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B Cell Competition for Restricted T Cell Help Suppresses Rare-Epitope Responses

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SUMMARY

The immune system responds preferentially to particular antigenic-epitopes contained within complex immunogens, such as proteins or microbes. This poorly understood phenomenon, termed ‘‘immunodominance,’’ remains an obstacle to achieving polyvalent immune responses against multiple anti-genic-epitopes through vaccination. We observed profound suppression in the hapten-specific antibody response in mice immunized with hapten-protein conjugate, mixed with an excess of protein, relative to that in mice immunized with hapten-protein alone. The suppression was robust (100-fold and 10-fold with a 10- or 2-fold excess of protein, respectively), stable over a 6-log range in antigen dose, observed within 10 days of vaccination, and resistant to boosting and adjuvants. Furthermore, there were reduced frequencies of antigen-specific germinal-center B cells and long-lived bone-marrow plasma cells. The mechanism of this ‘‘antigen-competition’’ was mediated largely by early access to T-helper cells. These results offer mechanistic in-sights into B cell competition during an immune response and suggest vaccination strategies against HIV, influenza, and dengue.

Graphical Abstract

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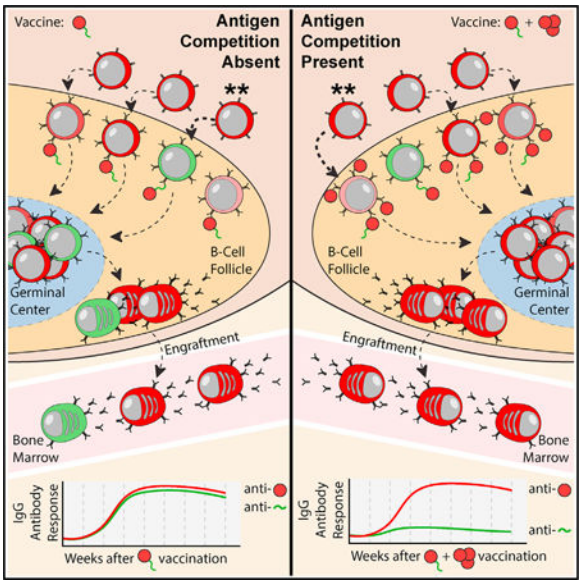
AUTHOR CONTRIBUTIONS

Conceptualization, M.C.W. and B.P.; Methodology, M.C.W. and B.P.; Formal Analysis, M.C.W.; Investigation, M.C.W., E.H.K., and W.L.; Writing – Original Draft, M.C.W.; Writing – Review & Editing, B.P.; Visualization, M.C.W.; Super-vision, B.P.; Funding Acquisition, M.C.W. and B.P.

DECLARATION OF INTERESTS

The authors declare no competing interests.



In Brief

Vaccination success depends on the immune system's ability to produce antibodies against highly specific pathogen targets, but the rules for how targets are selected remains poorly understood. Woodruff et al. describe a competition for resources among antibody producers that selects the cells, and thus targets, of the ensuing response.

INTRODUCTION

Germinal centers (GCs) are dynamic microenvironments providing infrastructure in the generation of high-affinity humoral responses (De Silva and Klein, 2015). GCs are highly organized, containing multiple hematopoietic and stromal subsets (Cremasco et al., 2014; Heesters et al., 2014) responsible for the selection and maturation of naive B cells. Through iterative cell-division (Ersching et al., 2017), somatic hypermutation (Gitlin et al., 2014), affinity testing (Anderson et al., 2009), survival factor competition (Wensveen et al., 2016), and GC re-entry (McHeyzer-Williams et al., 2015), individual B cell clones compete for inclusion into the final humoral responses critical for host protection. Each of these topics has attracted intense investigation, and recent studies have identified the fate of individual clones through GC selection with increasing resolution (Kuraoka et al., 2016; Tas et al., 2016). Applying these principles to a vaccination setting can be challenging, but great strides are being made in the generation of robust humoral responses through next-generation adjuvant development and vaccine delivery technology (Irvine et al., 2015; Kasturi et al., 2011; Liu et al., 2014). In addition, systems vaccinology promises to dig deeply into the mechanisms of human vaccine success (Pulendran, 2009), and our understanding of how to stimulate multiple branches of immunity for more complete host protection continues to grow (Pulendran and Ahmed, 2006).

Despite these advances, developing a vaccine designed to elicit epitope-specific responses has remained extremely challenging. It has become increasingly clear in diverse pathogens

such as HIV (Kwong et al., 2002), influenza (Henry et al., 2017), and dengue (Flipse and Smit, 2015) that achieving robust responses against subdominant epitopes within a single protein (e.g., HIV Env) or a mix of related proteins (e.g., dengue sero-types) is critical for the elicitation of effective protective immunity. Immunodominance has long plagued the vaccine community in guiding humoral response (Dale et al., 2017), and diverse approaches such as surface epitope masking (Angeletti et al., 2017) and ex-vivo stimulation (Sanjuan Nandin et al., 2017) have been attempted to address this problem. In many of these cases, however, the desired response epitope is ‘‘protected’’ in some way, making naive B cell targeting difficult (Kwong et al., 2002). This differential access to antigen is inherently understood to put these B cells at some sort of competitive disadvantage; however, the exact nature of that disadvantage remains poorly described. Further, work from Schwickert et al. (2011) has suggested that antigen-specific B cells can be denied access to the germinal center altogether, suggesting that early competition between B cells may have a large influence on eventual epitope targeting. By understanding the principles of that competition, we might leverage them toward promoting desired responses in a vaccine setting.

To this end, this study investigates the role of relative access to antigen by naive B cells on developing humoral responses. Using a simplified subunit vaccination system, it investigates the ability of an initiating immune response to identify and respond against low-frequency epitopes, and documents early competition among naive B cells in accessing T cell help. It details the long-term consequences of early B cell competition, and stresses the importance of vaccine design that elicits the correct response, and not simply the biggest. Finally, it discusses the application of these findings to currently used human vaccine strategies, and identifies potential ways forward in developing vaccines that make use of B cell competition to prompt humoral immune targeting of designer epitopes.

RESULTS

Low-Frequency Epitope Responses Are Suppressed in Complex Vaccine Settings

Understanding the capacity of an adaptive immune response to respond against low-frequency epitopes may be a critical step in the development of epitope-directed vaccines. To directly assess this capacity, a subunit hapten-carrier vaccination model was developed. The model makes use of the carrier protein rabbit serum albumin (RSA) conjugated to fluorescein isothiocyanate (FITC). Vaccinating C57BL/6 mice intra-muscularly (IM) in the calf with RSA-FITC at a 1:1 conjugation ratio (RSA-$FITC_{(1)}$), using a 1:1 alum suspension as an adjuvant, predictably results in a reliable IgG1 response against both carrier (RSA) and hapten (FITC) (Figure 1A). Surprisingly, adding 103 unconjugated RSA to the vaccine formulation, while keeping the RSA-$FITC_{(1)}$ dose constant, results in a loss in FITC responsiveness to near-unde-tectable levels (Figure 1A). The competition was specific to the anti-FITC response, as carrier-specific responses were un-changed, or even increased due to the increased total antigen dose (Figure 1A).

With the competition phenotype identified, several vaccination protocols were devised in an effort to understand the parameters under which it occurs. A dose escalation experiment that varied the total antigen dose over 6 orders of magnitude, but kept the ratio of RSA-$FITC_{(1)}$:RSA ratio constant, showed that even large vaccine doses were subject to

competition (Figure 1B). Prime and boost strategies failed to overcome competition, suggesting that repeated vaccination will not ‘‘rescue’’ outcompeted epitope responses (Figure 1C). The selection of adjuvant also appeared to have little impact on competition with the oil-in-water emulsion adjuvant, Addavax, failing to elicit an anti-hapten response under competition conditions (Figure 1D). Even boosting FITC-targeting through an increased RSA-FITCylation ratio (RSA-$FITC_{(4)}$) failed to rescue FITC targeting when mixed with unlabeled RSA. The only parameter that was identified that did have a large impact on FITC targeting was the ratio of RSA-$FITC_{(1)}$:RSA in the vaccine mix. Competition ratios of 1:1 consistently showed decreased (albeit non-significant) anti-FITC responses, while a 1:2 ratio resulted in more than a 10-fold decrease in FITC targeting. Competition ratios of 1:16 showed decreases of more than 100-fold and were inconsistently detectable above background. Altogether, these data suggest that select-epitope responsiveness is exquisitely sensitive to the frequency in which the epitope is present within the greater antigen pool, and low-frequency epitopes are at risk of being drowned out by overall response.

Rare-Epitope Suppression Occurs Early in Class-Switched B Cell Response

While competition was initially identified in the IgG compartment, it was unclear at what stage of the humoral response the competition was occurring. A serological time course tracking anti-FITC IgM and IgG responses in vaccinated animals suggested that the FITC-targeting deficit under competition conditions was restricted to the class-switched IgG response, and could already be identified at the earliest time points where anti-FITC IgG could be systemically detected (Figures 2A and 2B). Using a FITC-dextran ‘‘bait’’ probe to identify FITC-specific B cells (Figure S1), deficiencies in anti-FITC GC B cell responders could be identified by flow cytometry at both early (day 7) and peak (day 14) GC responses following vaccination (Figures 2C and 2D). Similar findings were reached in an independent vaccination/staining model using the well-established ova-NP system (Figure S2). Using *in vivo* bromodeoxyuridine (BrdU) labeling to identify early division, FITC-response defects under competition conditions could also be seen in proliferative class-switch responses as early as day 6 following vaccination (Figure S3).

Although the deficiency had been identified serologically through 6 weeks post-vaccination, long-lived plasma cell engraftment into the bone marrow remained unclear. Consistent with early and lasting defects in anti-FITC targeting, ELISpot analysis of the bone marrow 6 weeks following vaccination showed significantly decreased number of FITC-responsive IgG class-switched plasma cells under competition conditions (Figure 2E). Together, these data show a deficiency in rare-epitope responses at the earliest phases of class-switched humoral response, and persisting into long-term bone marrow engraftment.

Rare-Epitope Suppression Requires Highly Similar Competing Antigens

While the competition model system was robust and consistent, it is clear that humoral responses are capable of managing simultaneous GC reactions to independent antigens in a vaccination setting (Pabst et al., 1997). As a result, it was important to determine under which conditions antigens or epitopes might compete in this way. To confirm simultaneous response capacity, five protein antigens (hen egg lysozyme, ovalbumin, RSA, phycoeytherin,

and keyhole limpet hemocyanin) were administered in alum emulsions either individually or as a mixed group. As expected, serological responses to individual protein antigens were not diminished in the mixed-antigen group supporting the idea of competent simultaneous response (Figure 3A).

As these antigens are extremely diverse, it was possible that only antigens that contain cross-reactive B cell epitopes would be capable of competing. A benefit of the RSA carrier model is the availability of cross-species homologs containing varying degrees of primary sequence overlap. One homolog, human serum albumin (HSA), shares 76% primary sequence homology with its rabbit counterpart. Vaccinating mice with HSA results in a significantly cross-reactive anti-RSA response, albeit a lower response than vaccinating with RSA itself (Figure 3B). Despite this cross-reactivity, HSA was not competitive when mixed with RSA-$FITC_{(1)}$ at 1:2 or even 1:10 competition ratios (Figure 3C).

Rare-Epitope Suppression Is Enforced by Restrictions in T Cell Help

With B cell cross-reactivity (and thus, antigen availability) failing to explain the competition phenomenon, it was possible that competition was instead the result of B cell competition for limited T cell help. Consistent with this hypothesis, FITC-specific B cells under competition conditions analyzed by flow cytometry at day 7 expressed significantly less MHC II than their non-competition counterparts (Figure 4A). These results suggested that RSA and HSA might fail to compete not because of a lack of shared B cell epitopes, but because they drew on sufficiently exclusive T cell pools to prevent competition for T cell help (Figure 4B). This type of T cell help bottleneck has been observed previously in the case of B cell populations with significantly different receptor affinities (Schwickert et al., 2011).

Using the Immune Epitope Database and Analysis Resource (IEDB) peptide analysis tool, peptide hierarchies for both RSA and HSA were predicted using $I\text{-}A^b$ as a binding reference. The top 30% of predicted RSA peptides were mapped and then compared against the HSA sequence to identify conserved and non-conserved predicted peptides (Figure 4C). Surprisingly, while the RSA and HSA share 76% overall homology, they shared only one out of thirteen predicted ‘‘high-binding’’ peptides. This suggested that diversity in presented MHC II peptides, and thus, diversity in the responding CD4 T cell pool, may explain the failure of these proteins to compete.

To test this idea directly, two mutant RSA proteins were designed and synthesized. RSA_{mut1} was designed to be identical to RSA, excepting two point mutations in the peptide predicted to bind to $I\text{-}A^b$ with the highest affinity (Figures 4C and 4D). The point mutations selected were drawn from murine serum albumin (MSA) to conserve protein folding and render the peptide immunologically inert. The second mutant RSA_{mut2} was designed using a similar process, but used twelve point mutations borrowed from HSA or BSA to alter the top 10 predicted RSA peptides (Figures 4C and 4D) but maintain immunogenicity. The resulting two mutants shared 99.7%, and 98.0% sequence homology, respectively, and vaccinating with these proteins yielded identical anti-RSA responses in B6 mice (Figure 4E). Competing these proteins against a standard RSA-FITC vacci-nation, competition was seen to be titratable based on the extent of shared T cell epitopes (Figure 4F). Consistent with the T cell

bottleneck hypothesis, neither mutant was able to fully compete with RSA despite their extremely high level of similarity to the wild-type (WT) protein. RSA-FITC competition with RSA_{mut1}, despite only a single dominant T cell epitope difference, resulted in significantly less competition than the WT protein (Figure 4F). As expected, RSA_{mut2} exhibited even less ability to drive the competition phenomenon, and was statistically indistinguishable from HSA competition (although consistently trending lower) (Figure 4F).

These data suggest that by diversifying the T cell populations that individual antigens draw upon, a more diversified paratope set can be achieved. They also predict that by artificially inflating the number of $CD4^+$ T cells capable of responding to the antigen, the effects of competition might be mitigated. To test this idea, an ovalbumin (Ova) model was developed to take advantage of the ova-specific TCR transgenic OT-II mouse. An $Ova\text{-}FITC_{(3)}$ conjugate, vaccinated into B6 mice yielded anti-FITC, as well as anti-Ova IgG responses similarly to the RSA system (data not shown). By competing Ova-FITC with Ova, a similar competition phenomenon could be identified in accordance with previous RSA-FITC results (Figures 1A and 4G). Interestingly, adoptively transferring Ova-specific naive OT-II T cells 24 hr prior to vaccination resulted in decreased competition not seen with the transfer of WT cells (Figure 4G). These data further support the idea that while competition is readily seen at the B cell response level, it is a bottleneck in access to antigen-specific T cells that drives the competition phenomenon.

DISCUSSION

Despite the spectacular success of vaccines, there remains a critical need for effective vaccines against major global infections such as HIV, dengue, and influenza. There are numerous challenges in developing vaccines against such pathogens, but a major challenge is learning how to induce a broad antibody response against subdominant antigenic epitopes contained within a single protein (such as cryptic neutralization epitopes within the HIV Envelope protein [Sanders et al., 2015]) or a mixture or different proteins (such as the Envelope proteins in the four serotypes of dengue [Flipse and Smit, 2015]). This issue presents a major immunological challenge as the immune system has the propensity of responding in a hierarchical fashion to different epitopes contained within complex immunogens such as: (1) single protein (which contains multiple antigenic epitopes, such as the HIV Env protein), (2) a microbe (which contains multiple proteins each of which has multiple antigenic epitopes), (3) a mixture of different proteins with different B cell epitopes, but shared T helper epitopes (such as might occur in a microbe or in a vaccination regimen involving, for example, Env proteins from the four serotypes of dengue viruses), or (4) a rapidly mutating viral infection, where for example, the Env protein on only some virions contain the B cell epitope of interest but compete with other Env on other virions for shared T cell help. We would suggest that the immunodominance model presented in the current study, involving a mixture of hapten-protein plus protein, is similar to what might occur in examples (2), (3), or (4) above, where a mix of antigens of which only some contain the epitope of interest. Therefore, learning the mechanisms underlying such immunodominance is a critical challenge in vaccinology. The present study provides mechanistic insights underlying immunodominance and highlights an essential role for T cell help in this process.

Decades of work on GC reactions have stressed the importance of B cell receptor affinity (Schwickert et al., 2011), precursor frequency (Abbott et al., 2018), access to antigen (Kwong et al., 2002), and competition for survival signals (Wensveen et al., 2016) as critical variables in determining the clones that will come to dominate the humoral response. Indeed, previous studies (Schwickert et al., 2011) have identified restricted access to T cell help as the mediator of clonal restriction, in agreement of with the current work. The current study expands on that work to include an additional variable in B cell competition, relative local access to antigen, which plays an outsized role in selecting which B cells will ultimately receive sufficient shared T cell help.

While these determinants of immunodominance are often investigated and published as separate phenomena, a more integrated view of clonal selection is likely true. There is a clear bottleneck in access to T cell help as early as pre-GC B cell selection, and the dominant responders will be those B cells that most rigorously compete with their neighbors for co-stimulatory access. Whether due to relative access to antigen, affinity, kinetics, precursor frequency, or more likely a combination of all of those inputs, the relative activation state of a B cell in comparison with its neighbors will dictate its inclusion in the GC, and eventual role in the overall response. Such an integrative understanding helps clarify classical clonal selection findings such as Herzenberg's carrier-hapten/carrier experiments as complex models where increased affinity, increased precursor frequency, greater relative access to antigen, etc., all combine against a backdrop of T cell restriction to drive consistent immunodominant responses (Herzenberg et al., 1980).

Although all of these parameters likely combine to dictate clonal selection, only a few of these mechanisms might be harnessed to make better vaccines. Affinity of a naive polyclonal population to a specific epitope cannot be easily modified, nor can precursor frequency or kinetics of antigen acquisition in relation to neighboring B cells. Relative epitope abundance, however, might be leveraged in a vaccine cocktail setting with relative ease and may well offer a path forward in generating vaccines designed to narrow focus on an intended epitope set. Using the understanding that local antigen ratios are critical drivers in B cell activation and paratope selection, it may be possible to design vaccines to include a heterologous series of proteins with highly conserved epitopes only at regions of interest. In this way, specific B cells known to target epitopes critical for host protection will be given a competitive advantage over neighboring B cells, making them far more likely to enter into germinal centers and generate a productive response. This heterologous prime approach, in combination with adjuvants known to drive increased diversity in humoral response, could prove an important solution in targeting protection-critical, but normally sub-dominant, epitopes. Similarly, it is conceivable that immunization of HIV Env or influenza hemagglutinin proteins tagged with diverse and potent T helper epitopes may boost the immunogenicity of such proteins, particularly against subdominant epitopes.

Altogether, this study addresses important mechanistic issues into the early selection of epitope-specific responses. It is clear from these data that when the availability of resources is limiting, small advantages in antigen exposure and activation can have an outsized effect on humoral immune outcomes. Continued investigation into how these advantages might be leveraged in a vaccine setting provides an exciting path forward for the development of

epitope-targeting vaccine systems. In addition, these basic understandings of early B cell selection may impact diverse fields such as viral evasion and immuno-oncology where immune responses attempt to distinguish between highly similar, but genetically drifted, antigens.

STAR ★ METHODS

CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Dr. Bali Pulendran (bpulend@stanford.edu)

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice—C57BL/6 female mice were obtained from Jackson laboratory and received at 6 weeks of age. OT-II mice were bred and housed at Emory University in accordance with IACUC protocol under the care of Yerkes Animal Resources. All mice used in the study were selected female for the purposes of reproducibility, and used for experimentation between 6 and 12 weeks of age.

METHODS DETAILS

Vaccination—All vaccinations were performed intramuscularly (IM) in the calf to mimic human intramuscular vaccination. Protein antigens (ie RSA, HAS, etc.) were formulated in sterile PBS at a 1:1 mixture with a selected adjuvant (see KEY RESOURCES TABLE). Total injection volumes were limited to 20ul to minimize animal discomfort, and promote localized response in the draining lymph node. For Brdu incorporation experiments, mice were injected with 2mg Brdu stock solution I.P. 12h prior to subsequent vaccination (see KEY RESOURCES TABLE).

Adjuvants—Two adjuvants were used in this study: Aluminum hydroxide (Alum) and AddaVax. Both adjuvants were obtained through Invivogen (See KEY RESOURCES TABLE), and are readily available analogs to alum and oil-in-water emulsion adjuvants currently used in human vaccination. All vaccinations were carried out using alum, unless otherwise indicated in the figure legend.

Vaccine antigen generation—Ultra-pure rabbit serum albumin, human serum albumin, chicken ovalbumin, Hen egg lysozyme, and keyhole limpet hemocyanin were purchased from Sigma (see KEY RESOURCES TABLE). R-Phycoerythrin was purchased from ThermoFisher (see KEY RESOURCES TABLE). All carrier proteins were resuspended in sterile PBS according manufacturers instructions.

Selected carriers were conjugated to fluorescein isothiocyanate obtained from Sigma (see KEY RESOURCES TABLE) through simple amine modification. Briefly, carrier proteins were dissolved in 0.1M $NaHCO_3$ and mixed with a pre-determined amount of FITC dissolved in DMSO. Reaction tubes were rotated for 2h at room temp. Total reaction volumes were loaded onto PD-10 size exclusion columns obtained from GE healthcare, and unlabeled FITC was removed as per manufacturers instructions. Labeled protein was

assessed for conjugation ratio through spectral analysis, and concentrated to desired stock concentration.

ELISAs and ELISpots—Following vaccination, mice were bled at indicated time points for serological analysis. Serum was obtained by whole blood collection in eppendorf tubes, incubation at room temperature for 2 hours, centrifugation of at 14,000 g for 10 minutes, and collection of the serum fraction. ELISA or ELISpot plates were coated with either the vaccine carrier (such as RSA), or an unrelated carrier-FITC conjugate to assess carrier and FITC specific response (such as ova-FITC following RSA-FITC injection), respectively. Bio-rad blotting-grade blocker was used was used to prevent accidental albumin contamination (see KEY RESOURCES TABLE). Specific isotypes were identified using HRP-conjugated, human adsorbed, isotype-specific anti-mouse antibodies obtained from SouthernBiotech, and developed using Pierce TMB substrate kit (see KEY RESOURCES TABLE).

ELISA data was analyzed using an endpoint analysis. Positive control samples were titrated to produce an assay sensitivity curve, and biological samples were compared to that curve to assign a titer (AU) relative to the assays lower threshold. Groups were then compared by standard statistical testing using Prism statistical analysis software (see below and KEY RESOURCES TABLE). ELISpot data were collected through manual visual counting of positive spots following plate development. Groups were then compared by standard statistical testing using Prism statistical analysis software (see below and KEY RESOURCES TABLE).

Flow Cytometry—At indicated time points, draining lymph nodes (specifically the popliteal and inguinal lymph nodes) were collected from vaccinated animals, or unvaccinated controls. Single cell suspensions were obtained through physical disruption of the tissue, and filtration though a 20um mesh filter. Cells were stained with antibody panels as indicated (see Figures S1, S2, and S3, and KEY RESOURCES TABLE) for 30 minutes on ice, washed, and filtered.

Flow cytometry was carried out on BD FacsCanto flow cytometers and data was collected using BDs FacsDiva software. All data was analyzed using FlowJo flow cytometry visualization software, and resulting datasets were statistically analyzed using Prism (see below and KEY RESOURCES TABLE).

Adoptive transfers—C57BL/6, or OT-II female mice (see above) were sacrificed, and skin-draining LNs collected. LNs were processed into single cell suspension, and naive CD4+ T cells were enriched using Miltenyl naive CD4+ isolation kit (see KEY RESOURCES TABLE). Cells were counted, and 100k cells were transferred into naive C57BL/6 recipients. Recipients were vaccinated 24h following adoptive transfer to allow sufficient time for engraftment.

RSA mutant protein design and development—Primary RSA and HSA protein sequences were analyzed by the IEDB ‘consensus’ binding algorithm to predict peptide affinity for H-2-I-A^b. Peptides predicted to bind in the top 30^{th} percentile against a random

library (Wang et al., 2008) were designated as the most likely putative binders. Overlapping peptides were condensed to their predicted binding cores, and peptides originating from the signal sequence were eliminated from analysis. Peptides identical to murine serum albumin (MSA) homologs were eliminated as inert. The 13 resulting peptides were considered likely to contribute to T cell response.

RSAmut1 was generated through the introduction of two point mutations (437 K > Q, 439 V > A) converting the highest predicted binding peptide to its MSA homolog rendering it immunologically inert.

RSAmut2 was generated through the introduction of twelve point mutations (139 F > L, 166 V > I, 239 A > S, 241 V > A, 325 G > D, 359 S > A, 364 D > E, 426 N > K, 437 K > Q, 439 V > A, 517 p > V, 597 p > K) designed to alter the top ten predicted binding peptides. All point mutations were drawn from HSA or BSA peptide homologs to ensure proper protein folding.

RSA mutant proteins were cloned, expressed, and analyzed by ThermoFisher scientific to ensure protein folding and stability.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was carried out using Prism statistical analysis software (see KEY RESOURCES TABLE). For each experiment, the type of statistical testing, n values, summary statistics, and levels of significance can be found in the figures and corresponding figure legends.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Naive B cells compete for antigen-restricted T cell help
- Relative access to antigen determines clonal inclusion in humoral response
- Quantity of $CD4^+$ T cell helps directly modulate B cell clonal restriction

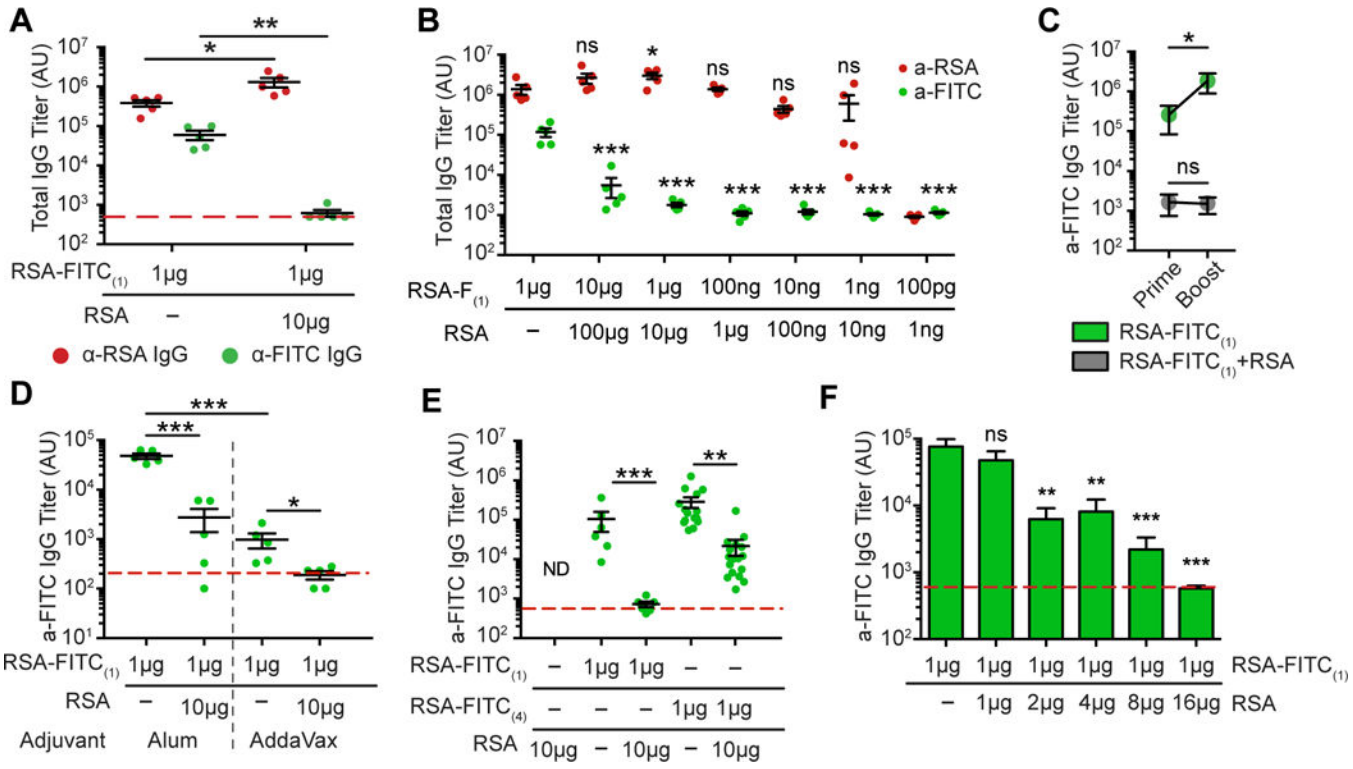

Figure 1. B Cell Competition Suppresses Humoral Response to Low-Frequency Epitopes

(A–E) B6 mice were vaccinated with indicated amounts of RSA and RSA-FITC(1). Day 42 serum analyzed for total IgG against RSA (red) or FITC (green) by ELISA.

(C) B6 mice were vaccinated with 1ug RSA-FITC(1) (green) or 1ug RSA-FITC + 10ug RSA (gray). Mice were boosted with the same at d42. Total IgG analyzed at d21 post-boost for anti-FITC antibody. n = 5 mice.

(D) Vaccines were delivered in alum (left) or addavax (right) suspensions.

(E) As in (A), with additional RSA-FITC(4) group.

(F) Bars representative of 5 mice/group.

(A–F) ns, not significant; *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. Means and standard deviations displayed. (A, D, and E) ANOVA analysis with multiple comparison testing among all groups. (B and F) ANOVA analysis with pairwise testing to non-competition group. (C) Student's t testing between prime and boost group. All results representative of at least 3 independent experiments.

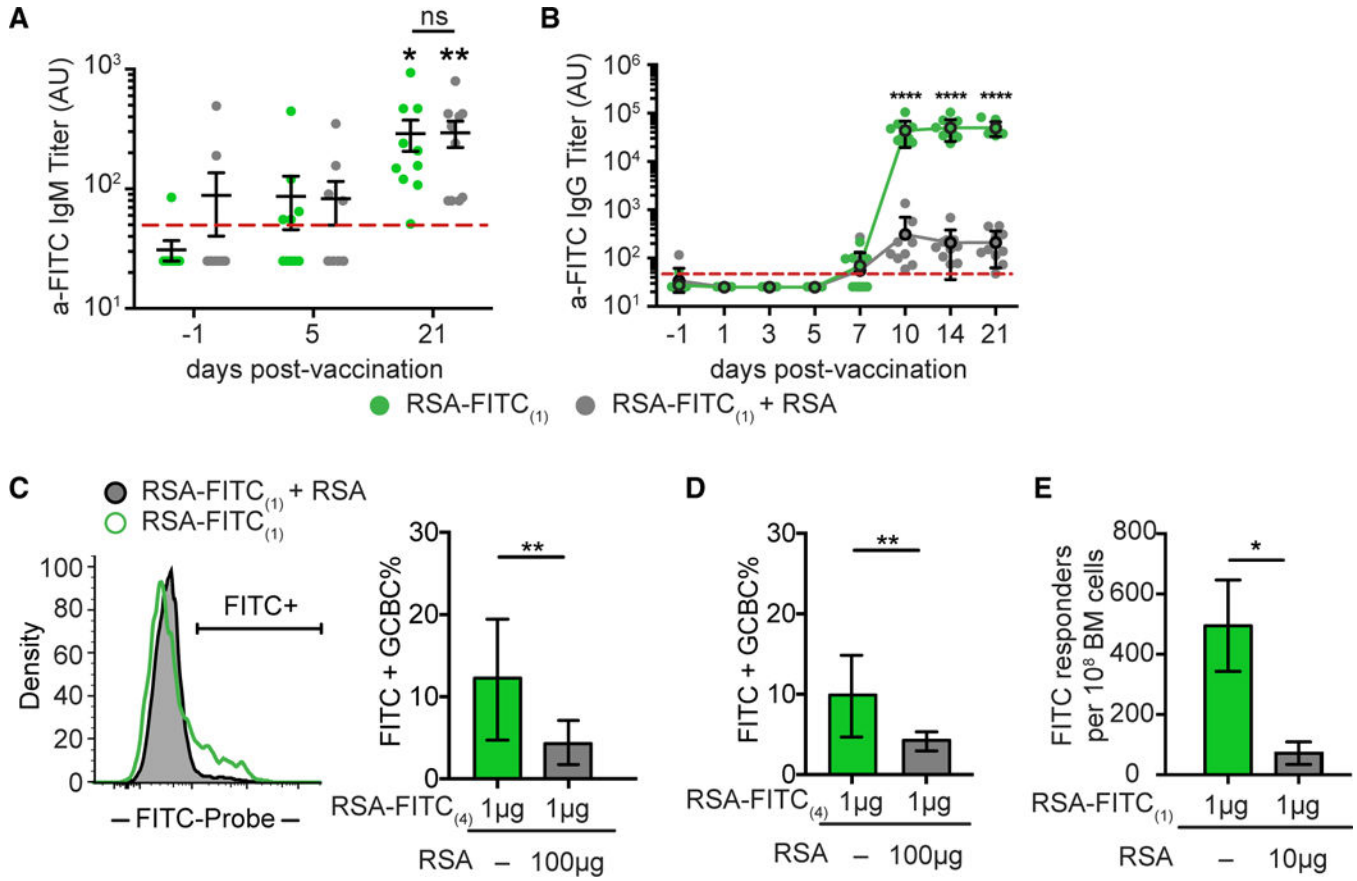

Figure 2. B Cell Competition Is Present at All Stages of T-Dependent B Cell Response

(A and B) B6 mice were vaccinated with 1 mg RSA-FITC(1) (green) or 1 mg RSA-FITC + 10ug RSA (gray). (A) IgM or (B) IgG anti-FITC serum response was assessed at indicated time points. ANOVA analysis with multiple comparison testing among all groups.

(A) Comparison testing to baseline (d-1) and between groups displayed.

(B) Comparison testing between groups displayed Student's t testing between groups as indicated.

(C) B6 mice were vaccinated with 1 mg RSA-FITC(6) (green) or 1 mg RSA-FITC(6) + 100 mg RSA (gray). PLNs analyzed by flow cytometry at day 7. Pre-gated on GC B cells (Figure S1). n = 5 mice/group.

(D) B6 mice were vaccinated with 1 mg RSA-FITC(1) (green) or 1 mg RSA-FITC(1) + 100 mg RSA (gray). PLNs analyzed by flow cytometry at day 14. Pre-gated on GC B cells (Figure S1). n = 5 mice/group.

(E) C57BL/6 mice were vaccinated with indicated amounts of RSA and RSA-FITC(1). Bone marrow was collected on day 42 and assessed for anti-FITC IgG reactivity by ELISpot. n = 5 mice/group. ns, not significant, *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. Means and SDs displayed. All results representative of at least 3 independent experiments.

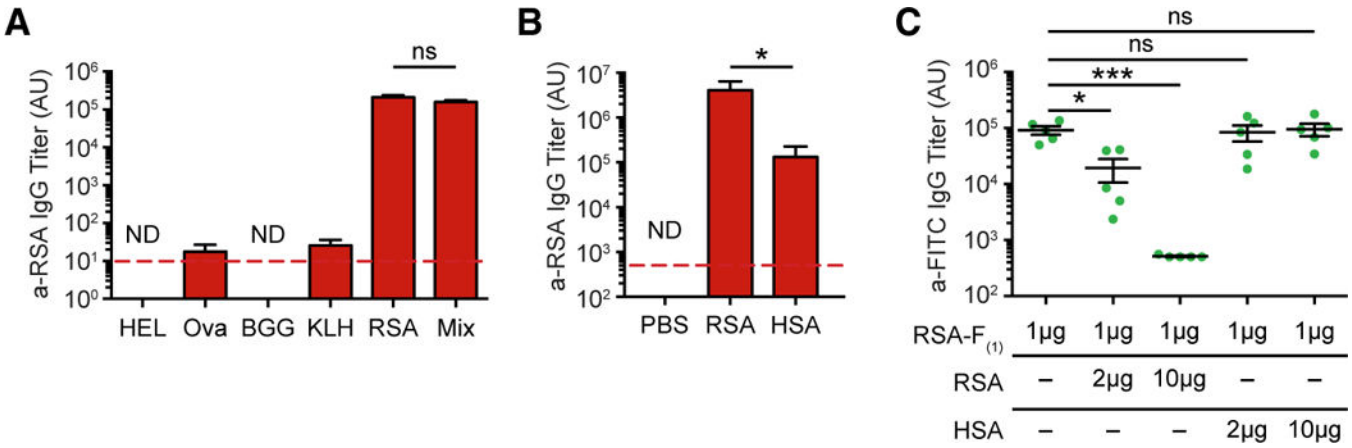

Figure 3. Competition Is Independent of B Cell Cross-Reactivity

(A) B6 mice were vaccinated with 10 mg indicated antigen. HEL, hen egg lysozyme; Ova, ovalbumin; BGG, bovine gamma globulin; KLH, keyhole limpet hemocyanin; HSA, human serum albumin. Day 42 serum analyzed for total IgG against RSA (red) by ELISA. n = 5 mice/group. Student's HEL Ova BGG KLH RSA Mix t testing between RSA and mix groups displayed.

(B and C) B6 mice were vaccinated with indicated amounts of RSA, HSA, and RSA-FITC(1). Day 42 serum analyzed for total IgG against or FITC (green) by ELISA. n = 5 mice/group.

(A and B) ns, not significant; *$p < 0.05$, **$p < 0.005$, ***$p < 0.001$. Means and SDs displayed. (B) Student's t testing between RSA and HSA groups displayed. (C) ANOVA analysis with multiple comparison testing among all groups. All results representative of at least 3 independent experiments.

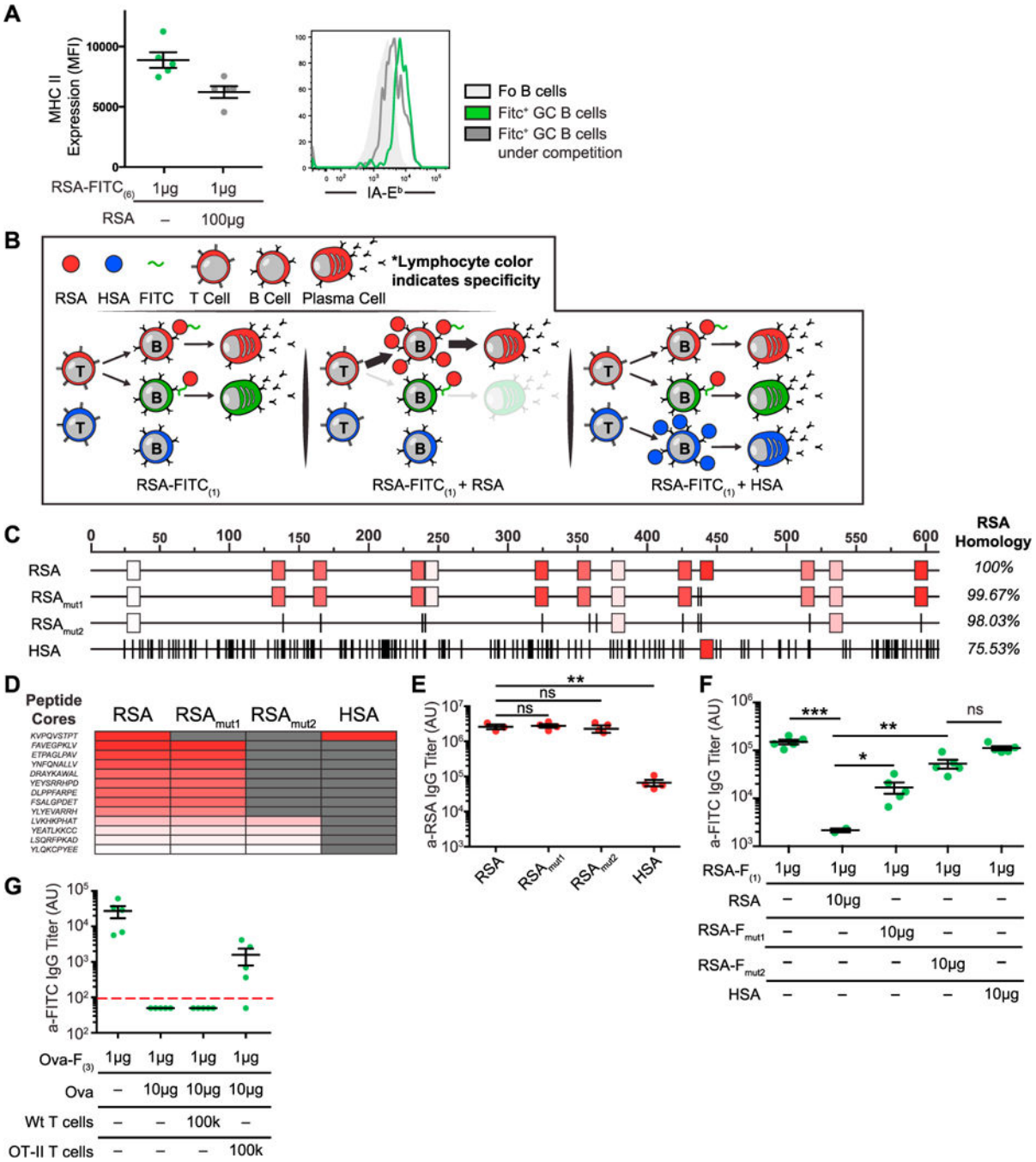

Figure 4. Diversification of the T Helper Pool Rescues B Cell Competition

(A) B6 mice were vaccinated with 1 µg RSA-FITC(6) (green) or 1 µg RSA-FITC(6) + 100 µg RSA (gray). PLNs analyzed by flow cytometry at day 7.

(B) Cartoon model of B cell competition for T cell help.

(C) Protein sequence map of RSA, RSAmut1, RSAmut2, and HSA. Predicted MHCII binding peptides indicated by boxes. Predicted binding strength indicated by box color (red, highest predicted binding). Point mutations as compared to RSA indicated by vertical lines.

(D) Comparison of predicted peptides to RSA. Grey boxes indicate that wild-type (WT) RSA peptide has been altered.
(E) B6 mice were vaccinated with 1 μg indicated antigen. Day 42 serum analyzed for total IgG against RSA (red) by ELISA.
(F) B6 mice were vaccinated with indicated amounts of RSA, RSAmut1, RSAmut2, HSA, and RSA-FITC(1). Day 42 serum analyzed for total IgG against or FITC (green) by ELISA.
(G) Indicated numbers of naive WT or OT-II $CD4^+$ T cells were transferred into B6 mice. 24 hr later, mice were vaccinated with indicated amounts of Ova and Ova-FITC(3). Day 42 serum analyzed for total IgG against or FITC (green) by ELISA.
(E and F) ns, not significant; $^*p < 0.05$, $^{**}p < 0.005$, $^{***}p < 0.001$. Means and SDs displayed. ANOVA analysis with multiple comparison testing among all groups. All results representative of at least 3 independent experiments.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Goat anti-mouse IgG HRP	Southern Biotech	Cat# 1030–05; RRID: AB_2619742
Goat anti-mouse IgG1 HRP	Southern Biotech	Cat# 1070–05; RRID: AB_2650509
Goat anti-mouse IgM HRP	Southern Biotech	Cat# 1021–05
Live/Dead Aqua	ThermoFisher	Cat# L34957
PerCP/Cy5.5 anti-mouse CD19	Biolegend	Cat# 115533; RRID: AB_2259869
PE anti-mouse CD95	Biolegend	Cat# 152607; RRID: AB_2632903
Alexa Fluor 647 anti-mouse/ human GL7 Antigen	Biolegend	Cat# 144605; RRID: AB_2562184
BUV496 Mouse Anti-Human CD38	BD	Cat# 564658; RRID: AB_2744376
Anti-FITC A488	EMD Millipore	Cat# MAB045X; RRID: AB_570395
Chemicals, Peptides, and Recombinant Proteins		
Rabbit serum albumin	Sigma	Cat# A0764
Human serum albumin	Sigma	Cat# A8763
Chicken ovalbumin	Sigma	Cat# A5503
Hen egg lysozyme	Sigma	Cat# L4919
Keyhole limpet hemocyanin	Sigma	Cat# H7017
R-Phycoerytherin	ThermoFisher	Cat# 46185
Fluorescein isothyocyanate	Sigma	Cat# F7250
Blotting-grade blocker	Bio-rad	Cat# Biorad1706404
RSAmut1	ThermoFisher	Custom expression
RSAmut2	ThermoFisher	Custom Expression
BRDU	BD	Cat# 550891
High MW FITC-Dextran	Sigma	Cat# 46946
Critical Commercial Assays		
TMB substrate kit	ThermoFisher	Cat# 34021
Naive $CD4^+$ isolation kit	Miltenyl	Cat# 130–104-453
Experimental Models: Organisms/Strains		
Mus: C57BL/6	Jackson Laboratory	Cat# 000664
Mus: OT-II	Jackson Laboratory	Cat# 004194
Software and Algorithms		
IEDB MHC-II consensus binding tool	N/A	http://tools.iedb.org/mhcii/
FacsDiva	BD	http://www.bdbiosciences.com/us/instruments/clinical/software/flow-cytometry-acquisition/bd-facsdiva-software/m/333333/overview
FlowJo	FlowJo	https://www.flowjo.com/solutions/flowjo/downloads
Prism	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
Alhydrogel	Invivogen	Cat# Vac-alu-250
AddaVax	Invivogen	Cat# Vac-adx-10