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DOCK8 mutations cripple B cell immune synapse, germinal centers and long-lived antibody production

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Abstract

To illuminate genes and mechanisms for humoral immunity we performed a mouse genetic screen for mutations that do not affect the first wave of antibody to immunization but disrupt response maturation and persistence. The first two mutants identified were loss-of-function mutations in DOCK8. DOCK8 mutant B cells were unable to form marginal zone B cells or to persist in germinal centers and undergo affinity maturation. DOCK8 mutation disrupted the concentration of ICAM-1 in the B cell immune synapse but did not alter other aspects of B cell antigen receptor signaling. Humoral immunodeficiency due to DOCK8 mutation provides evidence that

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Author Contributions:

TL, KR, AJ, TB-J & BW mapped and identified the mutations; KR performed analysis of *pri* and complementation crosses with EK, HD, LT & AE; TL performed analysis of *cpm* with TLC, AJ, TB-J, DA & JC; BT & FB performed synapse analysis; ML supervised early experiments on *cpm* by AJ; FM & KR analyzed effects of BAFF transgene; ED & ST analyzed role in SAP-dependent B-T conjugation; KR, TDC, TC & RB analyzed GC response in SW_{HEL} adoptive transfer system; CV & EK designed and established the genetic screen and identified *cpm* and *pri* founders; CG & RC initiated and contributed to all aspects of study design & interpretation. KR, TL, RC and CG wrote the paper in consultation with all co-authors.

Competing financial interests.

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organization of an immune synapse is critical for signaling the survival of B cell subsets required for long-lasting immunity.

Antigen receptor signaling coordinates the antibody response to infection or immunization, but the nature of the signals that guide the formation of high affinity memory B cells and long-lived humoral immunity remains unclear. Two classes of antibody deficiency syndromes are currently recognized as arising from defects in antigen receptor signaling: defects in early B cell development and common variable immunodeficiency¹. Defects in early B cell development result from mutations in the components of the B cell antigen receptor (BCR) and the Bruton's tyrosine kinase (BTK) signaling cascade immediately downstream. These syndromes are readily identified by agammaglobulinemia and reduced numbers of circulating B cells. By contrast, common variable immunodeficiency (CVID) syndromes have relatively normal B cell numbers and may have qualitative or quantitative defects in immunoglobulin (Ig) production². CVID is the most common clinically significant primary immunodeficiency, yet over 75% of cases cannot currently be explained genetically or mechanistically³. A small number of cases result from mutations in CD19⁴ [<http://www.signaling-gateway.org/molecule/query?afcsid=A000538>], a co-receptor for enhancing BCR signaling that recruits PI3-kinase⁵ [<http://www.signaling-gateway.org/molecule/query?afcsid=A001770>]. Other subsets are due to mutations in TACI, which is one of the B cell receptors for the TNF-related cytokines BAFF and APRIL^{6,7}, or mutations in ICOS, which serves as a costimulatory receptor for T follicular helper cells⁸. Understanding the variable antibody responses and deficiencies of switched memory B cells in the CVID spectrum of disorders⁹ is hindered by the limited understanding of genes and signaling mechanisms that are specifically required for maturation and longevity of the antibody response.

To identify new genes and mechanisms generating mature antibody responses, and animal models for understanding CVID and related immunodeficiencies, we describe here an immunization screen for selective defects in antibody response maturation and longevity in mouse pedigrees segregating thousands of induced single nucleotide substitutions. The first two pedigrees with humoral immune deficiency identified by this screen resulted from independent mutations in a previously obscure member of the DOCK family of Rho-Rac guanine exchange factors (GEFs; [<http://www.signaling-gateway.org/molecule/query?afcsid=A002062>]), Dedicator of cytokinesis 8 (DOCK8). The consequences of the mutation in B cells provide evidence that polarization of lymphocyte antigen receptors and integrins into an immune synapse is critical for signaling survival and establishment of high affinity antibody producing cells and long-lasting immunity, and yield a timely animal model for understanding human immunodeficiency.

Results

Immunization screen

To understand the generation of long-lived humoral immunity, we developed a genetic screen that could identify mutant mice with a failure to mount long-lived, high affinity antibody responses despite a relatively normal initial wave of antibody production. 301 pedigrees of C57BL/6 mice, each segregating thousands of N-ethyl-N-nitrosourea (ENU)-induced single nucleotide substitutions, were tested for initial and mature serum antibody after immunizing multiple siblings in each pedigree with a mixture of antigens. Two recessive mutations that disrupted antibody response maturation (Fig. 1a,b) were propagated from independent pedigrees and denoted *captain morgan* (*cpm*) and *primurus* (*pri*). During the initial antibody response, IgG antibodies against killed *Bordetella pertussis* bacteria and against the protein antigen chicken gamma globulin (CGG) were correctly polarized to

IgG2c and IgG1, respectively, and reached indistinguishable titers 14 days after immunization in *pri/pri* homozygous mice, *cpm/cpm* animals, and wild-type siblings (Fig. 1a, top; Fig. 1b; and data not shown). Since the majority of CGG-antibody and *B. pertussis* antibody produced at this time comes from extrafollicular B cell clonal expansion and plasma cell formation in collaboration with T_H1 and T_H2 T cells in the T cell zone¹⁰, the absence of a substantial effect of the mutations on this phase of antibody production establishes that this phase of B and T cell activation is intact. The homozygous mutations also did not have an appreciable effect on T cell-independent extrafollicular B cell responses to nitrophenyl (NP) hapten coupled to the polysaccharide antigen Ficoll (Fig. 1a). However during the mature phase of the antibody response, either after a booster immunization (44 days, Fig. 1a) or without boosting (28 or 56 days after primary immunization, Fig. 1b), IgG antibody to CGG failed to be sustained in *pri/pri*, *cpm/cpm* mice or in compound heterozygotes. The CGG immunogen carried a haptenic chemical group, azobenzenearsonate (ARS), which only elicits appreciable antibody in wild-type mice after 4 weeks (Fig. 1b) due to the need for antibody hypermutation and affinity maturation^{11, 12}. Antibody to the ARS hapten failed to develop in *pri* or *cpm* homozygotes or compound heterozygotes.

A comprehensive analysis of B cell subsets in blood and primary and secondary lymphoid organs revealed qualitatively normal development of circulating mature subsets, but near-absence of marginal zone B (MZB) cells (Fig. 2). Formation of immature B cells in the bone marrow and accumulation of mature follicular B cells appeared normal in mutant mice (Fig. 2a,b and data not shown), but there was profound deficiency of splenic MZB cells in *cpm* and *pri* homozygotes and *cpm/pri* compound heterozygotes (Fig. 2a-d, and Supplementary Fig. 1), which was cell autonomous to B cells bearing the mutation in mixed chimeras (Fig. 2e). MZB cell numbers are markedly elevated in transgenic mice over-expressing BAFF cytokine, but this failed to rescue MZB cell formation by mutant cells (Supplementary Fig. 2). The frequency of B1 B cells in the peritoneal cavity was decreased 60% in the mutant mice (Fig. 2f) and this was cell autonomous in mixed chimeras. Analysis of splenic T cells revealed normal numbers of activated or memory CD4⁺ and CD8⁺ T cells but a mean 50% decrease in the number of naïve T cells in *cpm* and *pri* homozygotes and in *cpm/pri* compound heterozygotes, although this trait overlapped the lower end of the normal range and was not reliable for identifying individual homozygotes (Fig. 2g). In 50:50 bone marrow chimeras with mutant T cells reconstituting in the presence of wild-type T cells, T cells bearing the mutation had a cell-autonomous competitive disadvantage and comprised only 5% of the total peripheral T cells (Fig. 2h).

DOCK8 mutations

MZ B cell deficiency provided a clear Mendelian recessive trait to map and identify the causative *cpm* and *pri* mutations. Both mutations mapped to the same region of chromosome 19 (Supplementary Fig. 3) and sequencing of B cell-expressed transcripts within the minimal 4.5 Mb interval identified independent mutations in *Dock8* (Fig. 3a). DOCK8 is a little studied member of a family of proteins that are critical activators of the Rho family of small GTPases (G-proteins) in worms, flies and mammals¹³. In other DOCK proteins, the Dock Homology Region 1 (DHR1) domain (also known as CDM-zizimin homology 1 or CZH1 domain) binds phosphatidylinositol-3,4,5-triphosphate (PtdIns(3,4,5)P₃) lipids generated by phosphatidylinositol-3-kinase (PI(3)K) activity to promote localized membrane binding and activation. The DHR2 (CZH2) domain binds to Rho-family G-proteins (Rho, Rac, Cdc42; [<http://www.signaling-gateway.org/molecule/query?afcsid=A002062>]), promoting GTP exchange and activating local cytoskeletal changes, integrin-mediated adhesion, lamellipodia formation, cell polarization, phagocytosis or cell fusion^{13, 14}.

DOCK8 has been isolated in a yeast two-hybrid screen for binding partners of Cdc42 and found to localize at lamellipodia in fibroblasts¹⁵, but its *in vivo* function was unknown.

The *cpm* mutation was a T to C substitution in the exon 20 splice donor sequence that caused aberrant mRNA splicing between cryptic 5' or 3' splice donors and the normal exon 21 splice acceptor (Fig. 3b,c). Sequencing of 74 cDNA clones from *cpm/cpm* splenocytes showed aberrant splicing to yield frame-shifts and premature stop codons that eliminate the DHR2 domain entirely (Fig. 3c). The *pri* mutation, in exon 43, was a T to C substitution changing codon 1827 from serine to proline (Fig. 3a). The mutated serine is absolutely conserved in vertebrate DOCK8 and in the DHR2 domain of DOCK6, 7 and 9 (Fig. 3d). In the structure of DOCK9 complexed with Cdc42¹⁶ this conserved serine lies in DHR2 lobe B alpha helix 6, which forms part of the Cdc42 binding site and provides four Cdc42 contact residues (triangles in Fig. 3d). The *pri* substitution of Ser 1827 to Pro would be expected to break this alpha helical structure and interfere with the GEF activity of the DHR2 domain. The nature of the mutations, their recessive inheritance, and their failure to complement in crosses establish the cause of the antibody response, B and T cell defects as being due to loss of normal DOCK8 function. *Dock8* mRNA was expressed ten times more abundantly in B and T lymphocytes than in other tissues (Supplementary Fig. 4). Since homozygotes for the truncating *Dock8^{cpm}* allele are weaned at normal Mendelian frequency and appear normal in growth and behavior, we conclude that *Dock8* has a relatively specialized role in adaptive immunity.

Crippled germinal centers

Since immunization of DOCK8 mutant mice elicited a normal early wave of antibody formation but the response failed to mature or be sustained, we sought to define the cellular basis for this variable immune deficiency. Following immunization with sheep red blood cells (SRBCs), wild-type mice formed large germinal centers, denoted by peanut agglutinin-positive (PNA⁺) IgD⁻ B cells, whereas *pri/pri* and *cpm/cpm* animals formed only small PNA⁺ clusters (Fig. 4a and data not shown). Phenotypically normal CD95⁺ GL7⁺ B220⁺ GC B cells could be readily detected by flow cytometry in homozygous mutants after immunization but their numbers were only 15% of normal (Fig. 4b). Immunization of competitive hematopoietic chimeras, constructed by reconstituting irradiated mice with a mixture of CD45.1⁺ wild-type and CD45.2⁺ *pri/pri* or *cpm/cpm* bone marrow, revealed that B cells bearing the DOCK8 mutation contributed normally to the main subsets of B220⁺ and IgD⁺ recirculating follicular B cells but contributed poorly to the B220⁺ CD95⁺ GL7⁺ GC subset particularly at day 11 after immunization when the response has begun maturing (Fig. 4c). The mutations therefore acted autonomously within individual B cells to interfere selectively with their capacity to differentiate into or sustain a GC state.

The precise step where the mutation interfered with GC B cells was determined by tracing a homogeneous population of B cells specific for hen egg lysozyme (HEL) antigen after adoptive transfer into wild-type mice immunized with SRBCs conjugated to HEL (Fig. 5,6). The *primus* mutation was crossed with CD45.1 SW_{HEL} transgenic mice, bearing a rearranged HEL-specific V(D)J_H element targeted into the *Igh* locus encoding immunoglobulin H chain combined with a HEL-specific kappa L-chain transgene¹⁷. B cells from *pri/pri* CD45.1 SW_{HEL} transgenic donors, or from wild-type CD45.1 SW_{HEL} transgenic controls, were adoptively transferred into wild-type C57BL/6 (CD45.2) recipients. The recipients were then immunized with a conjugate antigen comprising SRBCs covalently decorated with HEL protein bearing two substitutions (HEL^{2X}). In contrast with the very high affinity of Ig receptors on SW_{HEL} B cells for native HEL ($2 \times 10^{10} \text{ M}^{-1}$), HEL^{2X} is recognized with intermediate affinity ($8 \times 10^7 \text{ M}^{-1}$) by Ig receptors on SW_{HEL} B cells due largely to the effect of substituting Asp101 to Arg¹⁸⁻²⁰. Six amino acids in the complementarity determining region 2 (CDR2) loop of the SW_{HEL} V_H chain contact native

HEL at Asp101²¹, and selection of amino acid replacements in these CDR2 residues increases the affinity for D101R-substituted HEL^{2X} or HEL^{3X} in SW_{HEL} GC cells¹⁹ and unpublished data).

In HEL^{2X}-SRBC immunized wild-type recipients, both wild-type and *pri/pri* HEL-specific B cells underwent comparable activation into GL7⁺ Fas⁺ cells scattered within follicles 2.5 days after immunization, and comparable rates of isotype switching to IgG1 occurred between days 3.5 and 4.5 (Fig. 5a,b and Supplementary Fig. 5). Germinal centers formed within follicles on day 4.5, when the majority of both wild-type and mutant SW_{HEL} B cells had differentiated into Fas^{hi} GL7^{hi} GC cells. No accumulation or GC differentiation by donor B cells occurred when recipients were immunized with SRBCs lacking HEL^{2X} (groups marked by asterisks). Immunoglobulin class switching is a sensitive indicator of T helper cell signals²², and requires CD40 on SW_{HEL} B cells in immune responses to HEL^{2X}-SRBC²³. These data reinforce those in Fig. 1 to indicate that DOCK8 is not essential for the initial phase of B cell activation and collaboration with helper T cells *in vivo*.

By contrast, DOCK8 deficiency more profoundly disrupted the response 4.5-10 days after immunization when wild-type SW_{HEL} B cells undergo hypermutation and affinity maturation. Although there was no discernable difference in their location within germinal centers on days 4.5 and 5 post-immunization (Fig. 6a and Supplementary Fig. 5), accumulation of SW_{HEL} *pri/pri* GC cells at this time was one-third of wild-type controls at this time (Fig. 5b, 6b) and fell to one-fortieth of controls by day 9 despite ongoing germinal centers composed of wild-type host B and T cells (Fig. 6b). This difference was also observed upon adoptive transfer of lymph node cells from SW_{HEL} mice (Supplementary Fig. 6), excluding the possibility that it results from absence of MZB cells in transferred *pri/pri* SW_{HEL} spleen cells. IgG1-switched B cells doubled as a percentage of wild-type SW_{HEL} B cells on day 9 compared with day 7 or 5, whereas the percentage IgG1⁺ failed to increase in SW_{HEL} GC B cells bearing the *pri* mutation (Fig. 6b).

Nine days after HEL^{2X}-SRBC immunization, sequencing of *Igh* genes from individual flow-sorted SW_{HEL} GC B cells showed that wild-type cells had accumulated a high ratio of replacement to silent mutations (10.3) in the CDR2 residues contacting the D101R substitution in HEL^{2X} (Fig. 6c,d and Supplementary Fig. 7). Six of the 38 cells analyzed (16%) had Y58F replacements in CDR2 that have been shown to increase affinity²⁴. Flow cytometric staining with nanomolar concentrations of HEL^{3X} directly enumerated high affinity IgG⁺ variants for D101R-substituted HEL at this time, comprising 18% of wild-type IgG⁺ SW_{HEL} B cells, 3.4% of all SW_{HEL} B cells and 0.0082% of all spleen cells (Fig 6e). Sorted *pri/pri* GC cells on day 9 had accumulated the same or greater load of V_H nucleotide mutations (mean of 2.3 per cell) as their wild-type counterparts (mean of 2.0), including comparable numbers of mutations in the CDR2 codons. Despite undergoing comparable hypermutation, the mutant GC cells had a low ratio of replacement to silent mutations in CDR2 (2.4) and only 2 out of 44 cells (4.5%) had Y58F replacements (Fig. 6d and Supplementary Fig. 7). Flow cytometric staining with HEL^{3X} showed that *pri/pri* SW_{HEL} B cells generated few high affinity IgG⁺ variants to D101R-substituted HEL even when adjusted for their poor survival (Fig. 6e). These cells represented only 3% of IgG1⁺ SW_{HEL} B cells, 0.2% of the SW_{HEL} B cells and 0.00004% of all spleen cells. DOCK8 deficiency thus acts within GC B cells to allow normal immunoglobulin gene hypermutation but diminishing survival and selection so that the formation of higher affinity IgG⁺ B cells was decreased by 200-fold.

Defective B cell synapse

A systematic analysis of the effects of DOCK8 deficiency upon established molecular pathways for GC and MZ B cell formation revealed a selective disruption of immunological

synapse organization. When B cells recognize antigen on cell membranes, BCR-signaling triggers LFA-1 integrin to bind strongly to ICAM-1 [<http://www.signaling-gateway.org/molecule/query?afcsid=A002871>] on the antigen-presenting membrane, promoting strong B cell adhesion and spreading ²⁵. During the first 3 min of this process, BCRs become polarized in the center of the adhesion zone and cluster antigen into a central supramolecular cluster (cSMAC). LFA-1 becomes polarized into a surrounding peripheral supramolecular cluster (pSMAC) where it concentrates ICAM-1 in the antigen presenting membrane. DOCK8 mutant and wild-type B cells were isolated from MD4 transgenic mice expressing HEL-specific BCRs. The B cells were allowed to settle on planar membranes containing fluorescently-tagged HEL antigen and ICAM-1, and imaged by a combination of confocal, differential interference contrast (DIC) and interference-reflection microscopy (IRM). Both mutant and wild-type cells adhered and clustered antigen into a cSMAC, but the mutant cells exhibited an inability to cluster ICAM-1 into a pSMAC and consequently formed smaller areas of close membrane apposition (Fig. 7a).

By contrast with the disruption of B cell synapse formation, DOCK8 mutation had no effect on other BCR signaling events including anti-IgM stimulated elevation of intracellular calcium (Fig. 7b) or ERK phosphorylation (not shown), induction of activation markers CD25, CD86 and CD69 (Fig. 7c), and induction of DNA synthesis (Supplementary Fig. 8) or cell division (Fig. 7d), even under conditions of limiting BCR engagement (Fig. 7d and Supplementary Fig. 9,10). The mutation also had no discernable effect on CD40- or LPS-stimulated activation or proliferation (Fig. 7d, Supplementary Fig. 8-10 and data not shown). DOCK8 mutant B cells were also normal in forming stable conjugates during antigen presentation to specific CD4⁺ T cells, in a B–T cell adhesion process that depends upon the adaptor protein SAP (SLAM-associated protein) in T cells and is important for GC formation ²⁶ (Supplementary Fig. 11). DOCK8 mutation also did not decrease *in vivo* homing and accumulation of B cells in lymph nodes in competitive mixed chimeras (Supplementary Fig. 12), despite the dependency of this process on signaling of LFA-1 adhesion to ICAM-1 by CCR7 and CXCR4 chemokine receptors ²⁷. After adoptive transfer, DOCK8 mutant SW_{HEL} B cells migrated and positioned themselves normally in follicles and germinal centers (Fig. 6a and Supplementary Fig. 5). Mutant and wild-type spleen B cells were indistinguishable by *in vitro* chemotaxis to the three main chemotactic factors for B cells, namely S1P, CXCL12 and CXCL13 (Supplementary Fig. 13). DOCK8 therefore functions quite specifically in B cells, being necessary for BCR signaling of integrin–ICAM-1 polarization within the immunological synapse but not for many other BCR-, chemotactic, or integrin-mediated processes.

Discussion

The findings above reveal that mutations in DOCK8 cause humoral immunodeficiency that preserves T-independent antibody formation and the initial extrafollicular wave of T-dependent antibody formation, but severely cripples the longevity and affinity maturation of T-dependent antibody responses. The basis for these humoral deficits is a profound defect in germinal center B cell survival and selection during the affinity maturation phase of germinal center responses, due to an intrinsic requirement for DOCK8 within the GC B cells themselves. The GC B cell defect may be compounded by the decrease in the numbers of CD4⁺ helper T cells in DOCK8 mutant mice, although mixed chimera and adoptive transfer studies clearly establish that the mutation profoundly cripples the persistence and affinity maturation of mutant GC B cells even in the presence of a normal complement of helper T cells. Within B cells, DOCK8 plays a critical role in antigen recognition, being essential for recruiting ICAM-1 to the immunological synapse but unnecessary for the central events in BCR signaling to soluble ligands leading to activation and proliferation. The requirement for

DOCK8 in B cell synapse formation and for longevity and affinity maturation of T-dependent antibody illuminates an important step in the establishment of humoral immunity.

The requirement for DOCK8 to organize a normally polarized B cell immunological synapse is very similar to defects in B cell pSMAC formation caused by mutations in the Rho-family G protein, Rac2 [<http://www.signaling-gateway.org/molecule/query?afcsid=A002002>], or in the Rac GEFs Vav1 or Vav2²⁸ [<http://www.signaling-gateway.org/molecule/query?afcsid=A002361>]. In comparison to these proteins, DOCK8 appears to have a more restricted function for BCR-integrin coordination, because follicular B cell development, migration, chemotaxis, activation and T-independent type 2 antibody responses are normal in *Dock8* mutant B cells whereas pleiotropic defects in these processes are caused by mutations in Rac2 or Vav proteins. In addition to their role in BCR-integrin coordination, Rac2 and Vav proteins are critical for the initial events in BCR signaling of intracellular calcium and proliferation in response to soluble anti-IgM and for chemokine responsiveness²⁹⁻³². The specificity of DOCK8 function in BCR-integrin signaling and GC selection in B cells also contrasts with that of DOCK2, whose mutation in mice causes profound lymphopenia, disrupted lymphoid architecture, crippled B cell migration to lymph nodes and to CXCL12 and CXCL13 chemokines *in vitro*³³, and a generalized defect in B cell integrin activation in response to a range of stimuli³⁴. To understand how DOCK8 serves such a restricted role in B cells compared to Rac2, Vav or DOCK2, it will be interesting in future work to define the G-proteins and biochemical pathway regulated by DOCK8 for B cell immune synapse formation.

Mutations in CD19, a co-receptor that is phosphorylated by BCR engagement and then recruits PI(3)K⁵, cause a human CVID syndrome with normal numbers of IgD⁺ recirculating B cells, presence of germinal centers, but low numbers of CD27⁺ memory B cells and low avidity antibody to rabies vaccine⁴. In the mouse, CD19 mutations that eliminate the protein or inactivate its ability to recruit PI(3)K cause a humoral immunodeficiency syndrome that closely resembles that caused by DOCK8 mutation. These mice have normal numbers of recirculating IgD⁺ B cells, lack of MZB cells, normal calcium response and proliferation to soluble anti-IgM stimulation, relatively normal TI-2 antibody responses, and initiation of GC reactions and isotype switching but limited expansion of GCs and failure of affinity maturation or long-lived antibody³⁵⁻³⁸. The reduction in GCs and loss of MZB cells in CD19 mutant mice can be rescued by a mutation in the PtdIns(3,4,5)P₃ phosphatase, PTEN, underscoring the role of PtdIns(3,4,5)P₃ lipid products of PI(3)K in these processes³⁹. A distinction between mutations in CD19 and DOCK8 is that CD19 deficiency greatly diminished the early step of B cell adhesion and clustering of membrane HEL antigen in the bilayer assay used here⁴⁰, whereas DOCK8 deficiency was restricted to disrupting the later step of pSMAC formation. This difference may reflect the proximal role of CD19 in recruiting not only PI(3)K but also BCR signaling molecules with general roles in the initiation of BCR signaling such as Lyn⁴¹. The phenotypic similarities and the fact that the DHR1 domain of related DOCK proteins binds PtdIns(3,4,5)P₃ lipids to recruit these GEFs to the membrane and activate Rac at sites of PI(3)K activation¹³, make it logical to hypothesize that DOCK8 serves as an effector downstream of CD19 and PI(3)K to promote G-protein signaling events critical for integrin polarization at the synapse and for marginal zone and germinal center B cell survival.

The discovery here that DOCK8 mutations result in humoral immune deficiency in mice is complemented by the independent discovery of DOCK8 mutations associated with immunodeficiency in humans⁴². Recurrent sinopulmonary bacterial infections typical of humoral immunodeficiency were observed in DOCK8 deficient people, in addition to cutaneous viral infections usually associated with partial T cell deficiency. Despite normal or increased gammaglobulins, these individuals exhibited a variable lack of antibody to T-

dependent vaccines. The data here establish that DOCK8 deficiency indeed causes a primary humoral immunodeficiency selectively affecting the longevity and maturation of T-dependent antibody responses to protein antigens, and establish its cellular basis and its B cell intrinsic role. Although the focus of the current study was to understand the humoral defects caused by DOCK8 deficiency, an additional requirement for DOCK8 in T cells is demonstrated here and associated with the human syndrome. Future, separate studies will be needed to define the cellular basis for the decreased numbers of peripheral T cells caused by DOCK8 mutation.

The requirement for DOCK8 to form marginal zone B cells may be explained by the fact that this B cell subset is highly dependent upon both BCR-CD19-PI(3)K^{35, 38, 43-45} and integrin signaling^{46, 47}. Coordinated signaling by BCR and integrins has also been proposed as critical for GC B cell survival and selection, since ICAM-1 is highly expressed on follicular dendritic cells (FDCs) presenting antigen in germinal centers and inhibits apoptosis of GC B cells *in vitro*⁴⁸. ICAM-1 fails to be induced on FDCs when the *Ikbkb* gene is selectively inactivated in FDCs, and this leads to small germinal centers⁴⁹. *In vivo* soluble antigen has the opposite effect from FDC-bound antigen on germinal center B cells by triggering apoptosis within 4 h^{50, 51}. ICAM-1 costimulation has also been shown to modify the outcome of TCR stimulation in ways that are distinct from CD28 costimulation, converting a death signal into a survival signal in immature thymocytes^{52, 53} and being critical in formation of memory CD8⁺ T cells⁵⁴. It is tempting to speculate that DOCK8 immunodeficiency may result from failure of germinal center B lymphocytes and some classes of T cells to organize a normal immune synapse and thereby receive an integrin costimulus during antigen recognition that is needed to convert an antigen receptor-induced death signal into a survival signal.

METHODS

Mice and procedures

All experiments were approved by the ANU Animal Ethics and Experimentation Committee or the Oxford University Ethical Review Committee and performed under UK Home Office License. 100 mg/kg ENU was given IP to male C57BL/6 mice 3 times 1 wk apart. For bone marrow chimeras, B6.SJL-CD45.1 mice were irradiated with 10 Gy and injected with 2×10^6 bone marrow cells (50:50 mixture of wild-type B6.SJL-CD45.1 bone marrow and either mutant or wild-type B6 (CD45.2) marrow). They were allowed to reconstitute for 8-10 weeks before immunization and analysis.

Immunizations and ELISA

Primary immunization was 50 μ g ARS-CGG and 1×10^8 heat & formalin inactivated *B. pertussis* bacteria (BioSearch Technologies), coprecipitated in alum and PBS and given IP to each mouse divided into two 150 μ l injections to minimize false negative reactions. Booster IP immunizations were 50 μ g ARS-CGG and 25 μ g NP-Ficoll in 300 μ l PBS. Antibodies were measured by ELISA on plates coated with BSA-ARS, CGG, BSA-NP or sonicated *B. pertussis*, respectively, and detected using IgG subtype specific antibodies (Southern Biotech). SRBC immunization was by IP injection of 200 μ l SRBC cells diluted 1.5 times in phosphate buffered saline (PBS). Conjugation of SRBC, adoptive transfer of spleen cells containing 10^5 SW_{HEL} B cells/mouse, and immunization with conjugated SRBC was as previously described¹⁹.

Flow cytometry

The following antibodies were used (from BD Pharmingen unless otherwise stated): CD4-PerCPCy5.5, CD5-PE, CD5-PerCP, CD11b-FITC, CD19-PECy7 (eBioscience), CD21/35-

FITC, CD23-PE, CD23-PECy7 (eBioscience), CD25-FITC, CD38-PE, CD45.1-Biotin, CD45.1-FITC, CD45.1-AF700 (Biolegend), CD45.2-Pacific Blue™ (Biolegend), CD69-PE, CD86-APC, CD86-PE, CD93(AA4.1)-APC, CD95-PE, B220-FITC, B220-PerCP, B220-PerCPCy5.5, B220-APC-AF750 (eBioscience), B220-PECy7, IgM-APC, IgM-Biotin, IgD-PE (Southern Biotech), IgD-FITC, IgG1-PE, anti-GL7-FITC, Streptavidin-PerCPCy5.5, Streptavidin-PECy7 (eBioscience), Streptavidin-APCCy7, and HyHEL9 conjugated to AF647 using a Monoclonal Antibody Labelling Kit (Molecular Probes). Staining with HyHEL9 followed incubation with HEL (Sigma) or HEL^{3X} at 50 ng/ml. Data were acquired on a FACSCanto, FACS Calibur, FACS Sort or LSRII flow cytometer (BD) and analyzed using FlowJo Software (Tree Star Inc).

SHM analysis

SW_{HEL} GC B cells (B220⁺ CD45.1⁺ CD38⁻) were sorted from individual recipient animals 9 days after transfer and HEL^{2X}-SRBC immunization, deposited as single cells in 96 well plates, the SW_{HEL} heavy chain V region amplified from genomic DNA by nested PCR, and products sequenced without cloning, as described previously¹⁸.

Immunohistochemistry and immunofluorescence

Tissues were snap frozen in liquid nitrogen and embedded in OCT compound (Tissue-Tek). 5 µm sections were cut, air dried and fixed in ice cold acetone for 20 min. Sections were washed in 50 mM Tris-buffered saline (TBS) pH 7.6 or PBS between all steps and antibodies added for 30-45 min incubations at 25°C (immunohistochemistry) or 37°C (immunofluorescence).

Reagents for immunohistochemistry: Figure 4a: PNA-biotin (Vector) and sheep anti-mouse IgD (Binding Site), peroxidase-donkey anti-sheep IgG (Jackson ImmunoResearch), streptavidin-alkaline phosphatase (AP) (Vector). Peroxidase was revealed with 3,3'-diaminobenzidine (Sigma) and hydrogen peroxide, and AP activity using the AP-substrate III kit (SK 5300, Vector) per manufacturer's instructions. Figure 2d; CD1d-biotin (eBioscience) was detected using the AP-Substrate kit as above, followed by rat anti-mouse MAdCAM (Serotec), goat anti-rat IgG-biotin (Serotec), and binding detected using Peroxidase substrate kit DAB (SK-4100, Vector). For immunofluorescence, slides were incubated with HEL protein in 5% mouse serum, then HyHEL5-biotin, followed by IgD-FITC, CD3-PE and Streptavidin-APC (all from BD Pharmingen). Images were taken with an Olympus IX71 microscope, Nikon TE2000U or Leica confocal microscope (TCS SP5).

In vitro cell stimulation assays

Intracellular calcium levels were assayed using Indo-1 (Invitrogen) before and after stimulation with 50 µg/ml goat anti-IgM F(ab')₂ (Jackson ImmunoResearch) using an LSRII flow cytometer as described previously⁵⁵. Proliferation and overnight activation of lymph node lymphocytes was carried out in RPMI supplemented with 10% FCS, 55 µM β-mercaptoethanol and 50 units/ml penicillin/streptomycin and stimulated with goat anti-IgM F(ab')₂ (Jackson ImmunoResearch) at 0.33, 1, 3.3 and 10 µg/ml alone and together with anti-CD40 (10 µg/ml 1C10). DNA synthesis assays were carried out as previously described⁵⁶. For CFSE division assays, cells were labeled with 1.5µM CFSE for 5 min at 37°C and then cultured for 96 h. For chemotaxis assays, cells were washed in RPMI1640 with 0.5% fatty-acid-free BSA several times, re-sensitized for 30 min at 37°C, and then tested for transmigration across uncoated 5 mm transwell filters (Corning Costar) for 3 h to the indicated chemokines in the bottom chamber as described⁵⁷. Analysis of B cell synapse formation on lipid bilayers²⁸ and *in vitro* B:T cell conjugation assays (with peptide activated OT-II cells and LPS stimulated B cells)²⁶ were performed as previously described.

Mapping and sequencing

(B6.*cpm* × 129)F1 and (B6.*pri* × B10)F1 mice were intercrossed to generate F2 progeny, and strain specific microsatellite and SNP markers used to map the *cpm* and *pri* loci to chromosome 19. (B6.*cpm* × CBA)F2 and (B6.*pri* × CBA)F2 mice were analyzed to map to a 4.5Mb interval using the SNP markers rs3697576, rs13483577 and rs3717280. *Dock8* was amplified from genomic DNA or splenocyte cDNA (primer sequences available upon request) and sequenced using ABI Big-Dye terminators and analysis on an ABI 3830 capillary sequencer. Splenocyte cDNA was amplified with primers spanning *Dock8* exons 18–21, products resolved on agarose gels and TA-subcloned into Invitrogen TOPO TA Cloning® Kit (with pCR2.1 vector) and individual colonies picked were PCR amplified and sequenced to identify the splicing products produced by the *cpm/cpm* mutation.

Data analysis and Statistics

Statistical analyses were done using GraphPad Prism Software. Statistical comparison was done with unpaired *t* tests as noted in the text. ELISA data for immunization antibody titers was analyzed using non-linear regression (curve fit) and EC50 shift to allow comparison of titration curves.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1. Discovery of two independent, non-complementing ENU mutant mouse strains with humoral immunodeficiency characterized by failure to sustain a primary immune response and failure of maturation of the antibody response

(a) Representative screening assay results. IgG1 antibody to CGG and ARS, IgM antibody to NP, and IgG2c antibody to *B. pertussis* measured by ELISA optical density (OD) in serum collected 14 days post-primary immunization (d14) and 6 days after booster immunization (d44). To detect low responders among large numbers of mice, serum was tested at a fixed dilution yielding sub-saturating antibody titers in wild-type animals (CGG 1/2000, 1/20,000; BP 1/50; NP 1/50; ABA 1/2000). (b) Antibody titers to CGG and ARS followed longitudinally 14, 28 and 56 days after primary immunization, determined by titrating each serum sample against a standard reference serum whose titer was set as 1. Statistical comparison by Student's *t*-test: ns, not significant; * $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$). In a,b, columns are arithmetic means, bars standard error of the mean (s.e.m), and dots individual mice. Data are representative of 3 independent experiments.

Figure 2. Analysis of splenic and peritoneal lymphocyte subsets

(a, b) Number of immature transitional 1-3 B cells (T1-T3, Trans), mature follicular B cells (Foll), marginal zone B cell precursors (MZP) and marginal zone B cells (MZ) in spleen of individual mice of the indicated genotypes. MZ B cell numbers were statistically significant (unpaired, two-tailed *t* test, (a) $P = 0.006$, (b) $P = 0.038$), while other B subsets were not significantly different. Data representative of 2 independent experiments. (c) Representative plots (from >5 independent experiments) showing % of B cells in the MZ B cell subset. (d) Immunohistochemistry of splenic MZ B cell marker CD1d and marginal sinus marker MAdCAM. (e) Percentage of mutant or wild-type CD45.2⁺ cells reconstituting the indicated B cell subsets in the spleen of individual mixed chimera mice. Differences in MZ contribution are representative of two separate cohorts of *pri/pri* mixed chimeras and two cohorts of *cpm/cpm* mixed chimeras, while no consistent difference in contribution to follicular or transitional cells was observed. (f) Numbers of B1, B1a, B1b and B2 subsets in the peritoneum of mice of the indicated genotypes. Representative of two independent experiments. (g) Numbers of splenic T cells in individual mice of the indicated genotypes. Naïve T cell numbers (CD44^{lo}) were statistically significantly different (ANOVA) for both CD4⁺ ($P = 0.0003$) and CD8⁺ T cells ($P = 0.0001$). Differences in CD8CD44^{hi} cells were statistically significantly different ($P = 0.048$), but not in CD4CD44^{hi}. (h) Percentage of mutant or wild-type CD45.2⁺ cells reconstituting the indicated splenic cell subsets of individual mixed chimeras. Representative of three independent experiments.

(a) Conserved DOCK8 domains and mutations. (b) *Dock8* exon 18-21 cDNA amplification products from thymus (Thy) and spleen (Spl) of wild-type and *cpm* mice. Data are representative of 4 independent experiments. (c) Sequence of cDNA clones from *cpm/cpm* mice in (b), aligned to wild-type *Dock8* cDNA sequence. Number of clones of each type shown on left. //, segment of normal exon 20 sequence omitted. (d) Sequence alignment of DOCK8 orthologues and paralogues in the region surrounding the *pri* mutation. Mutated serine in bold; dashes indicate identity to mouse DOCK8; secondary structure elements based on the structure of DOCK9¹⁶; triangles show Cdc42 contact residues in DOCK9¹⁶.

Figure 4. DOCK8 mutation causes an intrinsic defect in germinal center B cells

(a) Immunohistochemistry of splenic PNA⁺ germinal centers and IgD⁺ follicular mantle 7 days after SRBC-immunization (scale bars: upper 500 μ m; lower 200 μ m). (b) Enumeration of GC B cells as percentage of B220⁺ in untreated mice (nil) or 5, 7 and 11 days after SRBC-immunization. Columns are arithmetic means, bars standard error of the mean (s.e.m), and dots individual mice (c) Contribution to different B cell subsets in 50:50 mixed bone marrow chimeras 7 or 11 days after SRBC immunization. Columns show percentage of CD45.2⁺ cells of the indicated genotypes in individual, numbered chimeric mice. Each bar represents an individual mouse. Numbers indicate total number of GC cells $\times 10^6$ per spleen (mean $\pm$ SD) in each group of mice. Data are from 3 independent experiments.

Figure 5. DOCK8 mutant B cells undergo normal T-cell dependent activation, switching and initial differentiation into GC cells *in vivo*

HEL-specific *pri/pri* or wild-type (+/+) B cells from SW_{HEL} transgenic mice, allotypically tagged by CD45.1, were adoptively transferred into wild-type mice that were immunized with HEL^{2X}-SRBC and analyzed on the indicated days after transfer and immunization. (a) Representative flow cytometric analyses of spleen gated on HEL⁺ CD45.1⁺ B220⁺ SW_{HEL} B cells, showing percentage of GL7⁺ Fas⁺ cells (left) and IgG1⁺ switched and unswitched cells (right). Control mice given SW_{HEL} B cells but immunized with unconjugated SRBC lacking HEL (SRBC) are marked (*). (b) Enumeration of number of HEL⁺ CD45.1⁺ B220⁺ SW_{HEL} B cells on the indicated days after immunization, and the percentage of SW_{HEL} B cells that were GL7⁺ Fas⁺ or switched to IgG1 in individual animals. Statistical analysis (wild-type vs. *pri/pri*) by unpaired *t*-test with Welch's correction. Unfilled circles represent wild-type, filled circles *pri/pri* and (*) control srbc mice. Early time-course experiment performed once.

Figure 6. Intrinsic failure of DOCK8 mutant germinal center B cells to persist or undergo affinity maturation

HEL-specific *pri/pri* or wild-type (+/+) B cells from SW_{HEL} transgenic mice, allotypically tagged by CD45.1, were injected into wild-type B6 mice and the recipients immunized with HEL^{2X}-SRBC. (a) Confocal immunofluorescence of spleen cryosections from recipient mice 5 or 10 days after immunization, stained for HEL, IgD and CD3. GCs (arrows) and extrafollicular plasma cell foci (PC), and T cell zones (T) are marked. (b) Representative flow cytometry plots and enumeration of CD45.1⁺ HEL-binding B cells and GC cells derived from the HEL⁺ CD45.1⁺ SW_{HEL} cells, of the indicated genotypes, 5-9 days after adoptive transfer. Control mice given SW_{HEL} B cells but immunized with unconjugated SRBC without HEL (srbc) are marked (*). Panels also show total number of GC B cells per spleen in individual mice and the percentage of GC B cells that were SW_{HEL} B cells (day 9, +/+ vs. *pri/pri*, *P* = 0.008) and the percentage and absolute numbers of HEL⁺ CD45.1⁺ SW_{HEL} FAS^{hi} HEL^{lo} GC cells and IgG1-switched cells. Unfilled circles represent wild-type, filled circles *pri/pri* and (*) control srbc mice. (c) Structure of HEL complexed with the SW_{HEL} antibody HyHEL10 showing the position of the two HEL residues mutated in HEL^{2X} and four of the D101 contact residues in the heavy chain CDR2. (d) Results of sequencing of heavy chain genes from individual flow-sorted SW_{HEL} GC B cells (both +/+ or *pri/pri*). Graphs show the percentage of cells containing the indicated number of mutations in the CDR2; the total number of cells sequenced is indicated in the center. The replacement to silent ratio (R/S) and percentage of cells with a Y58F replacement mutation are also shown. (e) Enumeration of high affinity variants on day 9 post-immunization by direct assay for binding to nanomolar concentrations of D101R mutant HEL^{3X} (independent experiment from b, d). Flow cytometry plots, gated on B220⁺ CD45.1⁺ SW_{HEL} B cells, are from concatenated data collected on 11-22 million spleen cells combined from four individual recipients of either +/+ or *pri/pri* SW_{HEL} B cells or from two recipients immunized with uncoupled SRBC (*). The mean % of spleen cells that are CD45.1⁺ SW_{HEL} B cells is shown in blue and the mean % of spleen cells switched to IgG1 is shown in red. Staining with nanomolar HEL^{3X} reveals mutated B cells with higher affinity BCRs (oval).

gate), and the mean % spleen cells in this gate shown in black. Panels on right show data for individual animals in each group. Statistical analysis by Student's t-test with Welch's correction indicated a significant difference between *pri/pri* and *+/+* SW_{HEL} cells in percentage of high affinity cells among either CD45.1+ B220+ cells ($P = 0.038$) or among CD45.1+ B220+ IgG1+ cells ($P = 0.031$) that remained on day 9. Data are representative of four independent experiments, except (d) and (e) which were each performed once but independently of each other.

Figure 7. DOCK8 mutations disrupt the formation of the B cell immunological synapse but not other aspects of signaling through the B cell antigen receptor

(a) HEL-specific MD4 wild-type or mutant B cells were settled onto lipid bilayers containing mono-biotinylated HEL (green) and Alexa-532-conjugated ICAM-1 (red) and imaged after 10 min of interaction. Differential interference contrast (DIC), confocal fluorescence for HEL and ICAM-1, and interference reflection microscopy (IRM) images of representative cells are shown. Right, quantification of the area of B cell contact with the bilayer, the relative amounts of antigen accumulated at the contact site, and the percentages of B cells forming a peripheral ring of ICAM-1 (pSMAC). Results representative of 3 independent experiments. Columns are means and error bars are s.e.m. (b) Intracellular calcium in mixtures of *pri/pri* CD45.2 (red trace) and wild-type CD45.1 spleen cells stimulated with anti-IgM (arrow). (c) Expression of CD25 and CD86 on lymph node B cells 18 h after stimulation with 1 or 10 $\mu\text{g/ml}$ anti-IgM. Unstimulated, grey histogram; wild-type, black line; *pri/pri*, red filled. Representative plots from 4 independent mice. (d) Division of LN B cells measured by dilution of CFSE in B220⁺ 7AAD⁻ cells after 4 d culture with the indicated stimuli. Mutant *pri/pri* or *cpm/cpm* CD45.2 cells (red histograms) were CFSE labeled and cultured with wild-type CD45.1 cells (black histograms) in the same wells to control for experimental variation. Representative of 6 out of 8 independent samples tested; in 2 samples there was a subtle decrease in CFSE dilution by *pri/pri* cells (Supplementary Fig. 9).