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Title

Lectin binding to surface Ig variable regions provides a universal persistent activating signal for follicular lymphoma cells

Running title

Lectin-mediated activation of FL via surface Ig

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Key Points

1. Unusual sugars on the tips of surface Ig of follicular lymphoma cells interact with a tissue lectin to activate tumor-specific signaling.

2. Activating lectin does not allow endocytosis of surface Ig, leading to persistent, essential and targetable antigen-independent stimulation.

Abstract

The vast majority of cases of follicular lymphoma (FL), but not normal B cells, acquire N-glycosylation sites in the immunoglobulin variable regions during somatic hypermutation. Glycans added to sites are unusual in terminating at high mannoses. We showed previously that the C-type lectins, DC-SIGN and mannose receptor, bound to FL surface Ig (sIg), generating an intracellular Ca^{2+} flux. We have now mapped further intracellular pathways activated by DC-SIGN in a range of primary FL cells with detection of phosphorylated ERK1/2, AKT and PLCγ2. The SYK inhibitor (tamatinib) or the BTK inhibitor (ibrutinib) each blocked phosphorylation. Activation by DC-SIGN occurred in both IgM+ and IgG+ cases and led to upregulation of MYC expression, with detection *in vivo* observed in lymph nodes. Unlike cells of chronic lymphocytic leukemia, FL cells expressed relatively high levels of sIg, unchanged by long-term incubation *in vitro,* indicating no antigen-mediated downregulation *in vivo*. In contrast, expression of CXCR4 increased *in vitro*. Engagement of sIg in FL cells or normal B cells by anti-Ig led to endocytosis *in vitro* as expected, but DC-SIGN, even when cross-linked, did not lead to significant endocytosis of sIg. These findings indicate that lectin binding generates signals via sIg but does not mediate endocytosis, potentially maintaining a supportive antigen-independent signal *in vivo*. Location of DC-SIGN in FL tissue revealed high levels sinusoid-like structures and in some co-localized mononuclear cells, suggesting a role for lectin-expressing cells in the life-style of FL.

Introduction

It is known that the t(14;18) translocation found in the majority of cases of FL, which leads to upregulation of BCL2 expression, is necessary but not sufficient for tumor development.[1] While further genomic changes are being revealed, particularly in histone-modifying genes,[2] the role of the microenvironment is also likely to be critical.[3] Another important feature of FL is that, in spite of the loss of one Ig allele by the t(14;18) translocation, surface Ig (sIg) is retained. Initially this retention and the on-going somatic hypermutation (SHM) led to speculation about a role for persistent antigen in FL, which was difficult to explain, given the high variability of *IGV* gene usage and the sequence changes resulting from SHM. A striking observation on the nature of sIg in FL has revealed a possible explanation both for retention of sIg and for an influence of microenvironmental factors. This involves a “universal” antigen-

independent mechanism able to engage sIg of all cases of FL.[4] Specifically, in FL, there is a tumor-specific structural change of sIg variable regions by introduction of motifs for N-addition of unusual glycans.[5,6] The placement of these glycans at the tip of the sIg on FL cells not only provides a common molecular feature for interaction with lectin, but, secondly, allows a functional bridge between the sIg and the microenvironmental cells which express candidate lectins.[4]

The introduced glycosylation motifs are in the sIg variable regions of most if not all FL cases, but rare in normal B cells.[7] Motifs are generated during SHM and appear to be positively selected by FL cells. They are also maintained over time, even while SHM continues[8] indicating an important role in pathogenesis and maintenance in the germinal center (GC) site. There is additional evidence for acquisition of motifs in other GC-located B-cell malignancies such as endemic Burkitt's lymphoma, and a proportion of diffuse large B-cell lymphoma, but not in multiple myeloma, mucosa-associated lymphoid tissue lymphomas or mutated chronic lymphocytic leukemia (CLL).[9]

We showed previously that the glycan added to the sites is unusual in terminating at the high mannose stage.[5] In contrast, available sites in the constant region are fully N-glycosylated,[10] suggesting that the motifs in *IGHV* region, although present at various sequence positions, and sometimes in *IGLV*, are sterically unavailable for glycosyl transferase activity in the Golgi. The outcome is expression in all cases of sIg with mannosylated sites in the V regions and fully glycosylated sites in the constant region.[5,4]

Candidate lectins in lymphoid tissue able to interact with FL sIg include DC-SIGN (dendritic cell-specific intercellular adhesion molecule-3 grabbing non-integrin) and mannose receptor.[11] These are both calcium-dependent (C-type) lectins with a major role in innate immunity, being able to bind to mannoses expressed by a range of pathogens and by self-ligands.[12] Both recombinant proteins were also able to bind to the mannosylated sIg and induced a tumor-specific sIg-mediated intracellular [Ca^{+2}] flux in primary FL cells, with no signal in normal B cells. A control protein, consisting of mannose receptor with the carbohydrate-recognition domain removed, was used to confirm the specificity for binding to glycan expressed by FL cells, and a blocking experiment indicated binding to sIgM.[4] We have now focused on DC-SIGN as a paradigm and have analyzed the intracellular pathways activated by binding of either the soluble lectin or after attachment to beads. Exposure of sIgM+ or sIgG+ FL cases to either form of DC-SIGN induced phosphorylation of ERK1/2, PLCγ and AKT. Bead-bound DC-SIGN also increased levels of MYC, with MYC protein detected in FL lymph nodes. In contrast, normal B cells which express similar levels of sIg, failed to respond to DC-SIGN. Another intriguing finding was that, unlike anti-Ig, soluble DC-SIGN, even when cross-linked, did not mediate significant endocytosis of sIg. These features may have been captured by opportunistic FL cells for long-term survival and proliferation in the hostile GC site.

Methods

Patient and Healthy Donor Material

Samples of primary cells from lymph nodes of 20 FL patients and PBMCs from blood of 5 healthy donors were isolated from frozen stocks.[7] Approval was obtained from the Southampton and South West Hampshire Research Ethics Committee. Informed consent was provided in accordance with the Declaration of Helsinki. . For experiments, cells were rapidly thawed at 37^{o}C prior to purification of viable cells using Lymphoprep. Cells were then washed in complete medium (RPMI1640 medium supplemented with 10% (v/v) FCS, 2 mM glutamine, and 1% (w/v) sodium pyruvate) and counted using Trypan Blue exclusion before being allowed to rest for 30 minutes at 37^{o}C/5%CO_2.

Phenotype and *IGHV* sequences

Live FL cells were used to access phenotype. anti-IgM-R-Phycoerythrin (PE), anti-IgD-Fluorescein isothiocyanate (FITC), anti-IgG-FITC (all DAKO, Ely, UK), anti-human CD19 Pacific Blue™ or allophycocyanin (APC), anti-human CD5 PerCP/Cy5.5 (BioLegend, Cambridge, UK). Cells were labelled with anti-IgM/IgD/CD19/CD5 or anti-IgM/IgG/CD19/CD5 or corresponding isotype controls. Surface Ig expression was measured in CD19+/CD5- population. Samples were acquired on FACSCanto II (BD Biosciences, Oxford, UK).

BCL2+ FL cells were identified for immunofluorescent analsysis after fixation in paraformaldehyde (final concentration 1.6%) (VWR, Lutterworth, UK), Cells were incubated for 5 min at room temperature then washed and resuspended in ice-cold 90% (v/v) methanol and incubated on ice for 30 min. Then cells were washed and reconstituted in Flow Buffer (1% bovine serum albumin, 4 mM EDTA, and 0.15 mM NaN_3 in phosphate buffered saline). Staining was with directly labelled anti-CD20 PerCP-Cy™ 5.5 (clone H1/FB1), anti-BCL2-FITC (clone 6C8). Samples were acquired on FACSCanto II. Gating was first for singlets (FSC-H/ FSC- A), then lymphocytes (SSC-A/FSC-A) before the malignant cells were gated based on CD20 and BCL2 double positivity.

Tumor-derived *IGV(D)J* gene sequences were identified in FL samples as described.[7]

DC-SIGN, anti-Ig and bead conjugation

Recombinant Human DC-SIGN/CD209-Fc chimera (referred to as soluble DC-SIGN) (R&D Systems, Abingdon, UK), consists of the extracellular portion (amino acid residues 64-404) expressed as a dimeric lectin covalently linked to human Fcγ, Carrier Free construct protein, goat F(ab')2 anti-μ, anti-γ or goat F(ab')2 isotype control (Southern Biotech, Cambridge Biosciences UK) were used as soluble proteins or were conjugated to Dynabeads M-280, 2.8-µm diameter, with epoxy surface groups (Invitrogen, Paisley, UK) according to the manufacturer's instructions. Briefly, beads were washed in the provided C1 buffer and proteins were added at 10 µg/mg of beads. The suspension was incubated on a rotator at 37°C overnight, then washed in the provided C2 buffer, and resuspended in the provided SB storage buffer at a final bead concentration of 10 mg of beads per ml so that 1.5μl equates to 10^6 beads. For long-term storage, 0.02% NaN_3 was added.

Binding of DC-SIGN to surface Ig on primary FL.

Primary FL cells were recovered and allowed to rest as described above. The method for measuring binding of DC-SIGN was provided by Drs K Tarte and R Amin-Ali. Cells were divided into FACS tubes and placed on ice prior to treatment with soluble DC-SIGN. Tubes were incubated on ice for 45 minutes before 4%PFA was carefully applied to all tubes to fix cells and incubated at room temperature for 15 minutes. Cells were washed twice using PBS and centrifuged at 1500rpm for 5 minutes following resuspension in Flow Buffer. To determine the level of DC-SIGN attached to the cells, an unconjugated monoclonal mouse anti human-CD209 IgG2b antibody (BD Biosciences) was applied to tubes and incubated at room temperature for 45 minutes. As a control, some cells were not treated with this antibody. Cells were washed and resuspended using Flow Buffer before the addition of Alexa Fluor^{488}-conjugated goat anti mouse-IgG Fcγ2b (Jackson ImmunoResearch, Newmarket, UK) to detect bound anti-CD209 and Dylight^{549}-conjugated goat anti-human IgM (Jackson ImmunoResearch) to detect sIgM. Tubes were incubated at room temperature in darkness for 30 minutes. Cells were washed and resuspended in Flow Buffer before analysis with a BD FACS Canto. Data were analysed using FlowJo (Tree Star, Ashland, Oregon, USA). Singlet, live cells were gated on to detect sIgM expression and cells positive for this were gated on to determine DC-SIGN binding.

Immunoblot analysis

Immunoblotting was performed as described elsewhere[13] using the following antibodies: anti-phospho-ERK1/2 (9101), anti-ERK1/2 (9102), anti-phospho-AKT (S473) (4060), anti-AKT (9272) (all from Cell Signaling Technology, Hitchin, UK), anti-MYC (clone 9E10) (Merck Millipore, Beeston, UK) and anti-β-actin (2066; Sigma-Aldrich, Poole, UK). Secondary horseradish peroxidase-conjugated antibodies were from GE Healthcare (Little Chalfont, UK). For quantitation, images were collected using a Fluor-S MultiImager (BioRad, Hemel Hempstead, UK) and quantified using Fluor-S software Quantity One Version 4.6.3 (BioRad). All values were normalized to the relevant loading control and relative fold change was calculated with the isotype control antibody-treated cells taken as a 1.0 fold of expression.

PhosFlow analysis

For the PhosFlow assay[14] FL cells were left in complete medium for 60 min to recover at 37°C. 0.5-1x10^6 cells in 0.1 mL of medium were left untreated or treated with anti-Ig mix (10 μg/mL goat F(ab')2 anti-human IgM and anti-human Fcγ) or DC-SIGN (10μg/mL) cross-linked with anti-human IgG (Fc specific), F(ab')2 fragment antibody (Sigma-Aldrich, Poole, UK) (3:1 (3 DC-SIGN 1 anti-IgG to avoid non-specific binding from anti-IgG in the experiment) molar ratio, 30 min preincubation) for indicated time points. H_2O_2 was added immediately prior to stimulation at final concentration 3.3mM (Irish, Blood) except for one unstimulated control. Stimulation was stopped by addition of paraformaldehyde (final concentration 1.6%) (VWR) and cells were incubated for 5 min at room temperature then washed and resuspended in ice-cold 90% (v/v) methanol and incubated on ice for 30 minutes. Then cells were washed and reconstituted in Flow Buffer. Cells were stained with directly labelled

anti-CD20 PerCP-Cy™ 5.5 (clone H1/FB1), anti-Bcl-2-FITC (clone 6C8), anti-ERK1/2 (pT202/pY204)-PE or PLC-γ2 (pY759) Alexa Fluor® 647 or appropriate isotype controls (All BD Biosciences) antibodies for 30 minutes on ice, then washed and reconstituted in the Flow Buffer. Samples were acquired on FACSCanto II. Change in phosphorylation was determined on the gated CD20+/Bcl2+ cells and geometric mean fluorescence intensity (GeoMFI) was calculated for PE (pERK) or Alexa Fluor® 647 (pPLC-γ2) at the different time points. The results were analysed by the on-line analytical platform Cytobank (Cytobank, Inc., Mountain View, USA). The resulting GeoMFI were normalised to controls samples (H_2O_2 treatment only) for each time point and fold changes in phosphorylation were calculated.

Immunohistochemistry

4μ-thick formalin-fixed, paraffin-embedded sections obtained from reactive tonsils and follicular lymphomas (encompassing grades 1, 2, and 3a) were stained with the following antibodies; CD20 (clone L26, Dako, Glostrup, Denmark, dil. 1:100), CD21 (clone EP3/93, cell Marque, Rocklin, USA, dil. 1:50), c-MYC (clone Y69 Abcam, Cambridge, UK, dil. 1:200) and CD209 (clone EPR5588, Epitomics, Burlingame, USA, dil 1:100). For CD20, CD21 and c-MYC antibodies, antigen retrieval was obtained with TRIS-EDTA at pH 9 for 30 minutes. CD209 and correspondent double stains were performed on frozen sections.

Kinase inhibitors

The clinical BTK and SYK inhibitors ibrutinib and tamatinib (the active form of fostamatinib) (Stratech Scientific Ltd, Suffolk, UK) were used at 10μM final concentration. Cells were pre-treated with the inhibitor for 30 minutes prior to stimulation.

Endocytosis of surface Immunoglobulin

Internalization of sIgM was assessed by measuring the remaining anti-Ig or DC-SIGN on the cell surface of CD19+ve cells with secondary FITC-labelled antibodies. Briefly, washed and pre-cooled cells were pre-incubated with 20 μg/mL goat F(ab')2 anti-human Ig or DC-SIGN for 40 min on ice. Also, the aliquots ($1x10^6$ cells) of cells were left for the unstained control and the secondary antibody staining control. Then the cells labelled with anti-Ig or DC-SIGN were divided into aliquots ($1x10^6$ cells) for incubation on ice or at 37ºC for 0, 10, 30 and 60 min. At each time point the internalization was stopped by addition of ice-cold Flow Buffer then the aliquots were left on ice. Next, cells were washed in ice-cold Flow Buffer and labelled with anti-CD19-APC and rabbit anti-goat F(ab')2-FITC (Jackson ImmunoResearch,) for anti-Ig labelled cells or goat anti-human IgM secondary antibody, FITC (Cambridge Bioscience, Cambridge, UK) for DC-SIGN labelled cells. The samples were acquired on FACSCanto II and GeoMFI for green channel in CD19+ve gated cells were used to calculate percentage of internalization. The GeoMFI value at the starting time (0 minutes) was calculated as 100% of sIg expression.

Results

Primary FL tissue analysis

The primary FL samples investigated were from lymph nodes of 18 cases of Grade 1-3a (Table 1S) and included 12 IgM+ and 6 IgG+ cases by phenotype. Analysis of the *IGHVHDHJ*-constant region by PCR analysis confirmed the isotype in 13/13 available cases and revealed Asn-X-Ser/Thr motifs in 14/14 cases, with 6 of these having additional sites in *IGLV*. Samples were analyzed for tumor cells by CD20/BCL2 staining (Fig.1A) and expression of surface Ig with representative IgM+ or IgG+ cases shown in Fig.1B. The percentage of B cells in the mononuclear cell fraction was usually 40-50% and the proportion of normal (BCL2-negative) cells in the B-cell population was low (~10-20%).

DC-SIGN binds to primary FL cells and specifically activates signalling pathways

Primary FL cells express relatively high but variable levels of sIg. We compared the level of binding of DC-SIGN with the amount of expressed sIg in 7 cases of sIgM+ FL. Results on 3 cases are illustrated in Fig.2A and data from the 7 patients indicated a correlation between sIgM levels and the ability to bind DC-SIGN (Fig.2B). In 2 cases (774 and 861) illustrated in Fig.1S, we detected two populations of BCL-2+ cells expressing different (high and low) levels of sIgM (A), and in a further case (590) there was a broad peak indicating a range of sIgM expression (A). Analysis of these samples for DC-SIGN binding showed double peaks (B) and back gating to determine the level of sIgM on the peaks revealed a correlation between DC-SIGN binding and the level of sIgM expression (C). This supports the conclusion that DC-SIGN is binding to sIgM and that the ability to bind varies with the level of sIgM expression. However, in addition to sIgM levels, another variable is the number of glycan addition sites/molecule. Some correlation with binding is apparent, with cases 86 and 590 having 5 and 3 sites respectively (Fig.2B) but case 221 with 4 sites was an exception being only a moderate binder, perhaps indicating heterogeneity in glycan addition.

Exposure of primary FL cell samples to DC-SIGN led to phosphorylation of ERK1/2 and AKT. This occurred in both IgM+ (Fig.3A) and IgG+ cases (Fig.3B). The level of response induced by bead-bound DC-SIGN was generally higher than the soluble form. Increasing the bead:cell ratio to 4:1 did generate higher responses against DC-SIGN, especially evident with p-AKT (Fig.2S). Since DC-SIGN is a calcium-dependent lectin, we measured responses in two cases over a range of calcium concentrations and found that the level in medium plus FCS (1.5mM) was adequate (Fig.3S), therefore this was used in all experiments.

Comparative data of stimulation with DC-SIGN or with anti-Ig on 16 cases are illustrated in Fig.3C. While responses to anti-Ig are higher, the responses to DC-SIGN appear to persist longer, with little reduction from 0.5 to 6h. In contrast to primary FL cells, normal B cells did not respond to bead-bound DC-SIGN, although

the response to anti-Ig was similar to FL cells (Fig.3D). The gel shows one example of the same results obtained from 4 normal donors.

Analysis of responses to DC-SIGN in single FL cells

Responses of single cells were analyzed by flow cytometry using soluble DC-SIGN crossed-linked via anti-Fcγ. Soluble anti-Ig was used as a control, and detection of responses was by PhosFlow, using FITC-labelled antibodies against either p-ERK or p-PLCγ. The basal phosphatase activity was inhibited by addition of hydrogen peroxide.[14] A typical result (Fig.4A) using an IgG+ sample (FL-33), gated on CD20 and BCL-2 double positive expression, confirms the early phosphorylation of ERK seen by Western blotting, with a similar pattern observed for PLCγ.

We constructed heat maps of scaleable geometric means of responses for 13 FL cases. Compared to the unstimulated controls (NT), all cases responded, but at variable levels (Fig.4S A). Higher responses to DC-SIGN were generally in cells with higher responses to anti-Ig. Compiled kinetic responses to lectin are similar to those with anti-Ig, but again tended to be more persistent (Fig.4B). We could find no consistent significant differences in the level of phosphorylation between IgM+ or IgG+ cases (Fig.4S B), although numbers are limited.

Inhibitors of BCR-associated proximal kinases decrease DC-SIGN dependent signalling in FL.

If DC-SIGN stimulation has a role in maintenance of FL cells, it is important to assess if new inhibitors will act against this pathway. We therefore tested inhibitors of SYK (tamatinib) and BTK (ibrutinib). Analysis by single cell flow cytometry of FL cells, for the effects of pre-exposure to inhibitor for 30 minutes on signalling, showed strong inhibition of responses against either anti-Ig or DC-SIGN. Compiled data for 6 cases (SYK) and 3 cases (BTK) are shown in Fig.4C.

DC-SIGN stimulates MYC expression in primary FL cells.

To investigate the downstream BCR-associated targets activated by DC-SIGN, we analyzed MYC expression in FL. Basal levels of MYC were mostly low, but were increased by exposure to bead-bound DC-SIGN (2 representative examples shown in Fig.5A). The response was again weaker than that induced by bead-bound anti-Ig but had a similar kinetic being maximal at the 6h time point. Data from 11 samples confirmed a significant increase in MYC levels following exposure to DC-SIGN (Fig.5B).

In contrast, normal B cells did not upregulate MYC after exposure to DC-SIGN, but did after bead-bound anti-Ig (Fig.5C). The potential relevance of this upregulation *in vivo* was investigated by staining of FL tissue for MYC protein. Immunohistochemistry revealed FL cells, with characteristic heterogeneous neoplastic cell nuclei, embedded within the CD21-positive follicular dendritic cell network, among which were detectable MYC-positive FL cells (Fig.5S).

Effects of incubation *in vitro* on expression of sIg or CXCR4

Levels of sIg for FL cells vary but are in the same range as normal memory B cells[4] (Fig.1A) and ~10x higher than cells of chronic lymphocytic leukemia (CLL). In CLL, expression of sIgM can be downregulated *in vivo* by antigen or *in vitro* by anti-Ig.[15] A second receptor with downregulated expression in CLL is the chemokine receptor, CXCR4.[16,17,18] Recovery of expression of sIgM by 48-72h and CXCR4 by ~8h can then occur in CLL cells following incubation *ex vivo.*[19] To gain insight into events *in vivo* which may have occurred in FL cells, expression of sIg and CXCR4 was tracked over time in 9 FL samples (6 IgM+, 3 IgG+). Only 1/9 showed any recovery of sIg expression, even when starting levels were relatively low (Fig.6A.B). In contrast, most cases (8/9) recovered expression of CXCR4 over a 24h period (Fig.6C,D). The high expression of sIg and its stability *in vitro* argue against downregulation of sIg by antigen *in vivo*. Recovery of CXCR4 expression reveals that the FL cells were able to reverse the expected chemokine-mediated downmodulation in tissue sites.

Engagement of sIg by DC-SIGN fails to lead to endocytosis

Following binding of anti-Ig, sIg of CLL cells undergoes rapid endocytosis.[15] The ability of DC-SIGN to induce endocytosis of sIg was first tested by measuring the sIgM remaining at the cell surface following incubation with either DC-SIGN or with anti-Ig. Endocytosis was clearly seen after exposure to anti-IgM as expected in both FL cells (Fig.7A) and normal B cells (Fig.7B) following incubation for 10-30 min. Assessment of the effects of engagement with DC-SIGN on sIgM expression has to take into account a partial blocking of access of anti-IgM by bound DC-SIGN.[4] The initial level of DC-SIGN-engaged sIgM in ice was then assigned a value of 100%. No significant change in this level was detectable following incubation for 60 minutes (Fig.7C) indicating minimal removal by endocytosis. To increase lectin avidity, a cross–linking secondary antibody against Fcγ was added to the DC-SIGN protein prior to addition to the cells. Even then, no significant endocytosis could be induced (Fig.7D).

To test whether binding of DC-SIGN, which failed to induce endocytosis, was sufficient to induce a signal, we analyzed two FL samples (FL86 and FL590) in depth. The results were very similar and are illustrated for FL86 in Fig.6S. After exposure to DC-SIGN, we measured binding, signaling and persistence of sIgM on the cell surface (A). It is clear that binding of DC-SIGN was high (B) and was able to mediate phosphorylation of ERK1/2 and especially AKT (C). However, there was no significant loss of expression of sIgM (D) by endocytosis. Comparison of endocytosis estimated by reduction of expression of sIgM at 30minutes indicated ~77% reduction via anti-IgM, but only ~29% reduction via DC-SIGN.

Localization of DC-SIGN in FL lymph node.

Expression of DC-SIGN in FL lymph node was assessed by IHC using anti-CD209 . The results (Fig.7S) show a background of CD20-positive FL cells with sinusoid-like

structures staining for CD209. There were also some mononuclear cells (arrowed) which were CD209-positive.

Discussion

Normal B cells do not linger in the GC, but go through a ruthless process of antigen selection or death. Selected B cells leave the site and, although revisiting does occur,[20] their fate is to become memory B cells or plasma cells. In contrast, FL cells remain mainly locked in the GC, partially protected by upregulated BCL-2, and apparently able to exploit the microenvironment for survival and proliferation. Candidate supporting cells are likely to be multiple, but appear to include IL-4-producing T-follicular helper cells (T_{FH}), tumor-associated macrophages and stromal cells.[21]

The distribution and number of macrophages varies in FL, but an overall increase has been associated with a poorer prognosis.[22,23] One problem of assessing a prognostic role is that pathogenesis and response to therapy both influence clinical outcome, possibly in opposite directions. Thus macrophages may promote tumor growth but, as mediators of antibody-dependent cellular cytotoxicity, they are required for antibody therapy.[24]

The positively selected expression of high mannose in the sIg variable regions is a clue to pathogenesis,[25] and strongly indicates an essential interaction with a presumed lectin in the lymph node. It appears that mannosylation motifs are acquired at the early stage of lymphomagenesis termed follicular lymphoma in situ (FLIS).[26] Although we cannot be certain which lectin is involved, we have used DC-SIGN, known to recognize mannoses both on pathogens and on self-ligands,[11] as a paradigm to probe the consequences of engaging the mannosylated sIg.

It is clear that DC-SIGN can activate intracellular phosphorylation pathways characteristic of sIg engagement, thereby offering a mechanism for antigen-independent B-cell receptor signalling in FL. However, in contrast to anti-Ig, DC-SIGN, even when multimerized, fails to induce endocytosis. This is consistent with the finding that FL cells, unlike CLL cells, show no evidence for downregulation of sIg *in vivo* or for the anergic phenotype characteristic of CLL. CLL cells have features of cells which reversibly engage antigen *in vivo*, apparently leading to endocytosis followed by recovery.[15] Since the antigen may be cell-bound,[27] CLL cells appear capable of “biting” it off cell surfaces as described for normal B cells.[28] None of this occurs in FL, but sIg expression remains essential, as does the upregulation of BCL-2. One possibility is that interaction with a lectin could provide a continuous low-level activatory signal for tumor cells.

Expression of DC-SIGN in FL tissue from our cases confirms a dominating high level on lymphatic sinusoidal structures,[26] but also indicates expression on other nearby mononuclear cells. Since IL-4 is detected in LN tissue, polarization to the M2 type occurs[21] and DC-SIGN is upregulated.[29] The situation *in vivo* is more complex. FL cells could encounter DC-SIGN-expressing macrophages as they crawl through the tissue, or, the DC-SIGN on lymphatic endothelium could bind FL cells and block egress, acting as a retention signal. Both adhesion and the signalling pathways activated *in vitro* were blocked by ihibitors of BTK or SYK. However, while it is likely

that continuous stimulation by local autolectins is vital, we have recently shown that lectins from certain bacteria can also induce an i[Ca^{2+}] flux[30] raising the possibility of tumor perturbation during infection.

The ability of receptor-associated glycan to modulate signalling pathways has been revealed by the effects of galectins on T or B lymphocytes.[31] In that case, binding of galectin to galactose residues on the receptors generates a "lattice" which affects oligomerization and signalling but prevents endocytosis.[32] It appears likely that a parallel array of lectins can target V-region mannoses in sIg and that FL cells have exploited this strategy for survival and proliferation. Opportunities for blocking this interaction include either antibodies against high mannoses which are being developed for targeting HIV[33] or potentially the clinical-grade galactomannans, aimed to release tumor-infiltrating lymphocytes from galectin-mediated suppression in patients with cancer.[34]

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Authorship Contributions

Contribution: A.L. and S.K. performed research and analyzed data; F.K.S, G.P.,S.K. and A.L. designed the research and analyzed data; M.P. performed and interpreted the immunohistochemical data; P.W.J. provided patient samples and analyzed clinical data; F.K.S., G.P. and SK wrote the initial draft of the manuscript; and all authors contributed to the modification of the draft and approved the final submission.

Conflict of Interest Disclosures

The authors declare no competing financial interests.

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Figure Legends

Figure 1: Characterization of follicular lymphoma cells.

Phenotyping of representative patients used in this study, including FACS plots detailing gating strategy of singlet CD20+ cells to determine BCL2 expression (A) and gating of singlet, live CD19+ FL cells expressing to determine IgM (FL134) or IgG (FL45) isotype (B).

Figure 2: DC-SIGN binding to surface IgM+ primary follicular lymphoma cells

Representative FACS plots of three FL samples simultaneously stained within the same tube to detect sIgM expression and soluble DC-SIGN binding (n=7). Analysis (FlowJo) of singlet, live cells initially allowed examination of sIgM expression (A). Populations positive for sIgM were gated on to determine DC-SIGN binding on these cells. (B) Analysis of GeoMFI data (GraphPad Prism), found that expression of sIgM was significantly correlated to binding of DC-SIGN (Spearman Rank test). This correlation was confirmed further within samples of FL cells showing double peaks of high and low sIgM expression.

Figure 3: Induction of FL-specific signalling pathways by DC-SIGN.

Western blotting demonstrating ERK and AKT phosphorylation in primary FL cells taken from sIgM+ and sIgG+ cases. Representative examples of 16 samples: (A) sIgM+ and (B) sIgG+ cases treated with soluble or bead isotype control (IC), DC-SIGN or anti-Ig for 0.5 and 3 hours. (C) Fold change analysis was used to measure extent of ERK and AKT phosphorylation induced in the 16 samples by either DC-SIGN or anti-Ig over time. (D) Nnormal B-cells were stimulated with either DC-SIGN or anti-IgM beads and western blotting was analyzed for ERK and AKT phosphorylation.

Figure 4: Downstream signaling induced by anti-Ig or DC-SIGN is ablated by kinase inhibitors:

Phosflow data analysis to examine phosphorylation of PLCγ2 and ERK in FL cells. Cells were pre-treated with H_2O_2 to inhibit phosphatases prior to stimulation with either cross-linked DC-SIGN (xDC-SIGN) or soluble anti-Ig. (A) Cytobank software (representative heat map traces shown) was used to measure phosphorylation of PLCγ2 and ERK for each treatment at each time point. (B) Fold change analysis was used to chart the level of phosphorylation over time in 13 samples tested. (C) Phosflow (representative heat traces shown) was used to measure impact of the SYK inhibitor tamatanib or BTK inhibitor ibrutinib

on phosphorylation of PLCγ2 and ERK. (D) Analysis of data for 9 samples (6 for SYK and 3 for BTK) was compiled (GraphPad Prism). (Student t-test; ****:<0.0001).

Figure 5: DC-SIGN treatment induces MYC expression in FL cells.

Comparison of the ability of DC-SIGN to upregulate MYC in primary FL cells in relation to anti-Ig stimulation. (A) Western blotting was used to measure MYC protein expression in FL cells treated with isotype control, DC-SIGN or anti-Ig beads over time (2 representative samples shown). (B) Fold change analysis of band densitometry was used to compare MYC expression induced by each treatment for 11 FL samples tested (Student t-test). (C) Western blotting was used to examine MYC expression in normal B cells treated with either DC-SIGN or anti-IgM beads over time.

Figure 6: FL cells do not increase expression of surface Ig following incubation i*n vitro*.

Recovery of surface marker expression of FL samples *in vitro* (n=9; 6 IgM-positive; 3 IgG-positive). FACS was used to determine expression (GeoMFI) of surface Ig and CXCR4 at zero time and at subsequent intervals up to 72 hours. Expression analysis of GeoMFI (CellQuest) was used to plot change of expression over time.

Figure 7: DC-SIGN fails to induce endocytosis of surface Ig by FL cells.

Analysis of surface IgM internalization in response to DC-SIGN or anti-IgM treatments. Primary FL or normal B cells were treated on ice for 45 minutes with either soluble anti-IgM F(ab')2 or DC-SIGN prior to incubation at 37^{o}C to allow internalization. Flow cytometry was used to determine surface IgM expression over time in relation to zero time (100%). (A) FL samples treated with anti-IgM; (B) Normal B cells treated with soluble anti-IgM. (C) FL samples treated with soluble DC-SIGN; (D) FL cells treated with cross-linked (x) DC-SIGN. Expression of sIgM determined by FACS was analysed using CellQuest.