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Supporting Online Material
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Materials and Methods
SOM Text
Fig. S1
Tables S1 and S2
References

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Crystal Structure of a Shark Single-Domain Antibody V Region in Complex with Lysozyme

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Cartilaginous fish are the phylogenetically oldest living organisms known to possess components of the vertebrate adaptive immune system. Key to their immune response are heavy-chain, homodimeric immunoglobulins called new antigen receptors (IgNARs), in which the variable (V) domains recognize antigens with only a single immunoglobulin domain, akin to camelid heavy-chain V domains. The 1.45 angstrom resolution crystal structure of the type I IgNAR V domain in complex with hen egg-white lysozyme (HEL) reveals a minimal antigen-binding domain that contains only two of the three conventional complementarity-determining regions but still binds HEL with nanomolar affinity by means of a binding interface comparable in size to conventional antibodies.

The cartilaginous fish (sharks, skates, rays, and chimeras) diverged from a common ancestor with other jawed vertebrates approximately 500 million years ago and include over 700 extant species. Nevertheless, they possess an adaptive immune system based on immunoglobulin (Ig), T cell receptors (TCRs), and the major histocompatibility complex. Three immunoglobulin isotypes have been identified in cartilaginous fish: two standard heavy (H)-light (L)-chain isotypes, designated IgM and IgW (IgW is also called IgX or IgNARC); and an atypical isotype, IgNAR. IgNAR is an H-chain homodimer that does not associate with L chain (*1*, *2*), unlike conventional human and murine antibodies (Fig. 1). Each H chain has one variable (V) and five constant (C) domains. Electron microscopy studies of IgNAR have revealed that their V regions are single domains, tethered to the C domains by means of flexible hinge-like regions (*2*). The V regions of IgNAR are not closely related to the V_H regions of either shark IgM or IgW in phylogenetic tree analyses; rather, they cluster with the V regions of TCR or immunoglobulin L chains (*1*, *3*).

In addition to the two canonical cysteines typical of immunoglobulin domains, IgNAR V domains carry a number of noncanonical cysteines that enable classification into two closely related subtypes, I and II. Type II V regions have an additional cysteine in complementarity-determining regions (CDRs) 1 and 3, which have been proposed to form a domain-constraining disulfide bond, akin to those observed in camelid H-chain V (V_HH) domains (*2*, *4*), whereas the extra cysteines in type I V regions are in framework regions (FRs) 2 and 4, and another two or four cysteines are

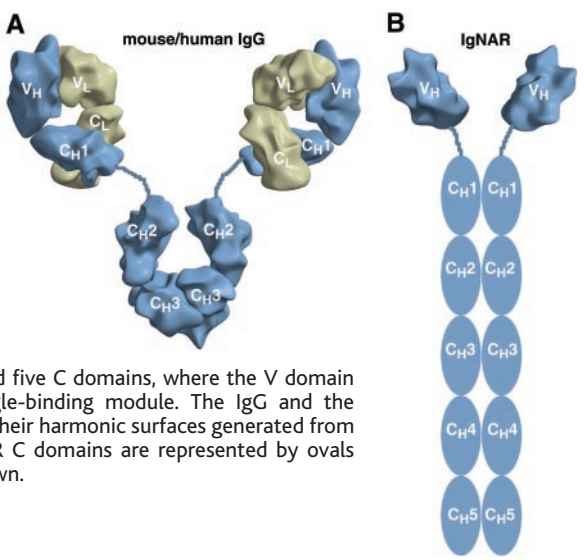

Fig. 1. Schematic representation of the overall IgG and IgNAR architectures. (**A**) A conventional IgG is composed of two H chains (blue) and two L chains (yellow) that assemble to form one Fc and two Fab regions or superdomains. The H chain has three C domains (C_H1, C_H2, and C_H3) and one V domain (V_H), whereas the L chain has one C domain (C_L) and one V domain (V_L). The V region is made up of two immunoglobulin domains (V_H and V_L). (**B**) IgNAR has only two H chains, each consisting of one V and five C domains, where the V domain is unpaired and constitutes a single-binding module. The IgG and the IgNAR domains are represented by their harmonic surfaces generated from atomic coordinates (*27*); the IgNAR C domains are represented by ovals because their structures are unknown.

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in CDR3. These CDR3 cysteines are usually encoded by the diversity (D) regions in their preferred reading frame (*2*) and are under strong selection in the primary repertoire (*5*). A deletion in the FR2-CDR2 region gives the IgNAR V domain its characteristically small size (~12 kD). Furthermore, somatic mutations that are found in the CDR1 of type II and in the shortened FR2-CDR2 region of type I appear to correlate with the acquisition and particular location of these extra disulfides in shark V regions (*5*).

IgNAR genes are found in the "cluster configuration" typical of cartilaginous fish immunoglobulins (*6*), with each cluster containing a single V region, three diversity elements, and a single joining gene (*1*). Because rearrangement only occurs within a cluster (*1*, *6*–*8*) and only a single functional cluster is present for each IgNAR type I or type II (*1*, *9*), diversity encoded by the V germline genes is severely limited. However, the repertoire is expanded greatly through the generation of enormous diversity in CDR3 through four rearrangement events, which include nucleotide (N)–region additions at each joining region, and by a high rate of antigen-driven somatic hypermutation (*10*).

Dimeric H-chain antibodies are also present in camels and llamas, where each H chain contains one V_HH domain and two C domains. Unlike IgNAR H chains, which are not known to have ever associated with L chains, camel H-chain antibodies have lost their ability to associate with L chain as a result of a deletion of their C_H1 domains and modification of V_H residues that would normally interact with V_L in conventional antibodies (*11*). Crystal and nuclear magnetic resonance structures for camel and llama V_HH single domains (*12*–*24*) have inspired antibody engineering of single-domain antigen-binding fragments for use in biotechnology and medicine (*25*).

The IgNAR V region clone HEL-5A7 was selected from a phage-displayed library derived from a nurse shark (*Ginglymostoma cirratum*) immunized with HEL. The HEL-5A7 single domain is highly stable, highly specific, and binds HEL with nanomolar affinity (*26*). The crystal structure of the IgNAR HEL-5A7 at 1.45 Å resolution is presented here (*27*) (Fig. 2 and table S1). Comparison of this shark IgNAR V domain with antibody and TCR immunoglobulin domains from higher vertebrates (Fig. 3) now permits more informed speculation about the origins and evolution of these immunologically important antigen-binding receptors.

The IgNAR V domain has an immunoglobulin β sandwich fold (Figs. 2A and 3A) consisting of only 8 β strands, rather than the 10 in a conventional antibody or TCR V domain, because of the deletion of the C′ and C′′ strands that normally comprise CDR2 (Fig. 3). As in C_L domains, the C strand connects directly to the D strand (Fig. 3, A and D). The CDR3 loop is long (*28*) compared with most human and especially murine CDR H3s, but it is of average length for IgNAR type I V regions [15 to 27 residues (*5*, *26*)]. The type I IgNAR CDR3 contains unusual disulfide bonds [N35 (FR2) to N92 (CDR3) and N97 (CDR3) to N104 (FR4)] (Fig. 2A) that constrain the CDR3 loop to fold over the outside sheet that would usually associate with V_L (IgNAR and lysozyme residues are designated by N- and L-chain identifiers, respectively). CDR3 residues N93 to N99 form a 3_{10} helix, similar to CDR3 conformations in several camelid V_HH domains.

The IgNAR V domain shares features with several types of vertebrate immunoglobulin domains, making it difficult to classify. Sequence homology is highest (~35% identity) between IgNAR V and TCR V_α or immunoglobulin V_κ chains, whereas structural homology is greatest with V_α, V_L, and V_H domains (*29*). From a gross topological comparison, IgNAR V (Fig. 3A) is similar to a C1-type domain (Fig. 3D), which does not have C′ and C″ strands. However, as in conventional V-type domains, the IgNAR A-strand hydrogen bonds to both "top" and "bottom" sheets, by means of a bend at Pro^{N7}. Strand A most resembles A strands in V_κ (Fig. 3B) and V_α (Fig. 3C) domains by

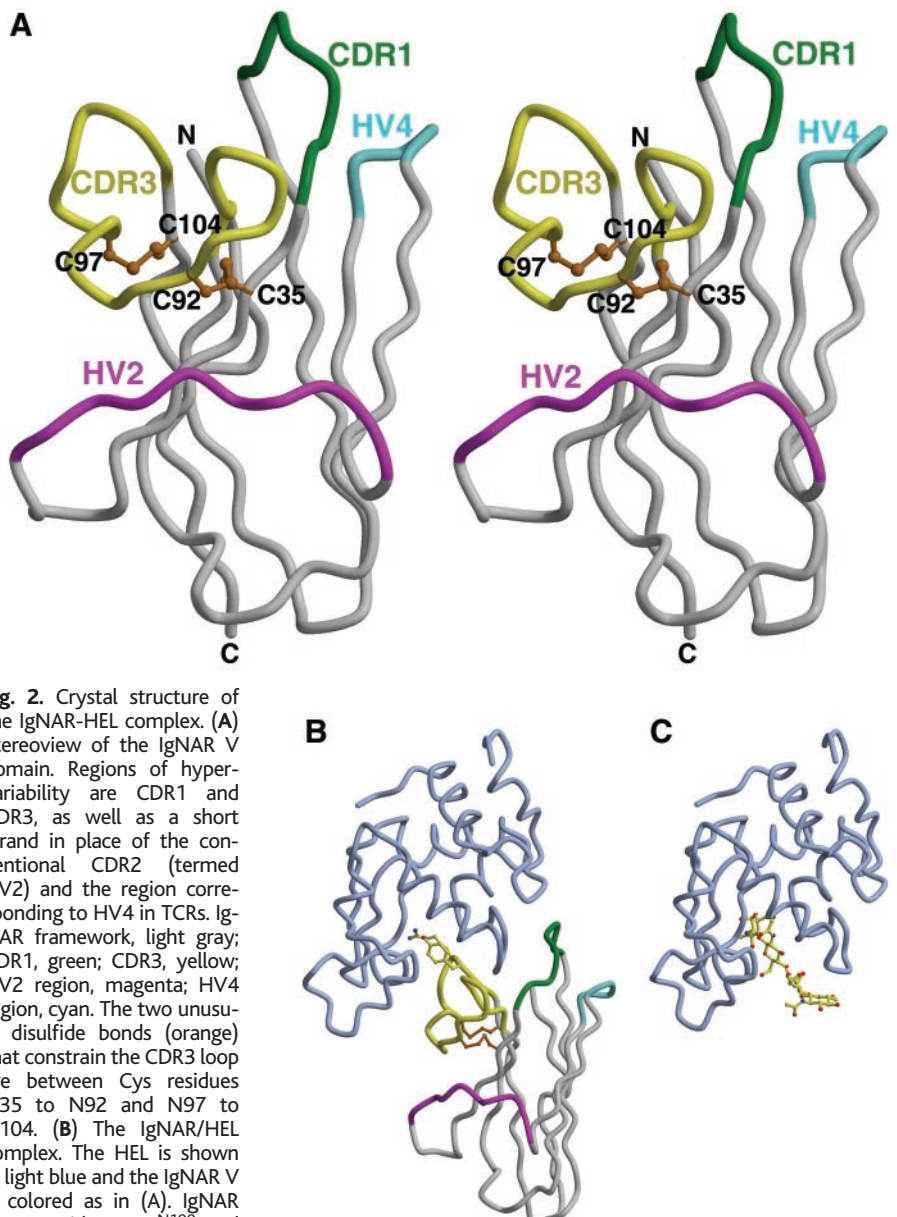

Fig. 2. Crystal structure of the IgNAR-HEL complex. (**A**) Stereoview of the IgNAR V domain. Regions of hypervariability are CDR1 and CDR3, as well as a short strand in place of the conventional CDR2 (termed HV2) and the region corresponding to HV4 in TCRs. IgNAR framework, light gray; CDR1, green; CDR3, yellow; HV2 region, magenta; HV4 region, cyan. The two unusual disulfide bonds (orange) that constrain the CDR3 loop are between Cys residues N35 to N92 and N97 to N104. (**B**) The IgNAR/HEL complex. The HEL is shown in light blue and the IgNAR V is colored as in (A). IgNAR CDR3 residues Arg^{N100} and Tyr^{N101} are deeply buried in the HEL active site. (**C**) The crystal structure of the HEL $Asp^{52}\rightarrow Ser^{52}$ mutant in complex with oligosaccharide [PDB accession code 1LSZ (*27*)] highlights the location of the lysozyme active site. The lysozyme is oriented as in (B).

length (one residue shorter than V_λ and V_H) and in the cis conformation of Pro^{N7} (highly conserved in V_κ domains). This structural homology with V_α or V_κ domains reflects the previous clustering of IgNAR V sequences with V regions of TCR and immunoglobulin L chains during the original phylogenetic analysis (*1*).

With the exception of CDR H3, the CDR loops of human and murine antibodies generally have "canonical" structures (*30*). However, the IgNAR CDR1 loop does not closely resemble human or murine canonical CDR1s (Fig. 3, A, B, and E) but instead converges on the "type 4" CDR1 conformation found in two camel V_HH domains, cAb-Lys3 and cAb-RN05 (*31*) (figs. S1 and S2). This similarity suggests that its conformation may be influenced by structural features and functional requirements specific to single-domain antibodies.

The IgNAR V region only contacts HEL by means of CDR1 and CDR3, with CDR3 residues inserted directly into the HEL active-site cleft (Figs. 2, B and C, and 4; tables S2 to S4). IgNAR Arg^{N100} forms a salt bridge with the catalytic Asp^{L52}, which explains its partial inhibition of HEL enzymatic activity (*9*) and lack of cross-reactivity with turkey egg-white lysozyme (*26*, *32*). The molecular surface area buried in the complex is extensive, considering that only two CDRs are used for antigen interaction (*33*), with 666 and 734 Å^2 of surface buried on IgNAR V and HEL, respectively (*34*).

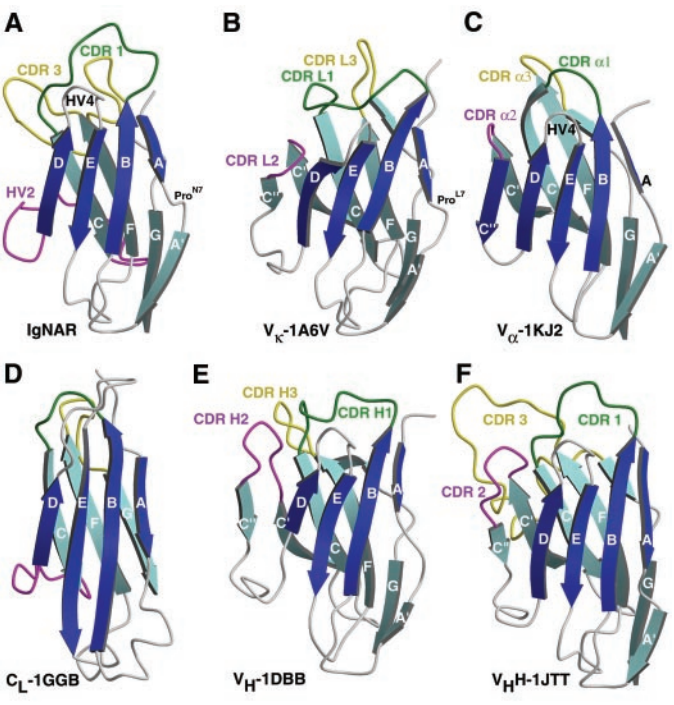

Fig. 3. Comparison of the IgNAR V domain with other immunoglobulin domains. (**A**) In IgNAR, the front sheet consists of strands A, B, E, and D, whereas the back sheet consists of strands A′, G, F, and C. The bend between A and A′ is similar to that seen in most V_κ domains, with a cis-Pro residue at position N7. (**B**) IgG V_κ domain from Fv B1-8 (PDB accession code 1A6V). (**C**) TCR V_α domain from TCR KB5-C20 (PDB accession code 1KJ2). (**D**) IgG C_L chain domain from Fab 50.1 (PDB accession code 1GGB). (**E**) IgG V_H domain from Fab DB3 (PDB accession code 1DBB) (**F**) Camel V_HH domain from cAb-Lys3 (PDB accession code 1JTT) (*27*).

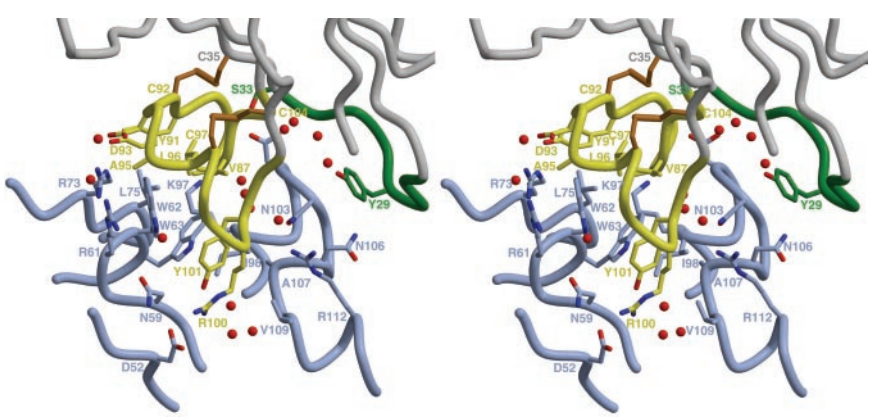

Fig. 4. Stereoview of the IgNAR V region/HEL interface. The main interactions of IgNAR HEL-5A7 (gray) are from CDR3 (yellow) and CDR1 (green) with lysozyme (light blue). Waters are shown as red balls and disulfides in orange. Noninteracting portions of the HEL have been omitted for clarity. The residues with side chains depicted are those that make van der Waals contacts or hydrogen bonds in the complex. A, Ala; C, Cys; D, Asp; I, Ile; K, Lys; L, Leu; N, Asn; R, Arg; S, Ser; V, Val; W, Trp; and Y, Tyr.

A model was constructed of the IgNAR type II V domain, primarily on the basis of this type I V region structure (fig. S3) (*35*), which supports a proposed noncanonical disulfide between CDR3 and CDR1. The type II CDR3 would then adopt a more extended conformation and protrude from the antibody framework akin to the camelid V_HH AMD10 CDR3 (fig. S3). Both the IgNAR Type II model and AMD10 have an exposed lysine at the structurally equivalent positions 84 (IgNAR) or 96 (AMD10) (*36*) rather than a Gly, as in IgNAR Type I and most other camelid V_HH structures. Glycines at this position are not solvent exposed, but instead are covered by the long CDR3 that folds over and masks this hydrophobic surface (the V_H/V_L interface in conventional antibodies).

In a study of random IgNAR V cDNAs (*5*), a number of residues were identified as having either high (positive selection) or low (negative selection) ratios of replacement to silent mutations. In addition to the CDR1 and CDR3 regions, residues in hypervariable region 2 (HV2) (N45 to N51) were found to be under strong positive selection (*37*). Their hypermutation among different IgNAR V regions could be either because of the direct recognition of the antigen [as observed in camelid domains AMB7 and AMD10 (*20*)] or because they maintain and influence the conformation of the CDR3, which does directly contact the antigen (supporting online material text).

Some species of shark respond readily to foreign antigens with a potent IgNAR response (*9*), and several libraries of IgNAR V regions displayed in bacteriophage have already been constructed (*26*, *38*, *39*), which should facilitate engineering of high affinity, minimal antigen-binding domains for biotechnological and biomedical use. The type I V region structure and type II model presented here suggest that these single-domain shark antibodies can generate sufficient structural diversity to recognize a wide array of antigens by varying the positions of their germline-encoded cysteines, which then markedly affect the conformation of CDR3 and the binding-site architecture. Whether this single-domain antibody did indeed evolve from a primordial antigen-binding receptor or whether it was derived later in phylogeny will remain unresolved until IgNAR (or IgNAR-like) molecules are found in more phylogenetically distant vertebrates, such as the jawless vertebrates, lamprey (*40*, *41*), and hagfish.

Note added in proof: While this paper was in production, a paper reporting the structures of the two unliganded type 2 IgNAR variable domains was published (*42*).

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24. Camel and llama V_HH domains share about 65% sequence identity with mouse and human V_H domains; however, several mutations in the canonical V_H interface region render the camelid V_HH domains more soluble than isolated human or murine V_H domains. Additionally, the long CDR3s in several of the camelid V_HH domains are folded back across this interface region, partially rescuing it from solvent exposure. The camelid CDR3 is sometimes anchored to the immunoglobulin core by formation of a disulfide bridge between CDR3 and CDR1 or FR2. Furthermore, some camelid V_HH domains can access recessed cavities, such as the HEL active-site cleft, by means of long CDR3 loops (*12*).
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28. The IgNAR CDR3 is 20 residues in length, spanning N84 to N103 (Gly-Leu-Gly-Val-Ala-Gly-Gly-Tyr-Cys-Asp-Tyr-Ala-Leu-Cys-Ser-Ser-Arg-Tyr-Ala-Glu).
29. Comparison of the IgNAR V domain structure with other antibody x-ray structures with the programs Dali and SSM (*27*) reveals similar root mean square deviations for the IgNAR V domain from V_α, V_L, and V_H domains of around 0.7 to 1.0 Å for 45 core Cα atoms.
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31. The IgNAR CDR1 has root mean square deviations from the cAb-Lys3 and cAb-RN05 CDR1s of 1.2 and 1.5 Å, respectively, for 15 Cα atoms (IgNAR residues N22 to N36).
32. Of the seven residues that differ between HEL and turkey egg-white lysozyme, two ($Arg^{73}\rightarrow Lys^{73}$ and $Asp^{101}\rightarrow Gly^{101}$) are found in the IgNAR-HEL interface. Arg^{L73} makes six van der Waals contacts, one hydrogen bond, and one salt bridge, and Asp^{L101} makes 17 van der Waals contacts and five hydrogen bonds in the interface with IgNAR. The camelid V_HH cAb-Lys3 also binds an epitope encompassing the recessed HEL active site, similarly using its CDR3 for access (*12*). Several murine antibodies to lysozyme (HyHEL10, HyHEL8, HyHEL26, and HyHEL63) recognize a common epitope around the active site, but do not penetrate this site as deeply as do cAb-Lys3 and IgNAR.
33. The camelid anti–ribonuclease A V_HH domain cAb-RN05 uses only CDR1 and CDR3 for binding antigen, whereas the camelid anticarbonic anhydrase V_HH domain cAb-CA05 uses CDR3 almost exclusively with only two van der Waals contacts to the antigen from CDR1. Similarly, the camelid anti–α-amylase V_HH domains AMB7 and AMD10 use all three CDR loops, but CDR1 contributes only 5% of the total buried surface area on the antibody upon antigen binding.
34. The IgNAR-lysozyme interface is comparable in size to those seen in lysozyme-Fab (Fv) complexes that range from 538 to 829 $Å^2$ for the Fab and 540 to 831 $Å^2$ for the lysozyme (table S3). Similar interface sizes have also been observed for lysozyme complexes with camel V_HH domains. A total of 122 van der Waals contacts (table S2), eight hydrogen bonds, and three charged interactions are made between HEL and the IgNAR (table S4). The majority of the buried surface on the IgNAR V domain is contributed by CDR3 residues N85 to N89, N91, N93, N95 to N96, and N98 to N103 (75%), with the remainder by CDR1 N26 to N33 (Fig. 4). The HEL-IgNAR V region interface has a good shape-complementarity index of 0.70 (0.72 and 0.70 for crystal form 2) (*27*), with waters filling several cavities in the interface (fig. S4). A total of 14 water molecules contact both the IgNAR V domain and HEL, with 7 of these (waters 2, 4, 7, 8, 60, 114, and 266) sequestered from contact with external solvent.
35. Type I and type II sequences are 90% identical. IgNAR type II V domains have only four conserved cysteines, rather than the six or more found in type I, and tend toward smaller CDR3 lengths [9 to 18 amino acids (*26*)].
36. The structurally equivalent positions Lys^{N84} (IgNAR) or Lys^{96} (AMD10) correspond to positions Leu^{89} or His^{93} of conventional V domains.
37. IgNAR residues Asn^{N45}, Glu^{N46}, Ser^{N48}, Ser^{N50}, Lys^{N51}, Gly^{N62}, Ser^{N63}, and Lys^{N64} are under strong positive selection.
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Supporting Online Material

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Materials and Methods
SOM Text
Figs. S1 to S4
Tables S1 to S4
References and Notes

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Defining a Link with Asthma in Mice Congenitally Deficient in Eosinophils

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Eosinophils are often dominant inflammatory cells present in the lungs of asthma patients. Nonetheless, the role of these leukocytes remains poorly understood. We have created a transgenic line of mice (PHIL) that are specifically devoid of eosinophils, but otherwise have a full complement of hematopoietically derived cells. Allergen challenge of PHIL mice demonstrated that eosinophils were required for pulmonary mucus accumulation and the airway hyperresponsiveness associated with asthma. The development of an eosinophil-less mouse now permits an unambiguous assessment of a number of human diseases that have been linked to this granulocyte, including allergic diseases, parasite infections, and tumorigenesis.

The underlying features of asthma display a marked heterogeneity (*1*, *2*), yet the presence of eosinophils in the airway lumen and lung tissue has been recognized even in the earliest studies (*3*) and is often regarded as a defining feature of this disease (*4*, *5*). Moreover, the recruitment of eosinophils occurs in animal models of allergen-mediated respiratory inflammation; in particular, mouse models have offered unique opportunities with which to examine detailed pathologic features of this disease. However, the availability of clinical studies and numerous mouse models of asthma have not led to an unambiguous description of eosinophil effector functions in asthma, and questions remain as to the specific role(s), if any, of these leukocytes (*6*).

A line of mice devoid of eosinophils was created to test hypotheses that link eosinophils and asthma-related pathogenesis. Transgenic mice devoid of eosinophils were created by lineage-specific expression of a cytocidal protein