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Soluble Antigen Prevents Generation of High-Affinity Memory B-Cell Precursors During the Germinal-Center Response

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Research Article

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Posted Date: November 25th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-11831149/v1

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Additional Declarations:
The authors declare no competing interests.

This study was approved by the Institutional Animal Ethics Committee (IAEC) of University of North Bengal (Protocol No.: IAEC/NBU/2025/04).

Soluble Antigen Prevents Generation of High-Affinity Memory B-Cell Precursors During the Germinal-Center Response

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Abstract

We assessed the splenic B-cell repertoire of unimmunized C57BL/6 mice to identify B cells producing relatively high-affinity antibodies to the hapten (4-hydroxy-3-nitrophenyl) acetyl (NP). Splenocytes were driven to IgG1 production by polyclonal stimulation with Escherichia coli lipopolysaccharide together with interleukins-2, -4, and -5 on 3T3 feeder layers. High-affinity, bivalent IgG1 responses were selectively detected by ELISA using lightly haptenated NP-conjugates as the capture antigen. Naïve spleens contained very few clonable high-affinity anti-NP B cells, whereas primary T-dependent immunization produced approximately 10^5 such precursors per spleen within two weeks. A single intraperitoneal dose (1 mg) of freshly deaggregated soluble NP2–human serum albumin (HSA) almost completely prevented development of clonable anti-NP IgG1 precursors when given before immunization and remained effective when administered up to six days after challenge. Soluble HSA alone produced only a partial effect; NP coupled to unrelated carriers gave inconsistent results. These observations indicate that continuing T-cell help is required for memory B-cell formation and that recently activated T and B cells can be functionally silenced in vivo by appropriately formulated soluble toleragen.

Keywords: memory B cells; immunological tolerance; germinal center; affinity maturation; T-cell help; hapten–carrier (NP–HSA); soluble antigen

Introduction

Affinity maturation in T-dependent antibody responses generates B cells whose receptors bind antigen with greatly increased strength. This process relies on somatic hypermutation of immunoglobulin variable-region genes and largely takes place within germinal centers (1–8). Because tolerance of the primary B-cell repertoire can be readily induced (9–12), it is important to ask how the secondary (memory) B-cell compartment is protected against potentially self-reactive variants that arise by random mutation during the germinal-center reaction (13). Soluble, deaggregated proteins have been used as model toleragens, and prior work demonstrated that such agents can block memory B-cell generation in a dose-dependent manner, acting on both T and B cells in adoptive-transfer models (14,15). Hapten–carrier systems provide a useful way to dissect T- and B-cell contributions because hapten-specific B cells require carrier-specific T-cell help for T-dependent antibody responses

(16–18). The NP response in C57BL/6 mice is particularly amenable to these analyses: a dominant VH gene and λ light chain are frequently used, facilitating study of V-gene hypermutation and affinity maturation, and high- versus low-affinity clones can be distinguished by ELISA using lightly versus heavily haptenated conjugates (22,23). Here, mice were immunized with heavily haptenated NP–HSA and tolerance was attempted using lightly haptenated, freshly deaggregated NP–HSA, HSA alone, or NP conjugated to irrelevant carriers. We quantified clonable, high-affinity anti-NP IgG1 precursors and examined the time window during which soluble toleragen could prevent memory cell formation, including administration after challenge immunization when germinal-center activity and initial V-gene mutation are underway.

Materials and Methods

Animals, antigens, and immunization

Specific-pathogen-free male C57BL/6 WEHI mice (8–10 weeks at first immunization) were used. Challenge immunizations comprised 100 μg alum-precipitated NP–HSA administered intraperitoneally together with 10^9 killed Bordetella pertussis organisms. Some groups received a single intraperitoneal dose of soluble antigen at times ranging from 7 days before to 13 days after challenge. Soluble antigen preparations were freshly deaggregated by centrifugation at 15,000 × g for 10 min. Blood samples were obtained serially from the orbital venous plexus for serum antibody assays.

NP conjugates NP conjugates were prepared as described previously (23). Briefly, NP-succinimide ester was dissolved in N,N-dimethylformamide at 20 mg/ml and coupled to carriers (BSA, HSA, HoSA, and pigeon heart cytochrome c) prepared at 5 mg/ml in 3% $NaHCO_3$. Low-density conjugates (≈2 NP groups per carrier; NP2-carrier) used 10–20 μg NP-succinimide per mg protein; high-density conjugates (≈18 NP groups per carrier; NP18-carrier) used ~800 μg/ml NP-succinimide. Reactions ran 2 hours at room temperature with rotation, then conjugates were dialyzed extensively against 3% $NaHCO_3$ and finally into mouse-tonicity phosphate-buffered saline (MT-PBS, pH 7.2) before determination of conjugation ratios. High-substitution NP–HSA was used for challenge immunizations; low-substitution NP conjugates served as toleragens.

Lymphokines recombinant interleukin-4 (rIL-4) and interleukin-5 (rIL-5) were obtained from monkey COS cell culture supernatants following transient transfection with appropriate constructs, and were used at final concentrations of 0.4% (vol/vol) and 2% (vol/vol) in culture, respectively. Highly purified human rIL-2 (E. coli–derived) was added at 10 units/ml.

Cell preparation and culture for antibody-forming cell precursor (AFCP) determination Five thousand BALB/c 3T3 fibroblasts were seeded per well in flat-bottom 96-well microtiter plates. Culture medium was RPMI 1640 containing 100 μM 2-mercaptoethanol and 5% fetal calf serum. Splenocytes were prepared, erythrocytes lysed with 0.83% NH_4Cl, and damaged/dead cells removed. Cells were plated in 200 μl/well of medium containing E. coli

0111:B4 LPS at 20 μg/ml plus rIL-2, rIL-4, rIL-5. To estimate IgG1 anti-NP precursor frequency, limiting-dilution cultures were set up with 300–10,000 splenocytes per well with 48 replicate wells per cell input; to examine cloning efficiency, cultures with 2.5–20 splenocytes per well (48 replicates) were used. Two spleens were pooled per test group. Cultures incubated at 37°C in 10% CO2/90% air for 7 days.

Antibody assays of serum samples and clonal supernatants ELISAs were used to detect IgM and IgG1 in culture supernatants and serum (29). U-bottom 96-well polyvinyl chloride plates were coated with affinity-purified sheep anti-mouse immunoglobulin or coated with NP2-BSA or NP18-BSA at 20 μg/ml in MT-PBS for antigen-specific assays. Culture supernatants were transferred to precoated plates, incubated overnight at room temperature, and developed after washing with horseradish peroxidase–conjugated anti-mouse immunoglobulin specific for IgM or IgG1 for 4 hours at room temperature. Substrate was ABTS at 0.55 mg/ml in 0.1 M citric acid with 0.1% H2O2 for 1 hour; absorbance was read at 414 nm with 492 nm reference. Serum anti-NP titers were measured in duplicate serial dilutions starting at 1:1,000 for anti-IgG1 NP2 and 1:10,000 for anti-IgG1 NP18.

Statistical analysis ELISA standardization used serial dilutions of myeloma proteins (MOPC 104E for IgM, MOPC 21 for IgG1) on each plate and hyperimmune anti-NP KLH as the antigen-specific standard. A cubic spline curve fit across standard points converted optical densities to antibody content. Frequencies of immunoglobulin-secreting precursors were estimated by Poisson analysis using maximum likelihood estimators for regression analyses (30,31).

Results

Soluble NP2–HSA reduces serum anti-NP IgG1 titers after NP18–HSA challenge Mice received 1 mg or 100 μg of deaggregated NP2–HSA, NP18–HSA, or no injection and were challenged 7 or 4 days later with alum-adsorbed NP18–HSA plus B. pertussis. Serum anti-NP IgG1 measured on NP2-BSA–coated plates showed dose-dependent reduction by NP2–HSA, with ~100-fold lowering at 1 mg when toleragen was given prior to challenge (Fig. 1). NP18–HSA failed to act effectively as a toleragen, possibly because of residual aggregation or rapid clearance, and was omitted from subsequent toleragen experiments. When 1 mg NP2–HSA was administered at various times after challenge and antibody levels measured 14 days after challenge, toleragen given up to 6 days after immunization caused at most ~4-fold lowering of high-affinity serum titers; administration at 8 or 13 days had no significant effect.

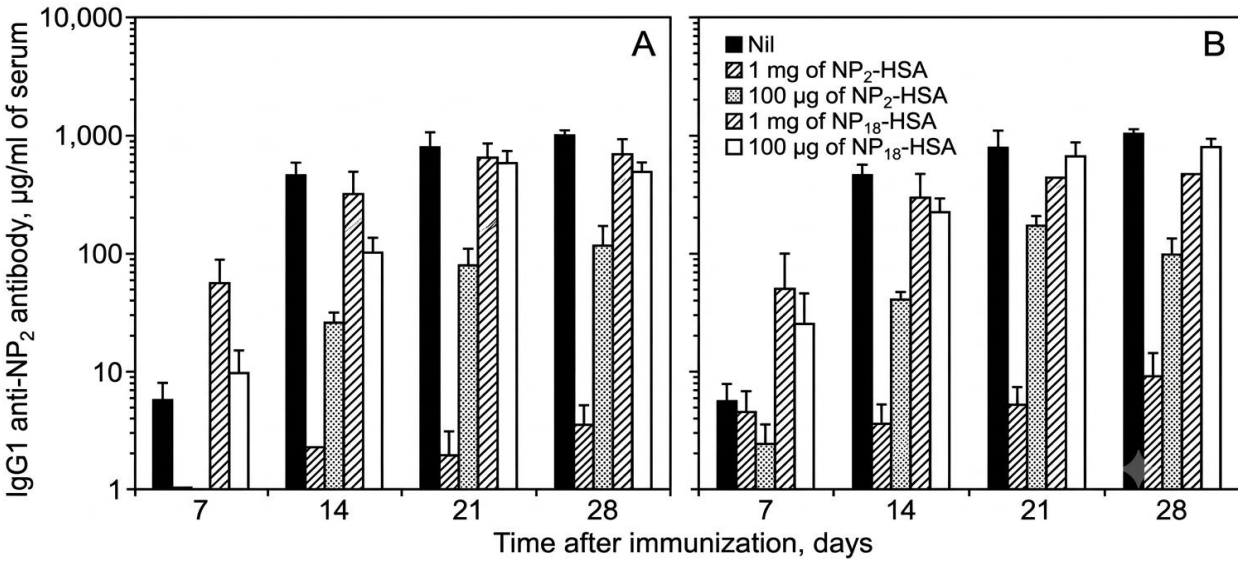


Figure 1. Serum IgG1 binding to NP2-BSA at multiple time points after challenge immunization. Mice were given soluble NP–HSA either 7 days (panel A) or 4 days (panel B) before immunogenic challenge. Error bars show standard deviations.

Appearance of higher-affinity anti-NP IgG1-forming precursors after immunization and suppression by preinjection of soluble antigen Limiting-dilution culture and selective ELISA for bivalent IgG1 binding to NP2- or NP18-BSA revealed very few high-affinity anti-NP B cells in naïve spleens (0, 93, 126, and 240 per spleen in four experiments). Following immunization with NP18–HSA, clonable high-affinity anti-NP IgG1 AFCPs rose significantly from day 5, peaking at day 14. Preinjection of 1 mg NP2–HSA four days before challenge reduced AFCP numbers substantially, particularly after day 6, with peak response AFCPs falling to ~2% of control values (Fig. 2). The tolerance effect was dose-dependent, but even 10 µg toleragen produced a significant reduction (Fig. 3).

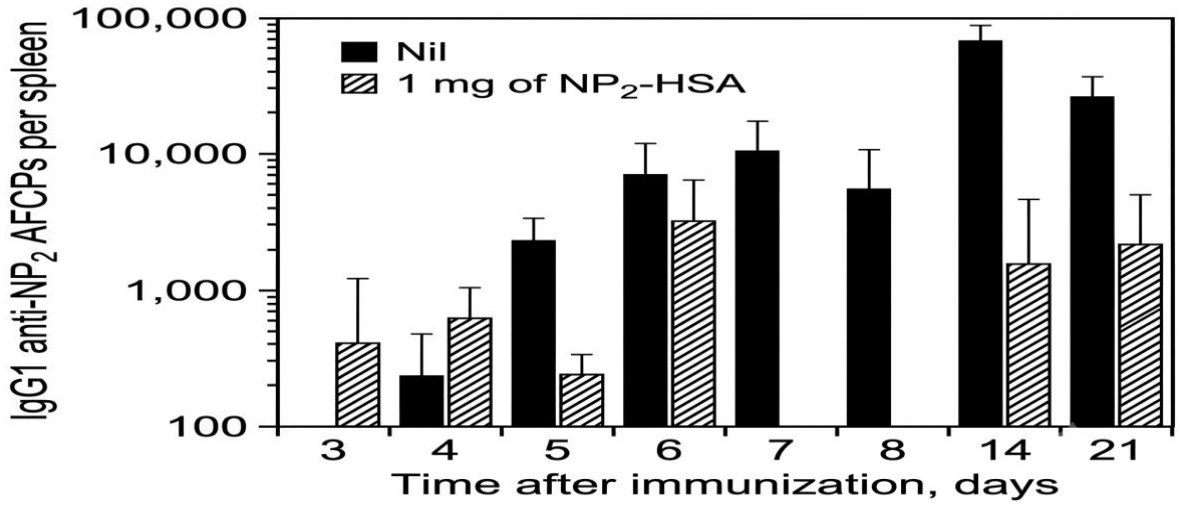


Figure 2. Effect of a 1 mg dose of soluble NP2–HSA given 4 days prior to immunization on splenic anti-NP2-BSA IgG1 AFCPs measured at multiple time points after challenge. Error

bars show 95% confidence limits. No solid bar at day 3 and hatched bars at days 7 and 8 indicate absence of detectable AFCPs.

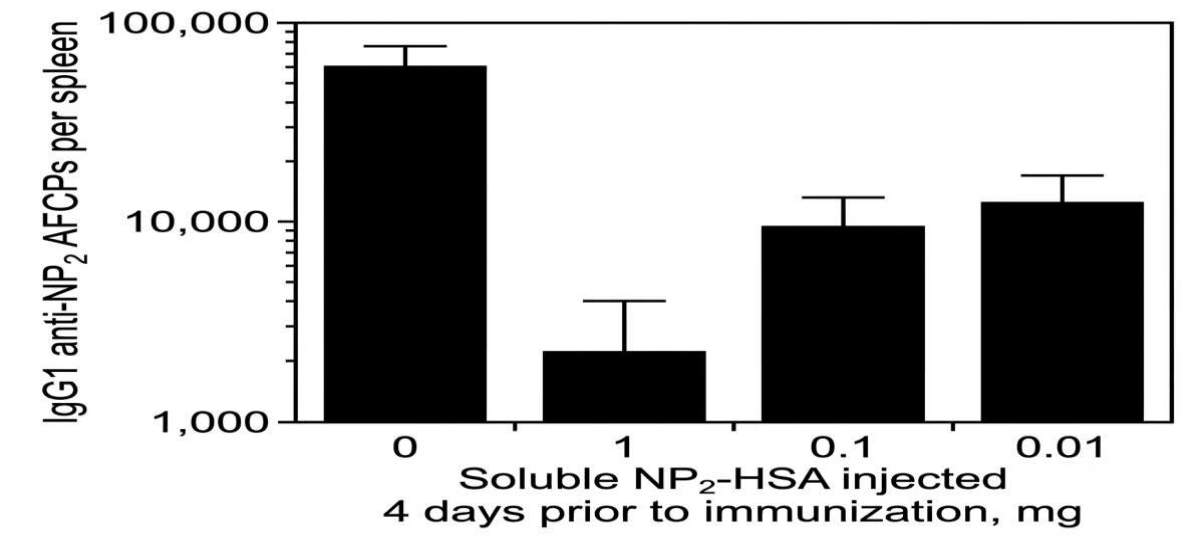


Figure 3. Dose–response of tolerance induced by NP2–HSA preinjection. Data points show group means; vertical bars indicate 95% confidence intervals.

Soluble antigen induces tolerance when given up to six days after challenge To mimic sudden emergence of potentially self-reactive B cells, NP2–HSA was administered at various times after immunization. Injection 6 days after NP18–HSA challenge significantly reduced anti-NP2 AFCP numbers measured at day 14, with both 1 mg and 100 µg producing marked suppression comparable to preinjection (Fig. 4). This strongly indicates that generation of

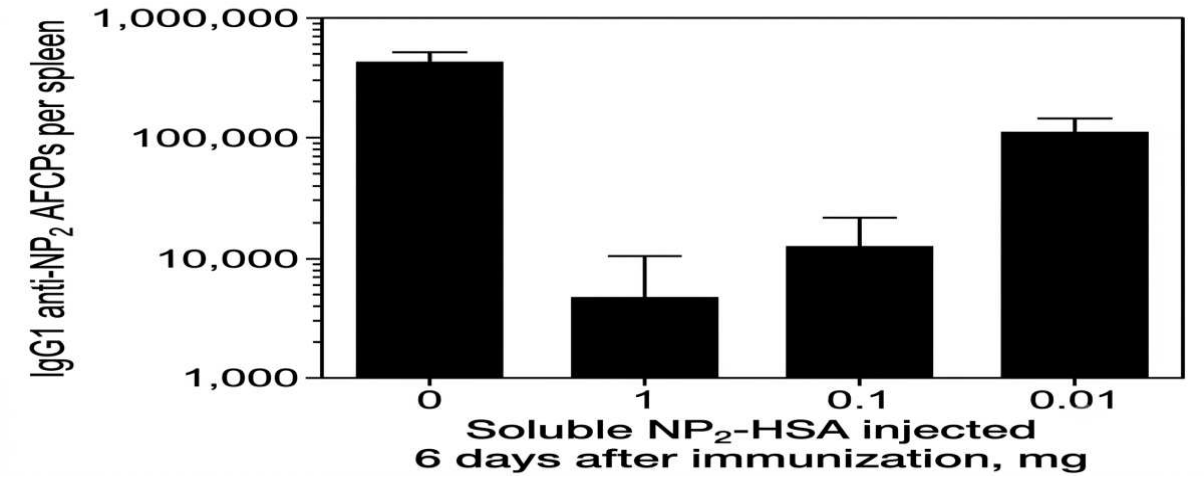


Figure 4. Dose-dependent effects of tolerance induction following NP2-HSA administration six days after challenge immunization. Vertical error bars represent the 95% confidence intervals.

AFCPs is especially susceptible to tolerization during this window. Repeating the 6-day, 1 mg NP2–HSA regimen in ten independent experiments consistently produced large reductions: control AFCPs averaged 76,100 ± 44,900 (higher-affinity, NP2-binding) and 502,000 ±

248,000 (lower-affinity, NP18-binding) per spleen, whereas tolerant groups averaged 4.6% ± 2.5% and 37% ± 12% of those control values, respectively. Effect of timing of toleragen administration Varying the time of toleragen injection after challenge showed effectiveness up to six days post-immunization; administration at day 8 produced variable reduction (one of two experiments), and injection at day 10 (four days before sacrifice) produced a marked booster effect, increasing AFCP numbers ~10-fold (Fig. 5).

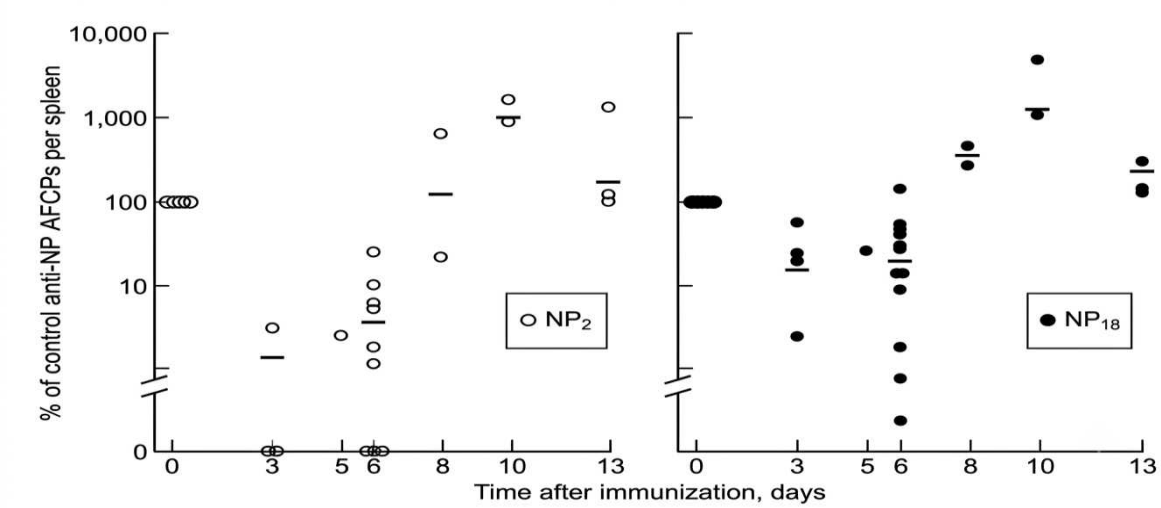


Figure 5. Influence of administering 1 mg of NP2-HSA at different time points following challenge immunization. All mice were sacrificed 14 days post-immunization. The numbers of high-affinity (left panel) and low-affinity (right panel) anti-NP-BSA IgG1 antibody-forming cells (AFCs) were expressed relative to those observed in the untreated control group. Horizontal lines indicate the normalized geometric mean values.

Maximal tolerance after challenge requires hapten and carrier identical to the immunogen
To dissect whether post-challenge toleragen acted primarily on T cells or B cells, mice were injected 6 days after challenge with 1 mg of various freshly deaggregated toleragens: NP2–HSA, HSA alone, NP conjugated to other carriers (NP3–HoSA, NP8–Cyt-c, NP3–Cyt-c). NP2–HSA consistently produced strong tolerance; HSA alone induced partial tolerance in two experiments but was less effective than NP2–HSA. NP coupled to other carriers produced tolerance only inconsistently (one of two experiments for NP3–HoSA), whereas NP8–Cyt-c and NP3–Cyt-c were ineffective (Fig. 6). These findings suggest a major contribution of loss of carrier-specific T-cell help when HSA is tolerized, but maximal suppression requires a toleragen capable of acting on both carrier-specific T cells and hapten-specific B cells.

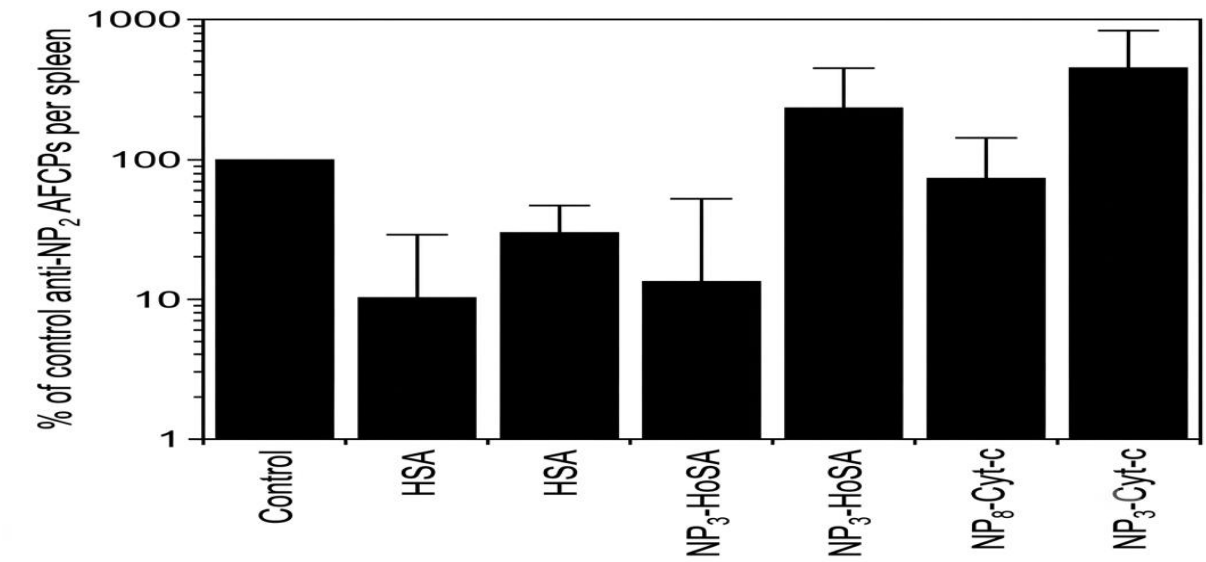


Figure 6. Effects of administering the carrier protein alone or different NP conjugates six days after challenge immunization on the number of anti-NP antibody-forming cells (AFCs). Each column represents an independent experimental result. The values were normalized against the corresponding positive control group (challenge immunization without soluble antigen) within each experiment. NP2-HSA served as the negative control in all experiments and consistently demonstrated tolerance induction, similar to the observations presented in Figure 5.

Discussion

Our data document two key findings. First, soluble, freshly deaggregated NP2–HSA can markedly lower primary anti-NP IgG1 antibody formation and, more strikingly, strongly block the generation of clonable memory B-cell precursors even when administered after immunogenic challenge. Second, the time window during which this post-challenge tolerization is effective extends to roughly six days after immunization — a period coincident with the onset of somatic hypermutation within germinal centers (first mutations appearing around day 6–7 and extensive mutation during the second week) (6,20,21,34).

The pronounced suppression of AFCPs by toleragen given up to day 6 suggests that continuing T-cell help is required for the build-up of the memory compartment and that recently activated T cells can be functionally silenced by soluble toleragen. That HSA alone produced partial suppression implies a substantial role for abrogation of carrier-specific T-cell help. However, NP2–HSA, capable of engaging both NP-specific B-cell receptors and carrier-specific T cells, produced deeper tolerance, indicating additive effects from direct action on B cells and on T-cell help. Why does the effect decline after day 6–8? By day 7 antibody is already abundant and soluble antigen could be rapidly sequestered on follicular dendritic cells and other accessory elements, possibly rendering it immunogenic rather than tolerogenic (as suggested by the booster effect observed when toleragen was given at day

10). Alternatively, processes that commit activated B cells to memory fate may have progressed beyond stages that are susceptible to modulation by soluble antigen.

These observations bear on mechanisms that prevent autoimmunity arising during somatic hypermutation. Memory B-cell generation in germinal centers appears to require ongoing T-cell support; consequently, a B cell that fortuitously acquires self-reactivity during mutation would lack necessary T-cell help unless corresponding T-cell populations were themselves autoreactive. The superior potency of NP2–HSA relative to HSA alone further supports the concept that direct tolerogenic signals to B cells can contribute to eliminating potentially self-reactive variants. Nonetheless, the occasional emergence of highly mutated autoantibodies in disease indicates that other pathways — such as presentation of intracellular particulate self-antigens in immunogenic contexts, defects in B-cell or T-cell tolerance, or molecular mimicry — can circumvent these safeguards (37,38).

Author contributions

AKS: Data curation, project administration, conceptualization, methodology, investigation, formal analysis, supervision, visualization, writing—original draft, writing—review & editing, validation, resources.

AD: Data curation, project administration, conceptualization, visualization, writing—review & editing.

Funding

No specific funding was received for this work.

Conflict of interest

The authors declare no commercial or financial conflicts of interest.

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