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Nonstereotyped Lymphoma B Cell Receptors Recognize Vimentin as a Shared Autoantigen

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Ag activation of the BCR may play a role in the pathogenesis of human follicular lymphoma (FL) and other B cell malignancies. However, the nature of the Ag(s) recognized by tumor BCRs has not been well studied. In this study, we used unbiased approaches to demonstrate that 42 (19.35%) of 217 tested FL Igs recognized vimentin as a shared autoantigen. The epitope was localized to the N-terminal region of vimentin for all vimentin-reactive tumor Igs. We confirmed specific binding to vimentin by using recombinant vimentin and by performing competitive inhibition studies. Furthermore, using indirect immunofluorescence staining, we showed that the vimentin-reactive tumor Igs colocalized with an anti-vimentin mAb in HEP-2 cells. The reactivity to N-terminal vimentin of IgG FL Igs was significantly higher than that of IgM FL Igs (30.4 versus 10%; p = 0.0022). However, vimentin-reactive FL Igs did not share CDR3 motifs and were not homologous. Vimentin was expressed in the T cell–rich regions of FL, suggesting that vimentin is available for binding with tumor BCRs within the tumor microenvironment. Vimentin was also frequently recognized by mantle cell lymphoma and multiple myeloma Igs. Our results demonstrate that vimentin is a shared autoantigen recognized by nonstereotyped FL BCRs and by the Igs of mantle cell lymphoma and multiple myeloma and suggest that vimentin may play a role in the pathogenesis of multiple B cell malignancies. These findings may lead to a better understanding of the biology and natural history of FL and other B cell malignancies. The Journal of Immunology, 2013, 190: 4887–4898.

Human B cell lymphomas are characterized by recurrent chromosomal translocations; however, they may not be sufficient for lymphomagenesis. The t(14;18) translocation in follicular lymphoma (FL), the most common indolent B cell lymphoma (1, 2), results in the juxtaposition of the BCL2 gene next to the Ig H chain locus, leading to hyperexpression of the antiapoptotic bcl-2 protein that provides a survival advantage to the tumor cells. However, the presence of t(14;18) translocation in a substantial proportion of healthy individuals suggests that it is not sufficient to initiate lymphomagenesis (3–5) and that other factors may be necessary. This suggestion is also supported by the observation that lymphoma developed in only 10–15% of transgenic mice, in which BCL2 expression was driven by an IgH enhancer (Eqμ) (6). Alternative factors that may promote lymphomagenesis include antigenic stimulation of the BCR, which may act in concert with intracellular genetic alterations by providing survival and proliferation signals (7).

Several reports suggest a possible role for Ag activation in the pathogenesis of FL. Most FL tumors express membrane-bound Igs, and the loss of BCRs is extremely rare in FL (8). FL tumors exhibit ongoing somatic hypermutation during tumor growth, and the patterns of these mutations suggest their selection by Ag activation (9, 10). Recently, crosslinking of BCRs was shown to activate MAPKs Erk1/2 and p38 more rapidly and for longer duration in FL tumor B cells than in nontumor B cells, thereby suggesting that BCR signaling may promote the survival and proliferation of tumor B cells (11). Taken together, these reports suggest a possible dependency of FL tumor cells on BCRs for...
survival and proliferative signals and support the need for the identification of Ag(s) recognized by FL BCRs to better understand the pathogenesis and natural history of FL.

Despite the observations mentioned above, the nature of the Ag(s) recognized by FL BCRs has not been well studied. Dighiero et al. (12) tested the reactivity of a small number of FL Igs against preselected autoantigens. They observed that at least 8 of 31 FL Igs reacted to a self-Ag. The purpose of our study was to define the characteristics of FL Igs and to identify potential self-Ags recognized by them using an unbiased approach and without preconceptions about the nature of the antigenic ligands. To determine whether other tumor Igs may also recognize self-Ags, we tested tumor Igs derived from mantle cell lymphoma (MCL) and multiple myeloma (MM).

Materials and Methods

Reagents

The following reagents were obtained from commercial sources: recombinant human vimentin (GenWay Biotech), anti-vimentin V9 and H84 Abs (Santa Cruz Biotechnology), HRP-conjugated mouse anti-6xHis (BD Biosciences), rituximab (Genentech), cetuximab (ImClone Systems), calf thymus dsDNA (Invitrogen), LPS and recombinant human insulin (Sigma-Aldrich), and goat anti-human Ig HRP-conjugated Ab (Jackson ImmunoResearch Laboratories). The human larynx epidermoid carcinoma (HEp-2) cell line was obtained from the American Type Culture Collection. ED38, mG0186, and mG053 mAbs were provided by Michel C. Nussenzieg (13).

Generation of tumor Igs

The Institutional Review Board at The University of Texas MD Anderson Cancer Center approved all studies. Tumor Igs were generated from patients previously enrolled in clinical trials of idiotype vaccine after obtaining informed consent (14–18). Tumor Igs from patients with previously untreated FL (n = 28) and MCL (n = 18) were generated by heterohybridoma fusion technology (15, 16, 18, 19). Additional tumor Igs from patients with FL (n = 211; 180 untreated and 31 relapsed) were generated by cloning tumor-derived VιH and VιL sequences into a baculovirus expression vector system containing the IgG1 and κ or λ C region coding sequences as previously described (14, 20). Tumor Igs from patients with previously untreated MM (n = 31) were purified from plasma by protein A affinity chromatography (17). The monoclonal tumor IgG and IgA represented >80% of the total IgG or IgA, respectively, in the plasma in all MM samples.

Sequencing and analysis of IgVιH and IgVιL

An Ig H and L chain gene library was constructed from fresh biopsy tissue from each patient using two different constant H and L chain cDNA primers with a commercially available 5’ RACE kit (Invitrogen). Variable region gene sequences that appeared multiple times from two different cDNA reactions were considered tumor derived (14, 20). Sequence analysis was performed using ImMunoGeneTics/V-QUEST analysis software (21). The usage of VH, JH, DH, VS, Jα, Vα, and Jα genes; the length of the CDR3 region; characteristics of each nucleotide mutation (replacement versus silent) in each framework region (FWR) and CDR; the number of positively charged amino acids; and sites of N-glycosylation were determined as previously described (21–24).

Analysis of amino acid frequencies

Amino acid frequencies were calculated from ImMunoGeneTics position 107–114, known as the “CDR-H3 loop” region. CDR-H3 loops derived from FL tumors were compared with a previously published compilation of normal, unique, human CDR-H3 loops obtained from the ImMunoGeneTics database (25), as previously described (26). CDR-H3 loops from FL tumors were also compared with normal human memory B cells; these sequences were further divided into IgM* or IgG* subsets (23, 24).

Analysis of CDR-H3 hydrophobicity

The average hydrophobicity of each CDR-H3 loop was calculated with values from the normalized Kyte-Doolittle hydrophobicity scale (27, 28), which ranges from arginine at −1.3 to isoleucine at +4.7. The distribution of average hydrophobicities was then plotted for normal, human memory B cells and for FL tumors.

Hep-2 lysate preparation

The Hep-2 cell line (American Type Culture Collection) was grown to ~70–80% confluence at 37°C in 5% CO2 and DMEM (Invitrogen) supplemented with 10% heat-inactivated ultra-low IgG FBS (Invitrogen), 20 U/ml penicillin, and 20 μg/ml streptomycin (Invitrogen). Whole-cell lysates were prepared by lysing Hep-2 cells in cold lysate buffer (1% Triton X-100, 150 mM NaCl, 10 mM Tris [pH 7.4], 1 mM EDTA, 1 mM ethylene glycol tetraacetic acid [pH 8.0], 0.2 mM sodium orthovanadate, 0.5% octylphenoxypolyethoxethanol [Sigma-Aldrich]) in the presence of a protease inhibitor mixture (Roche Applied Science). Hydrophilic cytoplasmic and nuclear proteins of Hep-2 cells were obtained by the use of NE-PER extraction reagents (Thermo Scientific) according to the manufacturer’s protocol. After the hydrophilic proteins were extracted, the hydrophobic fraction was dissolved in 8 M urea. All protein concentrations were determined by using a 2-D Quant kit (GE Healthcare).

ELISA

All proteins were dissolved in 100 mM sodium bicarbonate buffer (pH 9.6) to a concentration of 10 μg/ml, sonicated, coated onto 96-well ELISA plates, and stored overnight at 4°C. Coated plates were blocked with nonfat milk (Bio-Rad) for 1 h at room temperature, incubated with tumor Igs (12.5 μg/ml in SuperBlock T20 blocking buffer [Thermo Scientific]) for 1 h at room temperature, and washed with 0.05% Tween 20 in PBS. Bound tumor Igs were detected with goat HRP-conjugated anti-human Ig Ab diluted at 1:10,000 and 2.2’-azino-bis-3-ethylthiazolinedisulfonic acid peroxidase substrate (Kirkegaard & Perry Laboratories) and read at 405 nm in a microplate spectrophotometer (Molecular Devices). To determine whether vimentin inhibits the binding of tumor Igs to Hep-2 cell lysates, we preincubated tumor Igs (12.5 μg/ml) for 1 h with various concentrations of recombinant human vimentin protein or control GST protein and then used them in ELISA.

Western blotting and mass spectrometry

All proteins were dissolved in 8 M urea and boiled for 5 min in 0.4% SDS, 5% glycerol, 2.9 mM 2-βME, 0.025% bromophenol blue, and 12 mM Tris (pH 6.8). Proteins were analyzed by SDS-PAGE using 4–20% gradient gels (Bio-Rad) and stained with Coomassie brilliant blue R-250 (Thermo Scientific). Alternatively, after SDS-PAGE, proteins were transferred to a nitrocellulose membrane (Bio-Rad) for Western blotting with tumor Igs or V9 and H84 Abs. Bound tumor Igs were detected with goat anti-human IgG HRP-conjugated Ab (1:10,000 dilution) and a mixture of tetrazolium chloride and 5-bromo-4-chloro-3-indolyolphosphate p-toluidine salt (Thermo Scientific) as alkaline phosphatase substrates. Protein bands were excised from Coomassie blue–stained SDS-PAGE gels, digested with trypsin, and subjected to sequential mass spectrometry (liquid chromatography/mass spectrometry; Protein Chemistry Core Facility, Tufts University).

Immunofluorescence staining

Hep-2 cell–coated slides (Bion Enterprises) were incubated with tumor Igs (12.5 μg/ml) and anti-vimentin V9 mAb (1:250) in SuperBlock T20 Tris-buffered saline blocking buffer (Thermo Scientific) for 1 h at room temperature. The slides were washed three times in PBS and 0.05% Tween 20 and incubated with anti-human IgG or IgM Abs conjugated to Alexa Fluor 594 (Invitrogen) and anti-mouse IgG Ab conjugated to Alexa Fluor 488 (Invitrogen) in the blocking buffer for 1 h at room temperature. The slides were washed as before and mounted on slides with ProLong Gold antifade mounting medium (Invitrogen), and images of the stained sections were captured using a confocal laser-scanning microscope (Leica SP2 SE). The raw confocal data were converted to 8-bit red, green, and blue split mode, and green and red channels were isolated for colocalization analysis using ImageJ software and relevant plugins. A threshold of 50% was applied to exclude false signals and improve the signal-to-noise ratio. The resulting colocalized pixels were obtained as grayscale images. A Pearson correlation coefficient of >0.8 (as close to 1 as possible) was considered statistically significant. Laser intensities were compensated with single-color controls, and autofluorescence was eliminated by background correction based on secondary Ab-only controls. All experiments were performed in triplicate and repeated at least three times to ensure reproducibility.

Cloning of full-length and truncated vimentin cDNA fragments

Neil Porcelli (Thermo Scientific) provided full-length cDNA fragments of human vimentin. A 6×His tag bacterial expression vector, pQE-30 (Qiagen), was used to express full-length, N-terminal, and C-terminal human vimentin. The full-length vimentin gene was cloned into the TA PCR 2.1 vector (Invitrogen) from total RNA from the Hep-2 cell line by RT-PCR using vimentin-specific primers Fwd-Vim (5’-GGATCCATGTTTCAACAGCCTGATTG-3’) and Rev-Vim (5’-TCTAGATTCGTTGATTAGCTGCTG-3’) containing BamH I (underlined) and HindIII (double underlined) restriction sites, respectively. The vimentin-containing TA PCR 2.1 vector DNA was then digested with BamH I and HindIII restriction endonucleases, and the resulting fragment was subcloned into the pQE-30 vector.

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vector. To generate the C-terminal vimentin fragment (aa 260–467), we used 5'-AAGGATCCGATGTTTCAAGAGCTGACCTCAGGCGCT-3' as a forward primer and Rev-Vim-R as a reverse primer. To generate N-terminal vimentin fragments Vim-N1–259, Vim-5N1–224, and Vim-4N1–137, we used 5'-AAAAGCTTTAGTTAACATCGATGGACATGCTGTCC-3', 5'-AAAAGCTTTAGTTAACATCGATGGACATGCTGTCC-3' as reverse primers, respectively, and Fwd-Vim as a forward primer. The reverse primers were constructed with a stop codon after the RhoIII site in the sense strand. The start codon (ATG) and stop codon (TTA) are indicated in bold and italic in the respective primer sequences. The cloned full-length vimentin and truncated vimentin fragments were confirmed by sequencing.

**Generation of full-length and truncated vimentin protein**

Overnight cultures of BL21 (DE3) cells transformed with each plasmid were diluted to a ratio of 1:50 in fresh TB broth supplemented with 100 μg/ml ampicillin. Cells were grown at 37°C for 3 h or until a cell density of A600 of 0.5 was reached. Recombinant protein expression was induced by the addition of 1.0 mM isopropyl-β-D-thiogalactopyranoside (Invitrogen), and cultures were grown for 4 h at 37°C. Cells were harvested by centrifugation for 20 min at 10,000 × g and lysed at room temperature and lysed for 1 h in a lysis buffer (100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea, and Sigma-Aldrich protease inhibitor mixture [pH 5.9]), and pelleted. The recombinant full-length vimentin and truncated protein fragments were eluted with elution buffer E (100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea, and Sigma-Aldrich protease inhibitor mixture [pH 8.0]), and Sigma-Aldrich protease inhibitor mixture [pH 6.3]), repelleted, washed with wash buffer D (100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea, and Sigma-Aldrich protease inhibitor mixture [pH 5.9]), and loaded onto a column. The recombinant full-length vimentin and truncated protein fragments were eluted with elution buffer E (100 mM NaH2PO4, 10 mM Tris-Cl, 8 M urea, and Sigma-Aldrich protease inhibitor mixture [pH 4.5]). Each recombinant protein was characterized by SDS-PAGE and stained with Coomassie brilliant blue, and the protein concentration was determined using a 2-D Quant kit (GE Healthcare).

**Immunohistochemical staining**

Formalin-fixed, paraffin-embedded tissue sections of FL and normal tonsils were deparaffinized and rehydrated, and Ag retrieval was performed according to the manufacturer’s protocol (Vector Laboratories). Sections were treated for 5 min with 0.3% hydrogen peroxide solution to block endogenous peroxidase and incubated with a blocking buffer (1% BSA in PBS; Sigma-Aldrich) for 5 min at room temperature. Next, slides were incubated with anti-vimentin Ab (BD Biosciences) overnight at 4°C at a dilution of 1:100 in blocking buffer, washed, and incubated for 30 min with appropriate secondary Abs in blocking buffer. For CD3 and CD20

**FIGURE 1.** H and L chain usage, mutational status, and amino acid usage of tumor Igs in FL. (A) The percentage of tumors (n = 239) expressing different H chains as determined by flow cytometry. (B) The percentage of IgM+ and IgG+ FL tumors (n = 239) expressing Vκ and Vλ L chains as determined by flow cytometry. The p value was calculated using a 2 × 2 Fisher exact test. (C) The total number of mutations in VH, Vκ, and Vλ genes from FWR1 to FWR3 in individual IgM+ (n = 112) and IgG+ (n = 86) FL tumors. Each dot represents data from one tumor, and the horizontal lines represent the average number of mutations for the group. The p values were calculated using a two-tailed Student t test. (D) The frequency of replacement (R; black bar) and silent (S; gray) mutations in VH, Vκ, and Vλ were calculated and expressed as follows in the bar graphs: (number of nucleotide exchanges in each FWR and CDR/total number of nucleotides in the respective FWR and CDR) × 100. The R/S mutation ratio for each FWR and CDR is shown on the x-axis. (E) The frequency of amino acid usage in CDR-H3 loops of all FL Igs (middle) was compared with normal human B cells (top). Amino acids are listed in order of hydrophobicity as per a normalized Kyte–Doolittle hydrophobicity scale. Differences in amino acid frequencies between the two groups (bottom) were assessed by χ2 analysis. **p < 0.001, ***p < 0.0001.
double staining, a polymer mixture composed of anti-mouse IgG/alkaline phosphatase and anti-rabbit IgG/HRP (Thermo Scientific) was used in conjunction with primary Abs (Thermo Scientific). The sections were stained using diaminobenzidine solution (Vector Laboratories). Digital photomicrographs were acquired using DP Controller software (Olympus) mounted on a BX41 inverted microscope (Olympus).

Statistical analysis

Statistical significance of data was determined by a 2 × 2 or 2 × 5 Fisher exact test (for determining the reactivity of tumor Igs, analyzing tumor Ig gene repertoires, analyzing CDR3 charges, and identifying sites of N-glycosylation) or a Student t test (for determining the length of the CDR3 region and the number of mutations). Amino acid sequence analysis was performed in Microsoft Excel 2007. Once sequences were sorted into their respective subsets, macros and formulas were created to calculate amino acid frequencies. Differences between populations were assessed by χ² analysis or a Levene test for variance using JMP 9.0.0 (SAS, Cary, NC) and Prism 5.0 (GraphPad Software, La Jolla, CA).

Results

Characteristics of FL Igs

The isotype of the FL Igs used in this study was determined by flow cytometry of tumor samples derived from 239 patients (208 untreated and 31 relapsed). Consistent with previous reports (8, 29), we observed that IgM was expressed more frequently than IgG in FL (119 versus 98; 50 versus 41%) (Fig. 1A). A subset of the tumors expressed IgD, IgA, or dual Igs (4.3% IgD plus IgM, 2.6% IgA, 1.7% IgD, and 0.41% IgG plus IgM). Interestingly, the κ L chain was more frequently expressed in the IgM+ tumors, whereas the λ L chain was more commonly expressed in the IgG+ tumors (Fig. 1B; p = 0.0121). Sequencing of the variable regions of the H and L chains was performed on 198 FL tumors (167 untreated and 31 relapsed). The usage of VH, JH, DH, Vk, Jk, Vk, and Jλ genes did not differ significantly between IgM+ and IgG+ FL tumors except for the usage of VH3, which was more frequent in IgM+ FL tumors than in IgG+ FL (76 versus 56%; p = 0.012) (Supplemental Fig. 1A, Supplemental Table I).

FL Igs are highly mutated and have altered amino acid usage

To determine whether somatic mutations may correlate with Ag reactivity, we analyzed the V region sequences of FL Igs. We found that FL Igs had an average of 34.2, 19.7, and 20.4 somatic mutations in IgH, Igκ, and Igλ variable regions, respectively (Fig. 1C, Supplemental Table I). The number of somatic mutations was significantly higher in IgG variable H (p = 0.0195) and κ L chains (p = 0.006) than in IgM+ FL tumors, but did not differ in the λ L chain (p = 0.628). As expected, the mutations were more frequent in the CDRs than in the FWRs of both IgM+ and IgG+ FL tumors (Fig. 1D). Furthermore, the ratio of replacement mutations (nucleotide changes yielding amino acid replacements) to silent mutations (nucleotide changes not leading to amino acid replacements) was consistently higher in the CDRs than in the FWRs of both tumor types. The length of the CDR3 region and the number of positively charged amino acid residues did not differ significantly between the variable regions of IgM+ and IgG+ FL tumors (Supplemental Fig. 1B, 1C, Supplemental Table I). N-glycosylation sites were more commonly observed in IgM+ than in IgG+ tumors (Supplemental Fig. 1D, 1E; p = 0.0032). Comparison of tumor Igs from untreated and relapsed FL patients did not show significant differences in variable gene usage and the rate and pattern of somatic mutation. However, the high number of mutations in the tumor Ig variable regions and, in particular, the

![FIGURE 2](http://www.jimmunol.org/) Tumor Igs are frequently self-reactive. FL Igs were tested in triplicate for reactivity against HEp-2 whole-cell lysates using ELISA as described in Materials and Methods. (A) The percentage of IgM+ and IgG+ FL Igs reacting to HEp-2 whole-cell lysates. The p value was calculated using a 2 × 2 Fisher exact test. (B-E) Reactivity of individual tumor Igs from (B) IgM+ FL (n = 119), (C) IgG+ FL (n = 98), (D) MCL (n = 18), and (E) MM (n = 31) against HEp-2 whole-cell lysates is shown by OD determined at 405 nm. Each tumor Ig is indicated by a unique number, and a polyreactive ED38 Ab was used as a positive control (P). Nonreactive mG053 Ab (N) and rituximab (R) were used as negative controls. Tumor Igs were considered to be HEp-2–reactive if the OD was >5 times the OD of the nonreactive mG053 Ab (indicated by line). Error bars represent the SD for triplicate samples, and the four-pointed stars indicate HEp-2–reactive tumor Igs. Data were reproducible in three independent experiments.
high ratio of replacement to silent mutations in the CDRs suggests an Ag-driven process in FL pathogenesis. Analysis of amino acid frequencies in the CDR3 regions of H chains (CDR-H3) showed that amino acid usage differed between FL and normal human CDR-H3 loops (Fig. 1E). Amino acid usage was also different between IgM⁺ and IgG⁺ FL and their respective normal memory B cells (Supplemental Fig. 2A, 2B). However, the average CDR-H3 loop hydrophobicity was not significantly different between FL and normal memory B cells (Supplemental Fig. 2C).

**Tumor Igs are frequently self-reactive**

To determine whether tumor Igs are self-reactive, we tested their reactivity in an ELISA (30) against the HEp-2 cell line, which is commonly used to identify Ags recognized by autoantibodies (13). We observed that 24 (11.06%) of 217 FL Igs were self-reactive and that IgG⁺ FL Igs were more commonly self-reactive than those derived from IgM⁺ FL (20.4 versus 3.4%; \( p = 0.0001 \)) (Fig. 2A–C). Our results also showed that 6 (33.33%) of 18 MCL Igs and 14 (45.16%) of 31 MM Igs were self-reactive (Fig. 2D, 2E). We did not observe any L chain bias in HEp-2 reactivity in FL, MCL, or MM (Fig. 2B–D) \( (p > 0.05 \) for FL, MCL, and MM). Taken together, these data suggest that tumor Igs from FL, MCL, and MM are frequently reactive against a self-Ag.

**Tumor Igs recognized vimentin**

To identify the self-Ag recognized by the HEp-2-reactive tumor Igs, we extracted hydrophilic nuclear and cytoplasmic protein fractions and a hydrophobic fraction from Hep-2 cells and used them as antigenic material in separate ELISAs. We tested five randomly selected HEp-2-reactive tumor Igs (FL15, MCL29, MCL32, MCL43, and MM63) and found that they specifically recognized the hydrophobic fraction but not the hydrophilic nuclear or cytoplasmic fractions of HEp-2 cells (Fig. 3A). The hydrophobic fraction was dissolved in urea and subjected to SDS-PAGE and Western

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**FIGURE 3.** Tumor Igs recognize vimentin. Tumor Igs from FL (15), MCL (29, 32, 63), and MM (63) were tested in various assays to characterize the Ags they recognized. Polyreactive ED38 Ab was used as a positive control (P), and nonreactive mG053 Ab was used as a negative control (N) where appropriate. Error bars represent the SD for triplicate samples. (A) Tumor Igs were tested in triplicate for reactivity against hydrophilic cytoplasmic and nuclear proteins fractions and a hydrophobic fraction from HEp-2 cells using ELISA as described in Materials and Methods. (B) The hydrophobic fraction from HEp-2 cells was dissolved in urea, subjected to SDS-PAGE, and transferred to nitrocellulose membrane. Immunoblotting was performed with tumor Igs (5 mg/ml), and bound tumor Igs were detected by goat alkaline phosphatase–conjugated anti-human Ig. (C) The corresponding bands (arrows in (B)) were excised from Coomassie blue–stained gels and subjected to sequential mass spectrometry. Seven peptides (marked in red) that matched the vimentin protein sequence were observed. (D) Tumor Igs were tested in triplicate for reactivity against the HEp-2 hydrophobic fraction, commercially obtained recombinant human vimentin, and BSA (50 μl of 10 μg/ml) using ELISA. (E) Tumor Igs were tested for reactivity against commercially obtained recombinant human vimentin using Western blotting. (F) Tumor Igs were preincubated with various concentrations of recombinant human vimentin or GST and then tested for reactivity against the HEp-2 hydrophobic fraction.
blotting. The five tumor Igs recognized a single protein band of ∼56 kDa (Fig. 3B), which was then excised and analyzed by mass spectrometry. Using the Basic Local Alignment Search Tool to find regions of similarity and homology, we determined that seven peptide sequences matched and encompassed 23% of the vimentin protein (Fig. 3C). By performing ELISA (Fig. 3D) and Western blotting (Fig. 3E), we confirmed that the five tumor Igs specifically bound to recombinant human vimentin protein but not BSA (Fig. 3D, 3E). We did not observe any significant reactivity to vimentin by a non-HEp-2–reactive tumor Ig (FL1). To further validate the specificity, we preincubated the tumor Igs with recombinant human vimentin and observed that it significantly inhibited reactivity to the HEp-2 hydrophobic fraction in a dose-dependent manner, whereas preincubation with control GST protein did not (Fig. 3F). Additionally, using indirect immunofluorescence staining, we showed that the vimentin-reactive tumor Igs FL15, MCL32, and MM63 colocalized with a mouse anti-vimentin V9 mAb in HEp-2 cells but the non-HEp-2–reactive tumor Ig FL26 did not (Fig. 4). Taken together, these results suggest that the self-Ag recognized by the five HEp-2–reactive tumor Igs tested was vimentin.

Tumor Igs recognized N-terminal region of vimentin

To determine the location of the epitope recognized by the vimentin-reactive tumor Igs, we generated recombinant N-terminal (aa 1–259) and C-terminal (aa 260–466) 6xHis-tagged vimentin fusion proteins (Fig. 5A). We found that all five vimentin-reactive tumor Igs (FL15, MCL29, MCL32, MCL43, and MM63) bound to N- but not C-terminal vimentin fragments when tested by ELISA (Fig. 5B) and Western blotting (Fig. 5C). As expected, the C-terminal–specific mouse anti-vimentin V9 mAb recognized C-terminal vimentin, the N-terminal–specific rabbit anti-vimentin H84 polyclonal Ab recognized N-terminal vimentin, and the non-HEp-2–reactive FL1 Ig did not recognize either. To further define the precise location of the epitope, we generated additional truncated N-terminal vimentin protein fragments (Fig. 5A). Remarkably, four of the five HEp-2–reactive tumor Igs tested (FL15, MCL29, MCL32, and MCL43) bound to Vim-N (aa 1–259) protein fragments but not to Vim-5N (aa 1–224) or Vim-4N (aa 1–137) protein fragments (Fig. 5D). The MM63 Ig bound to Vim-N and Vim-5N protein fragments but not to Vim-4N protein fragment. These results suggest that the epitope recognized by four of the five vimentin-reactive tumor Igs derived from FL and MCL is located between amino acid residues 224 and 259 of the vimentin protein.
Nonstereotyped tumor Igs recognized N-terminal vimentin

Because the reactivity of some of the tumor Igs (MCL29, MCL32, and MCL43) was markedly higher to N-terminal–truncated vimentin than to full-length vimentin (Fig. 5B), we screened all available tumor Igs for reactivity against N-terminal vimentin (aa 1–259). We observed that 42 (19.35%) of 217 FL Igs, 9 (50%) of 18 MCL Igs, and 12 (38.7%) of 31 MM Igs were reactive with N-terminal–truncated vimentin (Fig. 6). However, only a fraction of the tumor Igs that bound to N-terminal vimentin bound to full-length vimentin (26 [61.9%] of 42 FL Igs, 3 [33.3%] of 9 MCL Igs, and 6 [50%] of 12 MM Igs) (Supplemental Fig. 3), and none of them bound to C-terminal vimentin (aa 260–466). Similar to the results of our experiments with HEp-2 cell reactivity (Fig. 2A–C), the N-terminal vimentin reactivity of IgG FL Igs was significantly higher than that of IgM FL Igs (30.4 versus 10%; p = 0.0022) (Fig. 6A–C). However, we did not observe any significant correlation between HEp-2 or vimentin reactivity and VH, JH, Vk, Jk, Vλ, and Jλ gene usage, CDR3 length, the number of positively charged amino acid residues in CDR3, the rate and pattern of mutations, or the rate and pattern of N-glycosylation (Table I, Supplemental Table I). Furthermore, the N-terminal vimentin-reactive FL Igs did not share CDR3 motifs and were not homologous (Table I). Similarly, the vimentin-nonreactive FL Igs were not homologous (Supplemental Table I). Interestingly, among the MM Igs, 11 (78.57%) of 14 IgGk tumor Igs bound to N-terminal vimentin as opposed to 1 (10%) of 10 IgGλ tumor Igs and 0 (0%) of 7 IgA tumor Igs (Fig. 6E). However, we did not see this pattern of L chain bias in FL and MCL. The reactivity with vimentin was variable between samples and suggests differences in the binding affinity of the tumor Igs.

To determine whether the N-terminal vimentin-reactive tumor Igs were polyreactive, we tested their reactivity against a panel of Ags, including dsDNA, insulin, and LPS, by ELISA as previously described (13). Tumor Igs that bound to at least one of these Ags were considered polyreactive. We observed that 14 (33.33%) of 42 FL, 0 (0%) of 7 MCL, and 2 (16.67%) of 12 MM N-terminal vimentin-reactive tumor Igs were polyreactive (Fig. 7). The IgG FL Igs were more commonly polyreactive than the IgM FL Igs but did not share recurrent CDR3 motifs (Table I). Interestingly, we did not observe polyreactivity in any of the non-vimentin–reactive or non-HEp-2–reactive FL Igs (Supplemental Table I). Taken together, these results suggest that N-terminal vimentin is frequently recognized by nonstereotyped FL BCRs as well as MCL and MM Igs.

We also examined whether the method of tumor Ig generation impacted their reactivity, as glycosylation can be different in insect

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**FIGURE 6.** Reactivity of tumor Igs with N-terminal vimentin. Tumor Igs were tested in triplicate for reactivity against N-terminal vimentin (Vim-N; aa 1–259) using ELISA as described in Materials and Methods. (A) The percentage of IgM+ and IgG+ FL Igs reacting to Vim-N. The p value was calculated using a 2 × 2 Fisher exact test. (B–E) Reactivity of individual tumor Igs from (B) IgM+ FL (n = 119), (C) IgG+ FL (n = 98), (D) MCL (n = 18), and (E) MM (n = 31) against Vim-N is shown by OD determined at 405 nm. Each tumor Ig is indicated by a unique number, and vimentin-reactive MM tumor Ig 63 was used as a positive control (P). Nonreactive mG053 mAb (N) and cetuximab (Cx) were used as negative controls. Tumor Igs were considered to be Vim-N–reactive when the OD was 5-fold the OD of the negative control cetuximab (indicated by line). Error bars represent the SD for triplicate samples. The black four-pointed stars indicate Vim-N–specific tumor Igs, and the gray four-pointed stars indicate polyreactive tumor Igs (Fig. 7). Results were reproducible in three independent experiments.
Table I. H and L chain CDR3 sequences of vimentin-reactive FL Igs

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Data are not available for patients 9, 23, and 27. Patient 23 had polyreactive tumor Ig. Polyreactive tumor Igs are indicated for patients 13, 25, 200, 245, 253, 266, 285, 294, 310, 314, 316, 317, and 323.

cells compared with mammalian cells. We found that 7 (25%) of 28 heterohybridoma-derived FL Igs were vimentin-reactive, of which 4 (14%) were vimentin-specific and 3 (11%) were polyreactive (Supplemental Table I). Of the 189 baculovirus system-derived FL Igs, 35 (19%) were vimentin-reactive, of which 22 (12%) were vimentin-specific and 13 (7%) were polyreactive (Supplemental Table I). Furthermore, 9 (50%) of 18 MCL Igs, all of which were generated by heterohybridoma technology, were vimentin-specific. Taken together, these results suggest that the expression system for the FL Igs did not appear to impact their reactivity.

Vimentin is expressed in lymphoma tissues

To determine whether vimentin is expressed in lymphoma tissues, we performed immunohistochemical staining of formalin-fixed, paraffin-embedded tissue sections. We found that vimentin expression was present predominantly in the T cell–rich interfollicular regions of FL (Fig. 8). Vimentin expression was cytoplasmic and possibly membranous on T cells, and this result suggested that vimentin may be available for binding to the BCR of tumor cells within the tumor microenvironment.

Discussion

Our results showed that a significant proportion of FL Igs recognized vimentin as a shared autoantigen, suggesting a possible role for self-Ags in the pathogenesis of FL. A recent report by Sachen et al. (31), which was published as this manuscript was under preparation, showed that 26% of FL Igs were Hep–2-reactive, and the self-Ag was identified as myoferlin for one tumor Ig. Using very stringent criteria and a different assay, we found that 11% of FL Igs were Hep–2-reactive and 19.35% were vimentin-reactive when tested using purified N-terminal vimentin protein. The higher reactivity seen with purified protein as compared with Hep–2 cell lysate may be due to better availability of antigenic epitopes. Under normal conditions, naïve mature B cells proliferate vigorously when their BCR is exposed to the cognate self or non-self Ags in secondary lymphoid organs. When the Ag-activated B cells already contain t(14;18) chromosomal translocation, they are likely to have a survival advantage because of the hyperexpressed antiapoptotic bcl-2 protein. When such Ag-activated t(14;18)-containing B cells are specific to abundantly expressed self-Ags such as vimentin, they are likely to undergo chronic stimulation, proliferate indefinitely, and develop into FL (Fig. 9). However, when such Ag-activated t(14;18)-containing B cells are specific to non-self Ags, they may differentiate normally into memory B cells and plasma cells and may undergo limited proliferation due to brief Ag exposure. The presence of multiple memory B cell clones with t(14;18) in up to 60% of healthy individuals without apparent development of lymphoma (3–5)
Vimentin is expressed in FL tissues. Immunohistochemical staining for vimentin (left three columns) was performed on formalin-fixed, paraffin-embedded FL (top row) and normal tonsil (bottom row) tissues. Original magnification, ×10, ×40, and ×100. Immunohistochemical staining was also performed for CD3 (red) and CD20 (blue) in the same tissues (farthest right column) and shown at original magnification ×10. The data represent one of two samples tested for FL and normal tonsil.

FIGURE 7. Polyreactive N-terminal vimentin-reactive tumor Igs. N-terminal vimentin-reactive tumor Igs (Fig. 6) were tested in triplicate for reactivity against insulin (inverted triangle), dsDNA (black circle), and LPS (gray circle) using ELISA as described in Materials and Methods. Reactivity of individual tumor Igs from (A) IgM+ FL (n = 12), (B) IgG+ FL (n = 30), (C) MCL (n = 7), and (D) MM (n = 12) against dsDNA, LPS, and insulin is shown by OD determined at 405 nm. Each tumor Ig is indicated by a unique number, and ED38 mAb was used as a positive control (P). Cetuximab (C) and rituximab (R) were used as negative controls. Tumor Igs were considered to be polyreactive if the OD at 405 nm was >5-fold compared with the negative control, cetuximab (indicated by line). Error bars represent the SD for triplicate samples. The results were reproducible in three independent experiments.

FIGURE 8. Vimentin is expressed in FL tissues. Immunohistochemical staining for vimentin (left three columns) was performed on formalin-fixed, paraffin-embedded FL (top row) and normal tonsil (bottom row) tissues. Original magnification, ×10, ×40, and ×100. Immunohistochemical staining was also performed for CD3 (red) and CD20 (blue) in the same tissues (farthest right column) and shown at original magnification ×10. The data represent one of two samples tested for FL and normal tonsil.
during apoptotic cell death because Asp\textsuperscript{259} in its L1-2 linker domain has been shown to be a cleavage site for caspase-6 and corresponds to similar cleavage sites in other intermediate filament proteins such as lamins and cytokeratins, which are also substrates for caspases during apoptosis (48). Additionally, the 1B domain from the N-terminal region of vimentin is rapidly expressed on the cell surface after activation of normal T cells (42). Taken together with our observation that vimentin is present in the T cell–rich interfollicular regions of FL (Fig. 8), these reports suggest that full-length vimentin or its N-terminal fragment may be secreted or expressed on the surface of nonmalignant cells under physiological conditions. Furthermore, they suggest that

![Diagram](http://www.jimmunol.org/)

**FIGURE 9.** Hypothetical model for the role of Ags in the pathogenesis of FL. First and second rows, During normal B cell development, naive mature B cells arise from B cell progenitors in the bone marrow and are released into the periphery. Mature B cells activated by a self or non–self-Ag undergo affinity maturation by a process of somatic hypermutation in the germinal centers of secondary lymphoid organs. A subset of these B cells with improved binding to the Ag may undergo clonal expansion and CSR and eventually differentiate into memory B cells and plasma cells. Chronic exposure to self-Ag may lead to autoimmunity depending on whether the self-Ag–specific B cells are subjected to mechanism of self-tolerance or not. Third row, Because of errors during the V(D)J recombination in early B cell development, chromosomal translocations such as t(14;18) may be introduced in the B cell progenitors in the bone marrow. Mature B cells containing t(14;18) hyperexpress the antiapoptotic bcl-2 protein and have a survival advantage following Ag activation in the periphery. If the Ag-activated B cells containing t(14;18) are specific to self-Ag, they undergo chronic stimulation and proliferation and may develop into FL. The FL may initially undergo Ag-dependent proliferation but may eventually become Ag independent as they acquire additional genetic alterations. Fourth row, Ag-activated B cells containing t(14;18) that are specific to non–self-Ags may differentiate normally into memory B cells and into plasma cells and may not develop into FL due to brief Ag exposure.
vimentin is potentially available for antigenic stimulation of the tumor BCRs in the tumor microenvironment.

The pattern of distribution of replacement and silent mutations in the CDRs and FRWs of FL (Fig. 1D) that we observed was similar to that reported in memory B cells in healthy individuals (23, 24). However, the average somatic mutation frequency in FL tumors was significantly higher than the average somatic mutation frequency in healthy donor memory B cells (33 versus 9.6 for IgH, 18.0 versus 4.0 for Igk, and 18.96 versus 5.4 for Igλ for IgM FL tumors versus IgM memory B cells and 36.8 versus 18.0 for IgH, 25.4 versus 10.0 for Igk, and 20.3 versus 7.9 for Igλ for IgG FL tumors versus IgG memory B cells) (23, 24). Consistent with previous reports (22), the number of N-glycosylation sites was significantly higher in FL IgGs than in normal IgGs. The usage of V and J genes in IgH, Igk, and Igλ was comparable between FL tumors and healthy donor memory B cells except for Jα-7, which was more common in FL (23, 24). We also found that the variation in CDR3 length, the average hydrophobicity, and the number of positively charged amino acid residues in FL were comparable to those in healthy donor memory B cells (23, 24). Nonetheless, we observed that the amino acid usage in FL is altered (Fig. 1E, Supplemental Fig. 2), suggesting that the FL repertoire may be distinct. However, without direct comparison with normal germinal center B cells, we cannot state with certainty whether the FL repertoire represents transformation of a distinct repertoire or Ag-driven selection from a normal B cell repertoire. It is most likely, however, that this repertoire represents Ag-driven selection for a specific type of reactivity, possibly self-reactivity such as CLL.

In conclusion, our results show that vimentin is a shared auto-antigen recognized by nonstereotyped FL BCRs as well as tumor IgGs from MCL and MM. These findings suggest that vimentin may play a role in the pathogenesis of these malignancies and may lead to better understanding of their biology and natural history. It is likely that other self-Ags are also recognized by tumor IgGs and additional studies are needed to identify them.

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Disclosures
The authors have no financial conflicts of interest.

References


