Affinity Maturation Is Impaired by Natural Killer Cell Suppression of Germinal Centers

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In Brief
Natural killer (NK) cells limit immunization-elicited follicular helper T cell and germinal center B cell responses. Rydyznski et al. linked perforin-dependent functions of NK cells to a reduced frequency and quality of somatic hypermutation within antigen-specific B cells. Strategies targeting this NK cell activity may enhance vaccination-induced generation of high-affinity protective antibodies.

SUMMARY

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AUTHOR CONTRIBUTIONS
Conceptualization, S.N.W. and S.N.W.; Methodology, C.E.R. and S.N.W.; Investigation, C.E.R. and S.A.C.; Formal Analysis, C.E.R., J.Q.Z., H.X., H.S., S.H.K., and S.N.W.; Writing – Original Draft, S.N.W. and C.E.R.; Visualization, S.N.W., C.E.R., and J.Q.Z.; Writing – Review & Editing, S.N.W., C.E.R., S.A.C., J.Q.Z., H.X., S.H.K., and H.S.; Funding Acquisition, S.N.W., C.E.R., J.Q.Z., and S.H.K.; Supervision, S.N.W.

DECLARATION OF INTERESTS
The authors declare no competing interests.

DATA AND SOFTWARE AVAILABILITY
The raw Vh186.2 DNA sequences can be accessed at https://tf.cchmc.org/external/rydlb5/Rydyznski_SHM_NK_data.zip.
Somatic hypermutation of immunoglobulin sequences in germinal center (GC) reactions must be optimized to elicit high-affinity, protective antibodies after vaccination. We expose natural killer (NK) cells as robust negative regulators of somatic hypermutation in antigen-reactive B cells. NK cells restrict follicular helper T cell (T\textsubscript{FH}) and GC B cell frequencies and titers of antigen-specific immunoglobulin after administration of alum-adjuvanted hapten-protein conjugate vaccines. This inhibition is perforin dependent, suggesting that NK cells kill one or more cells critical for GC development. In the presence of perforin-competent NK cells, antigen-specific GC B cells acquire fewer mutations, including less frequent generation of non-synonymous substitutions and mutations associated with increased antibody affinity. Thus, NK cells limit the magnitude of GC reactions and thereby restrain vaccine elicitation of high-affinity antibodies. Circumventing this activity of NK cells during vaccination has strong potential to enhance humoral immunity and facilitate vaccine-elicited prevention of disease.

**Graphical Abstract**

![Graphical Abstract Image]

**INTRODUCTION**

Infection and immunization induce formation of germinal centers (GCs), which facilitate follicular helper T cell (T\textsubscript{FH}) interaction with B cells to promote protective humoral immunity (Mesin et al., 2016). The GC crucially promotes affinity maturation of immunoglobulin responses through iterative rounds of somatic hypermutation (SHM) and Darwinian selection of mutant B cells with higher affinity immunoglobulin sequences. Thus, the GC aids generation of long-lived B cells, producing antibodies of greater affinity than would be possible in the germline immunoglobulin repertoire.

Multiple mechanisms contribute to regulating the formation and dissolution of GCs. This regulation is vital to optimize the output of long-lived protective B cells while preventing aberrant responses that can lead to autoimmunity. Several different cell types play either supportive or inhibitory roles in determining the development, maintenance, and resolution of GCs. Recently, natural killer (NK) cells were discovered to be an additional inhibitor of
NK cells are classically valued for their ability to kill virus-infected and transformed cells, but these innate cells can also suppress antiviral T cells to limit disease associated with chronic inflammation (Andrews et al., 2010; Crouse et al., 2015; Welsh and Waggoner, 2013). NK cell immunosuppressive function is contextually linked to secretion of the anti-inflammatory cytokine interleukin-10 (De Maria et al., 2007; Deniz et al., 2008; Lee et al., 2009; Perona-Wright et al., 2009), immune editing of dendritic cells (Ferlazzo et al., 2002; Piccioli et al., 2002; Wilson et al., 1999), and direct lysis of activated T cells (Crouse et al., 2014; Lang et al., 2012; Rabinovich et al., 2003; Waggoner et al., 2011; Xu et al., 2014). In the context of lymphocytic choriomeningitis (LCMV) virus infection, NK cells eliminate activated CD4 T cells (Waggoner et al., 2011), resulting in a diminished magnitude of GC responses (Cook et al., 2015; Rydyznski et al., 2015) as well as weak induction of both long-lived LCMV-specific B cells and virus-specific neutralizing antibodies (Rydyznski et al., 2015). Whether NK-cell-mediated decreases in GC magnitude translate to reduced SHM of immunoglobulin in antigen-specific B cells and whether this immunoregulatory function is generalizable to non-viral vaccine regimens remains unclear.

To determine whether NK-cell-regulatory activity inhibits SHM during immunization, we used the well-established mouse model of 4-hydroxy-3-nitrophenylacetyl (NP) conjugated to keyhole limpet hemocyanin (KLH) hapten-carrier conjugate (NP-KLH) immunization (Jack et al., 1977; Mäkelä and Karjalainen, 1977; Reth et al., 1978). Because previous analyses of immunoregulatory NK cells were performed in the context of highly inflammatory live-virus infection (Cook et al., 2015; Rydyznski et al., 2015; Waggoner et al., 2011; Xu et al., 2014), we adopted a regimen of repeat injections of NP-KLH (adapted from Schwickert et al., 2009) to ensure an adequate response by NK cells. The regulatory activity of NK cells was ablated using regimens of mono-clonal antibodies shown to selectively deplete NK cells (Waggoner et al., 2011) or via analysis of perforin-deficient (Prf1−/−) mice in which the granule pathway of NK cell cytolysis is inactivated (Kägi et al., 1994). We previously found that perforin is a crucial mediator of NK cell suppression of T and B cells (Rydyznski et al., 2015; Waggoner et al., 2011). The results of these experiments reveal that NK cells are important regulators of SHM in GC reactions.

RESULTS

NK Cells Restrain GC Responses following Immunization

To test whether NK cells suppress humoral immune responses after adjuvanted protein-conjugate immunization, we intraperitoneally inoculated mice with 100 µg NP-KLH in alum on day 0 and day 7. One day prior to immunization, groups of C57BL/6 mice were selectively and efficiently depleted of NK cells (Rydyznski et al., 2015; Waggoner et al., 2011) via intra-peritoneal (i.p.) administration of 25 µg of anti-NK1.1-depleting antibody (α-NK1.1) or mouse IgG2a isotype (control). The frequency of splenic CD4 NKp46+ NK cells remained significantly reduced for at least the duration of our immunization protocol (control = 1.78% ± 0.094% cells; α-NK1.1 = 0.52% ± 0.012%; n = 4/group; p < 0.0001; Student’s t test; day 8 post-depletion). NP-KLH-immunized, NK-cell-depleted animals

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harbored an increased frequency of splenic GC TFH (CD4+CD44hi and CXCR5+ PD-1hi) cells (Figures 1A and 1B) compared to immunized control mice. The total number of spleen CD4 cells (control = 1.1 ± 0.68 × 10^7; α-NK1.1 = 1.1 ± 0.11 × 10^7; n = 9 or 10/group; p = 0.85; Student’s t test) was similar between immunized α-NK1.1 and control mice. Consistent with enhanced frequencies of TFH after immunization in the absence of NK cells, there was a greater proportion (Figure 1C) and total number (Figure 1D) of B220+ CD19+ GL-7+ Fas+ GC B cell responses in the spleen of NK-cell-depleted mice relative to their NK-cell-sufficient counterparts after immunization. Thus, NK cells constrain GC responses following administration of an alum-adjuvanted non-replicating immunogen.

**Reduced NP-Specific B Cell Frequency and Immunoglobulin Titers in Presence of NK Cells**

Antigen-specific B cells generated in response to alum-adjuvanted immunogens predominately class switch to IgG1 (Lindblad et al., 1997; McHeyzer-Williams et al., 1993). Staining with fluorescently tagged NP reveals a two-fold increase in the proportion (Figure 2A) and total number (Figure 2B) of NP-specific IgG1+ GC B cells in NK-cell-depleted mice relative to control mice after immunization. In contrast, the proportion and total number of NP-specific IgG1neg GC B cells was similar in NK-cell-depleted and control mice (Figures 2A and 2C). Moreover, the proportions of NP-specific GC B cells expressing immuno-globulin M (IgM) (control = 3.3 ± 0.17; α-NK1.1 = 3.8% ± 0.40%; n = 4/group; p = 0.34; Student’s t test) or IgG3 (control = 2.0 ± 0.36; α-NK1.1 = 1.9 ± 0.33; n = 4/group; p = 0.82; Student’s t test) in the spleen at day 12 post-immunization were not enhanced with NK cell depletion. In accordance with increased frequencies of NP-specific IgG1+ GC B cells in the absence of NK cells, NK-cell-depleted animals had higher titers of sera NP-specific IgG1 captured by both high (NP4)- and low (NP20)-affinity conjugates of NP in ELISA (Figures 2D and 2E). Together, these data demonstrate that NK cell suppression predominately impacts IgG1-switched NP-specific B cells in the GC and subsequent elaboration of NP-specific IgG1.

**NK Cell Suppression Limits Quantity and Quality of Immunoglobulin Somatic Hypermutation**

We hypothesize that larger GC reactions following immunization in the absence of NK cells (Figures 1 and 2) will support a greater quantity and quality of SHM in GC B cells. The NP-KLH model is highly amenable to analysis of SHM in NP-specific B cells (Smith et al., 1997), in part because the majority of responding GC B cells carry the VH186.2 heavy chain (Cumano and Rajewsky, 1986). Moreover, mutations engendering a tryptophan to leucine switch at position 33 of VH186.2 confer an approximately ten-fold increase in antibody affinity for NP (Xu et al., 2015).

Sequencing of the VH186.2 heavy chain in sorted splenic NP-specific GC B cells 12 days after immunization reveals an increased average number of somatic heavy-chain mutations in B cells from NK-cell-depleted mice relative to control animals (Figure 3A). In addition, NP-KLH immunization in the absence of NK cells resulted in an enhanced proportion of sequences bearing the affinity-conferring W33L mutation compared to non-depleted control mice (Figures 3B and 3C). NP-specific GC B cells from NK-cell-depleted animals also exhibited more replacement mutations (p = 1.38 × 10^-4) across the entire length of V\textsubscript{H}186.2.
BCR sequences compared to controls when compared on a codon-by-codon basis (Figure 3C). Overall, these data suggest that NK cell suppression of GC responses constrains the frequency and quality of somatic mutations.

**Diminished GCs in Presence of NK Cells Is Driven by a Perforin-Dependent Mechanism**

Perforin-containing cytolytic granules represent a major mechanism by which NK cells trigger target cell death (Kägi et al., 1994) that is implicated in NK cell killing of activated CD4 T cells (Waggoner et al., 2011). Thus, we use perforin-deficient (Prf1−/−) mice to evaluate the role of perforin in NK cell suppression of GC-associated SHM. Similar to NK cell depletion in C57BL/6 mice (Figure 1), NP-KLH in alum immunization of non-depleted Prf1−/− mice results in increased frequencies of splenic GC T_{FH} (Figure 4A) and NP-specific IgG1+ GC B cells (Figure 4B) relative to C57BL/6 controls. Additionally, there was no enhancement of IgM+ (wild-type [WT] control = 12.0 ± 1.47 × 10^4; WT α-NK1.1 = 9.95 ± 1.34 × 10^4; Prf1−/− control = 9.73 ± 1.30 × 10^4; Prf1−/− α-NK1.1 = 13.6 ± 1.42 × 10^4; n = 4/group; p = 0.67; one-way ANOVA) or IgG3+ (WT control = 9.35 ± 2.50 × 10^4; WT α-NK1.1 = 7.90 ± 0.910 × 10^4; Prf1−/− control = 8.25 ± 1.51 × 10^4; Prf1−/− α-NK1.1 = 11.2 ± 0.810 × 10^4; n = 4/group; p = 0.65; one-way ANOVA) NP-specific GC B cells in Prf1−/− or anti-NK1.1-treated WT mice relative to non-depleted C57BL/6 WT controls. The elevated magnitudes of T_{FH} (control = 11.6 ± 0.97; α-NK1.1 = 10.6 ± 0.54; n = 3 or 4/group; p = 0.38; Student’s t test) and NP-specific IgG1+GC B cells (control = 29.5 ± 1.9; α-NK1.1 = 29.2 ± 1.5; n = 3 or 4/group; p = 0.91; Student’s t test) in Prf1−/− mice were not further enhanced by NK cell depletion.

Importantly, analysis of VH186.2 heavy-chain sequences in sorted splenic NP-specific GC B cells reveals a similar (p = 0.11) average number of VH186.2 mutations among mice lacking NK cells, perforin, or both, where all three groups of mice demonstrate enhanced mutation frequencies (p < 0.01) compared to control wild-type (C57BL/6) mice (Figure 4C). VH186.2 sequences in non-depleted Prf1−/− mice and NK-cell-depleted mice, regardless of strain, harbor mutations conferring the W33L change roughly two-fold more often than sequences from non-depleted control mice (Figure 4D). Together, these data suggest that NK-cell-derived perforin is important for suppression of the magnitude and quality of GC responses after immunization.

**DISCUSSION**

A major goal of vaccine development, particularly in the HIV field, is identification of mechanisms that limit affinity maturation of antibodies in GCs after immunization. We demonstrate that NK cells impose stringent limitations on GC B cell responses in the context of immunization with either live virus (Rydzynski et al., 2015) or alum-adjuvanted protein-conjugate immunogens. This NK-cell-mediated inhibition limits the efficiency of SHM by reducing the rate of mutation and by decreasing the frequency of replacement mutations, including those conferring large improvements in immunoglobulin affinity. Mechanistically, perforin-dependent functions of NK cells are critical in suppression of the GC. Our discovery of a crucial contribution of NK cells that limits vaccine-elicited SHM in antigen-specific GC B cells has major implications for vaccine design.
One goal of our study was to determine whether NK cells could suppress B cell responses against a non-replicating antigen coupled with a vaccine-relevant adjuvant (alum). In the context of LCMV infection, potent and sustained induction of type I interferon (IFN) and interleukin-15 (IL-15) activates NK cells (Nguyen et al., 2002) and likely provokes NK cell suppression of GC reactions (Cook et al., 2015; Rydzynski et al., 2015). In contrast, aluminum compounds (alum) used as adjuvants in human vaccines (Oleszycka and Lavelle, 2014) trigger markedly less inflammation than virus infection. Nevertheless, alum stimulates inflammasome-dependent release of IL-1 and IL-18 (Eisenbarth et al., 2008; Pollock et al., 2003), where the latter is a known stimulus for NK cell activity. This likely explained the capacity of alum to induce NK-cell-suppressive functions after NP-KLH immunization.

Overall, our results demonstrate that NK cells are sufficiently activated and GCs effectively receptive to NK cell suppression in the context of NP-KLH/alum immunization to provoke suboptimal affinity maturation of immunoglobulin in GCs. Suppression was most evident among IgG1-switched GC B cells that dominate the alum-adjuvanted NP-specific response. The focused effect of NK cells on IgG1 may simply reflect the IgG1-dominant nature of our schema or may be a consequence of the greater reliance of IgG1 responses on CD4 T cell help than has been noted for IgG3 responses (Mongini et al., 1981; Snapper et al., 1992). Of note, our sequencing experiments excluded analysis of non-IgG1 B cells, thereby precluding determination of the effect of NK cells on SHM in B cells using different isotypes.

Consistent with prior studies (Deauvieau et al., 2016), NK cells did not appreciably alter overall isotype usage in the NP-specific B cell response, suggesting that specific impacts of NK cells on B cells using discrete isotypes likely depends on adjuvant-stimulated class switching and the requirement for T cell help. Thus, NK cell suppression represents an innovative target for interventions to enhance SHM after immunization.

Importantly, depletion of NK cells or genetic ablation of perforin has similar, non-additive effects on GC responses. Therefore, GC suppression by NK cells is likely a product of NK cell killing of cells critical for GC formation, including T_{FH} cells. In fact, the earliest effect we observe in the absence of perforin or NK cells is an increased magnitude of activated CD4 T cells as well as a bias for T_{FH} versus T_{H1} differentiation of these cells (Rydzynski et al., 2015). Therefore, NK cells may shape the pool of T_{FH} cells available to support B cell expansion and affinity maturation in GCs. We did not observe any changes in the frequency of follicular regulatory CD4 T cells, suggesting that alterations in regulatory T cell development are not involved in GC suppression by NK cells. Nevertheless, we cannot rule out perforin-dependent lysis of antigen-presenting cells or even B cells themselves as phenomenon occurring in parallel to lysis of CD4 T cells, potentially creating a complex regulatory environment enforced by NK cells. The observation of perforin-dependent, NK-cell-mediated suppression of T_{FH} and GC B cell responses provides important mechanistic insights into how NK cells suppress humoral immune responses during immunization.

A deeper understanding of the impact of NK cell suppression of GC magnitude on the quality of resulting antigen-specific B cell responses was a major goal of this study. We adopted a two-dose immunization scheme in an effort to maintain a robust GC response from which we could measure and compare SHM rates. Importantly, we observed an increased number of mutations in the heavy-chain gene of NP-specific GC B cells following NP-KLH immunization of NK-cell-deficient mice. NK cells not only reduced mutation
rates, but their suppressive activity was associated with less efficient induction of affinity-conferring W33L mutations and a reduced ratio of replacement to silent mutations across all nucleotide positions within the complementarity determining regions of immunoglobulin heavy chains. Concurrent ELISA analysis revealed higher sera titers of NP-specific immunoglobulin in the absence of NK cells. However, measurements of both high- and low-affinity antibodies were elevated, with no significant skewing of the affinity ratio at the two-week time point. This result likely reflects a need for the more mutated GC B cells in NK-cell-depleted mice to egress from the GC before contributing to the peripheral pool of immunoglobulins.

Our results reveal that NK cells quantitatively and qualitatively restrain GC B cell responses following immunization. Of note, NP-KLH presents a relatively simple antigen necessitating a small number of specific mutations in a relatively large pool of naive NP-specific B cells to confer large increases in immunoglobulin affinity for antigen. By contrast, GC responses against HIV envelope originate from very small numbers of precursor cells and require both years of sustained inflammation and exposure to antigen to elicit very large numbers of mutations to enable development of broadly neutralizing antibodies. Despite these distinctions, our results highlight the potential for interventions that limit NK cell suppression of GC reactions as a strategy to potentiate vaccine-induced, GC-mediated generation of heavily mutated high-affinity antibodies capable of broad neutralization of HIV infection.

**STAR METHODS**

Detailed methods are provided in the online version of this paper and include the following:

**KEY RESOURCES TABLE**

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Mouse anti-NK1.1 (clone pk136) | Bio-X-Cell | BE0036; RRID: AB_1107737 |
| Mouse anti-IgG2a isotype control (clone C1.18.4) | Bio-X-Cell | BE0085; RRID: AB_1107771 |
| Mouse anti-CD4 AF700 | Biolegend | 100430; RRID: AB_493699 |
| Mouse anti-CD44 BV510 | Biolegend | 103044; RRID: AB_2650923 |
| Mouse anti-PD-1 e450 | eBiosciences | 48-9981-82; RRID: AB_2574139 |
| Mouse anti-CXCR5 PE-Cy7 | Biolegend | 145516; RRID: AB_2562209 |
| Mouse anti-CD49d FITC | Biolegend | 103606; RRID: AB_313037 |
| Mouse anti-IgM FITC | Biolegend | 406506; RRID: AB_315056 |
| Mouse anti-IgD FITC | Biolegend | 405704; RRID: AB_315025 |
| Mouse anti-CD95 PE-Cy7 | BD Biosciences | 557653; RRID: AB_396768 |
| Mouse/human anti-B220 AF700 | Biolegend | 103232; RRID: AB_493717 |
| Mouse anti-GL-7 e450 | eBiosciences | 48-5902-82; RRID: AB_10870775 |
| Mouse anti-IgG1 APC | Biolegend | 406610; RRID: AB_10696420 |
| Mouse anti-IgG3 BV711 | BD Biosciences | 565809 |
| Mouse anti-CD19 BV510 | Biolegend | 115546; RRID: AB_2562136 |
| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Bacterial and Virus Strains** | | |
| XL-10 Gold Ultracompetent e.coli | Agilent | 200314 |
| **Chemicals, Peptides, and Recombinant Proteins** | | |
| NP-KLH | Biosearch Technologies | N-5060-25 |
| TRIzol | ThermoFisher Scientific | 15596026 |
| NP-PE | Biosearch Technologies | N-5070-1 |
| NP-4-BSA | Biosearch Technologies | N-5050L-10 |
| NP-20-BSA | Biosearch Technologies | N-5050H-100 |
| LIVE/DEAD Fixable Aqua dead cell stain kit | Life Technologies | L34957 |
| GoTaq Flexi DNA polymerase | Promega | M8291 |
| **Critical Commercial Assays** | | |
| Superscript IV reverse transcription kit | Invitrogen | 18090010 |
| GeneJet gel extraction and cleanup kit | QIAGEN | K0691 |
| pGEM-T vector system | Promega | A3600 |
| Qiaprep spin miniprep kit | QIAGEN | 27104 |
| **Deposited Data** | | |
| Vh186.2 DNA sequences | This paper | https://tf.cchmc.org/external/rydbh5/Rydyznski_SHM_NK_data.zip |
| **Experimental Models: Organisms/Strains** | | |
| C57BL/6 mice | Jackson Labs | JAX:000664 |
| **Oligonucleotides** | | |
| CTCTTTGGCAGCAACAGC-Forward Primer 1 | This paper | N/A |
| GCTGCTCAAGTGTAGAGGTC-Reverse Primer 1 | This paper | N/A |
| GTGTCCACTCCCAGGTCCAAC-Forward Primer 2 | This paper | N/A |
| GTTCCAGTCTAGTCACTG-Reverse Primer 2 | This paper | N/A |
| **Software and Algorithms** | | |
| pRESTO | Vander Heiden et al., 2014 | https://prestodb.io/ |
| Change-O | Gupta et al., 2015 | https://changeo.readthedocs.io/en/version-0.4.1-airr-standards/ |
| R | R Development Core Team, 2014 | https://www.r-project.org/ |
| NCBI IgBlast package | Ye et al., 2013 | https://www.ncbi.nlm.nih.gov/igblast/ |
| IMGT/GENE-DB | Giudicelli et al., 2005 | https://www.imgt.org/genedb/ |
| **Other** | | |
| Xgal-IPTG-Ampicillin agar plates | KD Medical | BPL-2310 |

**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, Stephen Waggoner (stephen.waggoner@cchmc.org).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Mice**—C57BL/6 and Prf1<sup>-/-</sup> mice were purchased from the Jackson Laboratory (Bar Harbor, ME). Male mice 6–12 weeks of age (at onset of immunizations) were routinely utilized in experiments. Mice were housed under pathogen-free conditions, and experiments...
were performed using the ethical guidelines approved by the Institutional Animal Use and Care Committees of Cincinnati Children’s Hospital Medical Center. In many experiments, randomization was achieved by randomly assigning mice within a cage to different experimental groups or by modulating the processing order of the mice at the time of harvest.

**METHOD DETAILS**

**In vivo NK-cell depletion**—One day before infection, selective depletion of NK cells was attained through a single i.p. injection of 25 µg per mouse anti-NK1.1 monoclonal antibody (PK136) or 25 µg per mouse of a control mouse IgG2a (C1.18.4) produced by Bio-X-Cell (West Lebanon, NH).

**Immunizations**—4-hydroxy-3-nitrophenylacetyl conjugated to keyhole limpet hemocyanin (NP-KLH) was purchased from Biosearch Technologies (Petaluma, CA). Prior to immunization, NP-KLH was adsorbed to alum (Imject alum from Thermo Fisher) at a 1:1 volumetric ratio (100 µL of a 1 mg/ml NP-KLH stock with 100 µL alum) on a rotating mixer for one hour at room temperature. Mice were administered 200 µL of NP-KLH/alum intraperitoneally.

**Enzyme-linked immunosorbent assays (ELISAs):** NP-specific IgG1, ELISA plates were coated overnight with 5 µg/ml NP(4) or NP(20) conjugated to bovine serum albumin (BSA) in PBS. The next day, plates were washed and blocked with 2x PBS coating buffer at room temp for 2 hours. Following blocking, plates were washed and serum samples were serially diluted and plated in duplicate, and incubated for 2 hours. Plates were then washed 4x with PBS and biotinylated IgG or IgG1-HRP was added to the plates and incubated for 1 hour. Streptavidin-HRP was added to biotin-IgG plates and incubated for 30 minutes. All plates were washed and developed with the addition of TMB substrate for 15 minutes before addition of stop solution (2N H₂SO₄), and read in a plate reader at 450 nm. Values were reported as relative absorbance.

**Flow cytometry:** Single-cell leukocyte suspensions were prepared from spleens and LNs by mechanical homogenization of tissues between glass slides and filtered through nylon mesh. Red blood cells were then lysed via the addition of ACK lysis buffer (Thermo Fisher) for 5 minutes at 37 C. Cells were then washed, pelleted and resuspended in flow cytometry buffer (HBSS + 5% fetal bovine serum). Single-cell suspensions were plated at 2 × 10⁶ cells per well in 96-well plates and then incubated with a 1:200 dilution of anti-CD16/32 (clone 2.4G2 Tonbo Biosciences) in flow cytometry buffer. Subsequently, cells were stained for 20 minutes, or 90 minutes for NP-PE staining, at 4°C with various combinations of fluorescently-tagged antibodies. The following antibodies/reagents were used for flow cytometry: anti-CD4 AF700 (clone GK1.5 Biolegend), anti-CD44 BV510 (clone IM-7 Biolegend), anti-PD-1 ef450 (clone J43 eBiosciences), anti-CXCR5 PE-Cy7 (clone L138D7 Biologeund), anti-NK1.1 PE (clone PK136 Biologeund), anti-NKp46 APC (clone 29A1.4 Biologeund), anti-CD49d FITC (clone R1–2 Biologeund), anti-IgM FITC (clone RMM-1 Biologeund), anti-IgD FTTC (clone 11–26c.2a Biologeund), NP-PE (Biosearch Technologies), anti-CD95 PE-Cy7 (clone Jo.2 BD Biosciences), anti-B220 AF700 (clone RA3–6B2 Biologeund), anti-CD19 BV510 (clone 6D5 Biologeund), anti-GL-7 ef450 (clone GL7
eBiosciences), anti-IgG1 APC (clone RMG1–1 Biolegend), anti-IgG3 BV711 (clone R40–82 BD Biosciences). Following staining, cells were washed and fixed with BD fixation buffer (BD Biosciences) for 3 minutes at RT. Cells were washed twice and resuspended in 200µl flow cytometry buffer and analyzed on a BD Fortessa or LSRII flow cytometer.

**Cell sorting:** Single cell suspensions were prepared from spleens by mechanical homogenization between glass slides, filtered through nylon mesh, and RBC lysed with ACK lysis buffer (Thermo Fisher) for 5 minutes at 37°C. Cells were then washed and resuspended in cell sorting buffer (HBSS + 5% FBS + 5 mM EDTA). Cells were stained for 20–45 minutes at 4°C. The following antibodies/reagents were used for cell sorting experiments: anti-CD19 FITC (clone 6D5 Biolegend), anti-B220 AF700 (clone RA3–6B2 Biolegend), anti-CD95 PE-Cy7 (clone Jo.2 BD Biosciences), anti-GL7 ef450 (clone GL7 eBiosciences), NP-PE (Biosearch Technologies), anti-IgG1 APC (clone RMG1–1 Biolegend), LIVE/DEAD Fixable Aqua Dead Cell Stain Kit (Life Technologies). Cells were washed twice and resuspended in 500µl cell sorting buffer. Bulk GC B cells (300,000 – 500,000 cells) from pooled (n = 4/group) spleens of NK cell depleted or control treated animals or from spleens of individual mice (n = 4–8/group) receiving either NK-depleting antibody or isotype control were sorted on a BD FacsAria cell sorter into 1.5 mL Eppendorf tubes containing 200 µL of cell sorting buffer.

**VH186.2 BCR sequencing:** Following sorting, cells were pelleted and bulk RNA was isolated via Trizol/chloroform extraction. Extracted RNA was resuspended in 20 µL nuclease free water. Total cDNA was transcribed using the SuperScript IV reverse transcription kit. cDNA was then subjected to two rounds of nested PCR to amplify the Vh186.2 NP-specific BCR heavy chain (Forward primer 1: CTCTTCTTGGCCAGCACACGC, Reverse primer 1: GCTGCTCAGAGTGTAGAGGTC, Forward primer 2: GTGTCCACTCCCAGGTCAGTGTAGGTC, Forward primer GTTCCAGGTCACTGTCACTG). For the first PCR reaction, 2µl of cDNA was used for an amplification reaction in a total volume of 20µl containing 13.7µl nuclease-free H2O, 2µl 5x PCR buffer, 0.4µl each of 25mM MgCl2 and dNTPs, 0.5µl each of the first forward and first reverse primers, 0.5µl Taq polymerase and amplified with the following program: 94°C for 10 min, then 40 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, then 72°C for 10 min. For the second PCR reaction, 4µl of product from the first reaction was used for an amplification reaction in a total volume of 40µl containing 27.4µl nuclease-free H2O, 4µl 5x PCR buffer, 0.8µl each of 25mM MgCl2 and dNTPs, 1µl each of the first forward and first reverse primers, 1µl Taq polymerase and amplified with the following program: 94 C for 10 min, then 40 cycles of 94°C for 30 s, 56°C for 30 s, 72°C for 1 min, then 72°C for 10 min. Amplified sequences were then purified on a 1% agarose gel (product is 500 base pairs), and gel extracted using the GeneJet gel extraction (ThermoFisher Scientific) and cleanup kit. Purified products were then ligated into pGEM-T vectors (Promega) overnight at 4°C, transformed into XL-10 Gold ultracompetent E. coli (Agilent), plated on Xgal-IPTG-Amp agar plates (KD Medical) in triplicate, and incubated overnight at 37°C. The next day, white colonies were selected on plates and sent for direct colony sequencing at Genewiz, or white colonies were selected in the lab and grown overnight in 3ml LB with shaking at 37°C. When colonies were grown ON in house, the next morning, bacteria were pelleted and DNA extracted with the QIAprep
spin miniprep kit (QIAGEN). Purified DNA was sent to the CCHMC sequencing core for Sanger sequencing.

**V<sub>H</sub>186.2 sequence alignment:** Sequences were aligned to the Vh186.2 germline sequence using the NCBI IgBlast browser. Mutation data was only recorded if the entire (98aa) sequence was intact. We recorded the total number of mutations along the sequence as well as the type of mutation as either silent or amino acid changing. We also recorded whether the mutation occurred in framework regions or complementarity determining regions (CDRs) and whether the tryptophan to leucine (W33L) mutation was present. Due to an inability to determine whether multiple identical sequences came from truly separate cells representing a clonally dominant population or from potential biases introduced during the post-sorting processing steps, only unique sequences were plotted and used for statistical analysis.

**Mutation pattern analysis:** Raw sequencing data was processed using pRESTO (v0.3.5) (Vander Heiden et al., 2014), during which duplicate sequences were removed using ‘‘collapseSeq.py.’’ VDJ assignment was performed using ‘‘igblastn’’ from the standalone NCBI IgBlast package (v1.6.1) (Ye et al., 2013) against germline references from IMGT/GENE-DB (v.3.1.14) (Giudicelli et al., 2005). Default alignment parameters were used. For V, the mouse germline IGHV1–72*01 allele (UniProt: AC163348), which is identical to NCBI V186.2, was used. For D and J, the full IMGT mouse D- and J-references were used. Final processing was performed in Change-O (v0.3.5) (Gupta et al., 2015), during which non-productively rearranged sequences and sequences with V regions shorter than 294nt (98aa) were filtered. Mutation pattern analysis was performed for the V region using customized scripts in R (v3.3.3) (R Development Core Team, 2014). The IMGT unique numbering scheme (Lefranc et al., 2003) was used. Under this scheme, the V region of IGHV1-72*01 is numbered as follows: FWR1 = aa 1..26 (aa 10 unused); CDR1 = aa 27..38 (aa 31..34 unused); FWR2 = aa 39..55; CDR2 = aa 56..65 (aa 60–61 un-used); FWR3 = aa 66..104 (aa 73 unused); and CDR3 = aa 105..106 (Giudicelli et al., 2005). The codon known for Trp to Leu (W33L) mutation in NP mice is numbered aa 38. The amino acids encoded by the observed and germline sequences were compared codon-by-codon to determine the mutation type (silent or replacement). At each codon, the proportions of replacement and silent mutations across sequences were calculated. Two-sample, paired, and one-sided Wilcoxon signed rank tests were performed to compare the proportions across codons of silent mutations in Control and in ΔNK, and of replacement mutations in Control and in α-NK1.1 mice.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All statistical details are reported in the figure legends. For Figures 1 and 2, data were analyzed via two-tailed unpaired Student’s t test with biological replicates, n = 8–10 mice/group. Due to non-normal distributions in the data depicted in Figures 3 and 4, we performed non-parametric analyses. In Figure 3, differences in the total number of somatic mutations were analyzed via Mann-Whitney with median displayed with n = 67–97 unique VH186.2 sequences isolated from a total of 5–6 mice/group. In Figure 4, to compare non-normal data from more than two groups we used a Kruskal-Wallis test and Dunn’s multiple
testing correction with n = 74–99 unique VH186.2 sequences isolated from a total of 5–6 mice/group. In Figure 4, differences in T<sub>FH</sub> and GC B cell numbers between WT and Prf<sup>−/−</sup> mice were analyzed by two-tailed unpaired Student’s t test, n = 3–8 mice/group. Significance was accepted when p < 0.05.

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Highlights

- NK cells limit germinal center responses following protein immunization
- NK cells repress antigen-specific B cell and immunoglobulin production
- Quantity and quality of immunoglobulin somatic mutations are inhibited by NK cells
- NK cell suppression of affinity maturation is perforin dependent
Figure 1. NK-Cell-Depleted Mice Have Larger T_{FH} and GC B Cell Populations Relative to NK-Cell-Replete Controls

(A) Frequency of GC T_{FH} (CD4^{+} CD44^{hi} CXCR5^{+} PD-1^{hi}) cells in control or α-NK1.1 mice twelve days post-immunization with NP-KLH in alum.

(B) Total number of GC T_{FH} day 12 post-immunization in spleen.

(C and D) Frequency of germinal center B (CD19^{+}B220^{+}Fas^{+}GL-7^{+}) cells in the spleens of control or α-NK1.1 mice day 12 post-immunization (C) and total number of GC B cells in spleen day 12 post-immunization (D). Data were analyzed via Student’s t test; n = 9–10 mice/group. Experiments were repeated 3 times.
Figure 2. NK-Cell-Depleted Mice Have Higher Levels of NP-Specific GC B Cells and NP-Specific Immunoglobulin

(A) Frequency of NP\textsuperscript{+}IgG1\textsuperscript{+} GC B cells and NP\textsuperscript{+} IgG1 GC B cells in the spleens of control or α-NK1.1 mice day twelve post-immunization with NP-KLH in alum.

(B and C) Total number of NP\textsuperscript{+}IgG1\textsuperscript{+} (B) and NP\textsuperscript{+}IgG1 GC B cells day 12 post-immunization (C).

(D and E) Relative levels of high (NP4; D) and low (NP20; E) NP-specific IgG1 from sera of control or α-NK1.1 mice 12 days post-NP-KLH immunization. Data were analyzed via Student’s t test; n = 8–10 mice/group. Experiments were repeated 3 times.
Figure 3. NK Cell Suppression of GC Responses Has Consequences for Somatic Hypermutation
(A) Total number of mutations in unique VH186.2 BCR sequences isolated from spleens of control or α-NK1.1 mice day 12 post-immunization with NP-KLH in alum. Data were analyzed via Mann-Whitney with median displayed; n = 5–6 mice/group.
(B) The proportion of unique VH186.2 sequences from (A) containing the W33L mutation in control and α-NK1.1 mice.
(C) The frequency of either replacement or silent mutations at all positions within the VH186.2 sequence between control or α-NK1.1 mice. Experiments were repeated 2 or 3 times.
Figure 4. Perforin-Deficient Animals Have Enhanced GC Expansion and Somatic Mutation Rates Comparable to NK-Cell-Depleted Animals

(A) Total number of GC T<sub>FH</sub> (CD<sub>4</sub>^+CD44<sup>hi</sup>^CXCR5<sup>+</sup>PD-1<sup>hi</sup>) cells in the spleen of C57BL/6 (WT) or perforin-deficient (Prf1<sup>−/−</sup>) control-treated (mIgG2a) animals day 12 post-NP-KLH immunization.

(B) Total number of NP^+IgG1^+ GC B (CD19^+B220^+Fas^+GL-7^+) cells. Data were analyzed via Student’s t test; n = 3–8 mice/group.

(C) Total number of mutations in control or α-NK1.1-treated WT or Prf1<sup>−/−</sup>animals day 12 post-immunization. Data were analyzed via Kruskal-Wallis with multiple testing correction; n = 5–6 mice/group.

(D) Proportion of unique V<sub>H</sub>186.2 sequences from (C) bearing W33L mutation. Experiments were repeated 2 or 3 times.