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A Single Human V_H-gene Allows for a Broad-Spectrum Antibody Response Targeting Bacterial Lipopolysaccharides in the Blood

**Graphical Abstract**

**Highlights**
- Transgenic mice alter V_H-gene contribution to human-like BCR repertoires
- Specific human V_H-genes are needed to engage bloodborne bacterial LPS *in vivo*
- Germline-endowed antigen recognition targets a universally conserved LPS moiety

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**In Brief**
The ligand binding surface of antibodies is generated by diverse gene-encoded and hypervariable loops. Sangesland et al. demonstrate that certain gene-encoded loops are needed for human antibodies to accommodate the shapes of some bacterial antigens and naturally tune for recognition of conserved microbial features therein.

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A Single Human V_H-gene Allows for a Broad-Spectrum Antibody Response Targeting Bacterial Lipopolysaccharides in the Blood

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SUMMARY

B cell receptors (BCRs) display a combination of variable (V)-gene-encoded complementarity determining regions (CDRs) and adaptive/hypervariable CDR3 loops to engage antigens. It has long been proposed that the former tune for recognition of pathogens or groups of pathogens. To experimentally evaluate this within the human antibody repertoire, we perform immune challenges in transgenic mice that bear diverse human CDR3 and light chains but are constrained to different human VH-genes. We find that, of six commonly deployed VH sequences, only those CDRs encoded by IGHV1-2*02 enable polyclonal antibody responses against bacterial lipopolysaccharide (LPS) when introduced to the bloodstream. The LPS is from diverse strains of gram-negative bacteria, and the VH-gene-dependent responses are directed against the non-variable and universal saccharolipid substructure of this antigen. This reveals a broad-spectrum anti-LPS response in which germline-encoded CDRs naturally hardwire the human antibody repertoire for recognition of a conserved microbial target.

INTRODUCTION

The adaptive immune system generates diverse sets of antibodies through the recombination of B cell receptor (BCR) gene segments. For each germline BCR, affinity to incoming antigen is provided by complementarity determining regions (CDRs) that are either directly encoded by antibody variable (V) genes (CDR1 and CDR2) or assembled de novo through stochastic N-junctional diversification of antibody D and J segments (CDR3). Most BCR diversity is concentrated within the antibody heavy-chain CDR3 (CDRH3), which occupies a central position within the germline antigen binding site (Glanville et al., 2009; Schroeder and Cavacini, 2010). CDRH3 loops vary highly in length, sequence, and shape, and because of this diversity, they are the principal determinants of antigen specificity (Kuroda et al., 2008; Morea et al., 1998; North et al., 2011; Oliva et al., 1998; Saada et al., 2007; Schroeder and Cavacini, 2010; Shirai et al., 1999; Wedemayer et al., 1997; Xu and Davis, 2000). The remaining CDRs are genetically encoded and structurally restricted to a few canonical loop conformations that have generally been considered functionally redundant (Al-Lazikani et al., 1997; Martin and Thornton, 1996; North et al., 2011), although some bacterial and viral proteins bear natural affinities for V-gene-specific features outside the antigen binding surface, triggering non-productive/superantigen B cell responses (Silverman and Goodyear, 2006; Villar et al., 2016). In the current study, we harnessed mouse genetics to mechanistically interrogate natural antigen specificities endowed by human antibody genes in vivo. Our results show that despite their limited diversity, human heavy chain variable (V_H) genes are not functionally redundant and that specific germline-encoded input is both needed and tuned to accommodate conserved bacterial structures when present in the bloodstream.

It has long been hypothesized that the encoded CDR repertoire was shaped to endow antigen-specific recognition of conserved microbial features (Chothia and Lesk, 1987; Chothia...
Although this hypothesis has been refined and applied to account for human antibody specificities (Henry Dunand and Wilson, 2015; Lerner, 2011; Lonberg, 2005), experimental evaluation of germline-encoded recognition of recurrent microbial patterns has largely been explored within mice in which genetic manipulation has enabled mechanistic studies in vivo (Chen et al., 2009; Mi et al., 2000). Early reports noting Vδ-gene bias in hybridomas reactive to phosphorylcholine (PC), a lipid head group displayed by bacterial and eukaryotic cell membranes (Crews et al., 1981; Gearhart et al., 1981), revealed a germline-encoded target specificity that is now recognized as a core feature of natural antibodies: circulating polyreactive immunoglobulins that are produced constitutively by murine B1 cells and provide important bacterial defense activities (Chen et al., 2009; Grönwall et al., 2012; Holodick et al., 2017; Mi et al., 2000; Savage and Baumgarth, 2015). Natural antibodies with Vδ-gene-biased polyreactive substrate specificities have also been identified in humans that, like their murine counterparts, are produced from specialized B cell subsets (Grönwall et al., 2012; Nguyen and Baumgarth, 2016). However, in these cases, the microbial targets do not serve as the natural B cell priming agents, because natural antibodies are produced constitutively, possibly through BCR ligation by polyreactive self-antigens (Grönwall et al., 2012; Holodick et al., 2017; Savage and Baumgarth, 2015).

In this study, we sought to mechanistically evaluate whether conventional B cell responses from the human antibody repertoire (triggered by BCR ligation with exogenous antigen) bear natural specificities for conserved microbial substrate. Individual germline antibody CDRs have already shown clear evidence of differing utilities, because CDR sequences are differentially deployed following immune exposure to certain antigens (Broson et al., 1999; Hardy et al., 1989; Henry Dunand and Wilson, 2015; Hwang et al., 2014; Kirik et al., 2017; Lerner, 2011; Lonberg, 2005; Pape et al., 2018; Schickel et al., 2017; Wang et al., 2014; Willis et al., 2013), most recently observed for antibody responses to SARS-CoV-2 within convalescent individuals (Cao et al., 2020; Robbiani et al., 2020; Wec et al., 2020b). In humans, such germ-line-endowed affinity for antigen can also seed genetically reproducible or public broadly neutralizing antibody (bnAb) responses against numerous pathogens, including influenza virus, HIV, hepatitis C virus, hepatatis B virus, and yellow fever virus (Avnir et al., 2014; Flyak et al., 2018; Golsaz-Shirazi et al., 2015; Hehle et al., 2020; Joyce et al., 2016; Lerner, 2011; Lingwood et al., 2012; Lucas et al., 1991; Pappas et al., 2014; Scheid et al., 2011; Tzarum et al., 2019; Wec et al., 2020a; Wheatley et al., 2015; Wu et al., 2011; Zhou et al., 2013, 2015). We have experimentally demonstrated that such public epitope targeting by human BCRs enables vaccine amplification of pathway-reproducible antibody responses that broadly neutralize influenza virus (Sangesland et al., 2019). However, much of the work defining natural antigen substrate for human antibodies has relied on retrospective analyses of immune responses, in which patterns in antibody gene usage are first identified and then subsequently defined through biochemical activities in vitro.

To mechanistically evaluate human antibody Vδ-gene contribution to the polyclonal response within the living organism, we engineered transgenic animals that harbored human CDRH3 and light-chain (LC) diversities but were simultaneously constrained to different user-defined Vδ-genes (encoding CDRH1 + CDRH2). Using this approach, we defined the in vivo Vδ-gene contribution to extrafollicular responses against bacterial antigens in the blood, simple antiseptic B cell reactions that enable rapid humoral targeting of bacterial lipids and carbohydrates and in which response titers reflect antigen recognition by germ-line BCRs (Cerutti et al., 2013; Garraud et al., 2012; Limet et al., 1987, 1989; MacLennan et al., 2003; Shishido et al., 2012). We observed that specific Vδ-gene input was needed to engage and respond to lipopolysaccharide (LPS), a defining antigen of gram-negative bacteria. Notably, we found that the human Vδ-gene IGHV1-2*02, known for genetically endowing public bnAb responses against HIV (Gristick et al., 2016; Scheid et al., 2011; Wu et al., 2011; Zhou et al., 2013, 2015), was required to accommodate this key glycolipid antigen when prepared from diverse bacterial sources. We then mapped this Vδ-gene endowed polyclonal target specificity to the conserved substructure of LPS. Animals using non-IGHV1-2 Vδ-genes failed to accommodate LPS, despite bearing similar CDRH3 and LC diversities, B cell frequencies, and capacity to respond to other thymus-independent (TI) and thymus-dependent (TD) antigen geometries when present in the blood. Hence, we conclude that despite their limited diversity, individual human antibody Vδ sequences are not functionally redundant and that specific germline-encoded CDRs naturally endow the human antibody repertoire with a broad-spectrum response targeting a conserved bacterial feature.

### RESULTS

#### A System to Mechanistically Evaluate Human Vδ-gene Contribution to BCR Antigen Recognition In Vivo

We previously showed that mice homozygous for the HC2 locus are fully constrained to user-defined usage of single human Vδ-genes while containing a humanized CDRH3 length distribution and amino acid composition that is similar to that of the human repertoire (Sangesland et al., 2019). By systematically altering the Vδ sequence, this system provides an opportunity to mechanistically evaluate human Vδ-gene contribution to the polyclonal antibody response within the living organism. The number and frequency of B and T lymphocytes, antibody response titers, and somatic hypermutation within these mice are comparable to wild-type (WT) C57BL/6 (Sangesland et al., 2019). We previously used the HC2 locus to fully constrain the antibody response to single human Vδ-genes: IGHV1-2*02 or IGHV1-69*01, in which antibody LC diversity was provided by the murine repertoire (Sangesland et al., 2019).

To more closely recapitulate the human repertoire in the present study, we replaced the murine LC repertoire with KCo5, an engineered human kappa chain locus, as previously established for the HC2 platform (Fishwild et al., 1996; Lonberg, 2005; Xu and Davis, 2000) (Figure 1A). As expected, deep sequencing of the immunoglobulin M (IgM) BCR repertoires of these transgenic mice, termed IGHV1-2 + hlgK and IGHV1-69 + hlgK, revealed CDRH3 length and amino acid distributions that were similar to each other (>13.0 x 10^6 aligned BCR reads per genotype) and to the human CDRH3 profile, as mined from a recent human IgM repertoire analysis (DeWitt et al., 2016) (Figures 1B and 1C).
Hence, these animals provided a system to experimentally vary human antibody V<sub>H</sub>-gene usage to mechanistically evaluate V<sub>H</sub> contribution to antigen engagement in the context of an otherwise unconstrained polyclonal human-like BCR repertoire. We then applied this approach to evaluate B cell antigen recognition within the rapid blood-based extrafollicular antibody responses, normally triggered by bacterial lipids and carbohydrates (Cerutti et al., 2013; Garraud et al., 2012; Limet et al., 1987, 1989; MacLennan et al., 2003; Shishido et al., 2012).

**Transgenic Animals Have Similar Frequencies of B Lymphocytes**

Antisepsis responses to bloodborne bacterial lipids and carbohydrates are primarily TI IgM arising from B cells residing in the marginal zone (MZ) of the spleen (Cerutti et al., 2013; Garraud et al., 2012; Limet et al., 1987, 1989; MacLennan et al., 2003; Mond et al., 1995; Vos et al., 2000).

We measured the frequencies and total numbers of B cells (CD3<sup>-</sup>/CD19<sup>+</sup>), T cells (CD3<sup>+</sup>/CD19<sup>-</sup>), and MZ B cells (CD3<sup>-</sup>/CD19<sup>+</sup>/B220<sup>-</sup>/MHCI<sub>I</sub>''/CD23<sup>high</sup>/CD23<sup>low</sup>''; Garraud et al., 2012; Kanayama et al., 2005) within our transgenic animals (Figures 2A and 2B). We found that the animal genotypes contained similar numbers and frequencies of these immune cell subsets. Furthermore, to confirm that the intravenously (i.v.) introduced bacterial antigens trafficked to and perfused the extrafollicular space, we i.v. injected fluorescent bacterial LPS and then imaged the CD169 macrophage surface boundary layer encapsulating the spleen follicles. We found that the fluorescent antigen perfused the MZ and was captured within this space in both transgenic mouse genotypes (Figure 2C). Hence, B cells from our mice bearing human antibody repertoires with different V<sub>H</sub>-genes appeared similarly poised to initiate humoral responses against extrafollicular antigenic stimuli, particularly bacterial LPS.
A Specific Human \( \text{V}_{H} \) Sequence Is Needed to Accommodate Bacterial LPS In Vivo

Extrafollicular IgM responses emanating from spleen MZ B cells reflect antigen accommodation by germline BCRs (Cerutti et al., 2013; Garraud et al., 2012; MacLennan et al., 2003; Mond et al., 1995; Shishido et al., 2012; Vos et al., 2000; Öner et al., 2016; Tittle and Rittenberg, 1980; Vos et al., 2000) and can be tracked 3, 7, and 14 days after i.v. immune challenge (Cerutti et al., 2013; Chappell et al., 2012; MacLennan et al., 2003; Rubtsov et al., 2005). Moreover, sequential immunization with TI antigen will not boost antibody response titers, because there is no T cell help to coordinate B cell germinal center formation and development of B cell memory (Mond et al., 1995; Tittle and Rittenberg, 1980; Vos et al., 2000).

We found that both IGHV1-2 and IGHV1-69 mice produced robust extrafollicular IgM responses against TNP-ficoll, a standard TI-2 antigen that elicits antibodies through BCR crosslinking only, in the absence of innate immune receptor stimulation (Mond et al., 1995; Öner et al., 2016; Tittle and Rittenberg, 1980; Vos et al., 2000) (Figure 3A). We observed the same result following immunization of HCo12 mice, which use the same human CDRH3 and LC diversity but deploy five common human \( \text{V}_{H} \)-genes (IGHV5-51 + IGHV3-23 + IGHV3-30 + IGHV1-69 +IGHV4-34) (Figures 3A and 3B). All mouse genotypes also produced robust IgM responses against ovalbumin when this control TD antigen was administered i.v. (Figure 3A). Surprisingly, however, only the IGHV1-2 mice generated b
IgM responses against LPS, which were prepared from diverse bacterial strains spanning two phylogenetic orders: *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, and *Salmonella enterica* (Figures 3B and 3C). This demonstrated that despite housing comparable levels of CDRH3 and LC diversities and B cells receptive to other TI and TD antigen geometries, polyclonal accommodation of diverse LPS structures was functionally reliant on CDRs encoded by IGHV1-2.

**V<sub>H</sub>-gene Hardwired Antibody Specificity for LPS Maps to the Conserved Saccrolipid Core of This Antigen**

Given that V<sub>H</sub>-gene-dependent immunoreceptor activity was observed for diverse sources of LPS, we hypothesized that IGHV1-2-encoded CDRs may endow target solutions that recognize conserved features of this antigen, perhaps the conserved saccrolipid substructure of bacterial LPS (Figure 4A). To test this, we immunized our animals with a uniform Kdo2-lipid...
A preparation with chemically homogeneous acyl chain structures, and consequently the most potent LPS-based TLR4 agonist yet described, reported as 10-fold higher than conventional LPS preparations (Saito et al., 2010). As with the heterogeneous LPS inocula, we observed VH-gene-dependent accommodation of Kdo2-lipid A in vivo, as evidenced by robust IgM responses in IGHV1-2 animals and dampened immunogenicity in the IGHV1-69 and HCo12 genotypes (Figure 4B). We therefore surmised that the natural polyclonal B cell target specificity endowed by the IGHV1-2 sequence mapped to the conserved, universal building block of bacterial LPS.

**DISCUSSION**

Genetically hardwired specificities for conserved microbial patterns are hallmarks of the innate immune system and have long been proposed for B cell antigen receptors (Chothia and Lesk, 1987; Chothia et al., 1989; Cohn and Langman, 1990; Henry Dunand and Wilson, 2015; Hwang et al., 2014; Lerner, 2011; Lonberg, 2005). However, such germline-endowed affinities for specific features common to pathogens or groups of pathogens have been difficult to mechanistically prove in vivo. This is because the ligand binding surface of the BCR is composed of a chimeric display of encoded and non-encoded CDRs unique to each lymphocyte clone (Glanville et al., 2009; Kuroda et al., 2008; Morea et al., 1998; Oliva et al., 1998; Saada et al., 2007; Schroeder and Cavacini, 2010; Shirai et al., 1999; Wedemayer et al., 1997; Xu and Davis, 2000). How then can mechanistic causality be assigned to any one component in vivo? Much of the work defining natural human antibody substrate has relied on retrospective analyses of human immune responses in which patterns in antibody gene usage are first identified and then biochemically evaluated in vitro. In this study, we harnessed mouse genetics to mechanistically interrogate BCR antigen specificities.

**Figure 4. IGHV1-2 Endows Innate-like BCR Specificity for the Conserved Saccharolipid Core of Bacterial LPS**

(A) Kdo2-lipid A (Di[3-deoxy-D-manno-octulosonyl]-lipid A) from is a conserved LPS substructure and is the most homogeneous and potent LPS preparation available (Saito et al., 2010) (the defined hydrocarbon chain lengths are marked).

(B) Extrafollicular IgM responses against Kdo2-lipid A when introduced into the bloodstream of IGHV1-2, IGHV1-69, and HCo12 animals. IgM responses were monitored 3, 7, and 14 days post-immune challenge (mean ± SD, n = 3 per genotype). Pre-immune readings were subtracted from these curves.
endowed by individual human antibody genes. We engineered a system in which all antibody CDRs were humanized, allowing unconstrained diversity with the exception of those encoded by the \( V_{H} \) sequence, which were 100% restricted and systematically varied. Through this approach, we could experimentally define human \( V_{H} \)-gene contribution to the polyclonal antigen response as a whole and as a function of immune challenge within the living organism.

We evaluated this parameter within extrafollicular B cell responses to bloodborne LPS, simple and rapid antiseptic reactions in which antibody response titers reflect germline BCR engagement capacity (Cerutti et al., 2013; Garraud et al., 2012; MacLennan et al., 2003; Shishido et al., 2012). Our results indicated that of six common human \( V_{H} \) sequences, only IGHV1-2 could support robust polyclonal accommodation of LPS, which was prepared from diverse sources spanning two phylogenic orders of gram-negative bacteria. Importantly, this was achieved by genetically endowing for recognition of the non-variable saccharipolycid core of this antigen, revealing a broad-spectrum antiseptic response against a conserved microbial feature, akin to the pattern recognition receptor activities of the innate immune system.

In a pioneering study, Xu and Davis (2000) reported that a single \( V_{H} \)-gene mouse generated antibodies against any protein antigen through CDR3 diversity alone, functionally establishing this motif as the principle determinant of B cell antigen recognition in vivo. The centrally positioned CDR3 supplies the human BCR repertoire with \( \times 10^6 \) times the diversity of antibody V-gene-encoded CDRs and is thought to enable target solutions against any antigenic surface (Glanville et al., 2009; Kuroda et al., 2008; Morea et al., 1998; Oliva et al., 1998; Saada et al., 2007; Schroeder and Cavacini, 2010; Shirai et al., 1999; Wedemayer et al., 1997; Xu and Davis, 2000). Diverse CDR3- and LC-associated targeting solutions have been observed for antibody responses against LPS and other bacterial antigens (Haji-Ghassemi et al., 2015; Nguyen et al., 2003) and would also be expected for the polyclonal anti-LPS responses we observe. However, we demonstrate a deterministic feature that functionally gates the polyclonal response as a whole, namely, that specific human \( V_{H} \)-gene input is obligate for engaging bloodborne LPS, despite the presence of equivalent CDR3 and LC diversities across our transgenic mouse genotypes. The LPS response deficits occurred in animals that were otherwise able to respond to control TI and TD antigens and were not explained by the capacity to perfuse the spleen MZ with LPS or the number of responding B cells in residence. LPS is also a potent agonist for Toll-like receptor 4 (TLR4) and a powerful B cell adjuvant (Pone et al., 2012; Scheweffoer et al., 2017), and it is particularly potent in the Kdo2-lipid A format (Saato et al., 2010). The failure to accommodate these antigen geometries, even in the presence of such TLR stimulation, underscores our conclusions that despite their limited diversity, human \( V_{H} \)-genes are not functionally redundant and that some human antiseptic responses are germline encoded.

The amino acid sequence XXGG(S/T/G) within the CDR2 loops of mouse immunoglobulins has previously been reported as a lipid A binding motif (Haji-Ghassemi et al., 2016). This motif is present in the CDR2 from IGHV1-2*02; however, it is also present within IGHV3-23*01, contained within HC012 mice, which did not respond to bloodborne LPS. Consequently, LPS binding through this motif may not be broadly transplantable to the human \( V_{H} \)-genes of our study, although differential expression of \( V_{H} \) sequences within the HC012 mouse also cannot be ruled out.

In conclusion, we demonstrate that conventional B cell responses can be tuned by human-gene-endowed affinities for a conserved microbial pattern, akin to the receptors of the innate immune system. This discovery was enabled by manipulating mouse genetics to experimentally define \( V_{H} \)-gene contribution to B cell antigen recognition within an otherwise unrestricted human-like antibody repertoire. Beyond its potential clinical importance in combating sepsis, a germline-encoded anti-LPS response may reflect a primordial/evolutionarily retained capacity that, in the case of IGHV1-2*02, could have provided a foundation for later unanticipated germline utilities, such as the non-random use of this \( V_{H} \) sequence in human bnAbs against HIV (Grisick et al., 2016; Scheid et al., 2011; Wu et al., 2011; Zhou et al., 2013, 2015).

**STAR Methods**

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

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  - Materials Availability
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- **Experimental Model and Subject Details**
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  - Imaging Antigen Capture in the Spleen
  - Immune Challenge with Bloodborne Antigens
  - ELISA
  - Phylogenetic Reconstruction
- **Quantification and Statistical Analysis**

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**Author Contributions**

M.S., A.S.Y., and D.L. designed the research studies; M.S., A.S.Y., L.R., S.W.K., A.L.Z., G.J.G., M.R.H., R.M.B., M.Q.-C., D.R., N.L., D.K., A.K.S.,...
and D.L. performed the research; M.S., A.S.Y., D.K., A.K.S., and D.L. analyzed the data; and M.S. and D.L. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| Antibodies          |        |            |
| Rat Anti-CD19-PerCP-Cy5.5 | BioLegend | Cat#115533; RRID: AB_2259869 |
| Rat Anti-B20-FITC   | BD Biosciences | Cat#553087; RRID: AB_394617 |
| Rat Anti-MHCII-BV510 | BioLegend | Cat#107636; RRID: AB_2734168 |
| Rat Anti-CD3-BV785  | BioLegend | Cat#100232; RRID: AB_2562554 |
| Rat Anti-CD23-PE/Cy7 | BioLegend | Cat#101614; RRID: AB_2103036 |
| Rat Anti-CD169-FITC | BioRad | Cat#MCAB84FT; RRID: AB_1100895 |
| Rat Anti-CD21/CD35-BV421 | BD Biosciences | Cat#562756; RRID: AB_2737772 |
| Goat Anti-IgM-HRP   | Southern Biotech | Cat#1021-05; RRID: AB_2794240 |
| Chemicals, Peptides, and Recombinant Proteins |        |            |
| LIVE/DEAD Fixable Blue Dead Cell Stain | Thermo Fisher | Cat#L34961 |
| Sigma Adjuvant System | Sigma-Aldrich | Cat#S6322 |
| KAPA HiFi HotStart ReadyMix | KAPA Biosystems | Cat#KK2602 |
| Maxima H Minus Reverse Transcriptase | Thermo Fisher | Cat#EP0751 |
| HotStarTaq Plus DNA Polymerase | QIAGEN | Cat#203603 |
| RLT Buffer | QIAGEN | Cat#79216 |
| Collagenase D (Roche collagenase D) | Roche | Cat#1108858001 |
| ACK lysis buffer | Lonza | Cat#10-548E |
| Tissue-Tek O.C.T Compound | Sakura | Cat#4583 |
| Background Sniper Blocking Reagent | Biocare Medical | Cat#902-966-082317 |
| LPS (E.coli Serotype 055:B5, Fluor568 conjugate) | Thermo Fisher | Cat#L23352 |
| LPS (Escherichia coli O55:B5) | Sigma | Cat#L2880 |
| LPS (Klebsiella pneumoniae) | Sigma | Cat#L4268 |
| LPS (Pseudomonas aeruginosa 10) | Sigma | Cat#L9143 |
| LPS (Salmonella enterica serotype typhimurium) | Sigma | Cat#L6511 |
| kdo2-Lipid A | Avanti Polar Lipids | Cat#699500P |
| TNP-ficoll | Biosearch Technologies | Cat#F-1300-10 |
| Ovalbumin | Biosearch Technologies | Cat#O-1000-100 |
| Tetramethylbenzidine (TMB) | Sigma | Cat#T0440 |
| Critical Commercial Assays |        |            |
| MiSeq Reagent Kit, V2 500 cycles | Illumina | Cat#MS-102-2003 |
| Mouse B Cell Isolation Kit II | Miltenyi | Cat#130-104-443 |
| Deposited Data |        |            |
| igM HC sequences from IGHV1-69-hlgK and IGHV1-2-hlgK mice | This Paper | GEO: GSE154285 https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE154285 |
| Experimental Models: Organisms/Strains |        |            |
| Mouse: IGHV1-69*01+hlgK | This Paper | N/A |
| Mouse: IGHV1-2*02+hlgK | This Paper | N/A |
| Mouse: HCo12 | This Paper | N/A |
| Oligonucleotides |        |            |
| Primers for Bulk HC Amplification | Sangesland et al., 2019 | N/A |
| Software and Algorithms |        |            |
| FlowJo v.9.3.2 | TreeStar | https://www.flowjo.com; RRID: SCR_008520 |
| Prism 7 | GraphPad | https://www.graphpad.com; RRID: SCR_002798 |

(Continued on next page)
**RESOURCE AVAILABILITY**

**Lead Contact**
Further information and requests for reagents should be directed to and will be fulfilled by Daniel Lingwood (dlingwood@mgh.harvard.edu).

**Materials Availability**
There are restrictions to the availability of the IGHV1-69, IGHV1-2 and HCo12 mice due to a MTA with Bristol-Myers Squibb.

**Data and Code Availability**
Antibody sequences from this study were deposited in GEO (GSE154285).

**EXPERIMENTAL MODEL AND SUBJECT DETAILS**

**Transgenic Mice**
All transgenic mice were obtained as a gift to D.L. from Bristol-Myers Squibb (Redwood City, CA). The animals were generated using a pre-established strategy that fully constrains to user-defined human VH segments, while allowing for normal and random recombination with diverse human Dn and Jn segments, as encoded by the HC2 locus (Fishwild et al., 1996; Lonberg, 2005; Lonberg et al., 1994; Sangesland et al., 2019) (Figure 1). We have previously demonstrated that murine N-junctional diversification on the HC2-encoded Dn and Jn genes produces a germline CDRH3 repertoire in antigen naive IgM B cells that is similar to humans (Fishwild et al., 1996; Lonberg, 2005; Lonberg et al., 1994; Sangesland et al., 2019). Full IgH restriction to IGHV-69*01 or IGHV1-2*02 was also previously demonstrated using this approach (Sangesland et al., 2019), however, the light chain repertoire in those animals was provided by the murine kappa and lambda genes (Sangesland et al., 2019). The animals in the present study were further humanized by replacing the murine LC repertoire with the human kappa chain-encoded KCo5 cassette (= IGHV1-2 + hIgK and IGHV1-69 + hIgK), which provides for near full diversity in the human kappa locus (Fishwild et al., 1996; Lonberg, 2005; Xu and Davis, 2000). HCo12 animals were also deployed where the mice bear the same LC repertoire and humanized diversity in CDRH3, but are modified to include five common human VH-gene segments (IGHV5-51, IGHV3-23, IGHV3-30, IGHV1-69, and IGHV4-34) and have previously been applied in antibody drug discovery, where they have generated high-affinity therapeutic antibodies (US Patent 7247301). All transgenic animals were generated per standard techniques as described by Hogan et al. (1994). The animals were maintained within Ragon Institute’s HPPF barrier facility and all experiments were conducted with institutional IACUC approval (MGH protocol 2014N000252). In this study, both male and female animals, aged 6-10 weeks, were used.

**METHOD DETAILS**

**BCR sequencing**
CDRH3 diversity within the transgenic mice was measured by deep sequencing the BCRs from antigen naive IgM B cells in bulk according to our previously deployed method (Sangesland et al., 2019; Trombetta et al., 2014). Briefly, we enriched BCR libraries from B cell whole transcriptome amplification (WTA) products using the Smart-Seq2 protocol, as previously described (Sangesland et al., 2019; Trombetta et al., 2014). Following WTA, two 0.8x (v/v) SPRI bead-based cleanups and cDNA quantitation/normalization, we PCR amplified the BCR heavy chains (FR3 to CDR3) [HotStarTaq Plus; QIAGEN], using with a forward primer specific for the FR3 region within the Vn gene of interest (final concentration: 0.5 μM) and reverse primers against the heavy constant regions (final concentration: 1μM) (Sangesland et al., 2019). These primers were attached to the Illumina P7 (FR3 region) and P5 (constant region) as
described previously (Sangesland et al., 2019). Following BCR amplification, SPRI cleanup was performed, and we quantified and normalized the amplicons to 0.2–0.5 ng/μL. We then amplified the heavy chain sequences further using step-out PCR with the Illumina sequencing adapters [Kapa HiFi HotStart ReadyMix; Kapa Biosystems]. We sequenced these PCR products (FR3 through CDRH3) and aligned the resulting paired end FASTQs using MiXCR (Bolotin et al., 2015). Within this alignment step, we applied the following parameter: OvParameters.geneFeatureToAlign = {FR3Begin; Vend}, and constrained alignment to heavy chain matches only. We trimmed low sequence quality reads using the default settings within MiXCR. To measure the CDRH3 diversity in the human repertoire, we mined data from Donor 1A within a recent study of naive IgM BCR sequencing (DeWitt et al., 2016). Using custom scripts within immunarch (v0.5.5; https://zenodo.org/record/3613560#.XnzYjdNKiJQ) tcR (Nazarov et al., 2015), we reconfigured the sequenced naive IgM BCRs and the mined human data to generate frequency plots of CDRH3 length and assigned Kabat numbering for amino acid composition of 16aa length CDRH3s.

Flow cytometry
Mouse spleens were gently disrupted in R10 (RPMI with 10% FBS) in the presence of 1mg/ml collagenase D (Roche collagenase D) and then filtered through a 70μm cell strainer. The splenocytes were resuspended within ACK lysis buffer to remove red blood cells (Lonza) and then washed with PBS. The cells were then stained with Blue viability dye (0.025 mg/ml; Thermofisher) and then master mixes containing the following fluorescent antibodies: CD19 PerCP-Cy5.5; B220-FITC; MHCI-BV510; CD23-PECy7; CD21/CD35-BV421; CD3-BV785. Each antibody was used at a final dilution of 1:100 within the master mix. The samples were then run on a 5 laser LSR Fortessa (BD Biosciences) and data were analyzed using FlowJo software version 9.3.2 (TreeStar).

Imaging antigen capture in the spleen
To confirm that our transgenic mice could capture bacterial LPS within the spleen marginal zone, we intravenously injected 15μg fluorescent LPS (E.coli Serotype 055:B5; Fluor568 conjugate, Thermofisher) and then removed the spleens at 1 after the injection. We prepared the spleens for immunostaining by submerging ~5mm thick pieces within O.C.T Compound (Tissue-Tek; Sakura) and then freezing within a metal container that contained isobutene but was surrounded by liquid nitrogen. We then made tissue sections from the frozen block (40 μm) using a cryostat. These samples were air-dried overnight and then fixed in ice-cold acetone for 10 min. Following rehydration in PBS, we blocked the sections using “Background sniper” (Biocare) within a dark humid chamber and then incubated the chamber with anti-CD169-FITC diluted 1:500 in PBS containing 0.1% Tween20). This stained metallophilic macrophages to delineate the follicular versus extrafollicular space (Louie and Liao, 2019; Moran et al., 2019). The sections were then washed with PBS in a coplin jar (3x, five min each) and then slide-mounted using ProLong Gold Antifade (Invitrogen). Images of the fluorescent LPS and FITC were then acquired using a Zeiss Elyra PS.1 confocal/super resolution microscope.

Immune challenge with bloodborne antigens
Antigens were injected intravenously (IV) in 100μl inoculums containing: 15μg LPS (preparations from Escherichia, coli, Klebsiella pneumoniae, Pseudomonas aeruginosa, or Salmonella enterica; Sigma); 30μg TNP-ficol (Biosearch Technologies); 25μg ovalbumin (Biosearch Technologies); or 15μg of kdo2-Lipid A (Di[3-deoxy-D-manno-octulosonyl]-lipid A, Avanti Polar Lipids). Ovalbumin was adjuvanted with 50% w/v Sigma adjuvant, as described previously (Sangesland et al., 2019; Yassine et al., 2015). Blood was sampled pre-immunization and 3, 7 and 14 days post immunization.

ELISA
Serum antibody responses to LPS were measured according to a previously described method (Tobias et al., 1989). Briefly, 96 well microtiter plates were first coated with 30 μg/ml LPS in carbonate buffer (100mM Na2CO3, 20mM EDTA, pH 9.6) at 100μl/well for 3 h at 37°C. The plates were then washed (3x) with water and then dried overnight in a desiccator. Excess binding sites were then blocked by addition of 10mg/ml BSA in HS buffer (50mM HEPES, 0.15mM NaCl, pH 7.4) at 200μl/well. After blocking, the sera (pre-bleed and immune sera) were added through serial dilution in HS buffer containing 1mg/ml BSA, starting at 1:20 (v/v). This was allowed to bind for 3 h at 37°C. The plate was then washed (3x) with HS buffer containing 1mg/ml BSA and then incubated with anti-IgM-HRP at 1:5000 dilution in HS buffer + 1mg/ml BSA. The wells were then washed (3x) as above and developed by addition of TMB substrate followed by quenching with 1N sulphuric acid. The plates were then read at 450nm using the Teacan Infinite m1000 Pro microplate absorbance reader (Männedorf, Switzerland).

For the other antigens, 200ng/well of each antigen was coated to the microtiter plates by overnight incubation at 4°C. The plates were blocked with 2% BSA in PBS for 1 h and then washed (3x) with PBS. After blocking, the pre and post immune sera was serially diluted into PBS [starting at 1:20 (v/v)] and then added to the wells and incubated for 1 h. The plates were washed (3x) in PBS containing 0.5% Tween 20 (PBST) and incubated with the anti-IgM-HRP diluted at 1:5000. The wells were then washed in PBST and developed using TMB substrate followed by quenching by sulphuric acid, as detailed above.

Phylogenetic reconstruction
Genomes for type strains were downloaded from NCBI refSeq (O’Leary et al., 2016). Genes were called using Prodigal (Hyatt et al., 2010). Forty universal single copy marker genes were identified using fetchMG v1.0 (Sunagawa et al., 2015). ETE3 v3.1.1
(Huerta-Cepas et al., 2016) was used to produce a phylogenetic reconstruction. The tree was rooted to *Burkholderia cepacia* ATCC 25416 and visualized using iTOL v5 (Letunic and Bork, 2019).

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All statistical analysis was performed using Prism Graphpad software. Sample sizes of animals and specific tests to determine statistical significance used are indicated in the methods and corresponding figure legends. Data were considered statistically significant at $p < 0.05$. 