 80-90% of antigen-capturing cells.

358 We have previously found that FcγR-expression decreased on neutrophils and
359 monocytes during experimental anaphylaxis in mice expressing human FcγRs (31), and
360 in patients suffering from drug-induced anaphylaxis (8), due to receptor aggregation on
361 the cell surface and subsequent cell activation and endocytosis of antigen-IgG immune

362 complexes. As a simple readout of mouse FcγR engagement on lung resident or
363 infiltrating cells, we analyzed their surface expression of activating FcγRIII, the main IgG
364 receptor involved in IgG-anaphylaxis in mice (14), using an antibody recognizing both
365 FcγRIII and inhibitory FcγRIIB. FcγRIII/FcγRIIB expression decreased significantly
366 (1.5-2-fold) on neutrophils, Ly6C^{hi} and Ly6C^{lo} monocytes, eosinophils and B cells within
367 15 min of anaphylaxis induction, compared to cells from control animals immunized
368 against an alternate antigen (Fig.3A). Thus IgG-antigen immune complexes engage FcγR
369 on immune cells in the lung rapidly after challenge.

370 To understand if antigen accumulation in the lungs was dependent on immune
371 complex retention via FcγRs, we immunized and challenged FcγR^{null} mice, which are
372 knocked-out for FcγRI, FcγRIIB, FcγRIII and FcγRIV (18). Surprisingly, antigen
373 accumulation in the lung was unchanged in FcγR^{null} mice compared to wt mice (Fig.3B).
374 The same result was obtained in NOTAM mice (32) that express normal surface levels of
375 activating FcγRs, but lack functional signaling through the essential ITAM of the FcRγ
376 subunit associated with FcγRI, FcγRIII and FcγRIV. Complement component C1q-
377 deficient (C1q^{KO}) mice and double-deficient FcγR^{null}C1q^{KO} mice also demonstrated lung
378 antigen accumulation similar to wt mice after immunization and challenge (Fig.3B).
379 Neither FcγR^{null} mice nor NOTAM mice presented signs of anaphylaxis after antigen
380 challenge, whereas C1qKO mice developed temperature drop and mortality comparable
381 to wt mice (Fig.3C). Thus, antigen retention in the lung during anaphylaxis induction is
382 not contingent upon immune complex-mediated, FcγR-dependent cell activation, nor
383 complement component C1q (7, 33).

384 We previously reported that a concomitant depletion of neutrophils (using anti-
385 Ly6C/G antibodies) and monocytes/macrophages (using clodronate liposomes) in mice
386 protects from active systemic anaphylaxis (13). Here, we show that neutrophils and

monocytes/macrophages contribute to a large extent to antigen capture in the lung (Fig.2B-D; Supp.Fig.2B-C,3A). Antigen accumulation in the lung was, however, not impacted in the absence of mature neutrophils, using mice deficient for the transcriptional repressor Gfi-1 (26) (Fig.3D), or following the depletion of neutrophils alone or of both monocyte/macrophages and neutrophils before challenge (Fig.3E). Neutrophil-deficient Gfi-$1^{-/-}$ mice were nonetheless significantly protected from the loss of temperature and mortality associated with anaphylaxis challenge, compared to Gfi-$1^{+/-}$ littermate controls (Supp.Fig.3C). Accumulation of antigen in the lungs was also unaffected by PAF or histamine receptor blockade, although injection with PAF-receptor (ABT-491) or histamine-receptor (cetirizine) antagonists inhibited anaphylaxis symptoms (Supp.Fig.3D-E) (15, 33). Altogether, these results demonstrate that specific antigen accumulation in the lung after challenge of immunized mice does not rely on FcγRs, complement component C1q, neutrophils or monocyte/macrophages, nor on their activation, or on the anaphylaxis mediators PAF and histamine.

The composition or size of the immune complexes formed after challenge in this anaphylaxis model could determine antigen retention in the lung vasculature, conceivably via physical restraints in the small capillaries. We therefore investigated antigen retention after immunization with an alternative adjuvant, alum, that is expected to induce lower levels and different subclasses of IgG, compared to Freund's adjuvant (13); and that will likely result in the formation of immune complexes of a different composition or size after challenge. Anaphylactic reactions in alum-immunized C57BL/6J mice after antigen challenge were of similar intensity to that of CFA/IFA-immunized mice (Fig.4A) and also relied on PAF and histamine (Supp.Fig.3E); yet did not lead to specific antigen retention in the lung (Fig.4B-C). The difference in lung antigen retention between mice challenged after CFA/IFA compared to alum immunization was also observed in FcγR^{null}

412 mice bred on a C57BL/6 dominant mixed background (C57BL/6N + C57BL/6J +
413 129S6/SvEvTac) and in C57BL/6 Gfi-1$^{-/-}$ mice (Supp.Fig.4A-B). However, when we
414 performed alum immunization and challenge in BALB/c mice, we observed antigen
415 retention in the lungs with a strikingly similar pattern and intensity as that following
416 CFA/IFA immunization and challenge in C57BL/6J mice (Fig.4B-C). Alum
417 immunizations in C57BL/6J mice, compared to BALB/c mice, lead to significantly lower
418 antigen-specific total IgG responses, 4-fold lower IgG1 responses, undetectable IgG2a/c
419 and IgG2b responses (Fig.4D), as we reported earlier (13), and 2-fold lower IgG3
420 responses (Supp.Fig.4C).

421 Next, we tested if antigen-specific mouse IgG2 could be responsible for antigen
422 retention in the lung by employing a passive systemic anaphylaxis model based on
423 administration of anti-trinitrophenyl (TNP) IgG1, IgG2a and IgG2b mAbs, followed by
424 challenge with TNP-labeled BSA 18h later. Importantly, these antibodies all have similar
425 affinity for TNP (20). Whereas mouse IgG2a anti-TNP administered at doses of 1, 2 and
426 4 mg generated equivalent antigen aggregates in the liver, the 4 mg dose led to significant
427 antigen aggregates in the lung capillaries after antigen challenge (Supp.Fig.4D, Fig.4E).
428 Under the same conditions, neither anti-TNP IgG1 nor IgG2b lead to any detectable
429 antigen aggregates in the lung (Fig.4E). These results demonstrate that IgG2a antibodies
430 can mediate antigen retention in the lung; and suggest that IgG2a may also be responsible
431 for antigen retention in the lung in antigen-immunized and challenged mice.

432 We reported previously that active systemic anaphylaxis following CFA/IFA
433 immunization and challenge relied predominantly on neutrophils and PAF release, with
434 the release of the neutrophil enzyme myeloperoxidase (MPO) detected within minutes
435 after antigen challenge (13). MPO activity can be can monitored *in vivo* via luminescence
436 generated by the oxidation of luminol (28) and can thus be considered a real-time

neutrophil activation marker. We found that the main anatomical sites of MPO activity after challenge were the left and right lungs (Fig.5A): luminescence was readily detectable at 2 min and remained significantly elevated for at least 20 min after challenge (Fig.5B). Luminol oxidation was dependent on the presence of MPO and neutrophils: as expected, immunized MPO^{KO} mice and neutrophil-deficient Gfi-1^{KO} mice did not display elevated luminescence in the lungs over baseline unimmunized wt mice (Fig.5C), although both of these mouse strains do undergo ASA after antigen challenge (Supp.Fig.3C and Supp.Fig.4E). In wt mice undergoing ASA, MPO foci were detected in the lungs by immunofluorescence, but not in the lungs of control mice (Fig.5D). Altogether, these results suggest that antigen-specific antibodies within immune complexes allow antigen retention in the lung capillaries, leading to local neutrophil activation and degranulation.

DISCUSSION

We demonstrate here that the initiation of an IgG-dependent active systemic anaphylactic reaction in mice coincides with antigen retention specifically in the lungs. Lungs present with large antigen aggregates in the capillaries, myeloid cell recruitment and antigen capture predominantly by neutrophils and monocyte/macrophage populations. Antigen retention in the lung was observed independently of activating IgG receptors (FcγR), complement component C1q, neutrophil and monocyte/macrophage depletion. IgG2a-mediated passive systemic anaphylaxis, but neither IgG1- nor IgG2b-driven reactions, led to antigen retention in the lung. Antigen accumulation in the lung was associated with local neutrophil activation, which may contribute to pulmonary damage and anaphylaxis severity.

Clinical signs of anaphylaxis are driven by the release of anaphylactogenic mediators from myeloid cells (34, 35) downstream of specific antigen recognition by antibodies and their corresponding receptors. Active mouse models of anaphylaxis entail a polyclonal antibody response, the relative composition of which is determined, among other factors, by the use of particular adjuvants (16), and genetic backgrounds that favor certain antibody classes and subclasses. The large majority of these anaphylaxis models rely on IgG- rather than IgE-mediated mechanisms, because mice lacking IgG receptors are resistant to most anaphylaxis models, whereas mice lacking IgE receptors remain susceptible (14). The contribution of any given cell population is therefore determined by their expression of activating FcγRs and/or FcεRI, the capacity of the cells to release anaphylactogenic mediators, and a cells' potential for negative inhibition of FcR signaling by expression of the inhibitory IgG receptor FcγRIIB (20, 36). In wild-type mice, pathways of active systemic anaphylaxis and passive IgG anaphylaxis rely predominantly on monocyte and/or neutrophil activation via FcγRIII, with a minor

474 contribution of FcγRIV, and subsequent PAF release (13, 15, 37). Herein, we employed
475 a model of IgG-dependent active anaphylaxis. Mice were immunized with relatively large
476 amounts of antigen (200 μg) in Freund's adjuvant or alum, leading to an antibody
477 response and anaphylaxis induction upon antigen re-exposure. Anaphylaxis
478 predominantly relied on IgG, because FcγR^{null} mice were resistant to anaphylaxis,
479 excluding a significant contribution of antigen specific IgE and FcεRI. Although
480 complement component C1q may contribute to myeloid cell activation through bridging
481 immune complexes and complement receptors (38), C1q-deficiency did not alter
482 anaphylaxis kinetics or severity in this model.

483 Immune complexes can form in both circulation and tissues after antigen
484 encounter with circulating specific IgG. IgG concentrations are significantly higher in the
485 blood than in tissues, however, which would favor IgG-antigen encounter in circulation
486 or in highly vascularized tissues. Furthermore, as antigen was injected intravenously in
487 this study, it is likely that IC formation starts in the vasculature. We indeed detected
488 higher antigen levels in highly vascularized organs such as the lung, liver and spleen, but
489 also in the thymus of sensitized compared to non-sensitized mice; together with lower
490 detectable antigen levels in the kidney and heart. Surprisingly, among all these tissues,
491 only in the lungs did antigen accumulation increase dose-dependently, and >100-fold
492 more in sensitized compared to non-sensitized mice. Overall, lung antigen retention
493 reached >10-fold higher than any other tissue. Supporting our findings in mice, antigen-
494 antibody aggregates have been reported in the lungs of immunized rabbits that were
495 intravenously challenged with antigen, principally in regions of peri-bronchial vascular
496 dilatation and edema (39, 40). Apart from the lung, antigen accumulation was highest in
497 the liver compared to other tissues after intravenous antigen injection; yet liver antigen
498 levels were equivalent between naive and immunized mice.

Lung antigen retention required the presence of antigen-specific antibodies. Physical rather than biological constraints may provoke antigen retention in the lung, due to the small size of some lung vessels (41) that could favor aggregates or clots of immune complexes. In this study we used antigens of two different sizes, OVA (45 kD) and BSA (66 kD), however no difference in antigen accumulation was observed between mice challenged with the different antigens. Moreover, physical constraints alone could not explain our findings using a passive systemic anaphylaxis model in which neither IgG1- nor IgG2b-immune complexes, but only IgG2a-immune complexes, were retained in the lungs. Coherent with this finding, C57BL/6 mice immunized with alum – eliciting IgG1 but not IgG2 antibodies to antigen – also lacked antigen retention in the lung. On the contrary, BALB/c mice immunized with alum – generating IgG1, IgG2a and IgG2b antibodies (as in C57BL/6 mice immunized with Freund's adjuvant) – demonstrated antigen retention in the lung after challenge. Lung antigen retention during these anaphylaxis models might therefore rely on the quality of the antibody response, for example the presence of IgG2a antibodies. Certainly, the degree of antigen retention seems to depend on the amount of circulating antibody, because high levels of injected IgG2a were necessary to see antigen accumulation in the lung in the passive anaphylaxis model.

Reminiscent of our findings, Cloutier *et al* reported platelet sequestration and thrombi formation in the lungs in a mouse model of systemic immune complex-induced shock (17). Although this model required FcγR expression on platelets (via transgenic expression of human FcγRIIA/CD32A), and platelet activation led to local serotonin release and anaphylactic-like clinical symptoms, in parallel to our work these authors also identified that immune complex-containing thrombi in the lung were not required for anaphylaxis to occur. However, both in our study and in the Cloutier study (17), immune

524 complex accumulation in the lung did lead to local neutrophil activation and
525 degranulation that may contribute to pulmonary damage and the severity of immune
526 complex-induced anaphylaxis.

527 IgG-containing immune complexes are the primary ligands for FcγR. High-
528 affinity IgG receptors are able to bind monomeric IgG, like FcγRI for IgG2a and FcγRIV
529 for IgG2a and IgG2b, whereas low-affinity receptors like mouse FcγRIIB and FcγRIII
530 are unable to bind monomeric IgG (42). All these receptors bind immune complexes made
531 of mouse IgG2a and IgG2b, but only FcγRIIB and FcγRIII also bind immune complexes
532 made of mouse IgG1(43). In this study, we demonstrate significantly reduced
533 FcγRIIB/FcγRIII expression on neutrophils, Ly6C^{hi} and Ly6C^{lo} monocytes and
534 eosinophils during anaphylaxis, which is considered a marker of cell involvement in
535 immune complex-mediated reactions (20, 44). Among these cells, neutrophils and Ly6C^{hi}
536 monocytes were actively recruited to the lung, and bound and engulfed antigen, and
537 together constituted 40% of antigen-capturing cells. Supporting these findings, lung
538 neutrophilia was reported previously in a model of casein-induced anaphylaxis (45).

539 Both neutrophils and monocytes have been described, by us and others, to
540 contribute to IgG-dependent active anaphylaxis using depletion and reconstitution
541 experiments (13, 15). Yet here we demonstrate that neither cell type is required for
542 antigen retention in the lung. Neutrophils were necessary for local MPO release in the
543 lung during anaphylaxis that was detectable (and maximal) already 2 minutes post-
544 challenge. Nevertheless, MPO was not required for anaphylaxis to occur. MPO, together
545 with PAF release, by neutrophils in the lung and vasculature has been proposed to
546 contribute to platelet activation and favor neutrophil-platelet aggregates and clotting,
547 thereby participating in systemic mediator release (45).

Our data indicate that, within minutes of injection, circulating antigen formed aggregates in the lung and liver vasculature of immunized mice, with a dose-dependent accumulation exclusively in the lung. Neutrophils and monocytes recruited to the lung captured antigen and became activated. Antigen aggregation in the lung vasculature was not necessary for anaphylaxis to occur, but could be responsible for local damage. Results from a passive systemic anaphylaxis model suggest that antigen retention may rely on the presence of a antibodies of a single IgG subclass, IgG2a, that enable FcγR- and C1q-independent formation of large aggregates in the lung. The mechanism behind IgG2a-mediated lung-specific antigen retention remains to be determined, and if a similar mechanism exists in humans for one or several human IgG subclasses.

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AUTHORSHIP

BT performed most experiments, with contributions from OG, EC, CMG, DF, BI and ORL; AJM, LEM and JL designed mouse targeting and generated mouse strains; BT, LR, FJ and PB analyzed and discussed results; LR, FJ and PB supervised the research. PB and BT prepared the figures. PB designed the research and secured funding. PB wrote the manuscript with the help of CMG. All authors read and reviewed the manuscript before submission.

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FIGURE LEGENDS

FIG 1. Specific antigen accumulation in the lung during active systemic anaphylaxis. A, In vivo quantification of antigen in sensitized mice before (-) or 5 min after intravenous BSA-VT680 (500µg) challenge. B, 30 min post-challenge (500µg BSA-VT680) ex vivo quantification of BSA-VT680 in explanted organs. C, 15 min post-challenge (500µg BSA-VT680) immunofluorescence on frozen sections. Scale bar = 20 µm. Arrows indicate antigen aggregates. D, 30 min post-challenge ex vivo quantification of BSA-VT680 in explanted organs. E, Post-challenge (BSA) changes in body temperature. (B,D) Data are pooled from 2 or 3 independent experiments (n = 5-14 per group). (B,D) Individual measurements, (E) mean ±SEM and p values are indicated: ns: non-significant, *P < .05, ***P < .001 or ****P < .0001.

FIG 2. Infiltrating myeloid cells capture antigen in the lung during active systemic anaphylaxis. A-C, Flow cytometry quantification of (A) cell numbers, (B) proportion of antigen-positive cells of a given cell type (Ag^+) and (C) proportion of different cell types among Ag^+ cells in the lungs 15 min post-challenge with FITC-labeled BSA in (B) OVA- or BSA-sensitized, and (C) BSA-sensitized mice. D, Two-photon microscopy images of fresh lung slices 5 min post-challenge with BSA-VT680 in CX3CR1-GFP mice injected with anti-Ly6G-BV421 mAb to label neutrophils. Arrows indicate evidence of antigen uptake by $Ly6G^+$ cells (A-C) Data are pooled from 2 or 3 independent experiments (n = 6-11 per group). Individual measurements, mean ±SEM and (A-B) p values are indicated: ns: non-significant, *P < .05, **P < .01, ***P < .001 or ****P < .0001.

FIG 3. FcγR-, C1q- and effector cell-independent antigen retention in the lung. A, FcγRIIB/III expression on lung immune cells 15 min post-challenge of wt C57BL/6J mice. B, Post-challenge ex vivo quantification of BSA-VT680 in explanted lungs. C, Temperature drop and survival during experimental anaphylaxis. D-E, Post-challenge ex vivo quantification of BSA-VT680 in explanted lungs of (D) *gfi-1* mice and (E) C57BL/6 mice non-depleted (wt) or depleted of neutrophils (-neutro), or of neutrophils and monocyte/macrophages (-neutro-Mϕ). (A-E) Data are represented as mean ± SEM and are representative of at least 2 independent experiments. (A,B,D,E) Individual measurements, mean ± SEM and p values are indicated: ns: non-significant, *P < .05, **P < .01, ***P < .001 or ****P < .0001.

FIG 4. IgG2a antibody mediates antigen retention in the lung. A-C, (A) Body temperature drop (n≥7) in C57BL/6 mice, (B) in vivo quantification and (C) ex vivo quantification post-challenge of BSA-VT680 in explanted organs of C57BL/6 and BALB/c mice, during experimental anaphylaxis following BSA immunizations in CFA/IFA or alum (n≥6). D, Serum anti-BSA IgG titers of indicated mice immunized with BSA in CFA/IFA 10 days after the last immunization. E, 15 min post-challenge immunofluorescence on frozen sections during passive systemic IgG-subclass dependent anaphylaxis in C57BL/6 mice. Arrows indicate antigen aggregates. (C-D) Data are pooled from 2 or 3 independent experiments. Individual measurements, mean ± SEM and p values are indicated: ns: non-significant, *P < .05, **P < .01 or ****P < .0001.

FIG 5. Myeloperoxidase release in the lung during active systemic anaphylaxis. A, Representative images show color-coded maps of photon flux superimposed on black and white photographs of C57BL/6 mice 20 min post-challenge and injection of luminol

786 revealing myeloperoxidase activity. B, Time course of in vivo quantification of
787 myeloperoxidase activity after challenge or not (-) in C57BL/6 mice. C, In vivo
788 quantification on the lung area of myeloperoxidase activity 30 min after BSA challenge.
789 D, 15 min post-challenge immunofluorescence on lung slices from C57BL/6 wt mice;
790 arrows indicate MPO foci. (A-D) Data are representative of ≥2 independent experiments;
791 (B) mean ± SEM and (C) individual measurements, mean ± SEM, and p values are
792 indicated: ns: non-significant, ****P < .0001.