

ORIGINAL ARTICLE

ANAPHYLAXIS

Characteristics of anaphylaxis-inducing IgG immune complexes triggering murine passive systemic anaphylaxis

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Abstract

Background: With the broad and increasing application of therapeutic monoclonal antibodies (mAbs) in clinical settings, IgG-induced allergic reactions, including passive systemic anaphylaxis (PSA), have attracted significant attention. However, it is not clear which types of IgG mAb–antigen complexes or IgG aggregates formed by antigen binding can trigger PSA, as not all immune complexes (ICs) are capable of triggering PSA. Here, we characterise mAb–antigen complexes capable of inducing murine PSA to evaluate and predict which ICs are able to induce PSA.

Methods: Thirty-six combinatory reactions with eight antigens and 27 corresponding mAbs were used to trigger PSA, which was defined by rectal temperature. Sandwich ELISA, passive cutaneous anaphylaxis (PCA) induction and flow cytometry analysis of CD16/32 (FcγRIII/II) expression were used to characterise the ICs. The dynamic concentrations of antigen in the peripheral blood were measured by ELISA.

Results: Only 14 of the 36 ICs could trigger PSA and thus be termed anaphylaxis-inducing immune complexes (Ai-ICs). The Ai-ICs could be characterised by constructing sandwich ELISA, inducing PCA and down-regulating CD16/32 (FcγRIII/II) expression on blood neutrophils *in vitro* and *in vivo*. Additionally, the occurrence and severity of PSA was found to be associated with the instantaneous concentration of antigen in the peripheral blood in the presence of antibody.

Conclusions: Only Ai-ICs, not all ICs, could trigger IgG-mediated PSA, which could be characterised by the above simple methods. The occurrence and severity of PSA was associated with the instantaneous concentration of antigen in the peripheral blood in the presence of antibody.

Passive systemic anaphylaxis (PSA) is a life-threatening allergic reaction that can take place in hosts following antibody infusion (1–4). Recently, IgG-induced PSA, a severe adverse effect that can take place following therapeutic treatment with monoclonal antibodies (mAbs) and immunoglobulins, has attracted significant attention (2, 3, 5, 6). In clinical settings, antibody infusion-induced PSA is estimated to range in frequency from approximately 0.09 to 77% depending on the type of antibody used (7) and can occur rapidly, as quickly as several minutes after the first infusion of therapeutic mAbs (8–10). To date, 30 therapeutic

mAbs have been approved by the FDA, and more than 300 therapeutic antibodies have been studied in clinical trials (11).

In mouse models, IgE-induced anaphylaxis occurs through the classical pathway, while IgG-induced anaphylaxis uses an alternative pathway that can be mediated by basophils, macrophages and neutrophils (12–14). All elements in the murine alternative pathway are thought to play a pivotal role in human IgG-induced anaphylaxis, suggesting that this alternative pathway may exist in both mice and human (15). Notably, not all ICs or IgG aggregates that are formed by

binding with antigen can trigger PSA in mouse models, which is similar to what is observed in humans. Therefore, it is important to evaluate and predict what types of ICs are capable of triggering PSA.

In this study, we defined the characteristics of mAb–antigen complexes inducing PSA by observing 36 combinatory mAb–antigen reactions. We found that constructing sandwich ELISA, inducing PCA and down-regulating CD16/32 (FcγRIII/II) expression on blood neutrophils both *in vitro* and *in vivo* could be used to effectively identify ICs that are capable of inducing PSA. We also observed that the occurrence and severity of PSA was associated with the instantaneous concentration of antigen in the peripheral blood.

Methods

Mice

Female BALB/c mice (6–10-week-old) were purchased from the Laboratory Animal Centre of Sun Yat-Sen University (Guangzhou, Guangdong, China). All animal experiments were approved by the Ethics Committee for Experimental Animals at Southern Medical University and were performed according to the national guidelines for animal welfare.

Monoclonal antibodies for PSA

mAb No. 7 and No. 9 against ovomucoid (OVM) were provided by Li et al. (16); mAbs against human serum albumin (HSA) and recombinant mannoprotein 1 of penicillium (MP1P) were provided by Ma et al. (17) and Wang et al. (18); mAbs against recombinant human haemoglobin ζ (rHb zeta), human haemoglobin ζ (Hb zeta), human haemoglobin A2 (HbA2) and advanced oxidation protein products (AOPP) were prepared in our laboratory (19–22). The specificity and isotype of mAbs are listed in Table S1.

PSA model

Mice were sensitised with an intravenous (i.v.) injection of mAb in 200 μl PBS and challenged i.v. with antigen in 200 μl PBS 2 h later. Rectal temperature was measured using a digital thermometer (Model BAT-12; Physitemp Instruments, Inc., Clifton, NJ, USA) and recorded from 0 to 90 min postchallenge. Additionally, preformed ICs could be directly used for challenge without priming (Fig. 1C). To measure PSA-induced plasma exudation, the protocol was modified by challenging mice with antigen in 200 μl PBS containing 0.5% Evan's blue dye.

PCA model

PCA in the paw was induced as previously described with slight modifications (23). Mice were sensitised with an intradermal (i.d.) injection of mAb in 20 μl PBS in the hind paws and i.v. challenged with antigen in 200 μl PBS containing 0.5% Evan's blue 2 h later. Both front paws, used as

controls, and hind paws were photographed at 5 min post-challenge. The preformed ICs were also directly used for challenge (Fig. 2C and Table 2).

Determining mouse CD16/32 and human CD16 & CD32 expression on blood neutrophils

Both mouse CD16/32 and human CD16 & CD32 expression were analysed by flow cytometry as described in the supplement.

Detecting the instantaneous concentration of antigen in blood

The indicated doses of biotinylated OVM, biotinylated HSA and rHb zeta were injected i.v., and blood samples were taken at different time points postinjection. The serum biotinylated protein and rHb zeta were determined by either indirect or sandwich ELISA.

Statistical analysis

All statistical analyses were performed with SPSS 13.0 software (IBM Corporation, Armonk, NY, USA).

Further method details

The methods detailed in the supplement include the generation and identification of mAbs to OVM and ovalbumin (OVA), epitope mapping by the anti-OVM mAbs (Tables S2 and S3), the doses of mAb and antigen used for inducing PSA, PCA and blood neutrophil CD16/32 down-regulation (Table S4), and detailed protocols for ELISA and flow cytometry analysis.

Results

Murine PSA can only be induced with a fraction of anti-OVM mAbs or mAb cocktails reacted with OVM

OVM and anti-OVM mAbs were used to establish a PSA mouse model (Fig. 1A). After priming with 100 μg mAbs, as little as 5 μg OVM could induce PSA. Increasing OVM doses could enhance the severity of PSA, but no further severity was observed when the antigen doses were increased to between 100 and 1000 μg OVM per mouse (Fig. 1B). Thus, we chose 10 μg OVM as the working dose, which was also verified by measuring plasma exudation of dye in the mouse ears (Fig. 1D). PSA of similar severity to that observed using 10 μg OVM could also be induced using 100 μg OVA containing trace OVM (Fig. S2). In addition, PSA could also be induced using ICs preformed *in vitro* by mixing OVM with mAb 2C2 (Fig. 1C). Moreover, we found that only 3 mAbs, 2C2, 5G12 and 6H4, could initiate PSA following OVM challenge, whereas the other 4 anti-OVM mAbs could not (Fig. 1E). Interestingly, using cocktails of mAbs, No. 7 plus No. 9, No. 7 plus 1A6 or No. 7 plus 5B8 could initiate PSA, but the other combinations could not (Fig. 1F).

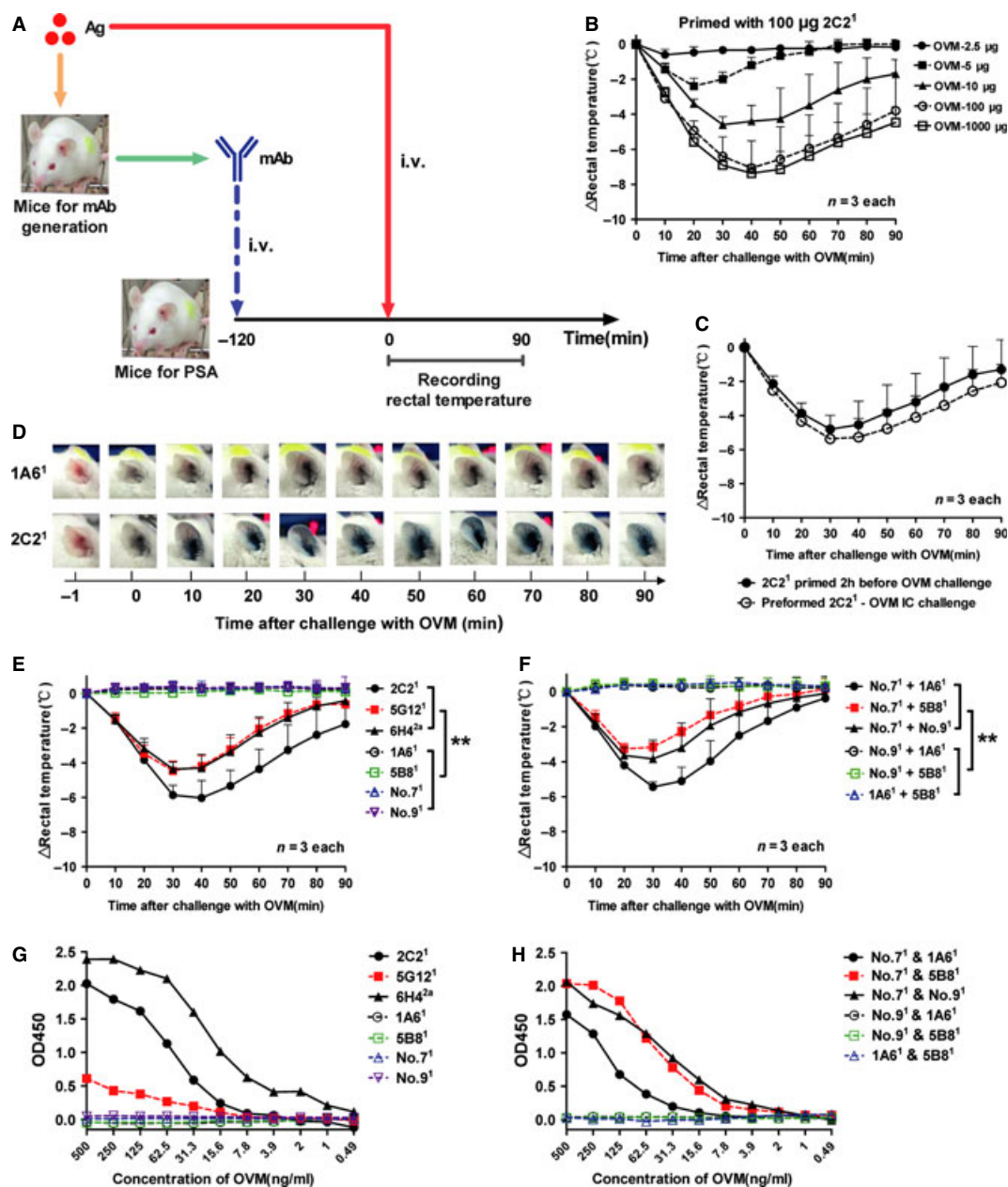


Figure 1 Establishing a PSA mouse model using OVM and OVM-specific IgG mAbs. (A) Procedure for establishing the PSA model. (B) Mice were primed with 2C2 and challenged with the indicated doses of OVM. $\Delta\text{Rectal temperature}$ is presented at 10-min interval for 90 min postchallenge. (C) Mice were primed with 100 μg 2C2 and challenged with 10 μg OVM or directly challenged with preformed ICs containing mAb 2C2 and OVM at same dose. (D) Mice were primed with 100 μg 1A6 or 2C2 and challenged with 10 μg

OVM. Plasma exudation was measured in the ears. (E and F) Mice were primed with 100 μg anti-OVM mAbs or mAb cocktails (1 : 1 ratio) and challenged with 10 μg OVM. (G and H) Sandwich ELISA consisting of OVM with one or two strains of IgG mAb used as the capture and detecting antibody. Superscripts on mAb names indicate the IgG isotypes. Data are represented as the mean $\pm$ SD (B, C, E and F) or mean OD 450 nm of duplicate samples (G and H). ** $P < 0.005$.

These results, further verified by testing the various mAb-antigen reactions shown in Table 1, indicated that only a fraction of mAbs or mAb cocktails bound with antigen could trigger PSA.

To define the ICs capable or incapable of inducing PSA, we termed the ICs capable of inducing PSA as anaphylaxis-inducing ICs (Ai-ICs) to distinguish from non-anaphylaxis-inducing ICs (nAi-ICs).

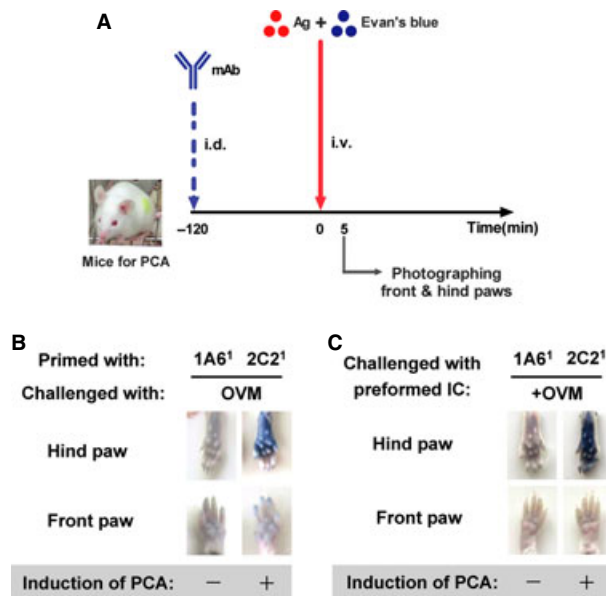


Figure 2 Establishing a mouse PCA model using OVM and 2C2. (A) Procedure for establishing the PCA model. (B) Mice were i.d. primed with 20 µg 2C2 or 1A6 in the hind paws and challenged i.v. with 500 µg OVM in 0.5% Evan's blue (200 µl total volume). (C) Mice were challenged i.d. with preformed ICs consisting of 20 µg mAb 2C2 or 1A6 with 2 µg OVM in hind paws followed by i.v. injection of 200 µl 0.5% Evan's blue in PBS 5 min later.

An association between PSA induction *in vivo* and sandwich ELISA formation *in vitro* with the same ICs

Based on the requirement of non-monomeric IgG binding with multivalent antigen for both triggering PSA and forming sandwich ELISA, we set up a sandwich ELISA using OVM and the mAbs or mAb cocktails shown in Fig. 1E,F. The results indicated that the individual mAbs 2C2, 5G12 and 6H4, but not the other 4 mAbs, could function as both capture antibodies and detecting antibodies to form sandwich ELISA (Fig. 1G). Moreover, the mAb cocktails No. 7 plus No. 9, No. 7 plus 1A6 and No. 7 plus 5B8, but not the other three cocktails, could also form a sandwich ELISA (Fig. 1H). Notably, the PSA results in Fig. 1E,F show an association with the sandwich ELISA in Fig. 1G,H, as both used the same mAbs or mAb cocktails and OVM as antigen. In addition, the epitope mapping using competition ELISA showed that seven anti-OVM mAbs recognised three types of distinct epitope on OVM (Table S3), and the mAbs of mAb cocktails inducing PSA and forming sandwich ELISA recognised two different epitopes on OVM.

We further wondered whether this association would also exist for other antigen–antibody reactions. The results in Table 2 show that, of 19 ICs containing antigens and mAbs capable of forming sandwich ELISA (Table 2, reaction No. 1–19), 14 of these ICs could induce PSA (Table 2, reaction No. 1–14), and the IgG mAbs involved in the Ai-ICs could be IgG1, IgG2a or IgG2b. None of the 17 ICs incapable of forming sandwich ELISA could induce PSA *in vivo* (Table 2,

reaction No. 20–36). Nevertheless, the sandwich ELISA was still useful as a preliminary criterion for determining Ai-ICs, as no ICs unable to form sandwich ELISA could induce PSA.

Ai-ICs induce PCA in parallel with the induction of PSA

Because IgG antibody–antigen complexes can also induce PCA, which is mediated by tissue mast cells expressing FcγRs (24, 25), PCA was used to evaluate the IgG ICs capable of triggering PSA. As shown in Fig. 2, PCA could be induced by i.d. injection of antibody followed by i.v. injection of antigen (Fig. 2B) or direct i.d. injection of preformed ICs (Fig. 2C). The results in Table 2 show full coincidence between PCA and PSA induction for all 14 ICs (Table 2, reaction No. 1–14, the primary photographic data are shown in Fig. S3). Importantly, inducing PCA with an i.d. injection of preformed ICs (approximately 2–20 µg antigens in ICs) might be a simple, sensitive and safe way to screen for Ai-ICs and predict PSA occurrence.

Ai-ICs induce down-regulation of blood neutrophil CD16/32 expression concurrent with PSA induction

Because the sandwich ELISA showed an incomplete coincidence with PSA induction, and PCA can only be evaluated *in vivo*, we tried to use an accurate and convenient *in vitro* test for Ai-IC prediction. The results in Fig. 3 show that 2C2–OVM complexes could induce significant down-regulation of mouse blood neutrophil CD16/32 expression *in vivo* (Fig. 3A) and *in vitro* (Fig. 3B) and could also induce down-regulation of CD16 and CD32 expression on human blood neutrophils (Fig. 3C). Furthermore, we confirmed that Ai-ICs, but not nAi-ICs composed of other antibodies and antigens, could induce down-regulation of CD16/32 expression on murine blood neutrophils *in vivo* and *in vitro* (Table 2, reaction No. 1–14, the primary data are shown in Fig. S4).

Collectively, we summarised the characteristics of Ai-ICs in Table 2, which indicated that the occurrence of PSA exhibited a strong association with the sandwich ELISA and notably complete coincided with PCA induction and down-regulation of blood neutrophil CD16/32 expression.

The occurrence and severity of PSA is associated with the instantaneous antigen concentration in the peripheral blood in the presence of antibody

Generally, the dynamic serum concentration of a protein is associated with its clearance rate or half-life. The results presented in Fig. 4A, E and G show that the clearance rates of OVM and rHb zeta were faster than that of HSA in the absence of antibody. A postinjection drop in murine rectal temperature was also correlated with the instantaneous concentration of OVM (Fig. 4A, B, C, D), HSA (Fig. 4E, F) and rHb zeta (Fig. 4G, H) in peripheral blood in the presence of their corresponding antibodies, indicating that lower instantaneous concentrations of antigen were

Table 1 PSA induction using 36 Antigen–mAb combinatory reactions

Antigen for challenge [†]	mAb [‡] or mAb [‡] cocktail for priming [†]	Induction of PSA	No. experiments (total no. of mice)	PSA Grade 1 [§]	PSA Grade 2 [§]	PSA Grade 3 [§]
OVM [¶]	1A6 ¹	—	2 (6)	6/6	0/6	0/6
	5B8 ¹	—	1 (3)	3/3	0/3	0/3
	2C2 ¹	+	20 (66)	0/66	7/66	59/66
	5G12 ¹	+	3 (8)	0/8	3/8	5/8
	6H4 ^{2a}	+	5 (14)	0/14	1/14	13/14
	No. 7 ¹	—	1 (3)	3/3	0/3	0/3
	No. 9 ¹	—	1 (3)	3/3	0/3	0/3
	No. 7 ¹ + No. 9 ¹	+	2 (5)	0/5	4/5	1/5
	No. 7 ¹ + 1A6 ¹	+	2 (5)	0/5	1/5	4/5
	No. 7 ¹ + 5B8 ¹	+	2 (5)	0/5	3/5	2/5
HSA ^{††}	H1A6 ^{2b}	—	1 (3)	3/3	0/3	0/3
	1G3 ^{2a}	—	1 (4)	4/4	0/4	0/4
	2E9 ¹	—	3 (9)	9/9	0/9	0/9
	3A5 ¹	—	1 (3)	3/3	0/3	0/3
	4A2 ¹	—	1 (3)	3/3	0/3	0/3
	4F12 ¹	—	1 (3)	3/3	0/3	0/3
	H1A6 ^{2b} + 3A11 ¹	+	2 (7)	0/7	7/7	0/7
	H1A6 ^{2b} + 4A2 ¹	+	2 (7)	0/7	6/7	1/7
	1G3 ^{2a} + 4F12 ¹	+	2 (6)	0/6	5/6	1/6
	3A5 ¹ + 2E9 ¹	+	2 (5)	0/5	0/5	5/5
	4A2 ¹ + 2E9 ¹	+	2 (5)	0/5	5/5	0/5
	4A2 ¹ + 3A5 ¹	+	4 (12)	0/12	1/12	11/12
MP1P ^{‡‡}	M4 ¹	—	1 (3)	3/3	0/3	0/3
	M12 ^{2b}	—	1 (3)	3/3	0/3	0/3
	M4 ¹ + M12 ^{2b}	+	3 (8)	0/8	0/8	8/8
rHb zeta ^{§§}	3H9 ¹	—	1 (3)	3/3	0/3	0/3
	4D11 ¹	—	1 (3)	3/3	0/3	0/3
	4D11 ¹ + 3H9 ¹	+	3 (8)	0/8	4/8	4/8
Hb zeta ^{§§}	4D11 ¹ + 3H9 ¹	—	2 (6)	6/6	0/6	0/6
HbA2 ^{¶¶}	2H4 ¹ + 2C9 ¹	—	2 (5)	5/5	0/5	0/5
AOPP ^{†††}	3F2 ¹ + 4C5 ^{2a}	—	2 (6)	6/6	0/6	0/6
OVA ^{‡‡‡}	1D1 ¹	—	4 (11)	11/11	0/11	0/11
	3C10 ¹	—	1 (3)	3/3	0/3	0/3
	3H8 ^{2a}	—	1 (3)	3/3	0/3	0/3
	4C10 ¹	—	2 (4)	4/4	0/4	0/4
	4F1 ¹	—	2 (5)	5/5	0/5	0/5

[†]The antigen–mAb dose for PSA is summarised in Table S4.

[‡]Superscripts on mAb names indicate the IgG isotypes.

[§]Maximum rectal temperature variation ($\Delta t_{\max}$) during the 90 min following challenge was classified according to the standard created by Jonsson F et al. (14) with modifications. Grade 1: no shock, $+1^{\circ}\text{C} > \Delta t_{\max} \geq -1^{\circ}\text{C}$, no mortality; Grade 2: mild shock, $-1^{\circ}\text{C} > \Delta t_{\max} \geq -4^{\circ}\text{C}$, no mortality; Grade 3: severe shock, $-4^{\circ}\text{C} > \Delta t_{\max}$, possible mortality. The results are presented as mice in each grade vs total number of mice used.

[¶]OVM, ovomucoid, from chicken egg white.

^{††}HSA, human serum albumin.

^{‡‡}MP1P, recombinant mannoprotein 1 of penicillium.

^{§§}rHb zeta, recombinant human haemoglobin zeta chain; Hb zeta, human haemoglobin zeta chain from cord blood of Bart's thalassaemia.

^{¶¶}HbA2, human haemoglobin A2 (Hb δ chain).

^{†††}AOPP, advanced oxidation protein products.

^{‡‡‡}OVA, ovalbumin.

associated with milder drops in rectal temperature, with no drop observed in some instances. These results suggested that the dynamics of instantaneous antigen concentration

change related to the clearance rate in peripheral blood could impact the occurrence and severity of IgG-induced PSA.

Table 2 Summary for characteristics of Ai-ICs inducing PSA and PCA

No. of reaction	Antigen–mAb [†]	Induction of PSA (no. experiments)	Formation of sandwich ELISA (no. experiments)	PCA (no. experiments): mAb priming i.d. followed by Ag i.v. ‡	PCA (no. experiments): preformed IC i.d.‡	Neutrophil CD16/32↓ (no. experiments) <i>in vivo</i> [§]	Neutrophil CD16/32↓ (no. experiments) <i>in vitro</i> [§]
1	OVM – 2C2 ¹	+ (20)	+ (3)	+ (7)	+ (5)	+ (3)	+ (5)
2	OVM – 5G12 ¹	+ (3)	+ (3)	+ (2)	+ (2)	+ (2)	+ (4)
3	OVM – 6H4 ^{2a}	+ (5)	+ (3)	+ (3)	+ (3)	+ (3)	+ (5)
4	OVM – No. 7 ¹ & 1A6 ¹	+ (2)	+ (3)	+ (2)	+ (2)	+ (2)	+ (3)
5	OVM – No. 7 ¹ & 5B8 ¹	+ (2)	+ (3)	+ (2)	+ (2)	+ (2)	+ (3)
6	OVM – No. 7 ¹ & No. 9 ¹	+ (2)	+ [¶]	+ (2)	+ (2)	+ (2)	+ (3)
7	HSA – 2E9 ¹ & 3A5 ¹	+ (2)	+ [¶]	+ (2)	+ (2)	+ (2)	+ (2)
8	HSA – 2E9 ¹ & 4A2 ¹	+ (2)	+ [¶]	+ (2)	+ (2)	+ (2)	+ (2)
9	HSA – 3A5 ¹ & 4A2 ¹	+ (4)	+ [¶]	+ (2)	+ (2)	+ (2)	+ (2)
10	HSA – H1A6 ^{2b} & 3A11 ¹	+ (2)	+ [¶]	+ (2)	+ (2)	Not done	+ (2)
11	HSA – H1A6 ^{2b} & 4A2 ¹	+ (2)	+ [¶]	+ (3)	+ (2)	+ (2)	+ (2)
12	HSA – 1G3 ^{2a} & 4F12 ¹	+ (2)	+ [¶]	+ (3)	+ (2)	+ (2)	+ (2)
13	MP1P – M4 ¹ & M12 ^{2b}	+ (3)	+ [¶]	+ (3)	+ (2)	+ (2)	+ (3)
14	rHb zeta – 3H9 ¹ & 4D11 ¹	+ (3)	+ [¶]	+ (3)	+ (2)	Not done	+ (2)
15	Hb zeta – 4D11 ¹ & 3H9 ¹	– (2)	+ [¶]	– (2)	– (2)	– (2)	– (3)
16	HSA – 2E9 ¹	– (3)	+ [¶]	– (3)	– (2)	– (2)	– (2)
17	HbA2 – 2H4 ¹ & 2C9 ¹	– (2)	+ [¶]	– (3)	– (2)	– (2)	– (2)
18	AOPP – 3F2 ¹ & 4C5 ^{2a}	– (2)	+ [¶]	– (3)	– (2)	– (2)	– (2)
19	OVA – 1D1 ¹	– (4)	+ (3) ^{††}	– (3)	– (2)	– (3)	– (2)
20	OVM – 1A6 ¹	– (2)	– (2)	– (3)	– (2)	– (3)	– (5)
21	OVM – 5B8 ¹	– (1)	– (2)	– (2)	– (2)	– (2)	– (4)
22	OVM – No.7 ¹	– (1)	– [¶]	– (2)	– (2)	– (2)	– (3)
23	OVM – No.9 ¹	– (1)	– [¶]	– (2)	– (2)	– (2)	– (3)
24	HSA – H1A6 ^{2b}	– (1)	– [¶]	– (3)	– (2)	– (2)	– (2)
25	HSA – 1G3 ^{2a}	– (1)	– [¶]	– (3)	Not done	– (2)	Not done
26	HSA – 3A5 ¹	– (1)	– [¶]	– (3)	– (2)	– (2)	– (2)
27	HSA – 4A2 ¹	– (1)	– [¶]	Not done	– (2)	Not done	– (2)
28	HSA – 4F12 ¹	– (1)	– [¶]	– (2)	– (2)	Not done	– (2)
29	rHb zeta – 3H9 ¹	– (1)	– [¶]	– (3)	– (2)	Not done	– (2)
30	rHb zeta – 4D11 ¹	– (1)	– [¶]	– (3)	Not done	Not done	– (2)
31	MP1P – M4 ¹	– (1)	– [¶]	– (3)	– (2)	– (2)	– (3)
32	MP1P – M12 ^{2b}	– (1)	– [¶]	– (3)	Not done	– (2)	– (3)
33	OVA – 3C10 ¹	– (1)	– (2)	– (2)	– (2)	– (2)	Not done
34	OVA – 3H8 ^{2a}	– (1)	– (2)	– (2)	– (2)	– (2)	– (2)
35	OVA – 4C10 ¹	– (2)	– (2)	– (2)	– (2)	– (2)	– (2)
36	OVA – 4F1 ¹	– (2)	– (2)	– (2)	– (2)	– (2)	– (2)

[†]Superscripts on mAb names indicate the IgG isotypes.

[‡]The antigen–mAb dose for PCA is summarised in Table S4, and the PCA photographs are presented in Fig. S3 in the supplement.

[§]The antigen–mAb dose for induction of CD16/32 down-regulation is summarised in Table S4, FCM figures are presented in Fig. S4 in the supplement.

[¶]The references on mAbs and sandwich ELISA pairs used in this work are noted in the Materials and Methods section of supplement.

^{††}All of the sandwich ELISA show specificity and high sensitivity (< 100 ng/ml) except for OVA with 1D1 (> 100 ng/ml).

Discussion

In this work, we proposed and demonstrated that only Ai-ICs, not all ICs, could trigger PSA. It should be emphasised that mAbs or mAb cocktails forming Ai-ICs must bind multiple epitopes to form non-monomer IgG aggregate to cross-link FcγRs on the effector cell membrane, and non-monomer IgG aggregate is also indispensable for the formation of sandwich ELISA. Therefore, sandwich ELISA as a simple

method could be used for preliminary screening to predict the formation of Ai-ICs, as none ICs incapable of forming sandwich ELISA induce PSA, although several ICs forming sandwich ELISA are incapable of triggering PSA. This incomplete coincidence between triggering PSA and forming sandwich ELISA may be associated with the size and conformation of antigen molecule. The sandwich ELISA only needs the binding of two epitopes on antigen with capture and detecting mAb, while triggering PSA requires at least two

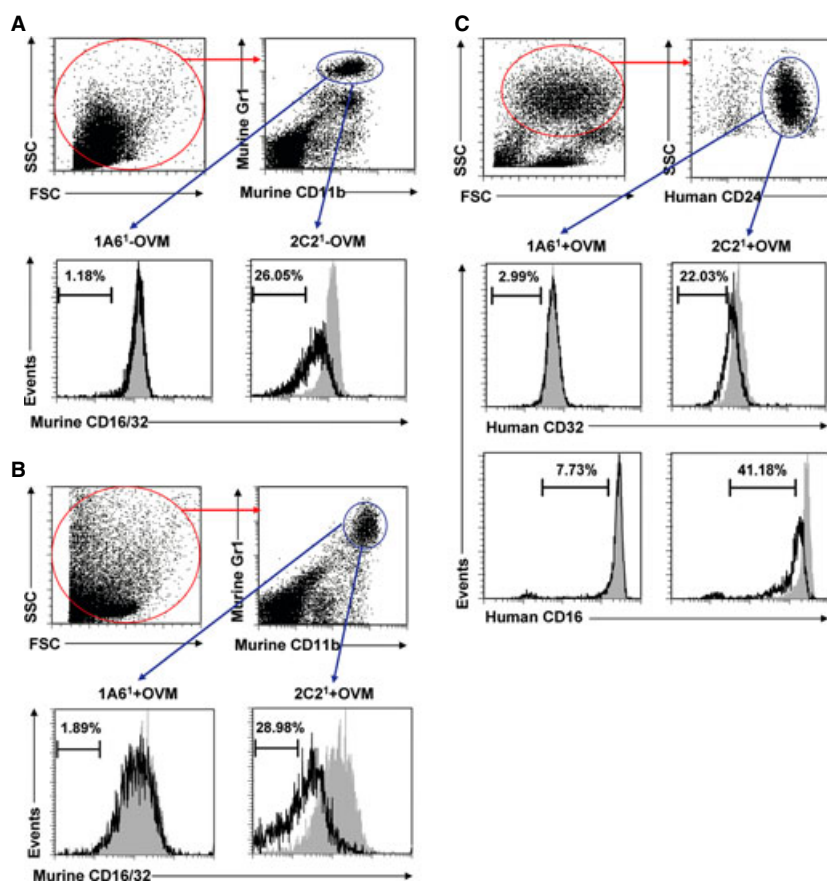


Figure 3 Induction of blood neutrophil CD16/32 down-regulation using OVM and mAb 2C2 *in vivo* and *in vitro*. (A) Blood collected 4-h post-PSA induction was analysed using flow cytometry for mouse neutrophil CD16/32 expression. (B) Mouse blood was stimulated *in vitro* with preformed ICs for 3 h before flow cytometry

analysis of neutrophil CD16/32 expression. (C) Venous blood from healthy individuals was stimulated *in vitro* with preformed ICs for 3 h before flow cytometry analysis for human neutrophil CD16 and CD32 expression. The shaded and unshaded peaks correspond to OVM group and 1A6/2C2-OVM group.

IgG Fc portions to bind Fc γ Rs on the effector cell membrane, in which the size and conformation of antigen as well as the distance between the two epitopes on antigen molecule may influence the ability of Fc portions to cross-link Fc γ Rs. For instance, both recombinant Hb zeta and native Hb zeta could be detected by the same sandwich ELISA system, but only rHb zeta could trigger PSA, which may be attributed to the fact that rHb zeta is a monomer, but native Hb zeta from cord blood can exist as a monomer, dimer, trimer or even tetramer.

PCA can also be used to evaluate whether ICs can trigger PSA as the ICs capable of inducing PCA completely coincide with those able to induce PSA. This coincidence implies that PCA induced by direct i.d. injection with *in vitro* preformed ICs could be a simple and safe method to predict PSA before i.v. administering therapeutic antibodies.

As PCA may not be suitable for human use *in vivo*, we used a more convenient *in vitro* way to predict the formation of Ai-IC by consulting Khodoun's work that showed a down-regulation of CD16/32 expression on mouse and

human neutrophils, but not on mouse monocytes, dendritic cells, NK cells or basophils during IgG-mediated PSA (26). In our work, noticeably, a decreased CD16/32 expression on mouse neutrophils and CD16 & CD32 on human blood neutrophils could only be induced by Ai-ICs containing murine IgG mAbs, but not by nAi-ICs. Furthermore, we found a correlation between severity of PSA and amplitude of decreased CD16/32 expression on mouse neutrophils (Fig. 1B and Fig. S5), in which a decreased CD16/32 expression could be detected by mAb 2.4G2 sensitively. As murine IgG1 cannot bind human CD16, but ICs containing murine IgGs can bind human CD32 that is an activating receptor for PSA (14, 27–29), it could be supposed that the decreased CD16 expression on human neutrophils induced by ICs containing murine IgG may be not a result directly from CD16 binding, but rather a result from the binding or action via CD32. Additionally, there was also a consideration that loss of CD16 during IgG-mediated anaphylaxis requires cell signalling involving receptor internalisation or shedding (26).

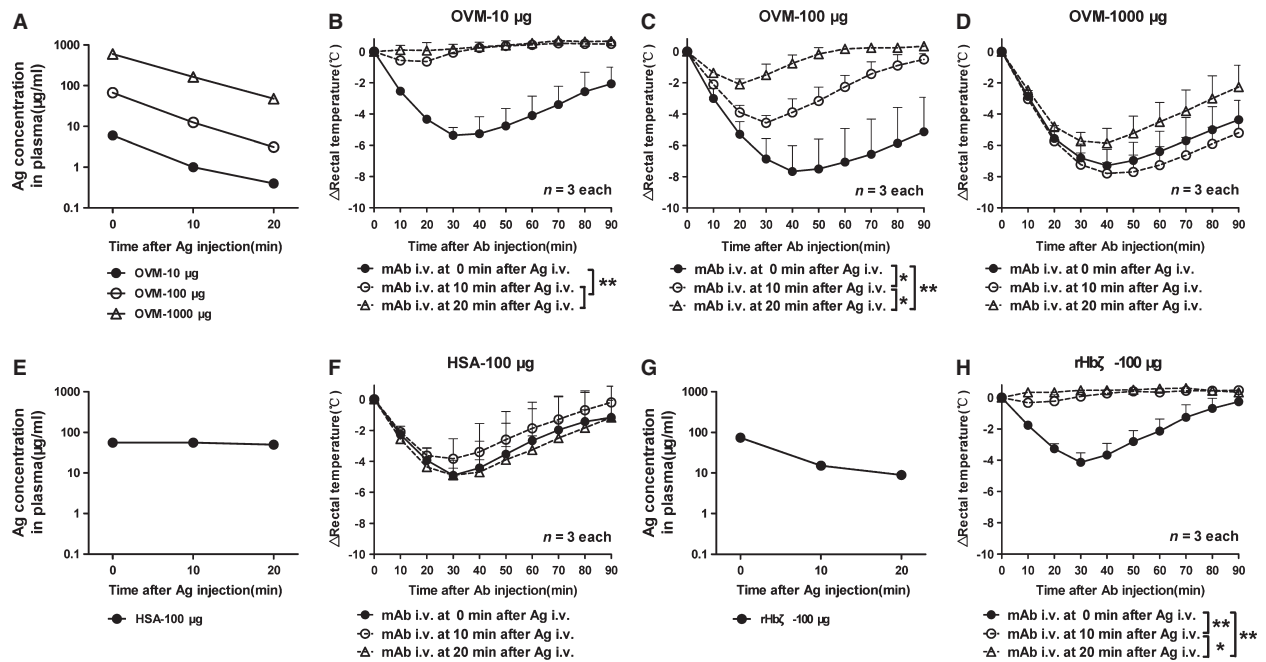


Figure 4 The occurrence and severity of PSA is associated with the instantaneous antigen concentration in the blood. (A, E and G) Blood was collected after i.v. injection with the indicated dose of biotinylated OVM, biotinylated HSA and rHb zeta, after which the antigen concentration in the plasma was measured by ELISA. (B–D) BALB/c mice were i.v. injected with 100 µg mAb 2C2 at 0, 10 and 20 min after injection with 10 µg (B), 100 µg (C) or 1000 µg (D) OVM, and rectal temperature was measured as previously mentioned. (F) 100 µg mAbs 2E9 and 3A5 (1 : 1 ratio) cocktail was i.v. injected at the indicated time points following injection with 100 µg HSA, and rectal temperature was recorded as previously mentioned. (H) BALB/c mice were i.v. injected with 100 µg mAb 3H9 and 4D11 (1 : 1 ratio) cocktail at the indicated time point after injection with 100 µg rHb zeta, and rectal temperature was recorded as previously mentioned. * $P < 0.05$, ** $P < 0.005$.

We also noticed that the clearance rate or instantaneous concentration of antigen in the peripheral blood in the presence of antibody could impact the occurrence and severity of PSA. These results suggest that it may be necessary to measure the target antigen concentration in the blood during treatment with therapeutic antibodies or IVIG. Particularly, cell components could act as antigens from dead tumour cells following antibody or chemical therapy, such as that observed in tumour lysis syndrome (30).

Moreover, some PSA models, using high-dose (approximately 0.5–4 mg) antigen or hapten conjugates (14, 31–33), led to a consideration that IgG-mediated PSA could not occur in human because it may be impossible for a person to have such high doses of antigen *in vivo* (15). However, Ishikawa's and our work using low doses antigen and mAbs suggest that it is more plausible that IgG-induced mouse PSA models can be used to investigate PSA resulting from the therapeutic antibodies infusion in humans (34).

In summary, we showed that only Ai-ICs could trigger IgG-induced PSA. Ai-ICs could be defined by construction of sandwich ELISA, induction of PCA and down-regulation of blood neutrophil CD16/32 expression. Our work is expected to provide new strategies for evaluating the safety in administration and development of therapeutic antibodies, including mAbs, polyclonal antibodies and immunoglobulins.

ously mentioned. (F) 100 µg mAbs 2E9 and 3A5 (1 : 1 ratio) cocktail was i.v. injected at the indicated time points following injection with 100 µg HSA, and rectal temperature was recorded as previously mentioned. (H) BALB/c mice were i.v. injected with 100 µg mAb 3H9 and 4D11 (1 : 1 ratio) cocktail at the indicated time point after injection with 100 µg rHb zeta, and rectal temperature was recorded as previously mentioned. * $P < 0.05$, ** $P < 0.005$.

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Author contributions

DL Jiao and Y Liu performed most of experiment; X Lu and QJ Pan performed cytologic analysis and a part of preliminary experiment; J Zheng and YJ Liu established animal model and revised the manuscript; YF Wang, BY Liu performed the generation and purification of monoclonal antibodies and recombinant proteins. N Fu with help from DL Jiao and J Zheng designed all research and wrote the manuscript.

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Conflict of interest statement

None of the authors has any conflict of interest to disclose regarding this manuscript.

Supporting Information

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

Figure S1. Identification of 10 mAbs binding OVM and OVA.

Figure S2. PSA induction by 10 mAbs and OVA.

Figure S3. Primary data corresponding to the PCA induction displayed in Table 2.

Figure S4. Primary data (for Table 2) corresponding to the down-regulation of murine blood neutrophil CD16/32.

Figure S5. The relationship between decline of rectal temperature and down-regulation of murine neutrophil CD16/32 expression.

Table S1. Antigens and mAbs used in murine PSA.

Table S2. The list of mAbs generated with OVA as the immunogen.

Table S3. Analysis of distinct epitope binding to anti-OVM mAbs by competition ELISA.

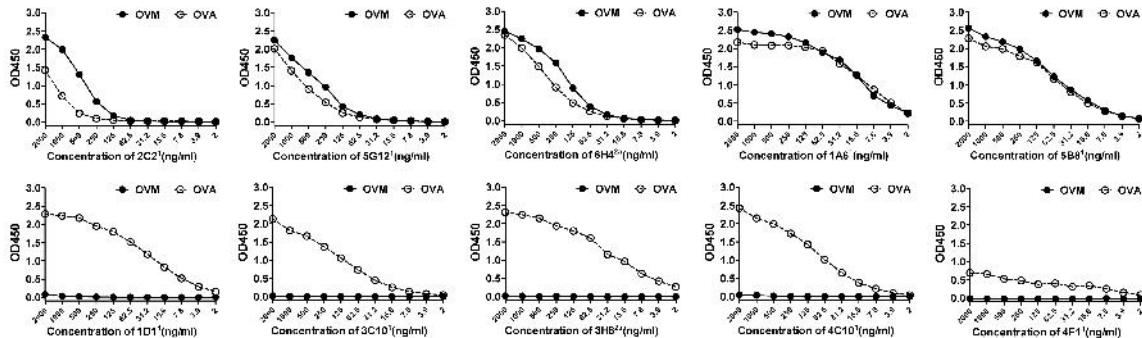
Table S4. Ag-mAb doses for PSA, PCA and induction of neutrophil CD16/32 down-regulation.

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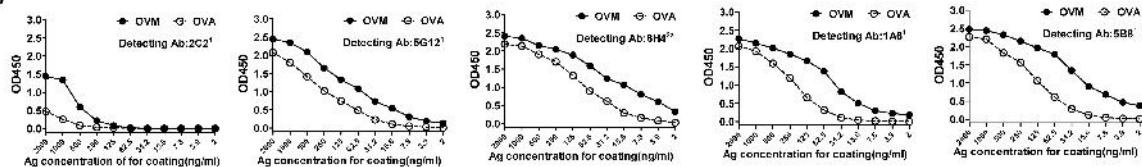
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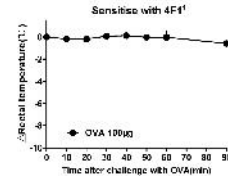
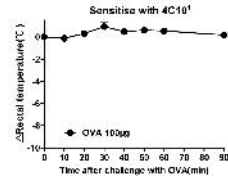
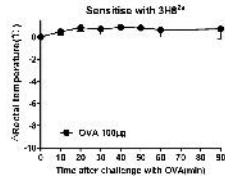
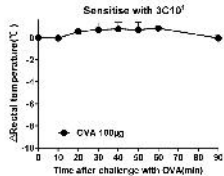
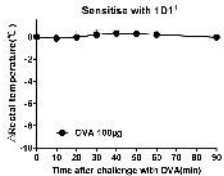
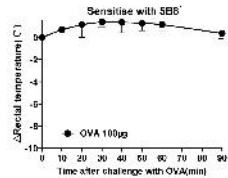
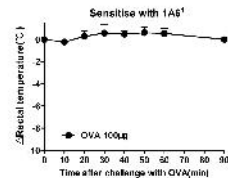
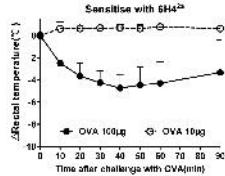
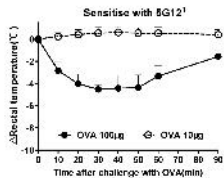
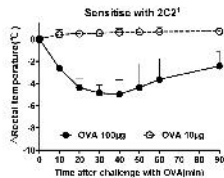
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A



B





A

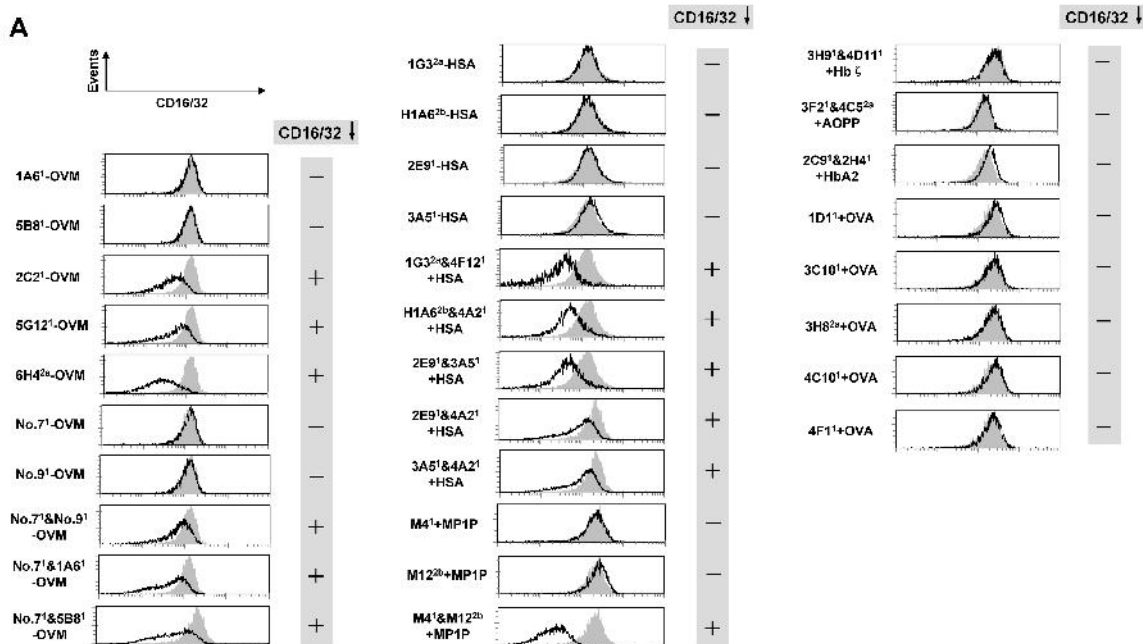
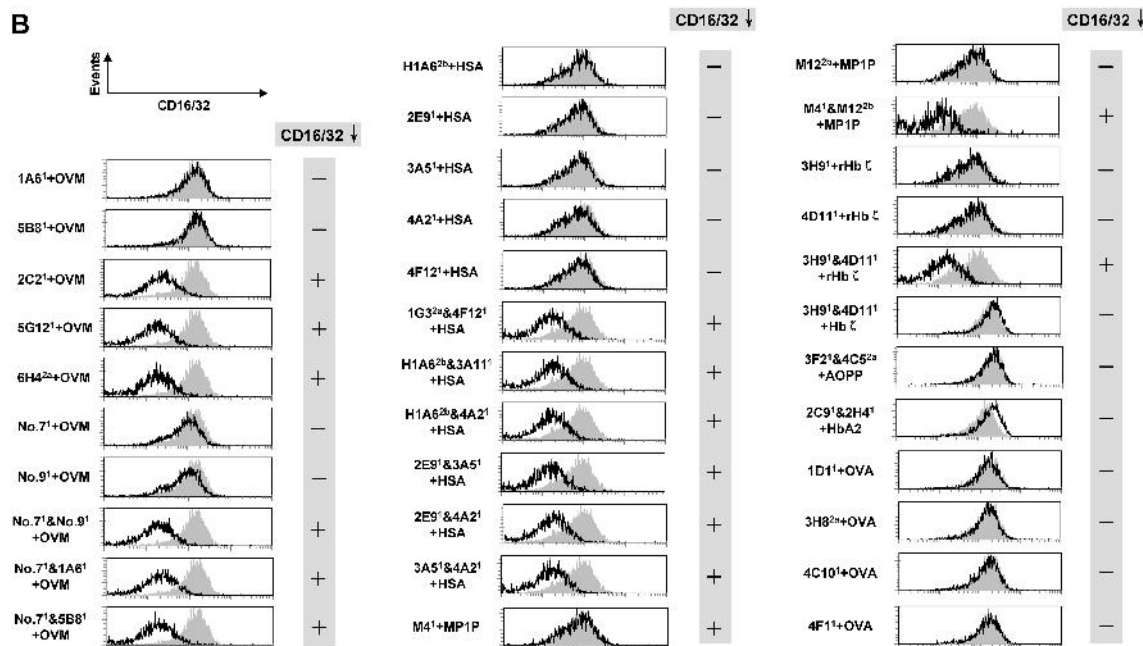
Primed with:	1A6 ¹	5B8 ¹	2C2 ¹	5G12 ¹	6H4 ^{2b}	No.7 ¹	No.9 ¹	No.7 ¹ &1A6 ¹	No.7 ¹ &5B8 ¹	No.7 ¹ &No.9 ¹	3A5 ¹	2E9 ¹	1G3 ^{2b}	H1A6 ^{2b}	4F12 ¹	1G3 ^{2b} &4F12 ¹	H1A6 ^{2b} &4A2 ¹	H1A6 ^{2b} &3A11 ¹	2E9 ¹ &3A5 ¹	2E9 ¹ &4A2 ¹	3A5 ¹ &4A2 ¹
Challenged with:	OVM										HSA										
Hind paw																					
Front paw																					
Induction of PCA:	-	-	+	+	+	-	-	+	+	+	-	-	-	-	-	+	+	+	+	+	+

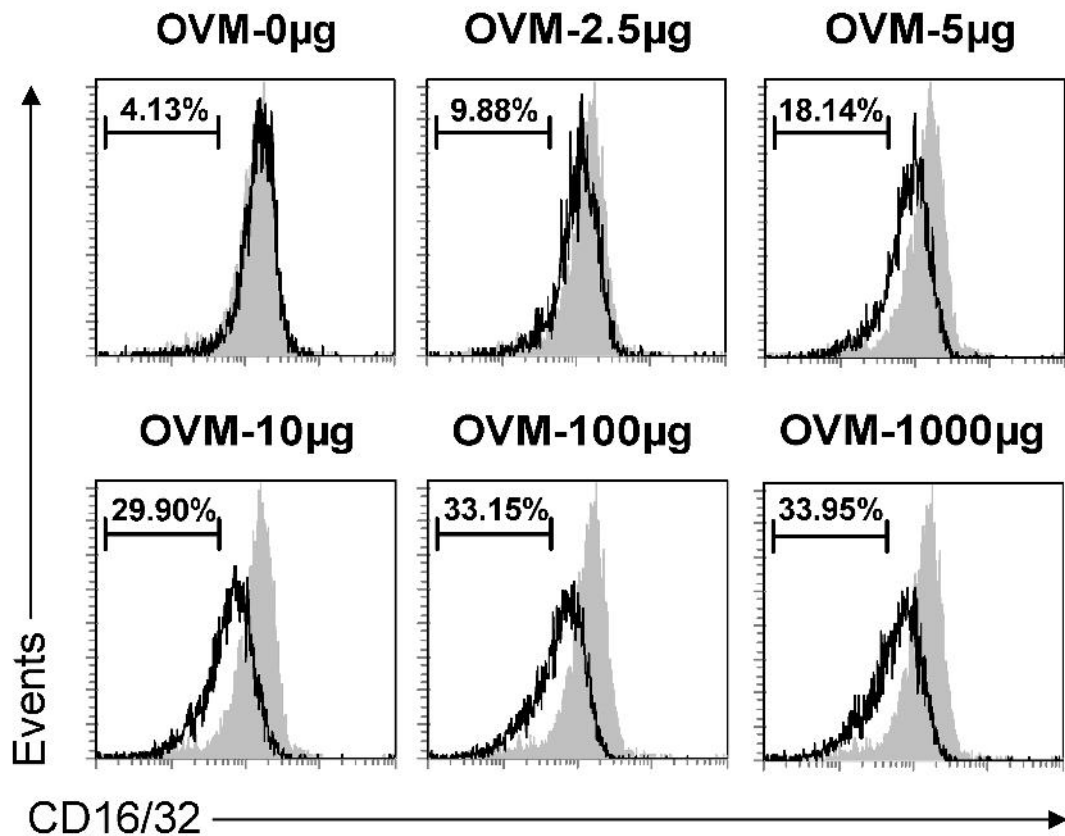
Primed with:	M4 ¹	M12 ^{2b}	M4 ¹ & M12 ^{2b}	3H9 ¹	4D11 ¹	3H9 ¹ & 4D11 ¹	3H9 ¹ & 4D11 ¹	2C9 ¹ & 2H4 ¹	3F2 ¹ & 4C5 ^{2b}	1D1 ¹	3C10 ¹	3H8 ¹	4C10 ¹	4F1 ¹
Challenged with:	MP1P			rHb ζ			Hb ζ	HbA2	AOPP	OVA				
Hind paw														
Front paw														
Induction of PCA:	-	-	+	-	-	+	-	-	-	-	-	-	-	-

B

Challenged with preformed IC:	1A6 ¹	5B8 ¹	2C2 ¹	5G12 ¹	6H4 ^{2b}	No.7 ¹	No.9 ¹	No.7 ¹ &1A6 ¹	No.7 ¹ &5B8 ¹	No.7 ¹ &No.9 ¹	3A5 ¹	2E9 ¹	4A2 ¹	H1A6 ^{2b}	4F12 ¹	1G3 ^{2b} &4F12 ¹	H1A6 ^{2b} &4A2 ¹	H1A6 ^{2b} &3A11 ¹	2E9 ¹ &3A5 ¹	2E9 ¹ &4A2 ¹	3A5 ¹ &4A2 ¹
	+OVM										+HSA										
Hind paw																					
Front paw																					
Induction of PCA:	-	-	+	+	+	-	-	+	+	+	-	-	-	-	-	+	+	+	+	+	+

Challenged with preformed IC:	M4 ¹	M4 ¹ & M12 ^{2b}	3H9 ¹	3H9 ¹ & 4D11 ¹	3H9 ¹ & 4D11 ¹	2C9 ¹ & 2H4 ¹	3F2 ¹ & 4C5 ^{2b}	1D1 ¹	3C10 ¹	3H8 ¹	4C10 ¹	4F1 ¹
	+MP1P		+rHb ζ		+Hb ζ	+HbA2	+AOPP	+OVA				
Hind paw												
Front paw												
Induction of PCA:	-	+	-	+	-	-	-	-	-	-	-	-

A**B**



Materials and methods

Human blood samples

The peripheral blood samples used to evaluate human neutrophil CD16 & CD32 expression were taken from 3 members in our lab upon informed consent. All of these performances were approved by the Ethical committee of Southern Medical University.

Reagents and antibodies

Grade V OVA, OVM and HSA were purchased from Sigma-Aldrich (St. Louis, MO, USA); antibodies used for flow cytometry analysis of mouse Gr1, human CD16 and human CD32 were obtained from BioLegend (San Diego, CA, USA); and antibodies for flow cytometry analysis of mouse CD11b, mouse CD16/32 (2.4G2, ATCC HB-197) and human CD24 were purchased from eBioscience (San Diego, CA, USA). The widely used mAb 2.4G2 was used to detect decreased CD16/32 expression with similar efficiency as an anti-CD16 mAb. The antigens and mAbs used for combinatory reactions are listed in Table S1. The anti-OVM mAb No. 7 and No. 9, and mAbs to HSA, MP1P, rHb zeta, Hb zeta, HbA2 and AOPP have already been published, and their ability to form sandwich ELISA has previously been established (1-7).

Preparation of preformed ICs

The indicated doses of mAbs and antigens were mixed and then incubated at 37 °C for 30 minutes to form ICs *in vitro*.

Monoclonal antibody preparation

Six-week-old female BALB/c mice were immunised subcutaneously (s.c.) and intraperitoneally (i.p.) with 100 µg OVA emulsified with complete Freund's adjuvant and were boosted by injection with 100 µg OVA in incomplete Freund's adjuvant 3 times at an interval of 3 weeks. A final boost by i.p injection with 50 µg OVA in PBS was administered 3 days before cell fusion. Hybridoma

generation and mAb production were performed as described previously (6, 8). In brief, spleen cells from immunised mice were fused with the NS-1 mouse myeloma cells to generate hybridoma cells. Due to trace amounts of OVM mixed in OVA, even in purity Grade VII OVA (Sigma A7641) (1). Specific antibodies in the hybridoma culture supernatant were detected by indirect ELISA using OVA and OVM as coated antigens, and antibody-secreting hybridoma cells were cloned by successive limiting dilution. Antibodies were obtained from ascites produced by i.p injection of hybridoma cells into mice pretreated with 0.5 ml of incomplete Freund's adjuvant and purified by affinity chromatography using HiTrap protein G HP (Amersham Pharmacia Biotech, Sweden). The mAb isotypes were determined using an ImmunoType Kit (Sigma). Both OVA and OVM were used to establish the mouse PSA model because of the 10 mAbs generated from mice immunised with OVA, 5 mAbs reacted only against OVA and 5 mAbs reacted against OVM with slight cross-reactivity to OVA (Sigma A5503 Grade V) (Table S2 and Fig. S1).

Indirect and sandwich ELISA

For indirect ELISA, microplates (Yijamei Company, Xiamen, Fujian, China) were coated with 100 µl/well OVA or OVM of 5 µg/ml in 0.01 M phosphate-buffered saline (PBS, pH 7.4) at 4° C overnight and blocked with 0.25% casein in 0.01 M PBS (pH 7.4) containing 0.1% Tween 20 (PBS-T) at 37° C for 2 h. After washing 5 times, 100 µl/well of antibody-containing samples was added and incubated at 37° C for 1 h, followed by washing with PBS-T. The microplates were then incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories, West Grove, PA) at 37 °C for 30 min, followed by thorough washing. Colour reactions were developed using tetramethylbenzidine (TMB) substrate solution and stopped with 2 M sulfuric acid. The optical density (OD) values were read at 450 nm using a microplate reader (PerkinElmer 2030). For sandwich ELISA, microplates were coated with mAbs in 0.01 M PBS (pH 7.4) at 4 °C overnight and blocked and washed as mentioned before. Next, 100

μl/well of antigen-containing samples at the indicated concentrations were added, incubated at 37 °C for 1 h and washed with PBS-T. HRP-labeled detection mAbs or biotinylated detection mAbs followed by HRP-streptavidin were added. Thorough washing was performed after each step, and a conventional colour reaction using TMB was developed as an indirect ELISA.

Analysis of distinct epitope on OVM by epitope mapping using competition ELISA

The biotinylated mAbs were mixed with equal volume of unlabeled, respectively, followed by adding 100 μl/well into microplates coated with 0.5 μg/ml OVM. The final biotinylated mAbs concentration were 0.016, 0.007, 0.035, 0.016, 1.0, 1.0, 0.3 μg/ml for 7 strain mAbs in order of Table S3, and the unlabeled mAb as competitor were 200 μg/ml for mAb 1A6, 5B8, No. 7 and No. 9, and 300 μg/ml for mAb 2C2, 5G12 and 6H4, which were designed based on ED50 and IC50 of all anti-OVM mAbs. Others procedures and conventional steps were the same as previously described (9). The percentage of inhibition was calculated by the following formula: $[1 - (\text{OD}_{450 \text{ nm of the test well}} / \text{OD}_{450 \text{ nm of the control well}})] \times 100\%$. The criteria for identifying a distinct or competitive binding epitope were judged by inhibition percentage: >75% inhibition represented competitive binding meaning same or overlapping epitope recognized by both labeled and inhibitory unlabeled mAb, 25 to 75% inhibition represented relatively competitive binding, and < 25% inhibition represented noncompetitive binding indicating different epitope recognized by labeled and inhibitory unlabeled mAb (10, 11).

Detection of the concentration of antigen in sera

An indirect ELISA was used to detect biotinylated OVM and biotinylated HSA in plasma. Briefly, microplates were coated with 5 μg/ml 2C2 or 2E9 and plasma samples containing biotinylated OVM or biotinylated HSA were added, respectively, followed by HRP-streptavidin (Jackson ImmunoResearch, 1:5,000). Next, a conventional colour reaction with TMB was performed. A sandwich ELISA was used to detect rHb zeta in plasma (4). Briefly, microplates were coated with

5 µg/ml mAb 4D11 and plasma samples containing rHb zeta were added, followed by incubation with 5 µg/ml HRP-3H9. A conventional TMB colour reaction was performed as above. The antigen concentration was calculated using standard curves of biotinylated OVM, biotinylated HSA and rHb zeta constructed with known concentrations.

Down-regulation of mouse CD16/32 and human CD16 & CD32 expression on neutrophils determined by flow cytometry

To detect blood neutrophils, whole blood cells were incubated with fluorescently labeled antibodies at 4 °C for 30 minutes, followed by removal of red blood cells (RBC) using RBC lysis buffer (BioLegend) before analysis using a flow cytometer (BD FACS Calibur). The data analyses were performed with FCS Express software. Gr1^{high} CD11b⁺ cells were gated as mouse blood neutrophils and human blood neutrophils were determined based on FSC/SSC properties and specific staining of human CD24 (12).

To measure mouse blood neutrophil CD16/32 down-regulation *in vitro*, heparinised blood was collected from normal mice and diluted 1:1 with RPMI 1640 (Gibco) supplemented with 100 ng/ml mouse GM-CSF (PeproTech). The diluted blood was then mixed with preformed ICs and incubated at 37 °C for 3 hours, followed by staining with anti-mouse Gr1, CD11b and CD16/32 antibodies. Gated mouse neutrophils (Gr1^{high} CD11b⁺) were analysed for their CD16/32 expression. To measure mouse blood neutrophil CD16/32 down-regulation *in vivo*, conventional PSA was induced in mice and heparinised blood was drawn at 4 hours after antigen challenge before staining with anti-mouse Gr1, CD11b and CD16/32 antibodies, gated mouse neutrophils were analysed for CD16/32 expression. To measure human blood neutrophil CD16 & CD32 down-regulation *in vitro*, heparinised venous blood samples was collected and diluted 1:1 with RPMI 1640, then the diluted blood was mixed with preformed ICs (with final concentration of 100µg/ml mAbs and 10µg/ml antigen) and incubated at 37° C for 3 hours, followed by staining with anti-human CD24 and CD16

& CD32 antibodies. Gated human neutrophils ($FSC^{high} SSC^{high} CD24^{+}$) were analysed for their CD16 & CD32 expression.

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Figure legends

Figure S1. Identification of 10 mAbs binding OVM and OVA. **(A)** The specificity of the mAbs in serial dilutions was determined by indirect ELISA. Microplates were coated with OVM or OVA and probed with the indicated mAb followed by HRP-labeled goat anti-mouse IgG. **(B)** The sensitivity of mAbs binding antigens in serial dilutions was determined by an indirect ELISA. Microplates were coated with serial dilutions of OVM or OVA and were probed with 1 µg/ml of the indicated mAbs followed by HRP-labeled goat anti-mouse IgG. The results represent the mean OD 450 nm of samples run in duplicate.

Figure S2. PSA induction by 10 mAbs and OVA. BALB/c mice were sensitised with 100 µg mAb and challenged with 100 µg OVA (or 10 µg OVA for some mAbs) 2 h later, and rectal temperature was recorded as previously described.

Figure S3. Primary data corresponding to the PCA induction displayed in Table 2. **(A)** PCA induced by mAbs priming prior to Ag challenge. **(B)** PCA induced by preformed ICs.

Figure S4. Primary data (for Table 2) corresponding to the down-regulation of murine blood neutrophil CD16/32. **(A)** *in vivo* inducing down-regulation of CD16/32 expression **(B)** *in vitro* inducing down-regulation of CD16/32 expression. The shaded and unshaded peaks correspond to antigen group and mAbs-antigen group.

Figure S5. Mice were primed with 100µg 2C2, challenged with the indicated doses of OVM, and blood neutrophil CD16/32 expression was analyzed 4 hour post-challenge. Shaded and unshaded peaks correspond to no challenge group and indicated doses of OVM challenge group.

Table S1. Antigens and mAbs used in murine PSA

Code	Name for Ab	Antigen	Subclass		Code	Name for Ab	Antigen	Subclass
1	1A6	OVM	IgG1(κ)		15	2E9	HSA	IgG1(κ)
2	2C2		IgG1(κ)		16	3A5		IgG1(κ)
3	5B8		IgG1(κ)		17	3A11		IgG1(κ)
4	5G12		IgG1(κ)		18	4A2		IgG1(κ)
5	6H4		IgG2a(κ)		19	4F12		IgG1(κ)
6	No.7		IgG1(κ)		20	H1A6		IgG2b(κ)
7	No.9	OVA	IgG1(κ)		21	1G3	MP1P	IgG2a(κ)
8	1D1		IgG1(κ)		22	M4		IgG1(κ)
9	3C10		IgG1(κ)		23	M12		IgG2b(κ)
10	3H8		IgG2a(κ)		24	2C9	HbA2	IgG1(κ)
11	4C10		IgG1(κ)		25	2H4		IgG1(κ)
12	4F1	rHb zeta; Hb zeta	IgG1(κ)		26	3F2	AOPP	IgG1(κ)
13	3H9		IgG1(κ)		27	4C5		IgG2a(κ)
14	4D11		IgG1(κ)					

Table S2. The list of mAbs generated with OVA as the immunogen

Code	mAb name	Ag bound with mAb	Subclass
1	1A6	OVA, OVM	IgG1(κ)
2	2C2	OVA, OVM	IgG1(κ)
3	5B8	OVA, OVM	IgG1(κ)
4	5G12	OVA, OVM	IgG1(κ)
5	6H4	OVA, OVM	IgG2a(κ)
6	1D1	OVA	IgG1(κ)
7	3C10	OVA	IgG1(κ)
8	3H8	OVA	IgG2a(κ)
9	4C10	OVA	IgG1(κ)
10	4F1	OVA	IgG1(κ)

Table S3. Analysis of distinct epitope binding to anti-OVM mAbs by competition ELISA.

mAb	Epitope group	Inhibition of unlabeled mAbs on binding of biotin-labeled mAb§ to OVM (%)						
		1A6	5B8	No.9	No.7	2C2	5G12	6H4
1A6	I	100 [†]	100	97	10	0	8	19
5B8	I	99	100	99	0	0	11	0
No.9	I	98	100	99	2	0	20	4
No.7	II	6	17	11	99	6	12	0
2C2	III	14	0	0	16	95	94	96
5G12	III	3	0	0	7	95	95	93
6H4	III	17	20	3	9	96	94	93

[†]The boldface represented the homologues mAb for both biotin labeled and unlabeled mAb as inhibitory mAb.

Table S4. Ag-mAb doses for PSA, PCA and induction of neutrophil CD16/32 down-regulation†

No. of reaction	Antigen – mAb‡	Antigen – mAb dose (µg-µg per mouse)§ for PSA	Antigen – mAb dose (µg-µg per mouse)§ for PCA: mAb priming i.d. followed by Ag i.v.	Antigen – mAb dose (µg-µg per mouse)§ for PCA: preformed IC i.d.	Antigen – mAb dose (µg-µg per mouse)§ for induction of neutrophil CD16/32↓ <i>in vivo</i>	Antigen – mAb concentration (µg/ml-µg/ml)§ for induction of neutrophil CD16/32↓ <i>in vitro</i>
1	OVM – 2C2 ¹	10 – 100§	500 – 20	2 – 20	10 – 100	10 – 100
2	OVM – 5G12 ¹	10 – 100	500 – 20	2 – 20	10 – 100	10 – 100
3	OVM – 6H4 ^{2a}	10 – 100	500 – 20	2 – 20	10 – 100	10 – 100
4	OVM – No.7 ¹ & 1A6 ¹	10 – 50 & 50	500 – 10 & 10	2 – 10 & 10	10 – 50 & 50	10 – 50 & 50
5	OVM – No.7 ¹ & 5B8 ¹	10 – 50 & 50	500 – 10 & 10	2 – 10 & 10	10 – 50 & 50	10 – 50 & 50
6	OVM – No.7 ¹ & No.9 ¹	10 – 50 & 50	500 – 10 & 10	2 – 10 & 10	10 – 50 & 50	10 – 50 & 50
7	HSA – 2E9 ¹ & 3A5 ¹	100 – 50 & 50	500 – 10 & 10	20 – 10 & 10	100 – 50 & 50	100 – 50 & 50
8	HSA – 2E9 ¹ & 4A2 ¹	100 – 50 & 50	500 – 10 & 10	20 – 10 & 10	100 – 50 & 50	100 – 50 & 50
9	HSA – 3A5 ¹ & 4A2 ¹	100 – 50 & 50	500 – 10 & 10	20 – 10 & 10	100 – 50 & 50	100 – 50 & 50
10	HSA – H1A6 ^{2b} & 3A11 ¹	100 – 50 & 50	500 – 10 & 10	20 – 10 & 10	Not done	100 – 50 & 50
11	HSA – H1A6 ^{2b} & 4A2 ¹	100 – 50 & 50	500 – 10 & 10	20 – 10 & 10	100 – 50 & 50	100 – 50 & 50
12	HSA – 1G3 ^{2a} & 4F12 ¹	100 – 50 & 50	500 – 10 & 10	20 – 10 & 10	100 – 50 & 50	100 – 50 & 50
13	MP1P – M4 ¹ & M12 ^{2b}	50 – 50 & 50	50 – 10 & 10	10 – 10 & 10	50 – 50 & 50	50 – 50 & 50
14	rHb zeta – 3H9 ¹ & 4D11 ¹	100 – 50 & 50	100 – 10 & 10	20 – 10 & 10	Not done	100 – 50 & 50
15	Hb zeta – 4D11 ¹ & 3H9 ¹	100 – 50 & 50; 200 – 100 & 100	500 – 10 & 10	20 – 10 & 10	100 – 50 & 50	100 – 50 & 50
16	HSA – 2E9 ¹	50 – 100; 100 – 100; 150 – 100; 500 – 500	500 – 20	20 – 20	100 – 100	100 – 100
17	HbA2 – 2H4 ¹ & 2C9 ¹	50 – 50 & 50; 100 – 50 & 50; 500 – 250 & 250	500 – 10 & 10	20 – 10 & 10	100 – 50 & 50	100 – 50 & 50
18	AOPP – 3F2 ¹ & 4C5 ^{2a}	50 – 50 & 50; 100 – 50 & 50; 500 – 250 & 250	500 – 10 & 10	20 – 10 & 10	100 – 50 & 50	100 – 50 & 50

19	OVA – 1D1 ¹	100 – 100; 200 – 100; 400 – 100	500 – 20	20 – 20	100 – 100	100 – 100
20	OVM – 1A6 ¹	10 – 100	500 – 20	2 – 20	10 – 100	10 – 100
21	OVM – 5B8 ¹	10 – 100	500 – 20	2 – 20	10 – 100	10 – 100
22	OVM – No.7 ¹	10 – 100	500 – 20	2 – 20	10 – 100	10 – 100
23	OVM – No.9 ¹	10 – 100	500 – 20	2 – 20	10 – 100	10 – 100
24	HSA – H1A6 ^{2b}	100 – 100; 200 – 200; 300 – 300	500 – 20	20 – 20	100 – 100	100 – 100
25	HSA – 1G3 ^{2a}	100 – 100; 200 – 200; 300 – 300	500 – 20	Not done	100 – 100	Not done
26	HSA – 3A5 ¹	300 – 300	500 – 20	20 – 20	100 – 100	100 – 100
27	HSA – 4A2 ¹	300 – 300	Not done	20 – 20	Not done	100 – 100
28	HSA – 4F12 ¹	300 – 300	500 – 20	20 – 20	Not done	100 – 100
29	rHb zeta – 3H9 ¹	100 – 100; 100 – 200; 100 – 300	100 – 20	20 – 20	Not done	100 – 100
30	rHb zeta – 4D11 ¹	100 – 100; 100 – 200; 100 – 300	100 – 20	Not done	Not done	100 – 100
31	MP1P – M4 ¹	50 – 100	50 – 20	10 – 20	50 – 100	50 – 100
32	MP1P – M12 ^{2a}	50 – 100	50 – 20	Not done	50 – 100	50 – 100
33	OVA – 3C10 ¹	100 – 100	500 – 20	20 – 20	100 – 100	Not done
34	OVA – 3H8 ^{2a}	100 – 100	500 – 20	20 – 20	100 – 100	100 – 100
35	OVA – 4C10 ¹	1000 – 300	500 – 20	20 – 20	100 – 100	100 – 100
36	OVA – 4F1 ¹	1000 – 300; 2000 – 1000	500 – 20	20 – 20	100 – 100	100 – 100

†Procedures for PSA, PCA, and induction of neutrophil CD16/32 down-regulation were described in methods.

‡Superscripts on mAb names indicate the IgG isotypes.

§Doses for Ag-mAb complexes were presented as Ag (µg or µg/ml)-mAb (µg or µg/ml). e.g., 10 – 100 in Reaction 1 of line 1 and rank 3 correlates to OVM 10µg – 2C2 100µg for induction of PSA.