

Aggregation makes a protein allergenic at the challenge phase of basophil-mediated allergy in mice

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Abstract

A hapten is a small molecule that is not immunogenic on its own but can stimulate the production of antibodies at the sensitization phase when conjugated to carrier proteins. The hapten then reacts specifically with the antibodies generated against it to elicit an immune or allergic response at the challenge phase. Here, we compared various carrier proteins conjugated with the same hapten in their ability to induce hapten-specific IgE-mediated allergic responses *in vitro* and *in vivo*, and characterized the nature of carrier proteins that determines the magnitude of response at the challenge phase of allergic reactions. Hapten 2,4,6-trinitrophenol (TNP)-conjugated ovalbumin (TNP-OVA) and bovine serum albumin (TNP-BSA) elicited TNP-specific, mast cell-dependent, immediate-type allergic reactions at a comparable level in mice that had been passively sensitized with TNP-specific IgE. In contrast, TNP-OVA but not TNP-BSA efficiently induced a basophil-dependent, IgE-mediated chronic allergic inflammation (IgE-CAI), even though both proteins could stimulate basophils *in vitro* at a comparable level. By comparing different carrier proteins and structurally modifying them, we found that the formation of large aggregates is crucial for TNP-conjugated carrier proteins to efficiently elicit IgE-CAI, regardless of the type of protein. Thus, the aggregation status of carrier proteins appears to determine the magnitude of allergic response at the challenge phase of hapten-specific IgE-CAI. Our findings suggest that the allergenicity of substances is a matter of importance not only at the sensitization but also at the challenge phase in a certain type of allergy including a basophil-mediated allergic inflammation.

Keywords: basophil, carrier protein, hapten, IgE, protein aggregation

Introduction

Allergy is considered as an unnecessary immune response to otherwise innocuous substances, including pollens, dust mites, food and medicine (1). Such substances are known as allergens. Allergic reactions typically occur when an individual who has produced IgE antibody in response to an allergen encounters the same allergen (2, 3). Therefore, allergic responses are often divided into two phases, namely the sensitization (or immunization) and challenge (or elicitation) phases (3–6). Once allergen-specific IgE is produced, mast cells and basophils bind IgE through the high-affinity IgE receptor FcεRI expressed on the cell surface. At the challenge phase, re-exposure to the allergen triggers activation of these cells by means of cross-linking of IgE-bound FcεRI, leading to the release of allergy-inducing substances including histamine and proteases (7–11).

A hapten is a small molecule that is not immunogenic on its own but can stimulate the production of antibodies when

conjugated to a larger molecule, most often a protein (carrier protein) (12, 13). The hapten then reacts specifically with the antibodies generated against it to elicit an immune or allergic response. Many drugs, such as penicillin, that cause allergic reactions including anaphylaxis are known to act as haptens (14). We previously developed the experimental system suitable for analysis of basophil function *in vivo* by using hapten 2,4,6-trinitrophenol (TNP)-specific IgE (15). Mice are passively sensitized with IgE by intravenous or intra-peritoneal administration of TNP-specific IgE and subsequently challenged with an intra-dermal injection of TNP-conjugated ovalbumin (TNP-OVA) in the ear skin. This induces three consecutive waves of ear swelling. The first two waves, early phase and late-phase ear swelling on day 1, are mast cell-dependent, immediate-type allergic reactions. The third-phase ear swelling, which starts on day 2 and peaks on day 4 post-challenge, is accompanied with massive infiltration of leukocytes, including

eosinophils, neutrophils, monocytes and macrophages. We designated this delayed-onset response IgE-mediated chronic allergic inflammation (IgE-CAI) (15). Of note, depletion of basophils but not mast cells abolishes the development of IgE-CAI, even though basophils account for only 1 or 2% of cellular infiltrates in the skin lesion, demonstrating that basophils but not mast cells play an essential role in IgE-CAI in contrast to the immediate-type ear swelling (15, 16). Thus, IgE-CAI is suitable for analyzing the challenge phase of the basophil-mediated allergic response.

In the present study, we examined whether the difference in carrier proteins has any impact on the challenge phase of hapten-specific IgE-mediated allergic reactions in the skin. We found that TNP-OVA and TNP-conjugated bovine serum albumin (TNP-BSA) behave differently at the challenge phase of TNP-specific IgE-mediated allergy, depending on the type of allergic reactions, in spite of fact that TNP-OVA and TNP-BSA showed a comparable ability to stimulate TNP-specific IgE-sensitized mast cells and basophils *in vitro*. We then addressed the question of what makes the difference between these proteins in terms of the allergenicity at the challenge phase.

Methods

Mice

C57BL/6J mice (7–9 weeks old) were purchased from Japan SLC. All animal studies were approved by the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University (Permission No. A2018-126A).

Antibodies

The following antibodies were purchased from BioLegend: biotinylated anti-CD49b (DX5), FITC-conjugated anti-F4/80 (BM8), PE-conjugated anti-CD200R3 (Ba13), APC-conjugated anti-Ly-6G (1A8), PE-Cy7-conjugated anti-CD45 (30-F11), Pacific Blue-conjugated anti-c-Kit (2B8) and anti-CD11b (M1/70). PE-conjugated anti-Siglec-F (E50-2440) and APC-conjugated anti-CD49b (HM α 2) were from BD Biosciences. IgE (IGELb4, ATCC-Tib141) specific to TNP and anti-CD16/32 (2.4G2) were prepared in our laboratory.

Modification of proteins to generate allergens reactive to anti-TNP IgE

OVA (Sigma-Aldrich), human serum albumin (HSA, Sigma-Aldrich), BSA (nacalai tesque), *N*-acetylglucosamine-conjugated BSA (GlcNAc-BSA, Dextra), DQ Green BSA (DQ-BSA, Thermo Fisher Scientific), bovine thyroglobulin (BTG, Sigma-Aldrich) and bovine γ -globulin (BGG, Sigma-Aldrich) were conjugated with hapten TNP as described previously (17). The valency of TNP in resulting proteins is within a range of 11–18. DyLight 633-conjugated proteins were prepared by incubating proteins with DyLight™ 633 NHS Ester (Thermo Fisher Scientific). Heat-aggregated proteins were generated by incubating proteins at 65 or 95°C for 1 h. To prepare large aggregates of proteins, proteins were first biotinylated by using EZ-Link™ Sulfo-NHS-Biotin (Thermo Fisher Scientific) and then incubated with avidin (Thermo Fisher Scientific). BSA- or TNP-BSA-coated beads were prepared

as described previously (18). Briefly, Polybead® Carboxylate Microspheres (3.0 μ m in diameter, Polysciences, Inc.) were activated and then incubated with BSA or TNP-BSA overnight. In some experiments, TNP-OVA was fractionated by size using Amicon Ultra-4 Centrifugal Filter Devices-50K (Merck) to obtain the fraction containing proteins <50 kD. The size of proteins was estimated by native-polyacrylamide gel electrophoresis (PAGE) (ATTO) under non-reducing conditions and Coomassie Brilliant Blue staining (nacalai tesque).

Generation and stimulation of bone marrow-derived basophils and mast cells

Bone marrow-derived basophils (BMBAs) were generated as described previously (19). In brief, mouse bone marrow cells were cultured with 300 pg ml⁻¹ of IL-3 (BioLegend) for 7 days, followed by purification of CD49b⁺ cells using IMag system with biotinylated anti-CD49b (DX5) and streptavidin-conjugated magnetic particles (BD Biosciences). To generate bone marrow-derived mast cells (BMMCs), mouse bone marrow cells were cultured with 10 ng ml⁻¹ of IL-3 and 15 ng ml⁻¹ of stem cell factor (BioLegend) for 4 weeks. BMBAs and BMMCs were sensitized with 1 μ g ml⁻¹ of anti-TNP IgE overnight and then stimulated with 300 ng ml⁻¹ of TNP-conjugated or unconjugated proteins, for 1 h (β -hexosaminidase assay) or 24 h (IL-6 assay). β -hexosaminidase release (%) was measured as described previously (20). IL-6 in cultured supernatant was detected using Mouse IL-6 ELISA MAX™ Standard (BioLegend).

Induction of immediate and delayed-onset allergic responses in the skin

Passive cutaneous anaphylaxis (PCA) was elicited as described previously (21). In brief, mice were locally sensitized with intra-dermal injection of 100 ng anti-TNP IgE or control phosphate-buffered saline (PBS) into the right and left ear, respectively. On the following day, mice were challenged with intravenous injection of 300 μ g TNP-OVA or TNP-BSA in 200 μ l of PBS containing 0.25% Evans blue dye. Ear thickness was measured at indicated time points after the challenge (Δ ear thickness = IgE – control PBS). For evaluation of microvascular permeability, ears were excised 30 min after the challenge and incubated in 0.7 ml formamide at 63°C overnight to extract the dye that had leaked into the skin. The amount of the dye in the extracts was determined by a spectrophotometer at 620 nm. IgE-CAI was induced as described previously (15). In brief, mice were sensitized with intravenous injection of 300 μ g anti-TNP IgE and challenged on the following day with intra-dermal injection of 10 μ g TNP-conjugated and unconjugated (control) proteins into the right and left ear, respectively. Ear thickness was measured at a 24-h interval for 7 days (Δ ear thickness = right – left). For evaluation of microvascular permeability, mice were treated with intravenous injection of 100 μ l PBS containing 0.5% Evans blue dye 3 days after the challenge. Two hours later, ears were excised and subjected to measurement of leaked dye.

Flow cytometric and histopathological analyses

For flow cytometric analysis, single cell suspensions were obtained from the ear skin by treatment with 150 U ml⁻¹

collagenase (Wako) at 37°C for 2 h. After pre-treatment with anti-CD16/32 (2.4G2) and normal rat serum to prevent the non-specific binding of irrelevant antibodies, cells were stained with indicated combination of antibodies for 30 min and analyzed by FACSCanto (BD Biosciences). Each cell lineage was defined as follows: hematopoietic cells (CD45⁺), basophils (c-kit⁺CD45^{int}CD49b⁺CD200R3⁺), eosinophils (SiglecF⁺SSC^{high}), neutrophils (Ly6G⁺CD11b⁺) and monocytes/macrophages (Ly6G[−]SiglecF-F4/80⁺CD11b⁺). For histopathological analysis, ear specimens were fixed and embedded in paraffin, and sections were stained with hematoxylin and eosin.

Protein uptake by bone marrow-derived macrophages

Bone marrow-derived macrophages (BMMφ) were generated as described previously with some modification (22). In brief, murine bone marrow cells were cultured for 7 days in the presence of 30% L929-cell conditioned medium as a source of macrophage colony-stimulating factor, and adherent cells were isolated and used as BMMφ. BMMφ were incubated with 300 ng ml^{−1} of DyLight-conjugated proteins at 37°C for 1 h, and the extent of protein uptake was measured by flow cytometry.

Statistical analysis

Comparisons between two groups were performed using unpaired Student's *t*-test. Comparisons between multiple treatment groups and a control group were performed using one-way analysis of variance with *post hoc* Tukey HSD (honestly significant difference) test. A *P* value <0.05 was considered statistically significant.

Results

TNP-OVA and TNP-BSA show a comparable ability to stimulate TNP-specific IgE-sensitized mast cells and basophils in vitro

To examine the possibility that parts of allergens, other than an epitope recognized by allergen-specific IgE, may have any impact on the effector phase of allergic responses, we compared carrier proteins, OVA and BSA, that were conjugated with hapten TNP (TNP-OVA and TNP-BSA) in their ability to elicit TNP-specific IgE-mediated allergic reactions. The valency of TNP in both proteins was almost comparable, within a range of 11–18. We first compared the ability of TNP-OVA and TNP-BSA to stimulate TNP-specific IgE-sensitized mast cells (BMMC) and basophils (BMBAs) *in vitro* (Fig. 1). As far as assessed by β-hexosaminidase release (Fig. 1A) and IL-6 secretion (Fig. 1B), TNP-OVA and TNP-BSA stimulated mast cells and basophils at a comparable level in a TNP-dependent manner, regardless of carrier proteins.

TNP-OVA but not TNP-BSA efficiently induces IgE-CAI in vivo even though both can elicit PCA at a comparable level

We next compared the ability of TNP-OVA and TNP-BSA to induce IgE-mediated allergic responses *in vivo*. To induce PCA that is a mast cell-mediated, immediate-type allergic

reaction, mice were locally sensitized with intra-dermal injection of TNP-specific IgE in the ear skin. On the following day, mice were challenged with intravenous injection of TNP-OVA or TNP-BSA in PBS containing Evans blue dye (Fig. 2). Both proteins elicited ear swelling at a comparable level with similar time courses (Fig. 2A). Moreover, both induced microvascular hyperpermeability to a comparable extent (Fig. 2B). Thus, no significant difference was observed between TNP-OVA and TNP-BSA in the induction of mast cell-mediated PCA.

We then examined the ability of both proteins to induce IgE-CAI that is a delayed-onset allergic reaction in the skin. A single intra-dermal administration of TNP-OVA, but not control OVA, in mice sensitized with TNP-specific IgE induces three consecutive waves of ear swelling as defined by Δear thickness (TNP-conjugated protein – unconjugated protein) (15): the first two waves (early- and late-phase ear swelling on day 1 post-challenge) are mast cell-dependent, immediate allergic reactions while the third one (IgE-CAI) is dependent on basophils and starts on day 2 and peaked on day 4. Of note, these allergic reactions, including IgE-CAI, are completely dependent on the passive sensitization with TNP-specific IgE in mice (15). In accordance with the observation in PCA, both TNP-OVA and TNP-BSA induced early phase ear swelling proceeding to IgE-CAI at a comparable level with similar time courses (Supplementary Figure S1). Unexpectedly, however, TNP-OVA and TNP-BSA behaved differently in the induction of IgE-CAI (Fig. 3). TNP-BSA elicited 3- or 4-fold weaker ear swelling and microvascular hyperpermeability compared to TNP-OVA (Fig. 3A–C and Supplementary Figure S2). Of note, the administration of even a 50 times higher dose (500 μg) of TNP-BSA failed to augment the IgE-CAI ear swelling (Supplementary Figure S3). The accumulation of hematopoietic cells, including basophils, eosinophils, monocytes/macrophages and neutrophils, was barely detected in the site of TNP-BSA injection in contrast to that of TNP-OVA injection (Fig. 3C and D). Thus, TNP-BSA induced little or no IgE-CAI-type inflammation unlike TNP-OVA.

Modification of TNP-BSA leading to its aggregation enhances its ability to elicit IgE-CAI

We sought to clarify the reason why TNP-OVA but not TNP-BSA efficiently elicited TNP-specific, basophil-mediated IgE-CAI response even though both could stimulate TNP-specific IgE-sensitized basophils *in vitro* at a comparable level. OVA is a glycoprotein, whereas BSA has no sugar (23, 24), suggesting the possible contribution of the sugar modification to the ability of proteins to elicit IgE-CAI. In accordance with this assumption, GlcNAc-conjugated TNP-BSA induced ear swelling of IgE-CAI as efficiently as TNP-OVA (Fig. 4A). Interestingly, the conjugation of DQ, a fluorescent chemical, to TNP-BSA also showed a similar enhancing effect (Fig. 4B), suggesting that the enhancing effect on the IgE-CAI induction is not restricted to the sugar modification. Of note, the conjugation of DyLight, another fluorescent chemical, to TNP-BSA showed little or no enhancing effect (Fig. 4C) in contrast to the DQ conjugation, demonstrating that the chemical modification of TNP-BSA does not necessarily augment its ability to elicit the IgE-CAI response.

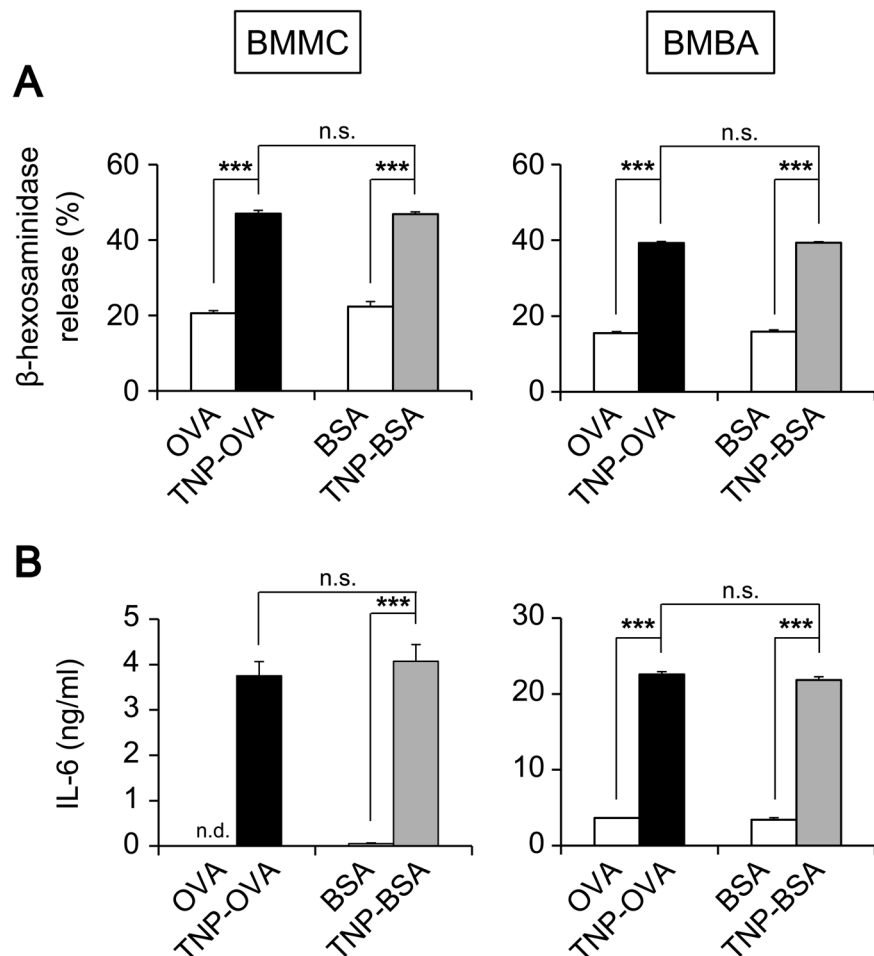


Fig. 1. TNP-OVA and TNP-BSA show a comparable ability to stimulate TNP-specific IgE-sensitized mast cells and basophils *in vitro*. Anti-TNP IgE-sensitized BMMCs and BMBAs were incubated with TNP-conjugated or unconjugated proteins (OVA or BSA) for 1 h (A) or for 24 h (B) at 37°C and subjected to the β -hexosaminidase assay (mean $\pm$ SEM, $n = 3$ each) in (A) or the IL-6 production assay (mean $\pm$ SEM, $n = 3$ or 4 each) in (B). Data shown are representative of three independent experiments. n.d., not detected; n.s., not significant; *** $P < 0.001$.

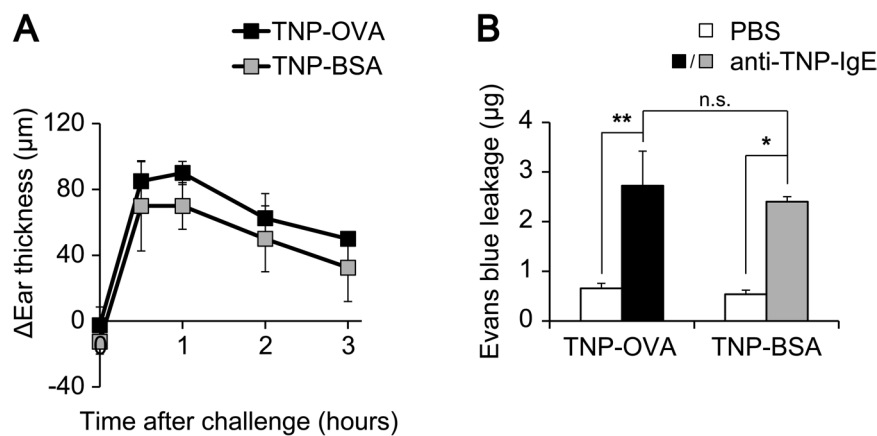


Fig. 2. TNP-OVA and TNP-BSA can elicit PCA *in vivo* at a comparable level. Mice were locally sensitized with intra-dermal injection of anti-TNP IgE or control PBS in the ear skin. On the following day, mice were challenged with intravenous injection of TNP-conjugated or unconjugated proteins (OVA or BSA) in PBS containing Evans blue dye. (A) Time course of ear swelling (Δ ear thickness = IgE – control PBS) is shown (mean $\pm$ SEM, $n = 4$ each). (B) Ears were excised 30 min after the challenge and subjected to the measurement of dye content (mean $\pm$ SEM, $n = 4$ or 5 each). Data shown in (A) and (B) are representative of at least three independent experiments. n.s., not significant; * $P < 0.05$; ** $P < 0.01$.

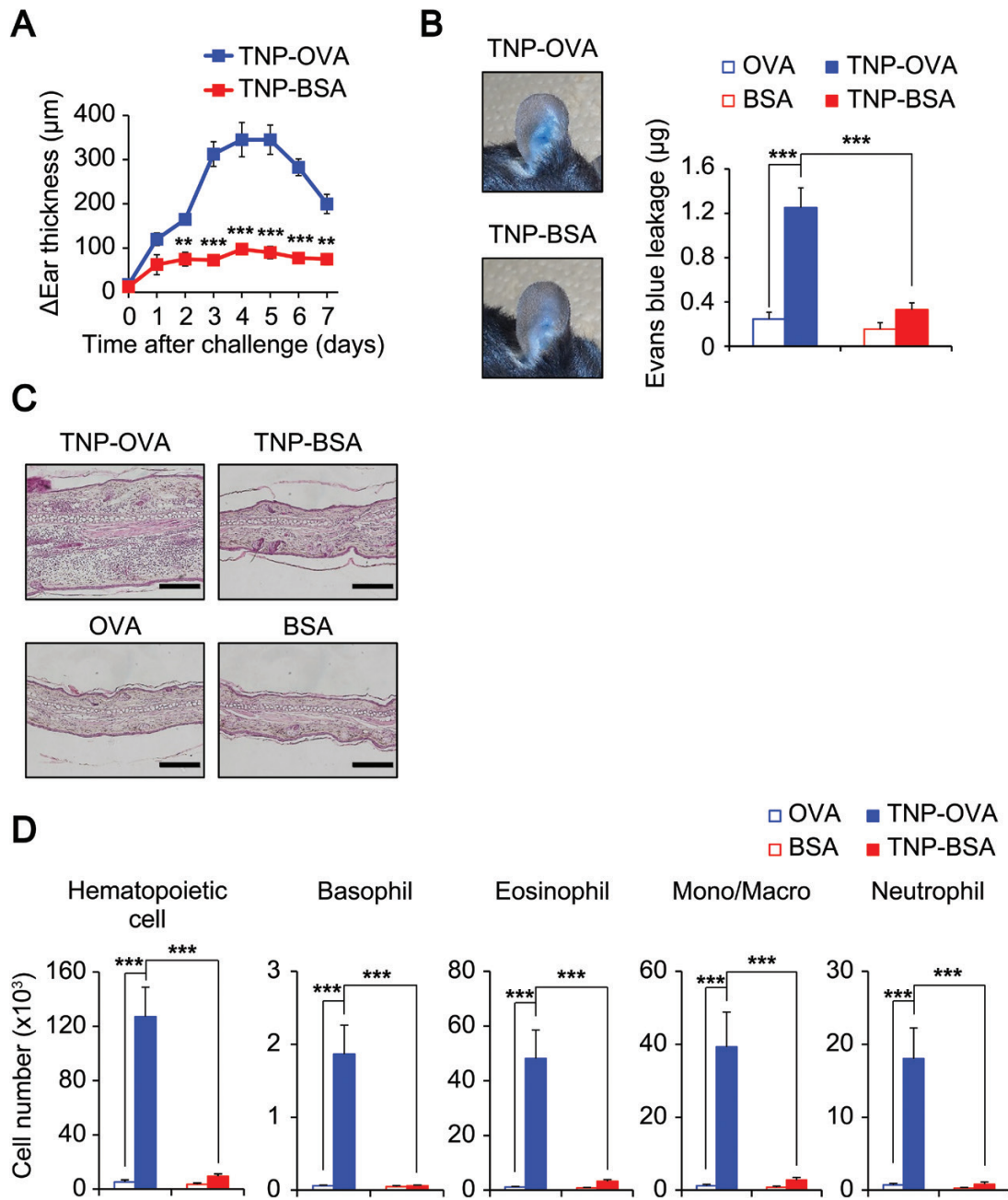


Fig. 3. TNP-BSA elicits much weaker IgE-CAI response *in vivo* than does TNP-OVA. To induce IgE-CAI, mice were passively sensitized with anti-TNP IgE and then challenged with intra-dermal injection of TNP-conjugated or unconjugated proteins (OVA or BSA). (A) Time course of ear swelling (Δ ear thickness = TNP-conjugated protein – unconjugated protein) is shown (mean $\pm$ SEM, $n = 4$ each). (B) Mice were treated with intravenous injection of PBS containing Evans blue dye on day 3 post-challenge. Two hours later, ears were excised and subjected to measurement of leaked dye (mean $\pm$ SEM, $n = 4$ each). (C) Sections of ear specimens isolated on day 3 post-challenge were stained with hematoxylin and eosin. Scale bars, 200 μm . (D) The numbers of each cell lineage accumulated in the ear skin on day 3 post-challenge are shown (mean $\pm$ SEM, $n = 4$ each). Data shown in (A–D) are representative of at least three independent experiments. ** $P < 0.01$; *** $P < 0.001$.

To address the question of what makes this difference between native and modified TNP-BSA, we compared their electrophoretic mobility in native-PAGE (Fig. 4D). All the proteins examined showed multiple bands corresponding to monomers and various multimer forms. Of note, TNP-GlcNAc-BSA and TNP-DQ-BSA but not TNP-BSA or TNP-DyLight-BSA showed a band near the gel top.

TNP-DQ-BSA also showed a smeared band >250 kD. The bands near the gel top were invisible when the proteins were analyzed with SDS-PAGE (data not shown), suggesting that high molecular forms of TNP-GlcNAc-BSA and TNP-DQ-BSA in native-PAGE are large aggregates of proteins. Thus, the formation of large aggregates appeared to correlate well with the ability to elicit the IgE-CAI response. If this is the case, forced formation

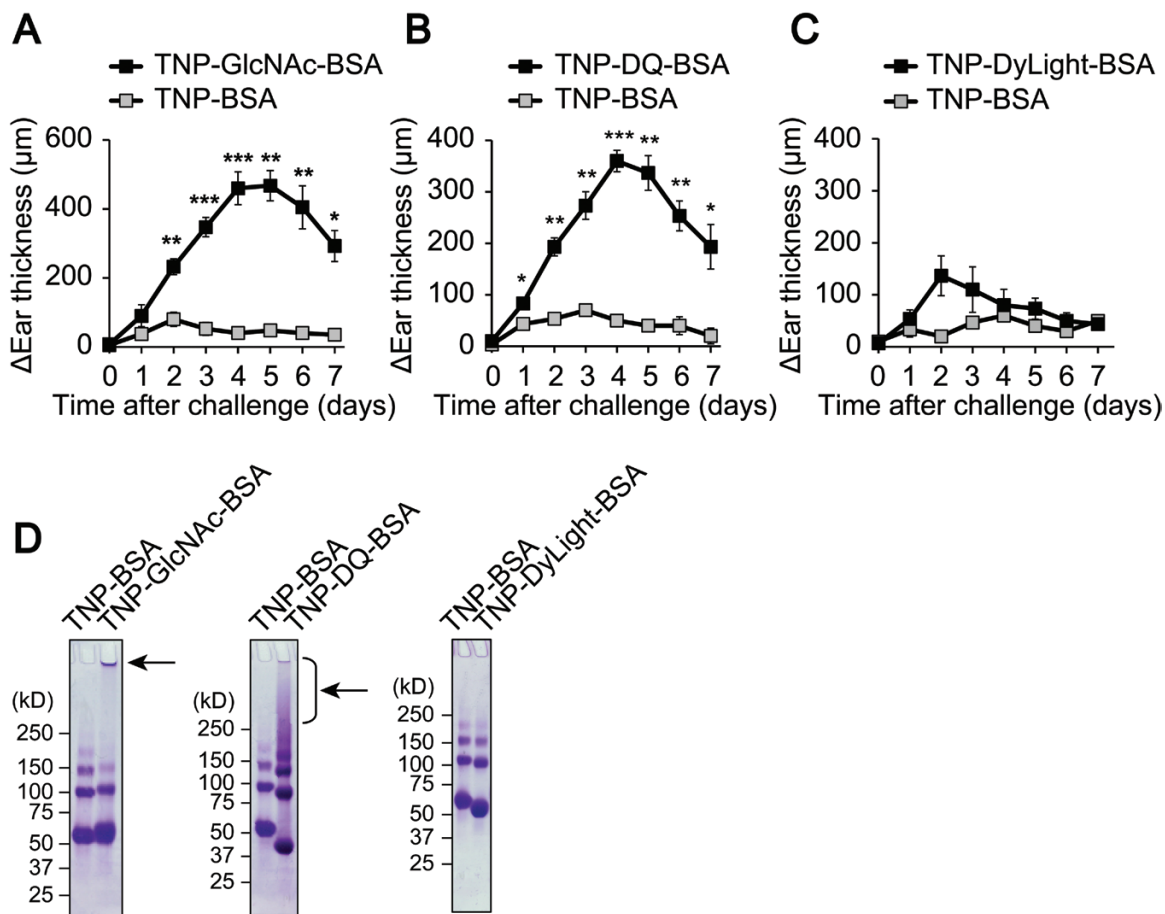


Fig. 4. Some but not other modifications of TNP-BSA enhance its ability to elicit IgE-CAI. (A–C) Mice were passively sensitized with anti-TNP IgE and then challenged with intra-dermal injection of TNP-conjugated or unconjugated proteins (BSA, GlcNAc-BSA, DQ-BSA or DyLight-BSA). Time courses of ear swelling (Δ ear thickness = TNP-conjugated protein – unconjugated protein) are shown (mean $\pm$ SEM, $n = 3$ or 4). (D) Indicated TNP-conjugated proteins were resolved on native-PAGE, followed by Coomassie Brilliant Blue staining. Arrows indicate high-molecular-weight forms of proteins. Data shown in (A–D) are representative of at least three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

of large aggregates should confer the ability on TNP-BSA. Indeed, heat treatment of TNP-BSA at 65 and 95°C enhanced the ability of TNP-BSA to elicit the IgE-CAI response moderately and strongly, respectively, in parallel with a degree of aggregate formation (Fig. 5A and B), even though heat treatment of TNP-BSA showed no apparent impact on the ability to stimulate basophils *in vitro* (Supplementary Figure S4). It was noted that heat-treated control (unconjugated) BSA was completely unable to elicit the IgE-CAI response, and that heat-treated TNP-BSA failed to elicit the IgE-CAI response in mice that had not been sensitized with TNP-specific IgE (data not shown). These results suggest that this IgE-CAI response is mediated through reaction between TNP-specific IgE and its antigen TNP.

It is well known that heat treatment renders proteins denatured (25). Of note, the formation of large aggregates through biotinylation of TNP-BSA followed by incubation with avidin also showed the enhancing effect (Fig. 5C), suggesting that denaturation of proteins may not be the main reason why heat treatment of TNP-BSA enhanced the ability of TNP-BSA to elicit the IgE-CAI response. Moreover, the conjugation of TNP-BSA

to polystyrene beads (3 μm in diameter) also enhanced the ability of TNP-BSA to elicit the IgE-CAI response (Fig. 5D), implying that the formation of a particulate including large self-aggregation may be a key factor to determine the ability of carrier proteins to elicit the IgE-CAI response.

TNP-conjugated proteins containing high-molecular-weight forms appear to elicit IgE-CAI

The observations in the experiments using the modified TNP-BSA prompted us to examine the possibility that the difference between TNP-OVA and TNP-BSA in the ability to elicit the IgE-CAI response can be attributed to the different degree of aggregation of proteins. In native-PAGE, TNP-OVA but not TNP-BSA showed a band near the gel top (Fig. 6A) as observed in TNP-GlcNAc-BSA (Fig. 4D). Elimination of high-molecular-weight forms of TNP-OVA by using centrifuge filtration (Fig. 6B) resulted in reduced ability to elicit the IgE-CAI response (Fig. 6C). Taken together, the high-molecular-weight fraction of TNP-OVA appears to be responsible for the elicitation of the IgE-CAI response.

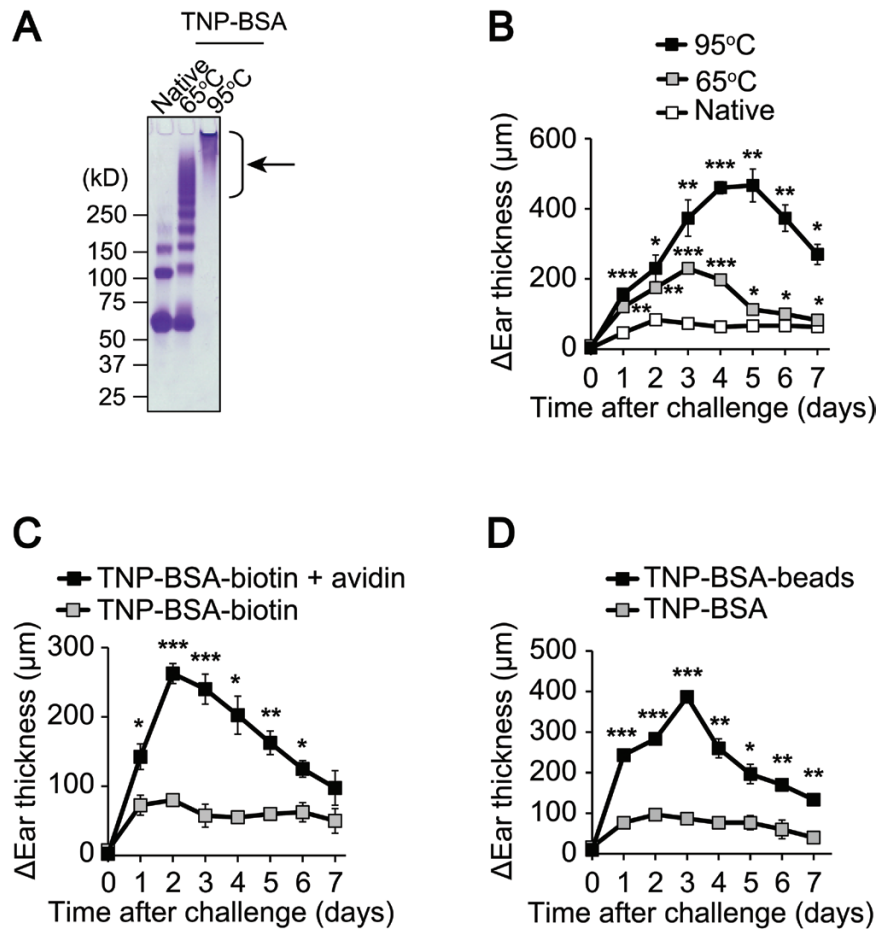


Fig. 5. Modification of TNP-BSA leading to its aggregation appears to enhance its ability to elicit IgE-CAI. (A, B) TNP-BSA was incubated at 65 or 95°C for 1 h. Native and heat-treated TNP-BSA were analyzed for their apparent size using native-PAGE in (A) and for their ability to elicit IgE-CAI in (B) (mean $\pm$ SEM, $n = 3$ or 4). An arrow indicates aggregated high-molecular-weight forms of proteins. (C) Proteins (biotin-conjugated BSA or biotin-BSA-avidin complex) with or without TNP conjugation were analyzed as in (B) (mean $\pm$ SEM, $n = 4$). (D) TNP-conjugated or unconjugated BSA-coated beads were analyzed as in (B) (mean $\pm$ SEM, $n = 3$). Data shown in (A–D) are representative of at least three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

We then examined a panel of TNP-conjugated proteins other than OVA and BSA in terms of their electrophoretic mobility in native-PAGE (Fig. 6D) and their ability to elicit the IgE-CAI response (Fig. 6E–G). TNP-BTG and TNP-BGG but not TNP-HSA showed bands near the gel top and induced IgE-CAI efficiently compared to TNP-BSA. Thus, TNP-conjugated proteins forming large aggregates appeared to be able to elicit the IgE-CAI response.

Discussion

The allergenicity of substances is commonly defined by their ability to induce IgE production at the sensitization phase of immune responses (26, 27). Therefore, it has been generally thought that once hapten-specific IgE has been produced at the sensitization phase, hapten-specific allergic response can be readily elicited at the challenge phase by hapten-conjugated carrier proteins, irrespective of the type of proteins. In the present study, we demonstrated that this notion is not always the case, and the aggregation status of carrier proteins determines the magnitude of allergic response

at the challenge phase of hapten-specific IgE-CAI. Thus, our findings suggest that the allergenicity of substances is not restricted to the sensitization phase and is also a matter of importance at the challenge phase of a certain type of allergy.

We found that TNP-OVA and TNP-BSA behave differently at the challenge phase of TNP-specific IgE-mediated allergy in the skin, depending on the type of allergic reactions, in spite of the fact that TNP-OVA and TNP-BSA showed a comparable ability to stimulate mast cells and basophils *in vitro*. Both proteins elicited TNP-specific, mast cell-mediated, immediate-type allergic responses at a comparable level in mice sensitized with TNP-specific IgE, whereas TNP-OVA but not TNP-BSA efficiently induced TNP-specific, basophil-mediated IgE-CAI. What makes this difference between TNP-OVA and TNP-BSA *in vivo* in mast cell- versus basophil-mediated allergic responses, both of which are specific to TNP? Distinct anatomical localizations of mast cells and basophils may account for this difference (8). Mast cells are skin-resident cells, and therefore IgE-armed mast cells can be readily stimulated with TNP-conjugated proteins that are delivered into the skin. In contrast, basophils circulate in the peripheral

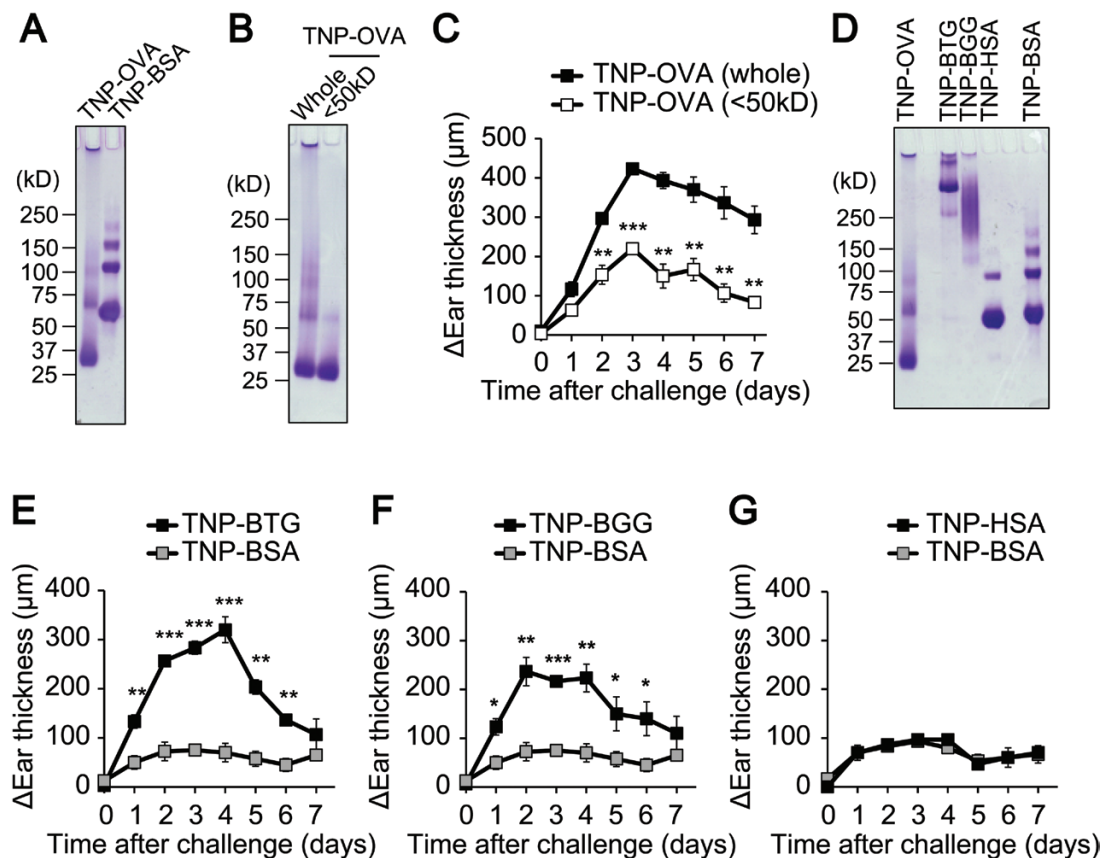


Fig. 6. TNP-conjugated proteins containing high-molecular-weight forms appear to efficiently elicit IgE-CAI. (A) TNP-OVA and TNP-BSA were analyzed by using native-PAGE. (B, C) TNP-OVA proteins were size-fractionated to obtain the fraction containing proteins with a molecular weight <50 kD. The whole and <50 kD fractions were analyzed by native-PAGE in (B) and for their ability to elicit IgE-CAI in (C) (mean $\pm$ SEM, $n = 3$ each). (D) TNP-conjugated proteins (OVA, BTG, BGG, HSA and BSA) were subjected to native-PAGE analysis. (E–G) TNP-conjugated or unconjugated proteins as indicated were analyzed for their ability to elicit IgE-CAI (mean $\pm$ SEM, $n = 3$ or 4). Data shown in A–G are representative of at least three independent experiments. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

blood under homeostatic conditions and migrate into the skin of the allergen injection site to elicit IgE-CAI. Intra-dermal administration of TNP-BSA induced little or no infiltration of leukocytes including basophils in mice sensitized with TNP-specific IgE, in contrast to TNP-OVA administration. Of note, intra-dermal administration of either TNP-BSA-coated polystyrene beads or heat-treated TNP-BSA in TNP-IgE-sensitized mice induced significant infiltration of leukocytes including basophils (Supplementary Figure S5, not all data shown) in association with ear swelling. These results suggest that the difference in the induction of IgE-CAI among various TNP-conjugated carrier proteins may stem from the difference in their ability to trigger the recruitment of basophils to the skin rather than the ability to activate basophils.

The present study revealed that carrier proteins forming large aggregates, irrespective of type of proteins, either at a native or artificially modified form, can elicit IgE-CAI, implying that a highly aggregated form of proteins may be able to trigger basophil recruitment to the skin. Previous studies reported that particulate antigens are more efficiently endocytosed by phagocytic cells than soluble antigens, and therefore function as a better inducer of antibody production at the sensitization phase (28, 29). In accordance with

this, we found that heat-treated TNP-DyLight-BSA was much more efficiently taken up by macrophages than TNP-DyLight-BSA, in parallel with their aggregation status (Supplementary Figure S6). Phagocytic cells, such as macrophages, dendritic cells and neutrophils, are known to produce chemotactic factors in response to various stimuli (30–32). Considering the tight association between the aggregation status of carrier proteins and their ability to induce IgE-CAI, one may assume that the aggregation status of carrier proteins determines the antigenicity of hapten-conjugated proteins at the challenge phase of the hapten-specific IgE-CAI response, perhaps because of differential uptake by phagocytic cells that regulate basophil recruitment to the skin. Alternatively, but not mutually exclusively, highly aggregated proteins may stay longer in the skin than do soluble ones, and hence have more chance to encounter and activate skin-infiltrating basophils, leading to the IgE-CAI response.

In conclusion, we demonstrated in the present study that carrier proteins play an important role in determining the magnitude of allergic response at the challenge phase of basophil-mediated IgE-CAI, in spite of the fact that the allergic response is specific to the hapten but not carrier proteins. Our findings cast new light on the definition of allergenicity

that has commonly been considered as the ability of substances to induce IgE production at the sensitization phase of immune responses. The allergenicity at the challenge phase is also a matter of importance in basophil-mediated allergy. Thus, the allergenicity at both sensitization and challenge phases needs to be taken into account in the preparation of reagents for medical use, including therapeutic protein products.

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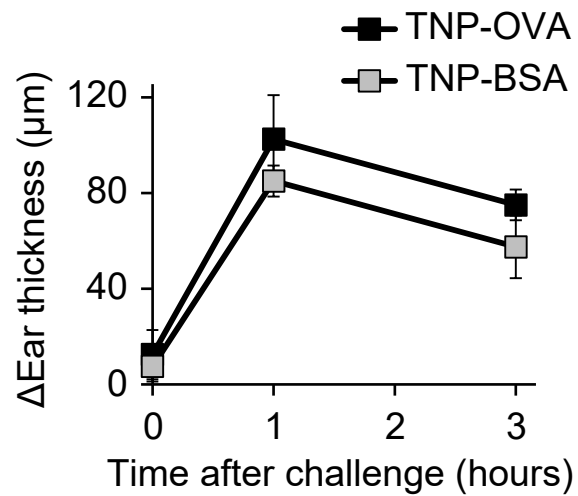
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Conflicts of interest statement: the authors declare no conflicts of interest.

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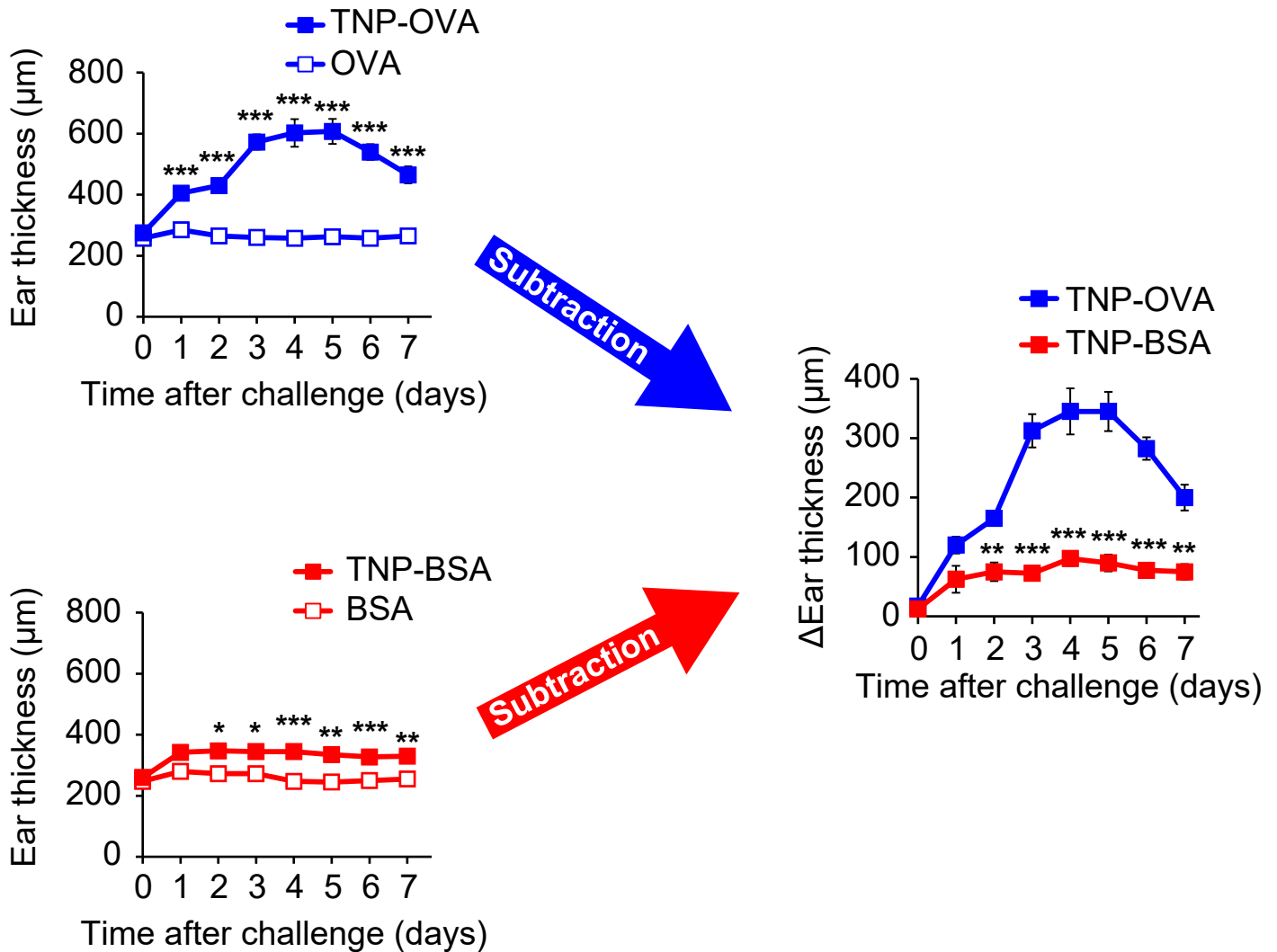
Supplementary Figure S1



Supplementary Figure S1. TNP-OVA and TNP-BSA can elicit the immediate ear swelling, that precedes the IgE-CAI response, at a comparable level

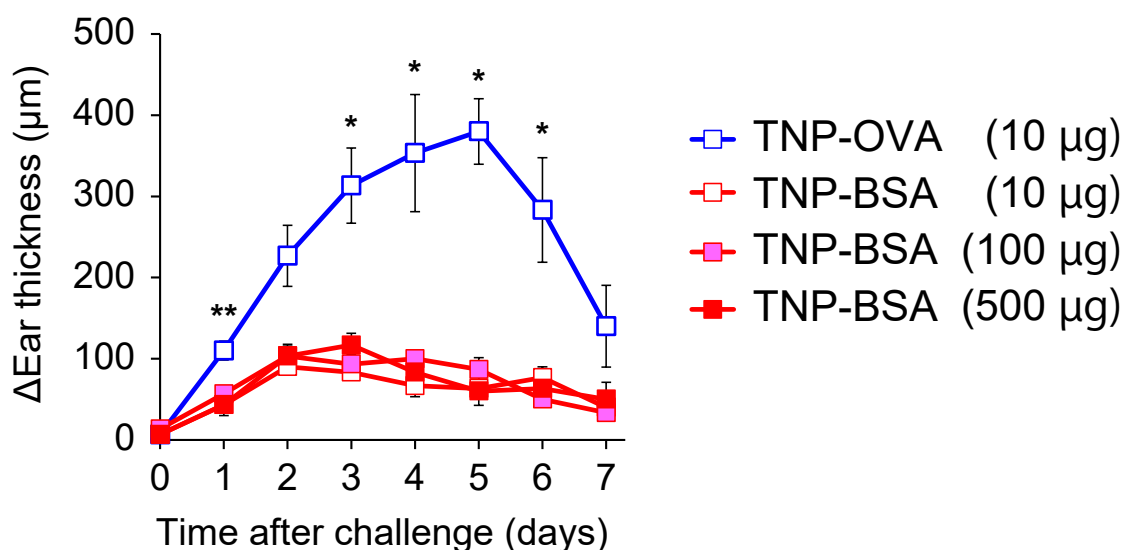
Mice were treated as in Fig. 3 to elicit IgE-CAI. Ear swelling (Δ ear thickness = TNP-conjugated protein - unconjugated protein) was measured just before and at the indicated time points after the allergen challenge (mean $\pm$ SEM, n=4 each). Data shown are representative of three independent experiments.

Supplementary Figure S2



Supplementary Figure S2. TNP-BSA elicits IgE-CAI response much more weakly than does TNP-OVA while unconjugated OVA and BSA are completely unable to do so Mice were treated as in Fig. 3 to elicit IgE-CAI. Time course of the actual thickness of ears injected with indicated proteins is shown in the left panels. Δ ear thickness was calculated as the subtraction between the actual thickness of ears injected with TNP-conjugated and unconjugated proteins, and is shown in the right panel (mean $\pm$ SEM, n=4 each). Data shown are representative of at least three independent experiments. *p<0.05; **p<0.01; ***p<0.001.

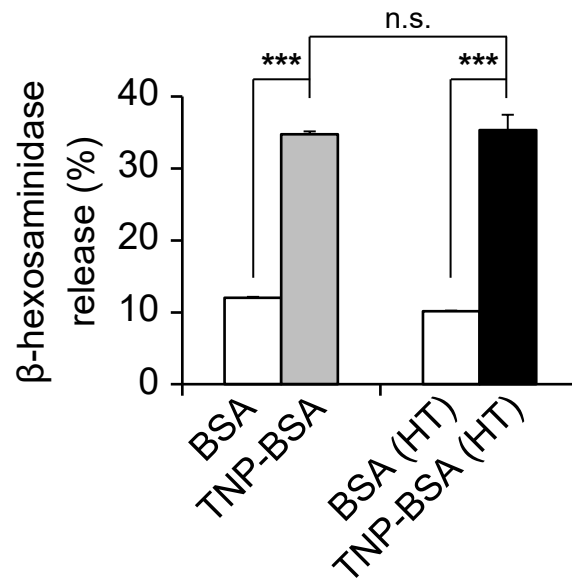
Supplementary Figure S3



Supplementary Figure S3. Excessive doses of TNP-BSA still fail to induce sufficient IgE-CAI response *in vivo*

Mice were treated with indicated doses of TNP-conjugated or unconjugated proteins (OVA or BSA) to induce IgE-CAI as in Figure 3. Time course of ear swelling (Δ ear thickness = TNP-conjugated protein - unconjugated protein) is shown (mean $\pm$ SEM, $n=3$ each). Data shown are representative of three independent experiments. * $p<0.05$; ** $p<0.01$.

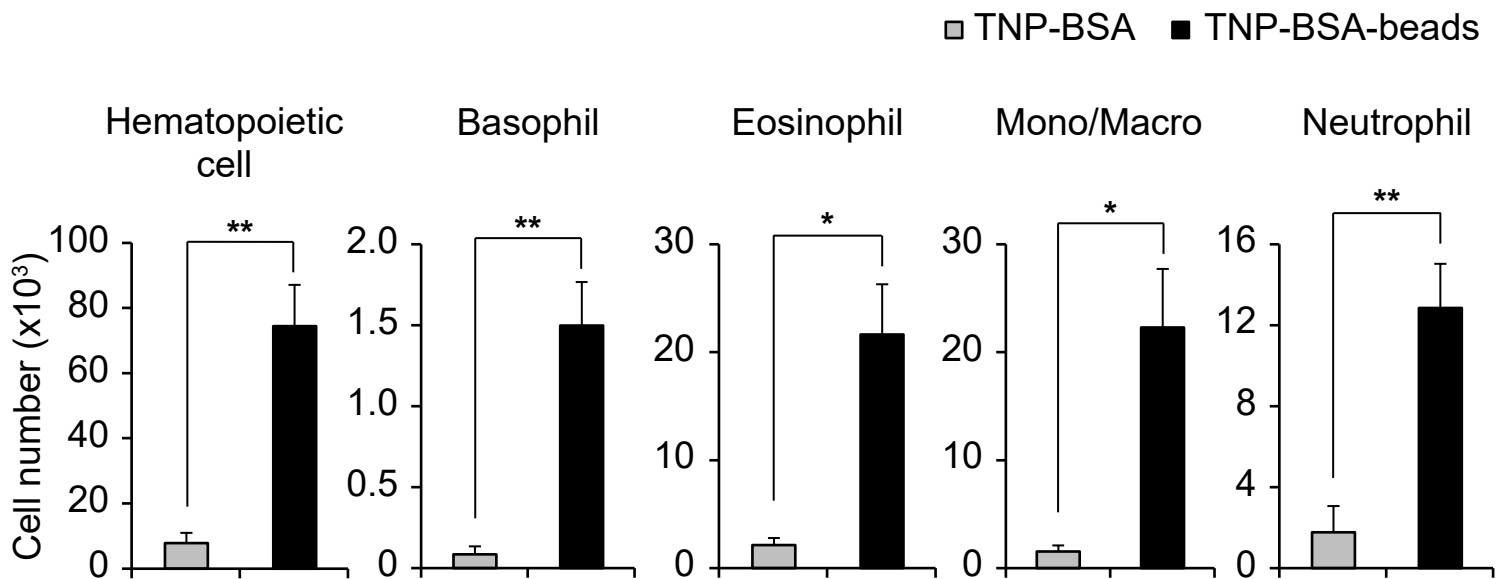
Supplementary Figure S4



Supplementary Figure S4. Native and heat-treated TNP-BSA can stimulate basophils *in vitro* at a comparable level

Anti-TNP IgE-sensitized BMBAAs were incubated with native or heat (95°C)-treated (HT) proteins (BSA and TNP-BSA) at 37°C for 1 hr and then subjected to β-hexosaminidase release assay (mean ± SEM, n=4 each). Data shown are representative of three independent experiments. n.s., not significant; ***p<0.001.

Supplementary Figure S5

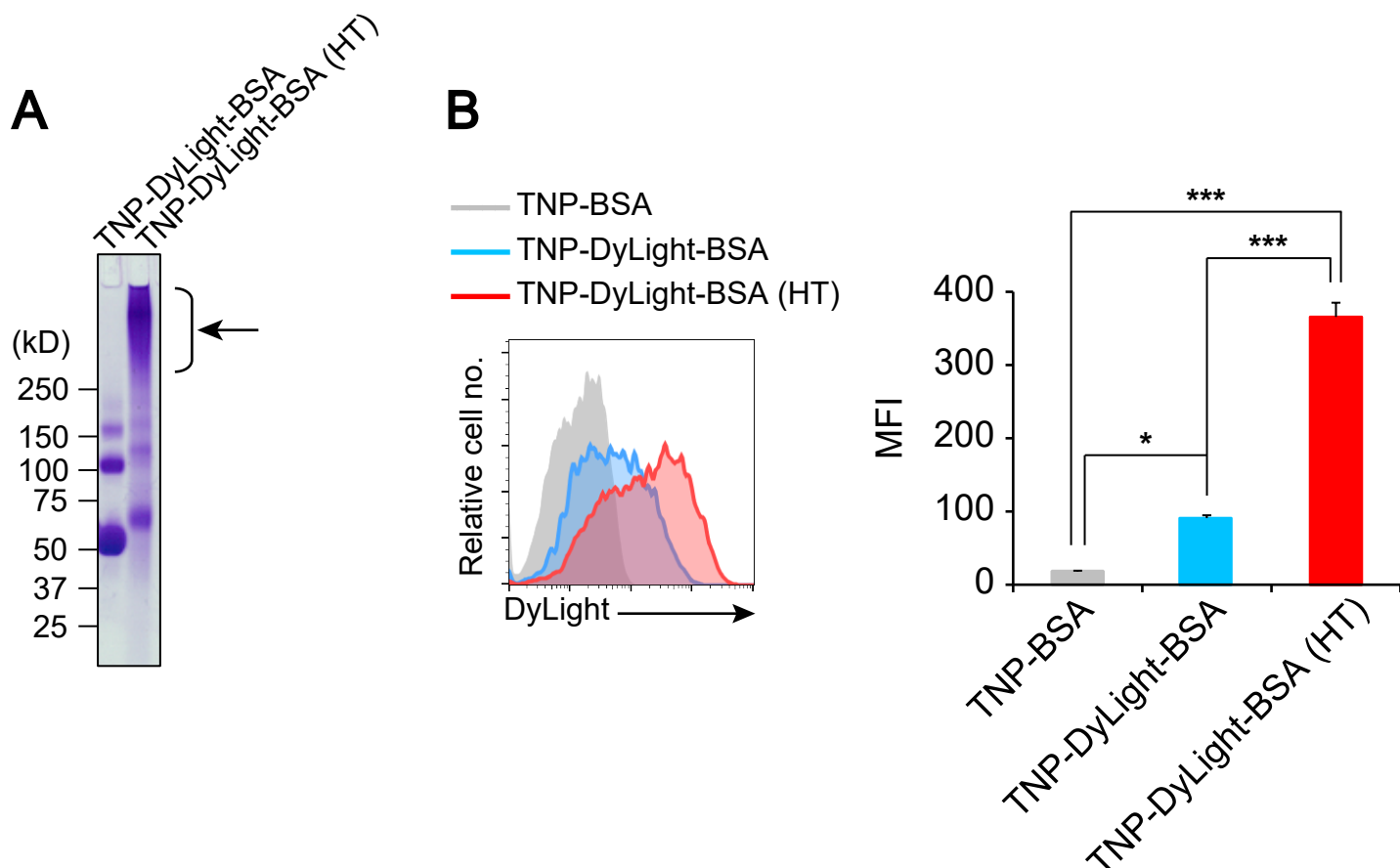


Supplementary Figure S5. TNP-BSA-coated beads but not TNP-BSA itself efficiently elicit leukocyte infiltration in the IgE-CAI skin lesion

Mice were passively sensitized with anti-TNP IgE and then challenged with intradermal injection of TNP-BSA (gray bars) or TNP-BSA-coated polystyrene beads (black bars). The numbers of indicated cell lineages accumulated in the ear skin on day 3 post-challenge are shown (mean $\pm$ SEM, n=5 each). Data shown are representative of at least three independent experiments.

*p<0.05; **p<0.01.

Supplementary Figure S6



Supplementary Figure S6. Heat-treated TNP-DyLight-BSA is more efficiently taken up by macrophages than native TNP-DyLight-BSA

(A) Native and heat-treated (at 95°C for 1 hr) TNP-DyLight-BSA were resolved on native-PAGE as in Figure 4D. An arrow indicates aggregated high molecular weight forms of proteins. (B) BMMΦ were incubated with 300 ng/ml TNP-BSA (gray histogram), TNP-DyLight-BSA (blue histogram) or heat-treated TNP-DyLight-BSA (red histogram) at 37°C for 1 hr. The extent of protein uptake was measured by flow cytometry (left panel). All the data are summarized in the right panel (mean $\pm$ SEM of mean fluorescence intensity (MFI), $n=3$ each). Data shown in A and B are representative of at least three independent experiments. * $p<0.05$, *** $p<0.001$.