OUTSTANDING OBSERVATION

Axon growth and guidance genes identify T-dependent germinal centre B cells

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Selection of B cells subjected to hypermutation in germinal centres (GC) during T cell-dependent (TD) antibody responses yields memory cells and long-lived plasma cells that produce high affinity antibodies biased to foreign antigens rather than self-antigens. GC also form in T-independent (TI) responses to polysaccharide antigens but failed selection results in GC involution and memory cells are not generated. To date there are no markers that allow phenotypic distinction of T-dependent and TI germinal centre B cells. We compared the global gene expression of GC B cells purified from mice immunized with either TD or TI antigens and identified eighty genes that are differentially expressed in TD GC. Significantly, the largest cluster comprises genes involved in growth and guidance of neuron axons such as *Plexin B2*, *Basp1*, *Nelf*, *Shh*, *Sc4mol* and *Sult4a*. This is consistent with formation of long neurite (axon and dendrite)-like structures by mouse and human GC B cells, which may facilitate T:B cell interactions within GC, affinity maturation and B cell memory formation. Expression of BASP1 and PLEXIN B2 protein is very low or undetectable in resting and TI GC B cells, but markedly upregulated in GC B cells induced in the presence of T cell help. Finally we show some of the axon growth genes upregulated in TD-GC B cells including *Basp1*, *Shh*, *Sult4a*, *Sc4mol* are also preferentially expressed in post-GC B cell neoplasms.

Immunology and Cell Biology (2008) **86**, 3–14; doi:10.1038/sj.icb.7100123; published online 16 October 2007

Keywords: axon; B cell memory; germinal centre; T-dependent

During T cell-dependent (TD) immune responses, recently stimulated B cells receive help from primed T cells in the outer T zones of secondary lymphoid tissues. B cells either differentiate extrafollicularly into mostly short-lived unmutated plasma cells or seed follicles and initiate germinal centre (GC) reactions.¹ GC are a hallmark of antibody-mediated immune responses to TD antigens, giving rise to memory B cells and long-lived plasma cells that express modified immunoglobulin (Ig) genes that encode antibody with high affinity for antigen (affinity maturation).

Mature GC segregate into dark and light zones. The prevailing model of GC function describes centroblast (CB) proliferation in the dark zone where somatic hypermutation takes place.¹ This represents the first step in affinity maturation, and since base changes are introduced stochastically, it results in Ig with a wide range of affinities for the immunizing antigen. CB differentiate into centrocytes (CC) that occupy the light zone. Recent evidence from live-imaging of GC dynamics confirms that there is constant B cell traffic between dark and light zones, and cell division occurs in both zones.^{2–4} Affinity

maturation depends on CC selection based on their ability to interact with follicular dendritic cells, take up antigen held in the form of immune complexes, present it to antigen-specific follicular helper T cells (T_{FH}) and elicit selection signals.⁵ T-B interactions are remarkably transient, unstable and promiscuous, and B cell competition for T cell help is likely to be crucial for affinity maturation.⁶ CC expressing high-affinity and non-self-reactive antibody are positively selected to differentiate into long-lived plasma cells or memory B cells. By contrast, low affinity^{7–9} or self-reactive CC that fail selection undergo apoptosis.^{10–12} Thus T cell-mediated CC selection within GC provides the basis for affinity maturation of the antibody response and B cell memory formation. The signals that initiate CC differentiation into either memory cells or plasma cells are only partially understood.

Together with others, we have previously observed that under exceptional circumstances, GC can also be induced by T-independent type 2 (TI-2) antigens,^{13–15} but unlike GC induced by TD antigens, T-independent (TI) GC abort at the time CC would normally undergo T_{FH}-driven selection. This happens after minimal hypermutation,¹⁶

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Received 30 June 2007; revised 30 August 2007; accepted 2 September 2007; published online 16 October 2007

and no cells are selected to become memory B cells. TI GC B cells, like their TD counterparts, express the known germinal centre B cell markers PNA and GL7 and downregulate IgD.¹⁷ Despite the different fate of TD and TI GC B cells, to date, there are no markers that can differentiate phenotypically TD vs TI GC B cells. These markers are likely to prove useful tools for understanding crucial aspects of immunology, since productive GC reactions underpin all successful vaccination strategies to date, and are essential for long-lasting immunity against certain life-threatening bacteria, viruses and toxins. Furthermore, productive GC may also be the source of pathogenic autoantibodies in diseases such as systemic lupus erythematosus.¹⁸ Also, identification of the molecular signature of TD GC B cells should provide important insights into the pathways that determine GC B cell survival, selection and differentiation, which all require T-cell help.

To this end, we have compared the gene expression signature of otherwise matched populations of TD and TI GC B cells. We show that a group of genes previously known to be involved in axonal growth and provision of axon guidance cues are selectively expressed in TD GC B cells. Among these, *Baspl* and *Plexin B2* emerge as new markers of productive GC. Furthermore, this pattern of gene expression coincides with the development of long neurite-like projections revealed by microscopic analysis of B cell subsets.

RESULTS

Selective gene expression on TD GC B cells

To compare the gene expression profiles of productive TD GC and non-productive TI GC, QMxB6 F1 mice were immunized with (4-hydroxy-3-nitrophenyl) acetyl (NP)-Ficoll (TI antigen) or NP-chicken gamma globulin (CGG) (TD antigen) as described in the methods. The latter group ('TD D4') had been primed with CGG 5 weeks earlier so that T-cell help was not limiting. Mice immunized with both TD and TI antigens developed PNA⁺ and GL-7⁺ GC B cells (Figures 1a and b) 3 days after immunization. Recirculating and GC B cells were sorted 4 days after immunization on the basis of GL-7 expression (Figure 1b). A third group of non-primed QMxB6 F1 mice were also immunized with NP-CGG, and GC B cells from these mice were sorted on day 10, at the peak of the GC reaction during a primary response ('TD D10'). GC B cells in all three groups expressed Bcl-6 (Supplementary Figure 1). RNA was extracted, biotin-labeled and hybridized onto Affymetrix microarrays. Comparison of expression profiles from resting, TI GC and TD GC B cells identified 55 genes that were selectively upregulated (over twofold) and 25 genes that were selectively downregulated in TD GC B cells (Figures 2a and b). These differentially expressed genes are candidate mediators of GC B cell selection by T cells and/or B cell memory formation.

Unique axon-related gene expression signature in TD GC B cells

Nine out of the 80 differentially expressed genes on TD GC B cells—Plexin B2 (*Plxnb2*), brain abundant membrane attached signal protein 1 (*Baspl*), nasal embryonic LHRH factor (*Nelf*), sonic hedgehog homolog (*Shh*), T-complex protein 1 (*Tcp1*), sterol-C4-methyl oxidase-like (*Sc4mol*), sulfotransferase family 4A, member 1 (*Sult4a1*), ligatin (*Lgtn*), myristoylated alanine-rich protein kinase C substrate (*Marcks*) and Solute carrier family 6 (neurotransmitter transporter, GABA), member 1 (*Slc6a1*) (Figure 3a)—are known to be involved in the regulation of neurite (axons and dendrites) growth and guidance (Table 1). With the exception of *Shh*, these genes have not been previously shown to have a function in immune responses. Quantitative reverse transcription-PCR analysis confirmed increased RNA levels of *Plexin B2*, *Baspl*, *Nelf*, *Tcp1* and *Sc4mol* in TD GC B cells (Figure 3b). This signature corresponds with the formation of long neurite-like

structures on GC B cells. Microscopic and ultrastructural analysis of mouse and human GC B cells revealed that GC, but not resting, B cells form a small number of cytoplasmic projections (Figure 4). Cytoplasmic outgrowths that were longer than the diameter of the cell body were observed in 1–6% of both TD and TI mouse GC B cells, but were virtually absent in resting B cells from unimmunized mice, in which only small microvilli could be seen. T-cell selection in GC is thought to operate on CC. Thus, dendrite formation by CB and CC was compared. Human CC and CB were sorted from tonsil single cell suspensions; recirculating B cells were sorted from peripheral blood and examined by scanning electron microscopy. Dendrites of variable lengths were more commonly observed in CC, occasionally found in CB and absent in resting B cells (Figures 4b and c). Dendrite formation has been previously reported in B cells stimulated with interleukin (IL)-4 in combination with lipopolysaccharide.¹⁹

BASP1 is expressed at high levels on TD GC B cells

BASP1 is known to promote neurite outgrowth and mediate axonal repair after axotomy.²⁰ To confirm that BASP1 protein is expressed in TD GC B cells, C57BL/6 mice were immunized with sheep red blood cells (SRBC) and GL-7^{high} Fas⁺ B220⁺ GC B cells were identified by fluorescence-activated cell sorting (FACS) 10 days later (Figure 5a). Polyclonal rabbit serum raised against BASP1 was used to detect intracellular BASP1 protein expression and pre-immune serum was used as the control. BASP1 was not detected in resting B cells but was expressed highly by GC B cells (Figure 5b). To confirm selective expression of BASP1 in the GC B cell subset, and assess expression on other lymphoid and myeloid subsets, immunohistochemistry was performed on spleen sections of mice 8–14 days after immunization with alum-precipitated NP-CGG plus heat-killed *Bordetella pertussis*. Highest expression of BASP1 was found in GC B cells, while BASP1⁺ B cells were not found in the primary follicles (resting B cells) (Figures 5c–e). Variable numbers of cells in the T zone, possibly including T cells and B blasts, expressed BASP1 (Figures 5c–e), but here expression was heterogeneous. Consistent with the microarray results, BASP1 was expressed at much higher levels on GC from spleens of mice immunized with TD antigens compared with those immunized with TI-2 antigens (Figure 5e). Finally, lysates from sorted GC B cells and resting B cells were separated by SDS-polyacrylamide gel electrophoresis and blotted with anti-BASP1 serum. A clear single band of the expected size²¹ was found in GC but not resting B cells (Figure 5f).

Next, we tested whether *Baspl* is induced upon B cell activation *in vitro*. No *Baspl* expression occurred after cells were activated with anti-IgM or anti-CD40 alone, but a fourfold increase in *Baspl* mRNA was observed after 24 h when these two stimuli were combined (Figure 5g), compared with an ~80-fold increase in BASP1 expression observed in freshly isolated GC B cells over that in resting B cells (Figure 3b). This difference in expression may be explained by the fact anti-IgM plus anti-CD40 stimulation does not generate GC B cells *in vitro*.

PLEXIN B2 is selectively and strongly expressed on GC B cells

Plexin family members are important for the detection of guidance cues that direct axon migration in the nervous system.²² One of these molecules, PLEXIN B2, is highly expressed in neurons, and mediates both attraction and repulsion reactions depending on the stimulus and type of neuron.^{23,24} PLEXIN B2 was not expressed in either resting or TI GC B cells, but is expressed at significant levels in TD GC (Figure 2). FACS analysis using a PLEXIN B2-specific monoclonal antibody confirmed that PLEXIN B2 protein was expressed highly on

Figure 1 Strategy for sorting GC B cells. (a) PNA and GL-7 selectively stain GC B cells on consecutive frozen sections from spleens of mice immunized with NP-CGG. (b) B220⁺GL-7^{high} splenocytes from mice immunized with TD or TI-2 antigen were sorted as GC B cells and B220⁺GL-7⁻ splenocytes from unimmunized mice were sorted as resting B cells. GC, germinal centre; NP, (4-hydroxy-3-nitrophenyl) acetyl; CGG, chicken gamma globulin; TD, T cell-dependent; TI-2, T-independent type 2.

GC B cells and absent on resting B cells (Figure 6a). Immunohistochemical staining of spleen sections from immunized mice showed that PLEXIN B2 was confined to B cells within GC. Specific PLEXIN B2 staining could also be seen in cells scattered through the red pulp and T zones, suggesting that other cell types—possibly dendritic cells and/or macrophages—may also express this protein (Figure 6b). No specific PLEXIN B2 could be detected in TI GCs by immunohistochemical staining (data not shown). Finally, expression of Plexin B2 on GC B cells was also confirmed by western blotting. As with BASP1, a band of the expected size was only observed in the GC B cell lysates but not in resting B cells (Figure 6c).

Besides the follicular/GC pathway of B-cell differentiation, activated B cells can also differentiate along the extrafollicular pathway to become short lived, low affinity plasma cells. Extrafollicular plasma cells form in both TD and TI B cell responses. PLEXIN B2 expression in this pathway was examined using a well-defined congenic adoptive transfer system in which CD45.2 SW_{HEL} B cells are transferred into CD45.1 recipients and immunization with SRBC conjugated with mutant HEL (hen egg lysozyme), HEL^{2x} results in extrafollicular CD45.2^{int} HEL^{int} Syn-1⁺ plasmablasts and plasma cells, and follicular/germinal centre B cells that are CD45.2^{high} HEL^{high} Syn-1⁻.²⁵ In this model, PLEXIN B2 expression was detectable on extrafollicular plasma cells, but the levels were more than twofold lower than those found on GC B cells (Figure 6d).

To assess PLEXIN B2 expression by B cells activated *in vitro*, purified B cells were cultured with mitogenic stimuli in the presence of IL-2. No

significant induction of PLEXIN B2 was detected after 48 h. However, after 6 days (when most B cells in the cultures have differentiated into plasmablasts and/or plasma cells), PLEXIN B2 was detected in cells stimulated under all conditions. The highest levels of PLEXIN B2 induced in culture were found on lipopolysaccharide-activated plasmablasts (Figure 6e). Nevertheless, PLEXIN B2 expression on GC B cells was still ~2-fold higher than that found in *in vitro*-generated plasmablasts. Taken together, these data show PLEXIN B2 is expressed selectively and highly on GC B cells although lower levels can be found on extrafollicular plasmablasts.

Human GC B cells express axon growth and guidance genes

Finally, we assessed *Basp1* and *Plexin B2* expression in human tonsillar GC cells (CC and CB) and memory B cells from peripheral blood (CD19⁺ IgD⁻ CD27⁺ cells) using gene expression data obtained by Affymetrix microarray analysis of each these B cell populations.¹⁵ Although *Basp1* itself was only minimally upregulated (Supplementary Figure 2), *Gap-43*, a protein that is closely related functionally to *Basp1* and can substitute for the latter *in vivo* to restore nerve sprouting,²⁰ was upregulated in both CC and CB. Expression of *Marcks*, the third member of the *Gap-43*-like protein family,²⁶ was downregulated in both CB and CC, thus following the same trend as that seen in mouse TD, but not TI, GC. In addition, in both CB and CC, *Plexin B2* expression was found to be upregulated by ~4-fold and remained ~2-fold higher in memory B cells compared to resting cells (Supplementary Figure 2).

Figure 2 Genes selectively regulated on TD GC B cells. Genes expressed differentially by at least twofold after analysis of variance analysis with $P < 0.01$ were studied. Upregulated genes were selected using the following algorithm: present in TD4 and TD10 with expression value > 2 -fold increase above the average expression of duplicate chips in a 4-way comparison (Resting vs TD D4, Resting vs TD D10, TI vs TD D4 and TI vs TD D10). Downregulated genes were selected using the following algorithm: present in R and TI with expression value > 2 -fold increase above the average expression of duplicate chips in a 4-way comparison (Resting vs TD D4, Resting vs TD D10, TI vs TD D4 and TI vs TD D10). Selected genes were normalized and clustered using CLUSTER software and visualized using Gene Tree View software. (a) Fifty-five genes were upregulated on TD GC B cells. (b) Twenty-five genes were downregulated on TD GC B cells. TD, T cell-dependent; GC, germinal centre; TI, T-independent.

Differential expression of TD GC B cell genes in pre- and post-GC B cell neoplasms

Mature mouse B cell lineage neoplasms constitute a spectrum, ranging from pre-GC, unmutated tumours of λ -MYC transgenic mice to fully differentiated, mutated post-GC plasmacytomas (PCT). Intermediate are tumours of SJL mice that originate in greatly enlarged GCs and retain an association with the follicular white pulp while undergoing plasma cell differentiation.^{27–29} For each neoplasm, a cell of origin is postulated with similarities between the paired normal and malignant cells varying for each lymphoma class. The possibility that genes involved in specifying cell localization and

interactions might contribute to the phenotypes of lymphomas prompted us to determine how TD GC genes might relate to tumours of known relation to GC passage. To this end, we used oligonucleotide microarrays (Figure 7) to study expression patterns of transcripts in pre-GC λ -MYC lymphomas (designated BL), and post-GC SJL tumours and PCT (designated PCT).

Hierarchical clustering of the genes identified two major subsets (Figure 7), one with genes expressed at higher levels in PCT and/or SJL lymphomas, and the second with higher levels of gene expression in pre-GC lymphomas. The genes preferentially expressed in the post-GC neoplasms as opposed to pre-GC neoplasms included a number of the

Figure 3 Expression of neurite-regulating genes by microarray and quantitative RT-PCR. (a) Signal value of selected genes by microarray analysis after normalization (y axis). (b) Quantitative RT-PCR in sorted resting and GC B cells from mice immunized by SRBC. The mRNA copy numbers were normalized by β -actin. The fold increase of GC B cells to resting B cells is shown (each dot represents an independent experiment and grey bars represent mean values). T14, T1 GC group; TD4, TD D4 GC group; TD10, TD D10 GC group. RT, reverse transcription; GC, germinal centre; SRBC, sheep red blood cells; T1, T-independent.

neuronal genes that were selectively upregulated in TD GC B cells—*Basp1*, *Shh*, *Sult4a1* and *Sc4mol*. *Plexin B2* was expressed by both types of neoplasms and expression differences were not statistically significant. Although the pre-GC neoplasms lack expression of most of the TD GC genes that are known to be involved in axon growth and guidance, they do express a number of genes that are also selectively expressed at high levels in TD GC B cells: *Mtf2*, *Dhfr*, *Cbx3*, *Hmgbl* and *Tmpo*. This suggests that they may derive from what has recently been described as the GC ‘founder cell’ subset of AID-positive but non-mutated, non-switched cells that have already acquired some GC features.³⁰ Interestingly, the GC marker gene, *Gcet*, which is also selectively expressed in TD GC B cells (Figure 2a) is ‘trapped’ for continually high expression in the SJL tumours but downregulated in the more mature PCT.

DISCUSSION

We have compared the gene expression signature from highly purified TD and T1 GC B cells generated *in vivo* and identified 80 genes differentially expressed in productive TD GC B cells. Remarkably, this hypothesis-free survey has identified a cluster of genes expressed selectively in GC B cells receiving T-cell selection signals that were previously only known to be involved in neuronal axon growth and migration. In addition, GC B cells and notably CC appear to be distinguished from resting B cell populations by their tendency to form dendritic cytoplasmic outgrowths. This finding supports and extends the recent demonstration of GC dynamics.^{2–4} Differentiation of GC B cells corresponds with morphological changes that appear to be crucial for affinity maturation to take place: the molecular machinery that drives axon growth and guidance in neurons is co-opted in GC B cells.

Table 1 Neurite growth genes differentially expressed in TD GC B cells

Gene symbol	Expression pattern	Subcellular localization	Function in neurons/CNS	Other functions	References
<i>Plxnb2</i> (<i>Plexin B2</i>)	Developing nervous system	Cell surface	Neurite growth and guidance	Not known	23,32,47,57
<i>Basp1</i>	Adult central and peripheral nervous system; early embryonic developing tissues	Plasmalemmal microdomains (rafts)	Neurite sprouting, synaptic plasticity, axon repair after axotomy, cell adhesion and spreading	Not known	20,21,42
<i>Nelf</i>	Embryonic developing nervous system	Cell surface	Axonal projection guidance and neuronal migration	Not known	58,59
<i>Shh</i>	Embryonic developing nervous system and limbs; adult organs	Secreted, diffuses; endosomes and lysosomes	Axon guidance; regulation of CNS polarity, neural stem cell proliferation, neuronal and glial cell survival	Regulates morphology of many tissues	60,61
<i>Tcp1</i>	Wide tissue expression	Cytoplasm, particularly at the leading edge of growth cones in neuritic processes	Axonal growth	Folding of cytoskeletal proteins	62–64
<i>Sc4mol</i>	Brain	Cytoplasm	Repair after axotomy	Not known	65
<i>Sult4a1</i>	Selectively in the brain	Cytoplasm	Related to schizophrenia	Not known	66–68
<i>Marcks</i>	Many tissues, particularly in the brain	Plasmalemmal microdomains (rafts)	Maintenance of dendritic spines	Cellular migration, adhesion	43
<i>Ligatin</i>	Mainly in the central nervous system	Cytoplasm, particularly in dendrites	Neuroplasticity, cell–cell interactions	Not known	69
<i>Slc6a1</i>	Neurons and glial cells in the brain	Processes and nerve endings	Neurotransmission regulation	Not known	70

Figure 4 Morphology of resting B cells and GC B cells. (a) Immunofluorescence of sorted resting (R) and GC B cells after fixing and staining with Oregon Green 488 phalloidin. (b, c) Scanning electron microscopic analysis of sorted human resting (R), centroblasts (CB) and centrocytes (CC). The strategy used for sorting R, CB and CC populations is shown in (b). M, memory B cells; GC, germinal centre.

We have taken advantage of a model in which GC can be induced with highly similar kinetics and characteristics up until the point of selection and differentiation. This allows the possibility of comparing two highly homogeneous GC populations with indistinguishable phenotype but polar opposite fates. In both TD and TI GC, CC emerge 3 days after immunization, but in the subsequent 48 h differentiation into plasma cells and memory cells, it only occurs in GC that contain antigen-specific T cells.¹³ This *in vivo* model is crucial, since recapitulating GC *in vitro* has been problematic, probably reflecting the complex reorganization of the follicular micro-environment that takes place during immune responses and governs the cellular interactions necessary for CC selection. *In vitro*, B cells can be induced to become short-lived unmutated plasma cells in the presence of various mitogenic stimuli. Although Bcl-6 overexpression in B cells can induce a GC phenotype *in vitro*,³¹ studies of these cells are hampered by their limited survival *in vitro*. Thus the molecular mechanisms that determine terminal B cell differentiation within GC remain elusive.

After identifying a cluster of genes expressed selectively in productive GC that were previously only known for their role in axonal growth and guidance, we focused our analysis on *Plexin B2* and *Basp1*, since the magnitude of differential expression was highly significant. PLEXIN B2 was selectively expressed in TD GC B cells and absent in both resting and TI GC B cells. BASP1 was not expressed at all by resting B cells, and was expressed only weakly on TI GC B cells, but was strongly expressed in TD GC B cells. *Nelf*, *Shh*, *Tcp1*, *Sc4mol*, *Sult4a1*, *Lgtn*, *Marcks*, and *Slc6a1* genes previously described to participate in neurite growth and guidance were also differentially expressed in TD GC B cells.

Plexins are a highly conserved family of neuronal receptors for the axon-guiding membrane-bound semaphorins. Soluble semaphorins can also signal through plexins but some of them require these to form complexes with neuropilins. Semaphorins direct axonal steering and branching by acting as chemorepellents or chemoattractants.²²

Figure 5 BASP1 is expressed by TD GC B cells. (a) Flow cytometric strategy for gating resting B cells and GC B cells from mice immunized with SRBCs 8–12 days before. (b) Shaded histograms show BASP1 staining by flow cytometry on resting B cells (left) and GC B cells (right). Pre-immunized rabbit serum was used as a staining control (open histograms). (c–e) Photomicrographs of spleen sections from (QMxC57BL/6 F1) mice from the TD D4 GC group (top panel: c, d, e) or from the TI GC group stained with PNA or BASP1 in blue and IgD in brown. BASP1 selectively stains TD GC, but not TI GC. G, germinal centres; T, T zones. (f) Western blots from lysates of sorted resting B cells and GC B cells probed with anti-BASP1 serum. β -Actin was used as a loading control. (g) Real time PCR indicating relative *Basp1* mRNA levels in MACS-purified B220⁺ spleen cells incubated for 24 h with anti-IgM, anti-CD40 or anti-IgM plus anti-CD40. TD, T cell-dependent; GC, germinal centre; SRBCs, sheep red blood cells; TI, T-independent; Ig, immunoglobulin.

Plexin family members B1, B2 and B3 are expressed in the central nervous system during development in a non-redundant manner.²³ To our knowledge, there have been no published reports of PLEXIN B2 expression in the immune system. In neurons, Semaphorin 4D (SEMA4D)/CD100 binds to PLEXIN B2.³² SEMA4D/CD100 is expressed on T cells,³³ and has been found within GC.³⁴ *Sema4d/Cd100* knockout mice have markedly reduced GC and defective affinity maturation during TD immune responses,³⁵ whereas *Sema4d/Cd100* transgenic mice display enhanced TD responses.³⁶ CD72 has been reported to be the receptor for T cell-derived SEMA4D/CD100 on lymphocytes.³⁷ Nevertheless, CD72-deficient

mice have no obvious defects in TD responses.³⁸ It is therefore possible that PLEXIN B2 mediates the effects of SEMA4D/CD100 in affinity maturation within GC. This contention is supported by selective upregulation of macrophage stimulating 1 receptor (*Mst1r*)/*Ron* in TD GC B cells (Figure 2); both *RON* and hepatocyte growth factor receptor (HGFR)/MET are known to form multimeric receptor complexes with plexins to mediate SEMA4D/CD100 signaling.^{39,40} Furthermore, HGFR/MET is known to be expressed on human GC B cells.⁴¹

BASP1 is also important for neurite outgrowth and regulates nerve sprouting. It has been shown that BASP1 and growth associated protein 43 (GAP-43) cooperate to induce axonal regeneration after axotomy.²⁰ BASP1 together with GAP43 and myristoylated alanine-rich C kinase substrate (MARCKS) are plasmalemma-associated PKC substrates and control the availability of PI(4,5)P₂ to regulate the actin cytoskeleton and growth of neuronal processes.⁴² Intriguingly, *Marcks* is selectively downregulated in TD GC B cells. Recent studies have shown that MARCKS regulates the stability and plasticity of dendritic spines and this is PKC-dependent.⁴³ The non-overlapping phenotypes of *Basp1*- and *Marcks*-deficient mice suggest they play related but nonredundant roles.^{20,44} Nevertheless, there have been no reports of inverse regulation of BASP1 and MARCKS in the central or peripheral nervous system. The known functions of other neurite growth and guidance genes are listed in Table 1. Products of these other genes are also involved in axon initiation and growth (TCP1, SC4MOL), axon guidance (NELF, SHH) and synaptic regulation (SLC6A1, LGTN).

The selective expression of PLEXIN B2 and other neuronal outgrowth molecules on TD GC B cells and the known expression of PLEXIN B2 ligand SEMA4D/CD100 on T cells and GC, together suggest that signals through some of these 'neuronal' genes may guide dendritic cytoplasmic outgrowths on GC B cells to establish interaction with T_{FH} cells. Microscopic analysis of highly purified GC B cells revealed long neurite-like structures (Figure 4a) in both TD and TI B220⁺ GL-7⁺ IgD[−] cells. Phalloidin staining confirmed that these are F-actin-based structures as opposed to tunneling nanotubes, which have been reported to form and facilitate intercellular communications between immune cells.^{45,46} In our experiments, neurite-like structures were found on only a small fraction of GC B cells *ex vivo* (~1–6%). *In vivo*, these processes appear to be more common;³ the predilection for apoptosis of GC B cells together with manipulation of GC B cells *ex vivo* may compromise the maintenance of these fragile and dynamic actin-based structures. As neurite-like structures are present in TI B cells without upregulation of *Basp1* nor *Plexin B2*, these genes are unlikely to be responsible for the formation of the processes *per se*, but are probably important for their growth and migration. This is consistent with the reported role of BASP1 and PLEXIN B2 in neurons. In the absence of BASP1, axon numbers in the peripheral nerves are normal but the nerve terminals display abnormalities suggestive of synapse instability and there is defective ultra-terminal nerve sprouting.²⁰ In the case of PLEXIN B2 deficiency, the regulation of migration of cerebellar granule cells is impaired, and the critical defect appears to lie in the orchestration of three processes: exit from cell cycle, differentiation and migration.⁴⁷

Neurite-like structures guided by T cell-derived cues appear attractive and efficient strategies to direct CC migration and enable establishment of probing interactions with T_{FH} cells before forming the stable cognate CC:T_{FH} cell interaction required for CC selection. Long-lasting, stable T–B interactions in GC are rare; instead, GC B cells and T cells form multiple short-lived interactions.³ Neurite-like extensions may also contribute to CC's capacity to bind many T cells, which are limiting, within the GC. It has been shown recently that in

Figure 6 PLEXIN B2 is expressed by GC B cells. (a) Flow cytometric analysis of PLEXIN B2 expression (open histograms) or isotype control (shaded histograms) on splenocytes from immunized mice gated on resting B cells (top panel) and GC B cells (bottom panel) following the strategy shown in Figure 5a. (b) Photomicrographs of serial spleen sections from immunized mice showing GC stained in blue for PNA (top) and PLEXIN B2 (bottom), and in brown for IgD. (c) Western blots from lysates of sorted resting B cells and GC B cells probed with anti-PLEXIN B2 serum. β -Actin was used as a loading control. (d) Flow cytometric analysis of PLEXIN B2 expression on splenocytes from SW_{HEL} mice 5 days after adoptive transfer into congenic CD45.1 mice and immunization with HEL² $\times$ -SRBC. Gates for extrafollicular CD45.2^{low} HEL-binding plasma cells (PC) and CD45.2^{high} HEL-binding GC B cells are shown in the dot blot (left). (e) Histograms show flow cytometric analysis of PLEXIN B2 expression after stimulation of purified B cells for 6 days under the conditions indicated. The expression of PLEXIN B2 on resting B and GC cells was examined in the same experiment. Shaded histograms indicate staining with an isotype control antibody. These results are representative from at least two independent experiments. GC, germinal centre; Ig, immunoglobulin; HEL, hen egg lysozyme; SRBC, sheep red blood cells.

the presence of limiting amounts of membrane-bound antigen, the process of B cell spreading can magnify differences in BCR affinity.⁴⁸ T_{FH}-dependent induction of genes that promote neurite growth in CC may enhance this spreading mechanism, providing a way to magnify further differences in BCR affinity in a way that is guided and proofread by T_{FH} specificity. This would result in a further increase in avidity and favour selection of high affinity mutants particularly as the amounts of antigen displayed by follicular dendritic cells becomes low at later time after immunization or infection.

Our findings suggest neurons and GC B cells share a set of neurite growth genes. There are numerous examples where certain proteins or

signaling pathways are shared between very different cells types to perform related functions including cell growth, survival, adhesion, and so on. Even signaling pathways regulating very specialized functions in one system can be used by another system to achieve similar outcomes. For example, molecules like neuropilin-1 known to be involved in synapse formation in the central nervous system have also been shown to participate in immunological synapses between T cells and antigen-presenting cells.⁴⁹ Also, common mechanisms of nerve and blood vessel wiring have been reported: a number of molecules that provide axon guidance cues or terminal arborization signals in neurons to direct neuronal cell migration and axon/dendrite growth

Figure 7 Expression of axon growth genes by mouse post-GC neoplasms. Hierarchical clustering of the genes shown in Figure 2, according to their differential expression in pre-GC neoplasms of λ -MYC transgenic mice (BL) and post-GC neoplasms: plasmacytomas (PCT) and the less mature post GC plasma cell neoplasms of SJL mice (SJL). The genes have been selected by *t*-test, $P < 0.01$. GC, germinal centre.

have also been found expressed in blood vessels, where they direct angiogenic sprouting.⁵⁰ Furthermore, recent reports suggest that this neurite growth and guidance machinery is not only shared by the nervous and immune systems, axonal guidance proteins and their signaling partners have also been identified in the developing mouse mammary gland where they appear to influence ductal growth and morphogenesis.⁵¹ Strikingly, both *BASP1* and *PLEXIN B2* appear to participate in this process.

We have shown that some of these neurite growth genes, including *Basp1*, are also highly expressed in mouse post-GC B cell lymphomas. This is consistent with other evidence that B-cell malignancies share many features with their normal counterparts during normal antigen-driven B cell differentiation and maturation. Interestingly, the neuronal signature was only found in tumours of clear-cut post-GC origin. By contrast, other GC-related genes were expressed in cells that could be the counterparts of GC founder cells, suggesting that the neuronal signature is a sign of late GC B-cell differentiation. This signature may be of value in future studies of mouse models of lymphoma, where classification by surface phenotyping is more difficult than in human lymphoma.

This work provides the markers to identify productive GC and possibly post-GC B cell malignancies. Further studies on the functions of the individual genes that comprise the axon growth and guidance signature of TD GC B cells will illuminate the molecular mechanisms that govern T:B cell interactions within GCs, affinity maturation and B cell memory formation.

METHODS

Mice and immunization

QM mice carry a recombinant VDJ transgene knocked into the IgH chain locus that encodes a heavy chain that when combined with any λ light chain binds NP with high affinity.⁵² In QMxB6 F₁ mice ~20% of B cells bind NP with

high affinity. SW_{HEL} mice have a knocked-in VDJ region in the IgH chain locus that in combination with a transgene-encoded light chain binds HEL with high affinity.⁵³ SW_{HEL} B cells can switch to all Ig isotypes. All mice were housed in specific pathogen-free conditions and all animal procedures were approved by the Australian National University Animal Ethics and Experimentation Committee.

To generate TI-2 and TD GC responses for microarray analysis, three groups of QMxB6 F₁ mice were immunized i.p. using the following strategy: (1) 'TI group'—mice were immunized with 30 μ g of the model TI-2 antigen NP conjugated to Ficoll; (2) 'TD D4 group'—to generate TD GC responses with comparable kinetics to the TI group, QMxB6 F₁ mice were primed with 50 μ g alum-precipitated CGG and then rechallenged 5 weeks later with 50 μ g of soluble NP-CGG so that T-cell help was not limiting. These mice were analysed on day 4 after the rechallenge. (3) 'TD D10 group'—to examine the peak of a primary TD GC reaction, mice were immunized with 50 μ g of alum-precipitated NP-CGG plus 1×10^9 heat-killed *B. pertussis*. Where indicated, TD responses and GC reactions were generated by i.p. immunization of 8- to 14-week old C57BL/6 mice with 2×10^9 SRBC. For experiments involving SW_{HEL} mice, 1×10^4 SW_{HEL} B cells were transferred into CD45.1 congenic C57BL/6 recipients, which were immunized i.v. with 2×10^8 SRBC conjugated with mutant HEL, HEL^{2x25}.

Antibodies

Anti-BASP1 rabbit serum was produced at Pico Caroni's laboratory. Anti-*PLEXIN B2* serum was made by immunizing rabbits with a *PLEXIN B2* peptide conjugated to ovalbumin (NeoMPS). Monoclonal hamster anti-*PLEXIN B2* antibody was produced at Wendy L Havran's laboratory. Antibodies for flow cytometry were from BD Pharmingen (San Diego, CA, USA) except where otherwise indicated: anti-mouse B220-PE, B220-APC, CD11b-biotin, CD43-biotin, CD45.2-FITC, Fas-PE, GL-7-FITC and Ter119-biotin; anti-human CD19-biotin, CD27-PE, CD38-PE, IgD-FITC, purified CD77 (Serotec, Oxford, UK). For immunohistochemistry, the following antibodies were used: rat anti-mouse IgD (Southern Biotech, Birmingham, AL, USA), goat anti-mouse IgD, PNA-biotin (Vector Laboratories, Burlingame, CA,

USA), hamster anti-PLEXIN B2, rabbit anti-BASP1, rat anti-BCL-6 (Santa Cruz, Santa Cruz, CA, USA) and rat anti-GL-7 (BD Pharmingen). Secondary antibodies used were rabbit anti-rat horseradish peroxidase (Dako, Glostrup, Denmark), goat anti-hamster-biotin (Jackson ImmunoResearch, West Grove, PA, USA), goat anti-rat horseradish peroxidase (Jackson ImmunoResearch) and swine anti-rabbit-biotin (Dako).

Cell isolation, culture and stimulation

In order to isolate resting and GC B cells for western blotting, single-cell suspensions were prepared from spleens of unimmunized and immunized mice, respectively, and stained with B220-PE and GL-7-FITC. Spleen cell suspensions from immunized mice were enriched for GC B cells by magnetic cell sorting with anti-FITC-magnetic beads and MACS LS columns (Miltenyi Biotec, Bergisch Gladbach, Germany) according to the manufacturer's instructions. GC B cells (B220⁺ GL-7^{high}) and resting B cells (B220⁺ GL-7⁻) were sorted to >95% purity on the FACS sorter (BD Vantage DiVa, San Jose, CA, USA). For microscopy, antibody-labeled cells were directly purified by FACS sorting without the magnetic enrichment to avoid damage of fine membrane structures. Human resting B cells (CD19⁺ IgD⁺ CD27⁻) were isolated by FACS sorting from peripheral blood, and CB (CD19⁺ CD38^{hi} CD77⁺) and CC (CD19⁺ CD38^{hi} CD77⁺) were isolated by FACS sorting from tonsils.

For *in vitro* stimulation assays, single cell suspensions were prepared from red blood cell-depleted splenocytes of unimmunized C57BL/6 mice and stained with biotinylated CD43, Ter119 and CD11b. Resting B cells were negatively selected by magnetic cell sorting with streptavidin magnetic beads and MACS LD columns (Miltenyi Biotec) following the manufacturer's instructions. Purity of sorted cells exceeded 95% by FACS. Purified resting B cells were cultured in RPMI media (JRH Bioscience) supplemented with 10% FCS, 10 mM HEPES, 55 μ M β -mercaptoethanol, 2 mM L-glutamine, 50 U ml⁻¹ penicillin, 50 μ g ml⁻¹ streptomycin, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (all from GIBCO) at 37 °C in a 5% CO₂-humidified incubator. Goat anti-IgM F(ab)₂ (10 μ g ml⁻¹, Jackson ImmunoResearch), anti-CD40 (10 μ g ml⁻¹, clone 1C10), lipopolysaccharide (20 μ g ml⁻¹; Fluka, Seelze, Germany) and recombinant IL-4 were used for stimulation and proliferation assays.

GC gene expression analysis

Mouse resting B cells, day 4 TI GC B cells, day 4 and day 10 TD GC B cells were isolated as described above. Human GC B cells were sorted from tonsils, whereas resting and memory B cells were sorted from peripheral blood. RNA was extracted from pellets of sorted cells by RNeasy (Qiagen, Crawley, UK) treatment. Total RNA was transcribed *in vitro* and biotin-labeled according to the recommended Affymetrix protocol, then hybridized on Affymetrix U74A arrays in the case of mouse cells, or U95A arrays in the case of human cells. Genes were considered differentially expressed when levels differed by at least twofold after analysis of variance analysis with $P < 0.01$. Microarray data comparing resting, TD day 4, TD day 10 and TI GC B cell profiles have been deposited in GEO (<http://www.ncbi.nlm.nih.gov/geo/>) under accession number GSE8906.

Lymphomas and oligonucleotide microarrays

NFS.V⁺ mice,⁵⁴ SJL mice, B6.λMYC²⁷ and IL-6 transgenic mice²⁷ were observed for development of tumours and necropsied. Samples were collected for histology and later preparation of RNA and DNA. Lymphomas were classified using a consensus nomenclature.⁵⁵ Pre-GC tumours (IgM⁺ IgD⁺ AA4.1^{+/−}; germ line IgH and IgL sequences) were from B6.λMYC mice. Post-GC tumours included plasma cell-rich tumours of SJL mice and PCT from IL-6 transgenic and *Myc*^{HIS} insertion mice.^{29,56} RNA prepared from the samples was used to probe 70-mer oligonucleotide arrays as described.²⁸ After normalization with an R-package LIMMA (www.r-project.org; bioinf.wehi.edu.au/limma/), Student's *t*-tests were conducted to identify differentially expressed genes between pre- and post-GC neoplasms.

Quantitative reverse transcription-PCR analysis

Total RNA was prepared from purified resting and GC B cells using TRIzol (Invitrogen, Carlsbad, CA, USA) and reverse-transcribed with oligo(dT). Quantitative reverse transcription-PCR was performed using the ABI Prism

7700 sequence detection system and TaqMan Syber-green reagents (PE Biosystems, Foster City, CA, USA). Primer sequences are available on request. Fluorescence signals were measured over 40 PCR cycles and the cycle (C_t) at which signals crossed a threshold set within the logarithmic phase was recorded. The C_t for the target gene was subtracted from the C_t for β -actin (ΔC_t). The relative amount of mRNA was calculated as $2^{-\Delta C_t}$.

Flow cytometry

Spleen cell suspensions were prepared by sieving and gentle pipetting. For surface staining, cells were maintained in the dark at 4 °C throughout. Cells were washed twice in ice-cold FACS buffer (2% fetal calf serum, 0.1% NaN₃ in PBS), then incubated with each antibody and conjugate layer for 30 min and washed thoroughly with FACS buffer between each layer. Intracellular staining used Cytotfix/Cytoperm kit (BD Biosciences) following the manufacturer's instructions. For detection of HEL-binding B cells, HEL was conjugated to Alexa 647 (Molecular Probes, Eugene, OR, USA).

Immunohistochemistry

Frozen sections of spleen were air-dried and washed in 0.1 M Tris-buffered saline (pH 7.6) and then primary antibodies were added in Tris-buffered saline and incubated for 45 min. After a further wash in Tris-buffered saline, secondary reagents that had been previously absorbed in 10% normal mouse serum were added to the sections for 45 min. When biotin-conjugated primary or secondary reagents were used, streptavidin alkaline phosphatase (Vector Laboratories) was added after a further wash in Tris-buffered saline and incubated for 20 min. Horseradish peroxidase activity was detected using diaminobenzidine tetrahydrochloride solution (Sigma, St Louis, MO, USA) and hydrogen peroxide. Alkaline phosphatase activity was detected using the AP-Substrate kit III (SK-5300, Vector Laboratories).

Western blotting

Isolated resting and GC B cells were lysed on ice in 20 μ l lysis buffer per 2 $\times 10^6$ cells (50 mM Tris-base, pH 8.0, 150 mM NaCl, 1% NP-40 plus protease inhibitor (Roche, Basel, Switzerland)). The lysates were centrifuged at 13 000 r.p.m., and the supernatants removed and boiled in 5 $\times$ SDS sample buffer (10% SDS, 50% glycerol, 0.2 M Tris-HCl, pH 6.8, 5% 2-mercaptoethanol and bromophenol blue to color). Protein samples from 2 $\times 10^6$ cells were separated on 10 or 12.5% SDS-polyacrylamide gel electrophoresis, transferred to a nitrocellulose membrane and then blotted with anti-PLEXIN B2, anti-BASP1 or unimmunized rabbit sera. Monoclonal anti-actin antibody (Sigma) was used as the loading control.

Morphological studies by microscopy

Sorted resting (B220⁺ IgD^{high} GL-7⁻) or GC B cells (B220⁺ IgD⁻ GL-7⁺) from mice 8–10 days after immunization with SRBC were allowed to settle onto poly-L-lysine-coated coverslips in cell culture media for 2 h and fixed in 2% paraformaldehyde. After one wash in PBS, cells were stained with Oregon Green 488 phalloidin (Molecular Probes) in the dark. After two further washes in PBS, the coverslips were mounted on slides with fluorescent mounting medium (Dako). The slides were observed at room temperature under an Olympus IX81 fluorescent microscope using an Olympus 60 $\times$ oil (N.A. 1.42) or 100 $\times$ oil (N.A. 1.35) PlanApo objective. Images were collected with an Olympus DP70 camera using software DP Controller (Version 1.2.1.108, Olympus). When necessary, the contrast of the pictures was adjusted. For scanning electron microscopy, cells were attached to Thermanix plastic coverslips coated with 0.1% poly-L-lysine. Cells were fixed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h, washed three times in 0.1 M sodium cacodylate buffer and post fixed in 1% OsO₄ in 0.1 M sodium cacodylate buffer for 20 min. After two further washes, the samples were dehydrated in a graded acetone series to 100% acetone and then 'critical point dried'. The samples were sputter coated with gold and photographed in a Cambridge S360 scanning electron microscope.

ABBREVIATIONS

CB, centroblast; CC, centrocytes; GC, germinal centre; HEL, hen egg lysozyme; NP, (4-hydroxy-3-nitrophenyl) acetyl; PCT, plasmacytomas; SRBC, sheep red

blood cells; TD, T cell-dependent; T_{FH}, follicular helper T cell; TI, T-independent; TI-2, T-independent type 2

ACKNOWLEDGEMENTS

We thank Robert Brink, Tri Giang Phan and Didrik Paus for their help with the experiments using SW_{HEL} mice; the Biomolecular Resource Facility founded by Australian Cancer Research Foundation for the instrumental and technical support with quantitative RT-PCR; Cathy Gillespie for microscopy; David Tarlinton and Roland Friedel for helpful discussions regarding the function of PLEXIN B2. This work was supported by a Wellcome Trust International Prize Travelling Research Fellowship to CGV, NHMRC project grants 224264 and 366771 and NHMRC program grant 427620 to CCG and CVG, and the Intramural Research Program of the NIH, National Institute of Allergy and Infectious Diseases (HCM).

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Supplementary Information accompanies the paper on Immunology and Cell Biology website (<http://www.nature.com/icb>).