

ORIGINAL ARTICLE Experimental Models of Allergic Disease

Monitoring neutrophils and platelets during casein-induced anaphylaxis in an experimental BALB/c mouse model

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Clinical & Experimental Allergy

Summary

Background With respect to the cellular players, mast cells and basophils have been well studied in experimental murine systemic anaphylaxis models, but the role of neutrophils and platelets is not fully understood today.

Objective We tested the hypothesis that neutrophils and platelets might participate in an antigen-induced anaphylaxis model.

Methods BALB/c mice were sensitized intraperitoneally with alum-adsorbed casein. A period of 2 weeks later, mice were challenged with 100 µg casein intravenously and immediate hypersensitivity reactions were assessed by rectal temperature measurements and monitoring the physical activity. Subsequently, leucocytes were counted in the peripheral blood as well as quantified *in situ* in typical shock organs like lung, liver and spleen, heart and kidney.

Results Mice sensitized with casein showed casein-specific IgG1, IgE, and IgG2a. When sensitized mice were specifically challenged with casein they developed immediate hypersensitivity reactions including drop of temperature and reduced activity. Furthermore, pronounced peripheral neutropenia and reduced platelet counts correlated with the severity of the hypersensitivity reactions. In the histological analyses of collected tissues we observed lung interstitial neutrophilia using Gr-1 staining. These events occurred specifically in mice sensitized and challenged with casein, in contrast to control groups.

Conclusions On the basis of our data we suggest that in addition to mast cells and basophils, neutrophils and platelets participate in the anaphylactic response in this BALB/c mouse model. Platelet and neutrophils expressing relevant immunoglobulin receptors may therefore have a synergistic effect with allergen specific IgE as well as IgG antibodies in food-induced anaphylaxis. We suggest that management of these cells could be of clinical importance to handle anaphylaxis.

Keywords anaphylaxis, casein, food allergy, IgE-independent mechanism, inflammation, neutrophils platelets

Submitted 10 June 2011; revised 03 February 2012; accepted 06 February 2012

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Cite this as: D. Krishnamurthy, P. Starkl, K. Szalai, F. Roth-Walter, S. C. Diesner, M. Mittlboeck, C. Mannhalter, E. Untersmayr and E. Jensen-Jarolim, *Clinical & Experimental Allergy*, 2012 (42) 1119–1128.

Introduction

Immunological anaphylaxis may occur in highly sensitive individuals when exposed to the specific allergens [1]. In mice, immune-mediated anaphylaxis has been explained by two different mechanisms [2]. The classical pathway involves IgE-sensitized mast cells releasing the vasoactive mediator histamine, and granular

enzymes such as mucosal mouse mast cell protease-1 and β -chymase upon allergen cross-linking of the surface bound IgE [3–6]. Similar clinical symptoms have been also described in association with the alternative pathway of mouse systemic anaphylaxis, being mediated through platelet activating factor (PAF), secretion by macrophages and/or basophils expressing low-affinity IgG receptor Fc γ RIII [7–9].

The involvement of mast cells, macrophages and basophils in systemic anaphylaxis has thus been studied intensively in both active and passive mouse models. Important for our study, recent work demonstrated that mouse and human neutrophils induce anaphylaxis in a mouse strain lacking all activating receptors for IgG and IgE. The mechanism was shown to be dependent on PAF, Fc γ RIII and newly reported Fc γ RIV [10]. This could partially explain why in some types of allergen-induced anaphylaxis, such as in severe drug adverse reactions, IgE diagnosis is extremely difficult, leading the classification of these reactions in 'idiopathic anaphylaxis' [11]. Much focus was put on platelets in earlier work, suggesting that they play a key role in anaphylaxis. In active systemic anaphylaxis for instance, it is well known that disseminated intravascular coagulation (DIC syndrome) leads to thrombocytopenia, and activation of the coagulation cascade with massive congestion; further, cellular infiltration in pulmonary interstitium and haemorrhagia in renal medullae as well as congestion in spleen and liver were observed [12]. Platelets express the high-affinity-IgE receptor Fc ϵ RI. Allergen-specific-IgE cross-linking causes cytotoxicity and active anaphylaxis in a rat model, was shown to be associated with IgE-dependent, antigen-mediated aggregation in microvasculature [13]. Platelets express various receptors that are of interest for pharmacological targeting with respect to allergic reactions, among them are alpha-adreno receptors, beta-adreno receptors and receptors for histamine, serotonin, prostacyclin and ADP [14]. *Vice versa*, the effects of anaphylaxis on platelet function have been described [15].

In accordance with these numerous arguments for the participation of neutrophils and platelets in anaphylaxis, we addressed the role of these non-classical effector cells in this study in a typical BALB/c mouse model using casein as paradigm for an important food allergen. Our hypothesis is that the compilation of knowledge about the participation of neutrophils and platelets could impact the management of anaphylaxis.

Materials and methods

Animals

For this study, 6 to 8-week-old female BALB/c mice were obtained from the Institute for Laboratory Animal Science and Genetics (Medical University of Vienna) and treated according to European Community rules of animal care with the permission of the Austrian Ministry of Science (BWMF-66009/0123-II/10b/2008).

Casein-induced systemic anaphylaxis model

A casein-specific BALB/c mouse anaphylaxis model was established by intraperitoneally injecting twice, in

2 weeks interval, with 100 μ g of alum-adsorbed Casein (Sigma, Vienna, Austria) per mouse, as depicted in the scheme (supplementary Fig. S1a). All groups contained eight animals each. A control group received phosphate-buffered saline (PBS). Anaphylaxis was induced by i.v. injecting 100 μ g casein/mouse and rectal temperature was assessed using digital thermometer 20 min after the challenge. Anaphylaxis was classified according to the standard symptom scores as previously described [16]. Temperature measurements and symptom scoring were performed in a blinded fashion. All experiments were performed twice for reproducibility. Analysis on end-time point includes allergen challenge, rectal temperature measurements, monitoring the physical activity, peripheral leucocyte counts, *in vitro* splenocyte stimulation and histological analysis of lung, spleen, liver, heart and kidney.

Measurement of allergen-specific antibodies

Casein-specific IgG1, IgG2a, and IgE serum levels were measured using ELISA. Microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) were coated with casein (5 μ g/well in 100 μ l) in 50 mM NaHCO₃ pH 9.6) overnight at 4°C, blocked with Tris-buffered saline with 0.05% Tween 20 (TBST) and 1% Bovine serum albumin (BSA) at room temperature. The plates were washed with TBST. Serum samples were diluted 1 : 10 for IgE, 1 : 100 for IgG1 and IgG2a and incubated in respective wells for 1 h at 4°C. Isotype standard antibodies (BD Pharmingen, Schwenhart, Austria) were diluted in series. Isotype-specific rat anti-mouse antibodies (BD Pharmingen) were 1 : 1000 diluted in TBST/0.1% BSA (RT, 2 h). Plates were again washed and incubated with peroxidase labelled rat anti-mouse IgG1, IgG2a and IgE (GE Healthcare, Buckinghamshire, UK) 1 : 2000 (RT, 2 h). Plates were developed using 3,3',5,5'-Tetramethylbenzidine (TMB, BD Bioscience, Vienna, Austria) substrate and optical density was measured at 450–630 nm using microplate reader.

Total blood cell counts

Peripheral leucocyte counts were performed using CELL-DYN® (Abbott Diagn, Wiesbaden, Germany) 3500 at the Institute of Biomedical Research, Medical University of Vienna. Mice blood was collected in potassium EDTA-tubes and subjected to automated peripheral leucocyte cell counts. Baseline leucocyte counts were acquired 1 week before the anaphylaxis experiments and compared with blood collected 40 min after the allergen challenge.

Spleen cell culture and cytokine measurements

Control groups or allergen-sensitized mouse groups (each n = 8) were divided and mice were either *in vivo*

allergen-challenged ($n = 4$) or saline-challenged ($n = 4$). Spleens were excised under sterile conditions from all mice. Single cell suspension was prepared by RBC lysis method and stimulated as previously described [17]. Following 72 h of incubation, cell culture supernatants were harvested and stored at -80°C until use.

The harvested splenocyte supernatants were subjected to the quantification of mouse IL-4, IL-5, IL-10 and IFN- γ using ELISA kit (Bendermed, Vienna, Austria) according to the manufacturer's protocol.

Immunohistochemistry

Spleen, liver, kidney, heart and lung were excised and fixed in buffered 4% paraformaldehyde following paraffin embedding using a standard protocol. Spleen, lung, and liver sections from control and allergic mice were deparafinised, rehydrated and Haematoxylin and Eosin stained. Moreover, they were also stained for CD41 (clone ebioMwreg30; ebioscience, Vienna, Austria) and Gr-1 (Vienna, Austria, clone RB6-8C5; BD PharmingenTM). Briefly 5 μm sections were deparafinised using xylol and rehydrated using ethanol descending gradient. Deparafinised lung sections were fixed and endogenous tissue peroxidase activity was quenched by 3% hydrogen peroxide and Gr-1 stained as previously described [18]. The sections were analysed using light microscope and staining quantification was performed using HistoQuest version 2.02.0252, tissue analysis software from TissueGnostics, Vienna, Austria.

β -hexosaminidase assay

C1.MC/C57.1 (C57) mouse mast cells were kindly provided by Prof. Stephen Galli, Stanford University School of Medicine [19]. C57 cells were washed twice with PBS and seeded in 96well plates, 2×10^4 cells/well. Mast cells were sensitized overnight with 1 : 10 diluted, pooled mouse sera. Excess IgE was removed by washing twice with Tyrode's buffer (100 mM HEPES, 130 mM NaCl, 1.8 mM CaCl_2 , 5 mM KCl, 2 mM MgCl_2 , 5.5 mM Glucose, 1 g/L BSA; pH 7.4). Next, cells were stimulated with 10 $\mu\text{g}/\text{mL}$ of casein. A combination of 25 ng/mL PMA and 5 μM compound A23187 (both from Sigma) diluted in Tyrode's buffer and incubated for 1 h at 37°C . Supernatants of cells and lysates of the corresponding centrifuged cells (by 0.1% Triton X-100 in Tyrode's buffer) were harvested and incubated with 4 mM p-Nitrophenyl-N-acetyl- β -D-glucosaminide (Sigma) in Na-phosphate/citrate buffer, pH 4.5 for 1 h at 37°C . The reaction was stopped by addition of 0.2 mM Glycine pH 10.7. β -hexosaminidase content was then analysed by absorbance measurement at 405 nm using a SpectraMax microplate reader (Molecu-

lar Devices). The results were calculated as percentage of total release [$\% \text{ degranulation} = \text{O.D.}_{\text{supernatant}} / (\text{O.D.}_{\text{supernatant}} + \text{O.D.}_{\text{lysate}})$].

Quantification of fibrinogen levels

Sera samples were analysed for fibrinogen levels according to the manufacturer protocol using the kit AssayMax Mouse fibrinogen (FBG) ELISA kit from LOXO GmbH, Dossenheim, Germany. The sensitivity of the assay is 0.625 $\mu\text{g}/\text{mL}$.

Data analysis

Data are graphically shown by scatterplots of single values within groups. This gives full information about the distribution of data (mean/median, spread and potential outliers) for small groups and enables easy visual comparison. Data were expressed as mean $\pm$ sem in case of normally distributed data. Group differences for normally distributed data were modelled by analysis of variance taking repetitions into account. Heterogeneity was addressed by a degrees-of-freedom adjustment by Kenward and Roger. For mIL-4 CAS2 was compared to the two CAS1 groups and an adjustment for multiple comparisons was made by Dunnett's method. Statistical comparisons between groups in mouse experiments with non-normally distributed data were performed by the non-parametric Wilcoxon's rank-sum test for experiments without repetitions and by vanElteren's test for experiments with a repetition of the experiment, that is a stratified version of the Wilcoxon rank-sum test. Correlations between continuous data were assessed by Spearman's rank order correlation coefficient. All statistical calculations and graphs were made by the software GraphPad Prism 4 (La Jolla, California) and SAS (©SAS Institute Inc., Version 9.2, Cary, NC, USA). All P -values given are two-sided and P -values ≤ 0.05 were considered statistically significant.

Results

Assessment of the specific and functional sensitization

Mice systemically immunized with casein according to the protocol given in supplementary Fig. S1b-d showed higher levels of allergen specific-IgG1 (mean $\pm$ sem: $41.5 \pm 1.1 \mu\text{g}/\text{mL}$), IgE (mean $\pm$ sem: $7.3 \pm 0.32 \mu\text{g}/\text{mL}$) and IgG2a (mean $\pm$ sem: $0.024 \pm 0.001 \mu\text{g}/\text{mL}$) in ELISA than control groups, which did not develop casein specific antibodies. IgG1 was the predominant isotype. To assess the biological relevance of the antibody titres in the sensitized groups, we performed a degranulation assay using C57 mouse mast cells and monitored the β -hexosaminidase release *in vitro*. Casein

stimulation induced enhanced β -hexosaminidase release when C57 cells were sensitized with pooled sera from casein-sensitized animals as compared to control group sera (supplementary Fig. S1e).

Furthermore, we assessed the effector potential of the induced casein-specific antibodies. All groups were challenged i.v. using 100 μ g casein and monitored for anaphylactic symptoms (physical activity and rectal temperature) for 20 min. All casein-immunized mice developed hypothermia and symptoms such as scratching, mouth swelling and laboured respiration upon allergen challenge (Fig. 1).

Anaphylactic mice display acute peripheral neutropenia and neutrophil influx into lungs

Peripheral leucocyte cells were counted 1 week before and 40 min after the allergen challenge and compared to baseline peripheral counts in naïve mice. In casein-sensitized mice (CAS), neutrophils in the circulation were significantly decreased in casein-challenged (CAS2) compared to saline (CAS1) challenged group. In contrast, non-allergic control animals (PBS2) showed increased peripheral neutrophil counts 40 min after i.v. allergen challenge compared to PBS1 (PBS-sensitized and saline-challenged) (Fig. 2a).

Migration of animals neutrophils to peripheral organs would explain the neutropenia observed in specifically challenged mice. We investigated different organs by analysis and could observe an accumulation of Gr-1⁺ neutrophils in lung interstitium of the casein-challenged immunized mice (Fig. 3a) accompanied by pulmonary congestion. Gr-1 staining on lung sections was quantified by HistoQuest analysis. In anaphylactic mice (CAS2), a significant increase in Gr-1 staining com-

pared to the controls was observed (Fig. 3b) (P -value 0.0079).

Furthermore, we observed significant thrombocytopenia in casein-challenged immunized mice (CAS2) compared to non-allergic control groups (CAS1; Fig. 2b) along with reduced body temperature. Basophil numbers were not statistically significant between CAS1 and CAS2 (Fig. 2d). Levels of casein-specific IgG1, IgE, and IgG2a did not correlate with neutrophil and platelet numbers analysed using Spearman correlation test.

In addition to the lower peripheral neutrophil and platelet numbers we also observed a statistically significant decrease in peripheral red blood cells in anaphylactic mice (CAS2) compared with PBS-challenged animals (CAS1); (Fig. 2c). Thrombi in lung, liver, and heart could be visualized by H&E staining only in the severely affected mice (supplementary Fig. S2), but not in control animals.

Splenocytes of allergen-sensitized, allergen-challenged anaphylactic mice release large amounts of cytokines

To assess possible cytokine participation during the anaphylactic reaction we allergen-challenged 4/8 of the casein allergic mice with allergen and kept 4/8 of each group with saline and analysed the response of isolated spleen cells. We characterized the T cell cytokine response from cultured splenocytes after 72 h of *in vitro* stimulation with casein, Concanavalin A or culture medium. Spleen cells of casein-challenged immunized mice released increased levels of IL-4, IL-5, IFN- γ and IL-10 as compared to saline-challenged casein-immunized mice, which resembled or surmounted the levels detected after stimulation with non-specific mitogen ConA (Fig. 4). There were relatively lower amounts of

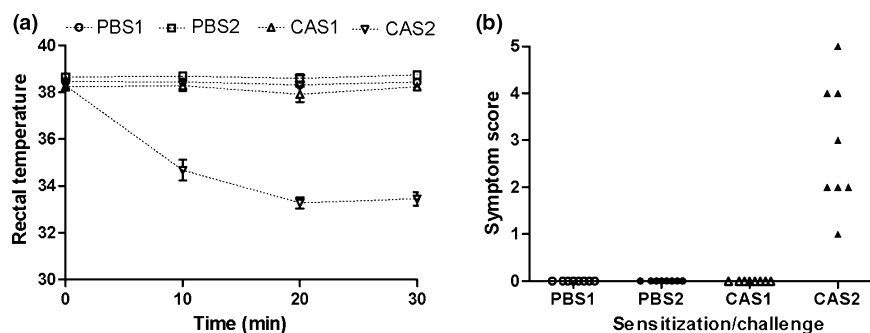


Fig. 1. Assessing the allergic symptoms and body temperature. Mice were sensitized with casein (100 μ g per dose) or phosphate-buffered saline (PBS) 2 times at biweekly intervals using Alum as Th2 adjuvant to induce specific hypersensitivity. On day 42 mice were challenged by intravenous injection of specific allergen or saline and typical anaphylaxis readouts were taken. (a) Rectal thermometry. i.v. casein challenge induces hypothermia in casein-sensitized mice, whereas the saline challenged mice remain unchanged. Casein-immunized and casein-challenged (CAS2) mice elicited anaphylactic symptoms assessed by blinded observer and by measuring rectal temperature. (b) Anaphylactic symptom score. Groups were assessed in a blinded fashion for anaphylactic symptoms by observing standard hypersensitivity scores. Casein sensitized and challenged (CAS2) mice showed allergic symptoms indicating the effector potential of allergen specific antibody titres. PBS1: PBS-sensitized and saline-challenged; PBS2: PBS-sensitized and casein-challenged; CAS1: Casein-sensitized and saline-challenged.

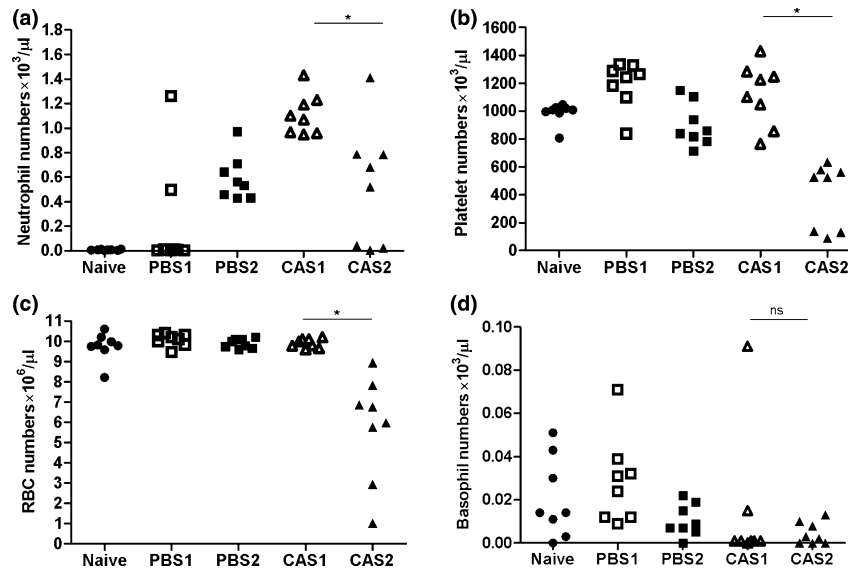


Fig. 2. Neutrophils, platelets and RBC counts significantly decreases from circulation. Mice sensitized with casein or phosphate-buffered saline (PBS) using Alum as Th2 adjuvant, were challenged by intravenous injection of specific allergen or saline. Blood was collected 40 min after the challenge from the tail vein in potassium EDTA-tubes and subjected to automated peripheral leucocyte cell counts. Leucocyte counts were compared to baseline levels acquired 1 week before the anaphylaxis experiments. (a) Neutrophil numbers in peripheral blood decrease during anaphylaxis: Casein-sensitized & casein i.v. (CAS2) challenged mice show a statistically significant reduction ($P = 0.0080$). (b) Allergen challenge reduces platelet numbers in circulation of immunized mice: Casein sensitized (CAS1) and casein i.v. (CAS2) challenged mice show a statistically significant reduction ($P = 0.0011$). (c) RBC numbers decreased from anaphylactic mice: Casein sensitized (CAS1) and casein i.v. challenged (CAS2) mice show a statistically significant reduction ($P = 0.0011$). (d) No differences in basophil numbers are seen between the groups: Casein sensitized (CAS1) and casein i.v. challenged (CAS2) mice do not show a statistically significant difference ($P = 1.0000$). VanElteren test was used for Casein group comparisons. PBS1: PBS-sensitized and saline-challenged; PBS2: PBS-sensitized and casein-challenged.

IL-4 and IFN- γ detectable in splenocyte supernatants from saline-challenged casein-immunized mice. Splenocytes from control mice being *in vivo* challenged with casein showed some, but significantly less production of cytokines *in vitro*.

Discussion

In addition to the well defined IgE associated mechanisms, the consideration of potential roles for non-classical allergy effector cells and IgE-independent mechanisms in systemic anaphylaxis may be important. IgG-dependent anaphylaxis usually requires higher doses of antigen as compared with IgE mediated reactions [5], but a recent study using a passive model of anaphylaxis showed that IgG1-dependent anaphylaxis can be induced by limited amounts of complexed antigen and antibody [20]. In our study, we show that besides specific IgE, predominantly antigen-specific IgG1 was induced in BALB/c mice upon i.p. allergen sensitization. We further observed that peripheral platelet and neutrophil numbers become significantly reduced in the systemic reaction. This might be the key observation associated with anaphylaxis in our model as the reports on the contributions of neutrophils and platelets to anaphylaxis steadily increase. Circulating

IgG1 may form immune complexes with the antigen upon challenge and act on Fc γ RIII expressed by various circulating leucocytes including neutrophils or tissue resident mast cells, thereby rendering the release of inflammatory mediators [21]. It was recently demonstrated in mice lacking all activating receptors of IgG and IgE that anaphylaxis could still be induced in a neutrophil-derived PAF and Fc γ RIV dependent pathway [10]. Notably, Fc γ RIV binds not only IgG, but also acts as low-affinity IgE receptor in mice [22], whereas on human neutrophils the expression of the high affinity receptor Fc ϵ RI itself has been demonstrated [23].

The decrease in neutrophils and platelets from blood was accompanied by an accumulation of neutrophils in pulmonary interstitium. These observations are new and to the best of our knowledge no evidence for correlation of neutrophils cooperating with thrombocytes in anaphylaxis in human subjects has been reported so far. It was surprising to note that i.v. casein-injection into non-immunized mice was sufficient enough to induce twofold increase in neutrophil numbers. This observation is supported by reports on casein as chemotactic factor for neutrophils [24, 25]. The direct participation of platelets in systemic anaphylaxis has been shown in human subjects as well as in rabbit and rat models [4, 13, 26, 27]. Despite reports on support of

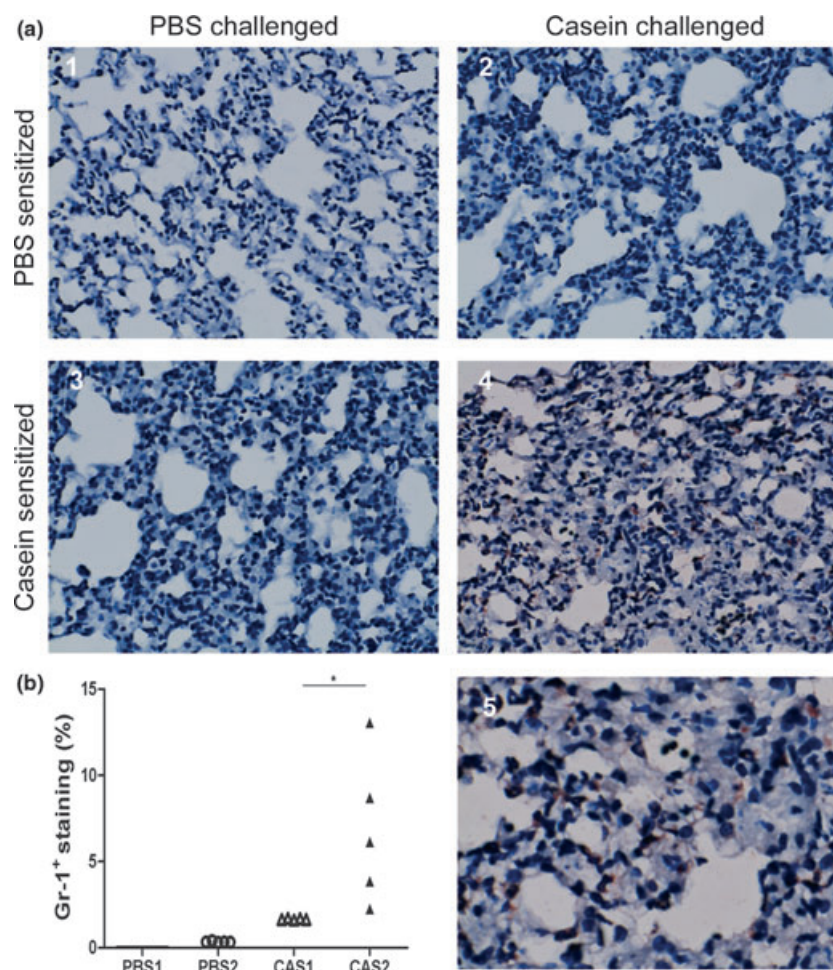


Fig. 3. (a) Lung Neutrophilia in anaphylactic mice. Lungs were collected from mice being challenged with specific allergen or control. After staining, numbers of Gr-1⁺ neutrophils were evaluated using the TissueFAXs technology. Neutropenia was associated with lung neutrophilia only in anaphylactic mice associated with congested lung capillaries, whereas saline and casein-challenged saline group remain unchanged and showed no characteristic anaphylactic symptoms. (1) phosphate-buffered saline (PBS)-sensitized and saline-challenged (PBS1), (2) PBS-sensitized and casein-challenged (PBS2), (3) Casein-sensitized and saline-challenged (CAS1), (4) Casein-sensitized and casein-challenged mice (CAS2), and (5) Higher magnification of the Gr-1 staining of the lung section 4 (b) Tissue FAXs- Histogram analysis. Lung neutrophilia was quantified using TissueFAXs, by Histoquest software. Lung tissue of anaphylactic mice (CAS2) showed remarkable infiltration of Gr-1⁺ neutrophils compared to saline challenged casein-immunized mice (CAS1) ($P = 0.0079$; Wilcoxon's rank sum test).

induction and maintenance of inflammatory immune responses by platelets, a direct role of platelets in mouse anaphylaxis has not been shown so far [28–32]. In contrast to the previous mouse model of anaphylaxis, we observed an accumulation of platelets in lungs and liver upon specific allergen challenge. Interestingly, platelet degranulation was reported to occur even before that of mast cells or basophils and might therefore contribute to the very early-phase of anaphylaxis [33]. It is known that fibrinogen levels are significantly reduced during DIC in an active systemic anaphylaxis mouse model [12]. Taking this parameter into consideration, we observed reduction of serum fibrinogen levels at time point 60 min in an independent, but similar mouse model of anaphylaxis using peanut allergen Ara

h 2 [data not shown], pointing indeed towards DIC occurrence. The late measurement time point of 40 min after allergen restricts our data interpretation as we cannot exclude that drop of platelets could be a secondary effect caused by the inflammatory and vasoactive mediators.

Both IL-4 and IL-13 enhance vascular permeability and anaphylaxis in mice [34]. In accordance, we observed dramatically higher production of IL-4, IFN- γ , IL-5, and IL-10 by *in vitro* stimulated splenocytes of casein-sensitized mice. This cytokine pattern is neither biased towards Th1 nor Th2 and resembles a cytokine storm which may cause endothelial injury and is associated with multiple organ dysfunction syndrome, especially in the early-phase of systemic inflammation [35].

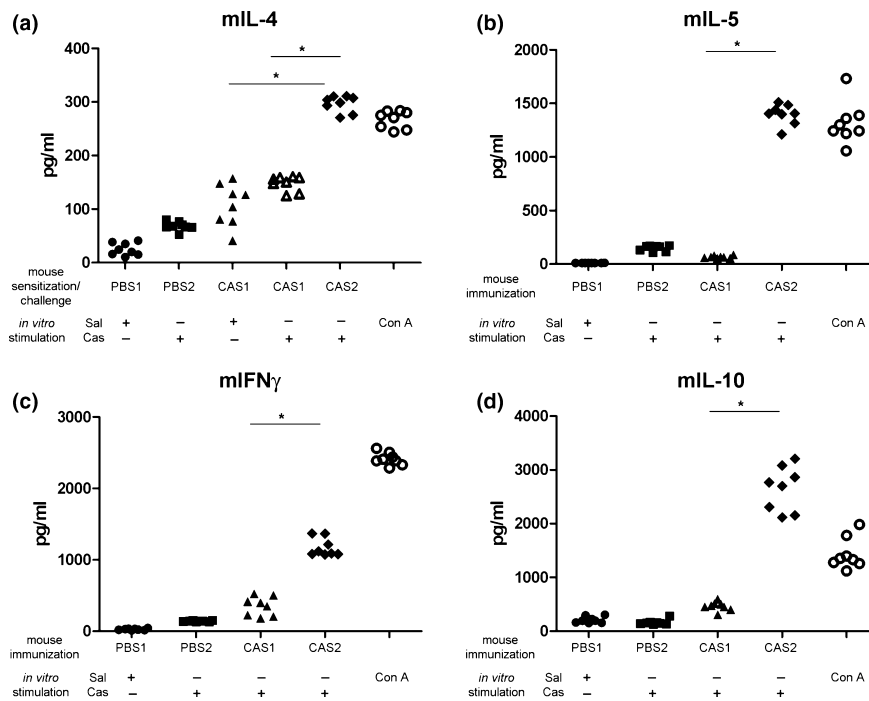


Fig. 4. Allergen stimulated splenocytes shows dominant Th2 phenotype with associated Th1 cytokine production. The *in vitro* cytokine production of splenocytes from mice being hypersensitized and challenged by specific allergen or controls was evaluated. *In vitro* with casein (Cas) stimulated splenocytes, derived from casein-sensitized/casein challenged (CAS2) mice, showed significantly higher levels of (a) IL-4, (b) IL-5, (d) IL-10, and (c) IFN- γ as compared to casein stimulated splenocytes derived from casein-sensitized/saline-challenged (CAS1) mice ($P < 0.0001$ for all 4 cytokines). Although being remarkably increased as compared to splenocytes derived from phosphate-buffered saline (PBS) sensitized/saline challenged (PBS1) mice, the release of IL-4 by saline stimulated splenocytes of CAS1 mice was still significantly lower than by casein stimulated splenocytes derived from CAS2 mice ($P < 0.0001$, Dunnett adjustment for multiple comparisons). X-axis labels indicate the sensitization and *in vivo* challenge regimen, the substances used for *in vitro* stimulation of splenocytes are shown below the axis. The statistical calculations are based on an analysis of variance. PBS2: PBS-sensitized and casein-challenged. Con A: Concanavalin A.

Interestingly, we observed substantially reduced RBC counts upon anaphylaxis in mice. RBCs have been recently demonstrated to cause lung leucocyte infiltration by producing hydrogen peroxide, thereby partially contributing to hypoxia-induced inflammation and impairment of the organ function [36]. We show in our anaphylactic mouse model that Gr-1⁺ neutrophils infiltrate in lung associated with thrombi, possibly contributing to RBC-induced hypoxemia associated symptoms. Furthermore, RBC adhesion to activated platelets and activated neutrophils has been previously reported to cause thrombosis in settings of lowered blood flow [37], especially in hypoxia [38] and increased haematocrit [34]. This effect was specifically dependent on interaction via glycoprotein VI expressed by activated thrombocytes, and it was further pronounced by fibrin.

Conclusion

On the basis of the collected histological evidence combined with the observed lowered blood cell counts we assume that the thrombi observed in the anaphylactic state consist of aggregated leucocytes, platelets and

erythrocytes. Figure 5 illustrates the concept of the interplay between platelets, neutrophils, RBCs and soluble factors in allergen-induced anaphylaxis based on the literature and our own data. However, because of the restriction of our experimental study, we are not able to draw a conclusion whether these cells play an active role or possibly represent a secondary effect.

We demonstrate in this study that besides clotting phenomena, neutrophils and platelets redistribute during anaphylaxis, adhere to the vasculature, associated with significant reduction of these cells from the circulation. On the basis of the presented data we propose that monitoring of neutrophil and platelet activation during the systemic inflammatory episodes as well as indirect measurement of their accumulation in different organs might be worth monitoring to improve management of anaphylaxis.

Acknowledgements

The authors thank Thomas Perkman from the Department of Medical and Chemical Laboratory Diagnostics, Ingrid Raab from Department of Clinical Pathology and

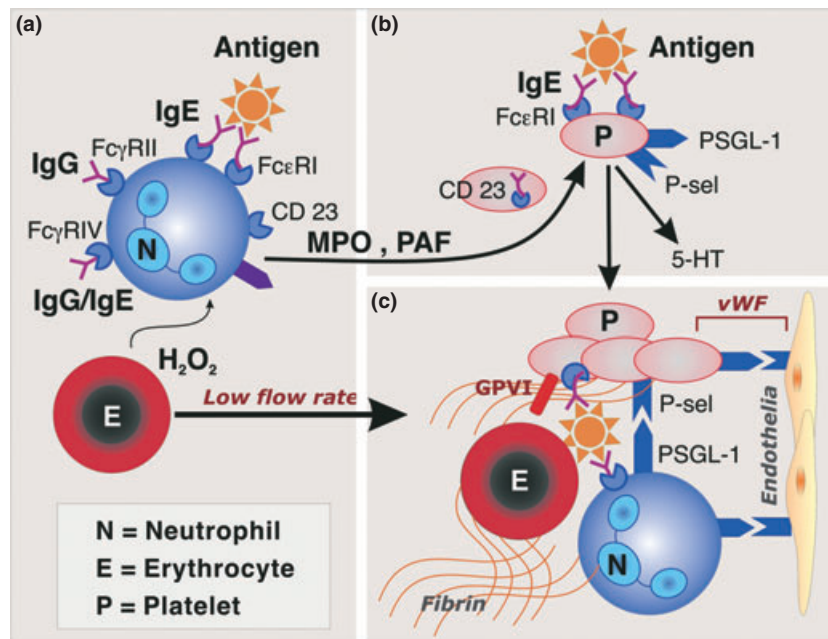


Fig. 5. Proposed model of molecular and cellular mechanisms during acute inflammation. (a) Human and/or mouse neutrophils express a panel of immune receptors including FcγRIII (binds complexed IgG1), FcγRIV (binds IgG2 and IgG-complexed IgE), FcεRI (IgE), CD23 (IgE), and P-selectin glycoprotein ligand-1 (PSGL-1). Upon encounter of the specific antigen, IgG and IgE antibodies bound to the respective receptors trigger neutrophils to release myeloperoxidase (MPO) and platelet activating factor (PAF). In parallel, anaphylaxis-associated hypoxia leads to H₂O₂ release by erythrocytes which acts as chemoattractant for neutrophils [38]. (b) Platelets constitutively express FcεRI and CD23 (associated with IgE endocytosis and storage). Neutrophil-derived MPO and PAF pre-activate platelets. Allergen cross-linking of FcεRI bound IgE on platelets this leads to release of mediators, such as 5-hydroxytryptamine (5-HT) antigen complexes may activate and aggregate platelets as well as induce the subsequent release of 5-HT [33]. (c) The anaphylaxis-associated hypoxic condition leads to P-selectin (P-sel) exposure by activated endothelial cells, leading to platelet aggregation via PSGL-1 which is further stabilized by von Willebrand factor (vWF) on activated endothelial cells [39]. [The participation of vWF and CD41 platelets in thrombi observed in shock-organs lung and liver in our anaphylactic mice is illustrated in supplementary Fig. S3.] Further, also highly activated, aggregated platelets express P-selectin, *vice versa* enabling interaction with neutrophils via PSGL-1. In addition, hypoxia associated low shear rate [38], fibrin formation [37] and increased haematocrit [34] enhance specific erythrocyte-platelet-aggregation via glycoprotein VI (GPVI). These mechanisms ultimately lead to formation of large vascular thrombi consisting of platelets, neutrophils and erythrocytes.

Marianne Leisser from Center for Brain Research for their technical guidance and Maria Wies-Pröbstl from Institute of Biomedical Research for the blood cell counts, Radu Rogojanu from TissueGnostics, Sarah Schwarz from the Department of Clinical Urology for tissue analysis using HistoQuest software and Prof. Sylvia Knapp for sharing the Gr-1 staining protocol. We further would like to thank Prof. Stephen J. Galli for providing C57 mast cells, Erika Bajna for assisting in imaging the histology slides and members of the

Division 1 at the Department of Pathophysiology and Allergy Research for helpful discussions. This work was supported by the CCHD PhD programme of the Austrian Science Fund FWF, project #APW01205FW and #SFB F 4606-B19. PS was supported by SFB F1808-B013. KS was supported by Biomedical International R+D, Vienna, Austria.

Conflict of interest: The authors declare no conflict of interests.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

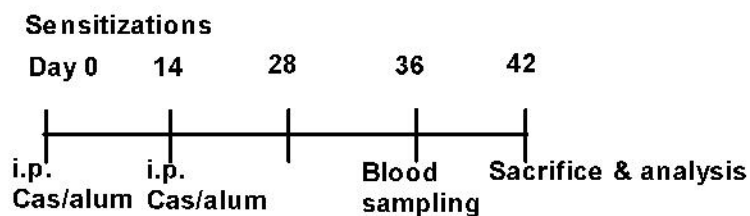
Figure S1. Characterization of the anaphylaxis mouse model.

Figure S2. Mice sensitized with casein using Alum.

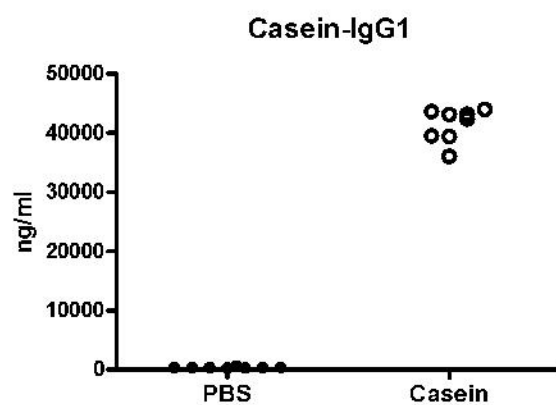
Figure S3. CD41 and von Willebrand factor (vWF) staining in shock organs of anaphylactic mice.

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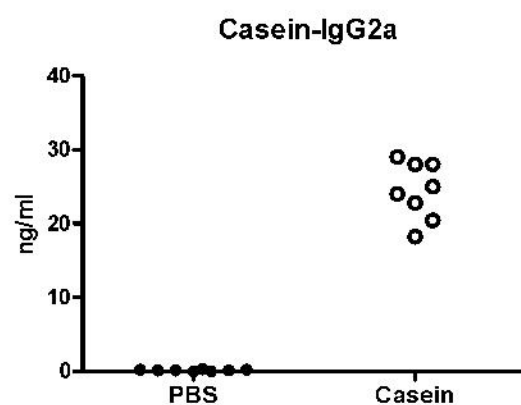
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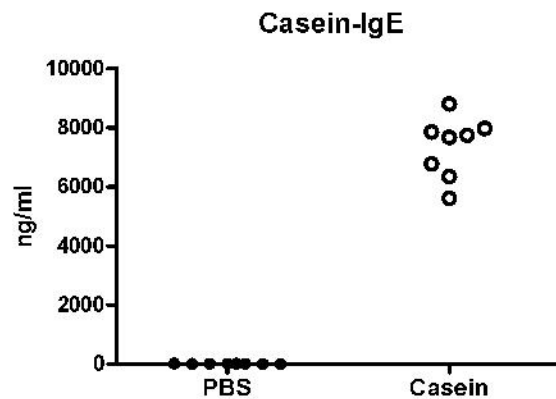
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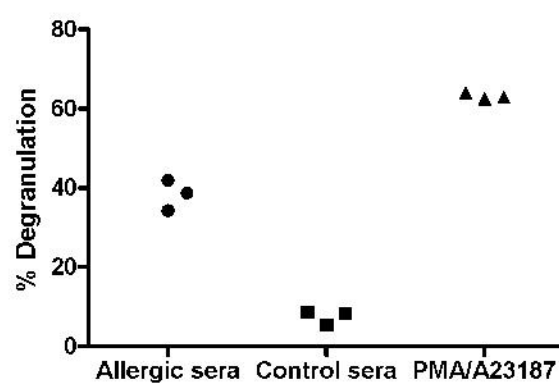
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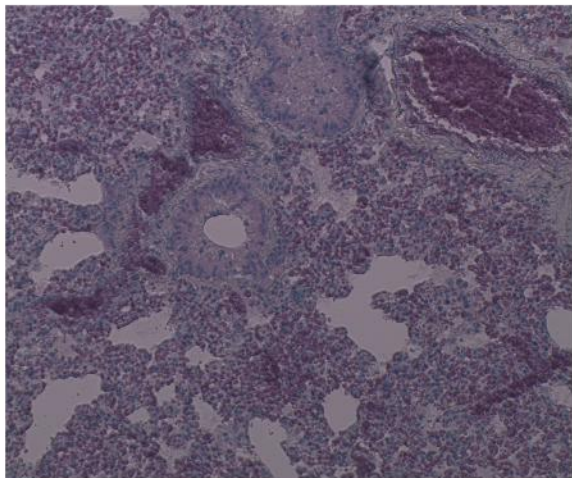
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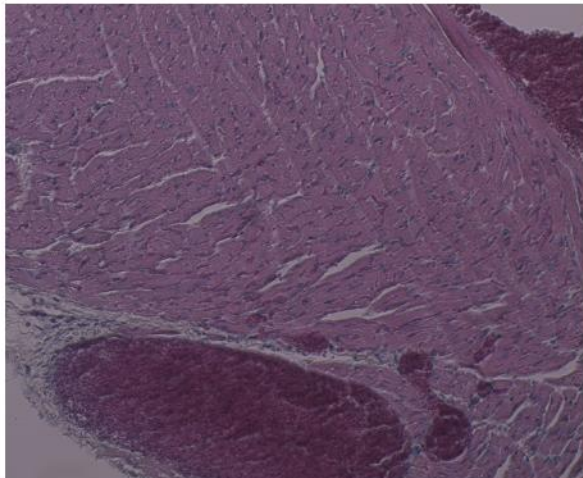
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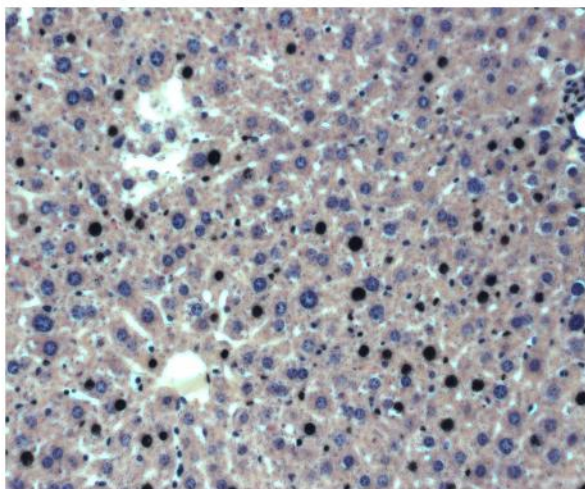
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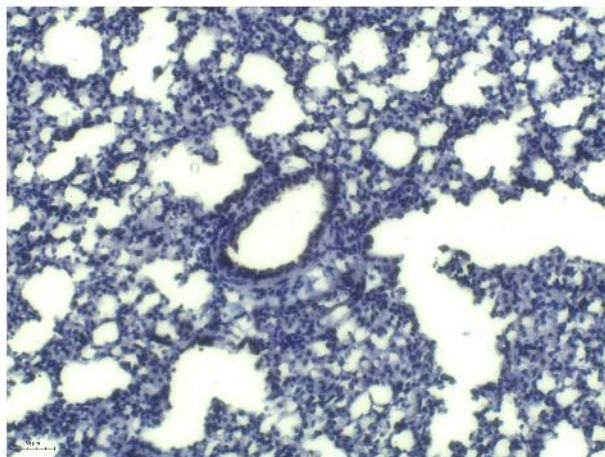
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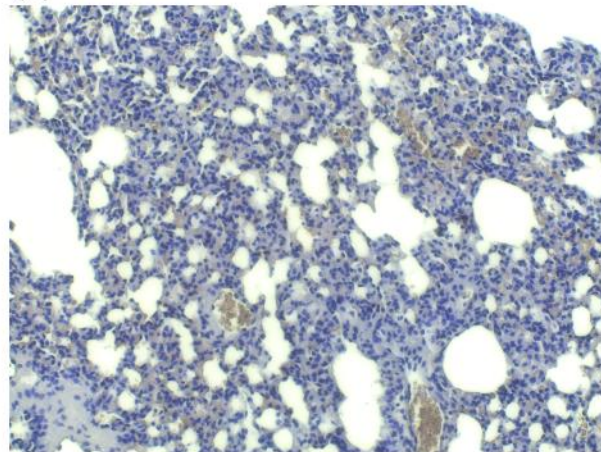
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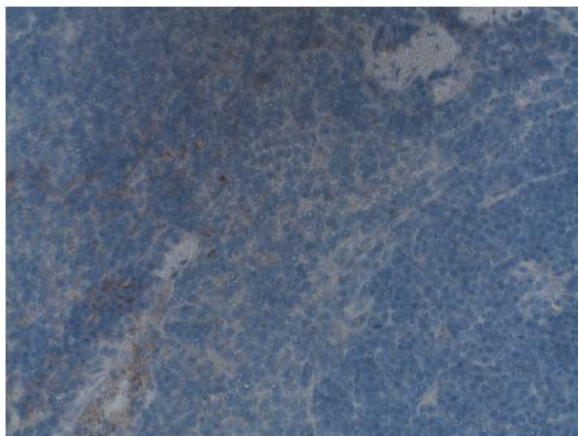
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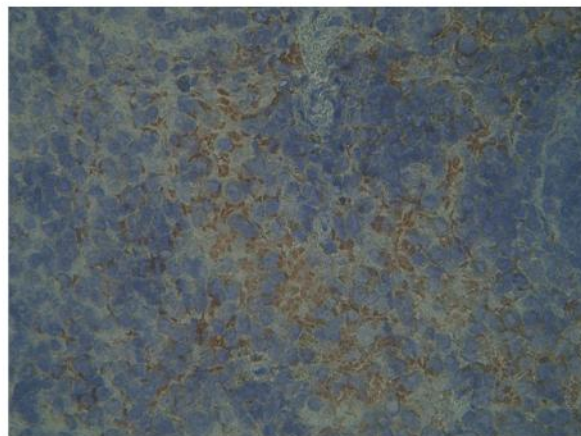
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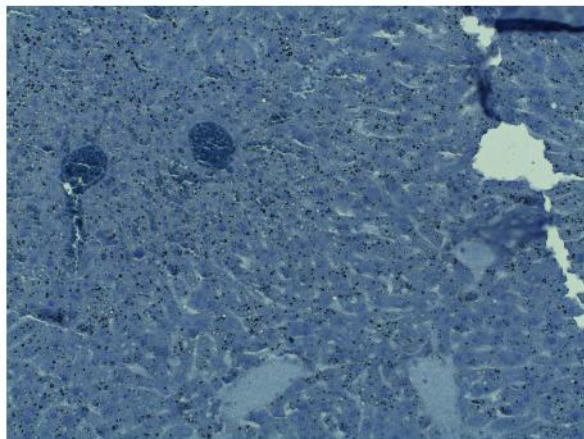
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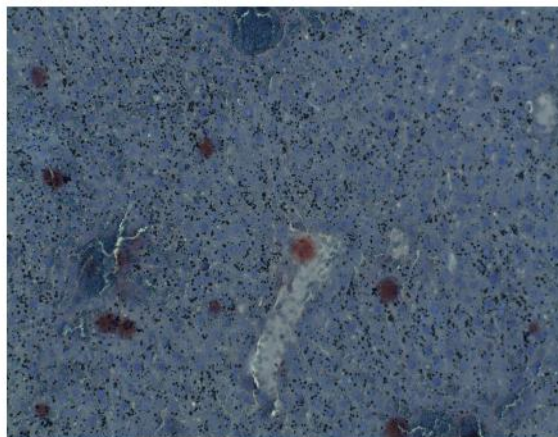
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(e)



(f)



Supplementary figure legends

Fig. S1 Characterization of the anaphylaxis mouse model

(a) shows the experimental scheme of the active systemic anaphylaxis model which is explained in detail in the materials and methods section. Sera were collected as indicated and antibody levels were analyzed by ELISA. Casein-sensitized mice showed specific (b) IgG1, IgG2a (c) and IgE titres (d), in comparison to controls. (e) *β-hexosaminidase assay*. Sensitized groups showed a significant mast degranulation compared to control group. C57 cells were assessed for β -hexosaminidase release with the casein-sensitized and non-sensitized sera samples. X axis indicates stimuli and y axis the percentage of degranulation.

Fig. S2

Mice sensitized with casein using Alum as Th2 adjuvant were challenged by intravenous injection of specific allergen as compared to control. Lung, heart and liver were collected and tissue sections were stained by Hematoxylin and Eosin. Thrombi were found in (a) lung, (b) heart and (c) liver of severe anaphylactic mice.

Fig. S3 CD41 and von Willebrand Factor (vWF) staining in shock organs of anaphylactic mice.

Casein sensitized mice were challenged with saline [left panels: (a), (c), (e)] or the specific allergen, casein [right panels: (b), (d), (f)]. CD41 distribution on lung [(a) and (b)] and spleen sections [(c) and (d)] and the presence of vWF [(e) and (f)] on liver sections was analyzed by immunohistochemistry.