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# TSPAN33 is a novel marker of activated and malignant B cells



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## KEYWORDS

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Biomarker

**Abstract** We have identified *Tspan33* as a gene encoding a transmembrane protein exhibiting a restricted expression pattern including expression in activated B cells. TSPAN33 is a member of the tetraspanin family. TSPAN33 is not expressed in resting B cells, but is strongly induced in primary human B cells following activation. Human 2E2 cells, a Burkitt's lymphoma-derived B cell model of activation and differentiation, also upregulate TSPAN33 upon activation. TSPAN33 is expressed in several lymphomas including Hodgkin's and Diffuse large B cell lymphoma. TSPAN33 is also expressed in some autoimmune diseases where B cells participate in the pathology, including rheumatoid arthritis patients, systemic lupus erythematosus (SLE), and in spleen B cells from MRL/Fas<sup>lpr/lpr</sup> mice (a mouse model of SLE). We conclude that TSPAN33 may be used as a diagnostic biomarker or as a target for therapeutic antibodies for treatment of certain B cell lymphomas or autoimmune diseases.

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**Abbreviations:** BCMA, B cell maturation antigen; BIGE, body index of gene expression (database); TSPAN33, tetraspanin 33; BL, Burkitt's lymphoma; RA, rheumatoid arthritis; NHL, non-Hodgkin's lymphoma; DLBCL, Diffuse large B cell lymphoma; HL, Hodgkin's lymphoma; SLE, systemic lupus erythematosus.

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## 1. Introduction

The discovery and characterization of lineage specific markers have been instrumental for the identification of cell subsets that underlie the complexity of the immune system. Cell surface markers, such as CD3 $\epsilon$  (pan T cell marker), CD4 (helper T cells), CD8 (cytotoxic T cells), and B220/CD45R (B cells), are routinely used to differentiate lymphocyte populations [1,2]. Advances in flow cytometry labeling techniques led to the characterization of CD4 subtypes (Th1, Th2, Th17 and Treg cells) based on the detection of lineage-specific transcription factors [3]. The discovery of regulatory 'B10 cells' was based on the identification of a small subset of B cells that are CD1<sup>hi</sup>CD5<sup>+</sup> and secrete IL-10 [4–6]. In addition, lineage specific surface markers (such as the B cell marker CD20), represent useful targets for the development of therapeutic mAbs that have proven effective against various lymphomas as well as autoimmune diseases like rheumatoid arthritis (RA) through their ability to delete pathogenic B cells [7,8].

### 1.1. TSPAN33 is a novel B cell activation marker

We sought to identify novel markers of human leukocytes. To this end, we analyzed a comprehensive database of human gene expression from 105 different human tissues including cells of the immune system (known as the body index of gene expression (BIGE) database) [9,10]. This database is useful for the identification of novel genes associated with specific organs or cells [11]. We identified a gene (*Tspan33*) that encodes a transmembrane protein not previously associated with B cells. The tetraspanin superfamily is defined by a conserved domain structure (Pfam00335) with a cysteine-rich long extracellular loop (LEL) containing a highly conserved cysteine–cysteine–glycine (CCG) motif [12]. These features facilitate the formation of large molecular complexes with other proteins, such as integrins or other tetraspanins and mediate diverse functions including proliferation, adhesion, motility, and differentiation. Some tetraspanins are widely expressed in adult tissues while

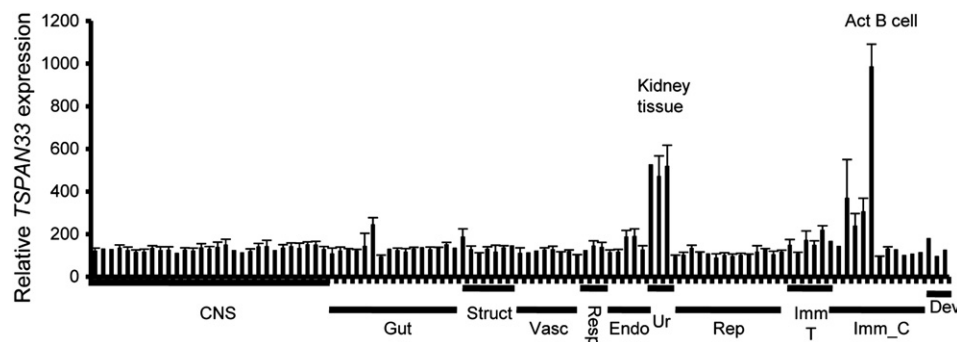
others, (including CD82, CD151 and CD37), exhibit a more limited expression profile and are highly expressed in specific cell lineages of the immune system [13].

### 1.2. Previous reports on TSPAN33

TSPAN33 has been previously reported as Penumbra (proerythroblast nu membrane), since it was originally detected in a subpopulation of erythrocyte progenitors in murine bone marrow suggesting that it was involved in hematopoiesis [14]. *Tspan33* expression in the mouse bone marrow was detected in the TER 119<sup>+</sup> fraction of bone marrow cells (erythroblasts), but not in neutrophils, T cells, monocytes, NK cells, or (resting) B cells [14]. Indeed, it is expressed in mouse pre-CFU erythroid cells and in mouse bone marrow [15]. These results may be explained by the small contribution that these *Tspan33*<sup>+</sup> erythrocyte progenitors make to total bone marrow RNA. Interestingly, Heikens et al. [14] generated a *Tspan33*<sup>-/-</sup> mouse, and some of these mice displayed abnormal erythropoiesis within 3 months and splenomegaly at 1 year of age. However, as we show here, the expression of TSPAN33 in normal human bone marrow is very low (Fig. 1) and is instead specifically and strongly expressed by activated B lymphocytes.

### 1.3. Approach

We have confirmed the expression of TSPAN33 in both mouse and human B cells. Taken together, these results indicate that TSPAN33 is a novel marker of activated B cells. In contrast to other B cell specific antigens (i.e. CD20, CD19) that are present on both resting and activated B cells, TSPAN33 is only expressed by activated B cells. We next sought to determine if TSPAN33 was also expressed in human diseases that involved activated malignant B cells. To this end we measured TSPAN33 expression in Hodgkin's lymphoma (HL), various types of non-Hodgkin's lymphoma (NHL), and in two autoimmune diseases, systemic lupus erythematosus (SLE) and rheumatoid arthritis (RA).



**Figure 1** TSPAN33 expression is restricted to activated B cells in normal human tissues. Affymetrix gene array (U133 plus 2.0) data compiled from the human body index of gene expression database observing *TSPAN33* expression in normal human tissue (n = 8) and immune cells. X axis is organized by organ systems: CNS (central nervous system), Gut (gastrointestinal), Struct (structural), Vasc (vasculature), Resp (respiratory), Endo (endocrine), Ur (urinary), Rep (reproductive), Imm\_T (immune tissue), Imm\_C (immune cells), and Dev (developmental).

## 2. Materials and methods

### 2.1. Microarray analyses

The generation of the body index of gene expression database (BIGE) has been described [9,10]. Briefly, total RNAs were obtained from 4 male and 4 female human donors, between 3 and 5 h post-mortem or augmented with commercially available human tissue RNAs (Clontech, Palo Alto, CA). Genome-wide gene expression data was obtained using Affymetrix Human Genome U133 Plus 2.0 gene arrays (Affymetrix, Santa Clara, CA) and data normalization, and summarization were done in ArrayAssist software (lobion Labs, La Jolla, CA).

### 2.2. qRT-PCR

RNA was isolated from human cell lines/cells or tissue using the QiagenRNeasy® kit according to the manufacturer's instructions (Qiagen, CA). The RNA was converted to cDNA using the *QuantiTect® Reverse Transcription* (Qiagen, CA). qPCR was performed using the Roche *LightCycler® 480* Real-Time PCR system with probes designed to detect TSPAN33, CD19, CD20, CD138 and GAPDH (Roche, Pleasanton, CA).

### 2.3. Detection of TSPAN33 protein

Polyclonal rabbit antibodies against human beta actin (Santa Cruz biotech, Santa Cruz, CA), beta tubulin (MP Biomedicals, Santa Ana, CA) and Tspan33/TSPAN33 (Abcam, Cambridge, MA) were used for western blotting.

### 2.4. Cell lines

The human B cell line 2E2 has been described [16]. The human T cell line Jurkat, was obtained from the ATCC (American Type Culture Collection, Manassas, VA). The murine cell line A20-2J has been described [17]. All DLBCL lines were a kind gift of David Fruman (UC Irvine Institute for Immunology). PBMCs from human donors were isolated by Ficoll density gradient. Mouse spleen B cells were enriched using Ficoll density gradient separation followed by panning with anti-CD3 mAb (Biolegend, San Diego, CA) and anti-CD11c mAb (Biolegend) coated plates. Briefly, 10 cm tissue culture plates were coated with anti-CD3 and anti-CD11c for 2 h at 37 °C. Splenocytes isolated by Ficoll density gradient separation were incubated on the coated plates for 2 h and the non-adherent cells were collected and passed through a second round of enrichment.

### 2.5. Reagents

B cells were stimulated using either LPS (Sigma Aldrich, St Louis, MO) + mouse or human rIL-4 (Sigma), anti-CD40 mAb clone G38.5 (Invitrogen, Carlsbad, CA) + rIL-4 or CpG + pokeweed mitogen (PWM) + pansorbin (Sigma). T cells were stimulated using anti-CD3 mAb + anti-CD28 mAb (Biolegend) or phorbol 12-myristate 13-acetate (PMA) + ionomycin (Sigma).

### 2.6. Mice

C57Bl/6j (stock number 000664) and MRL/*fas<sup>lpr/lpr</sup>* mice (stock number 000485) were obtained from the Jackson Laboratory (Bar Harbor, ME). All animal protocols were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California, Irvine.

### 2.7. Human samples

Human PBMCs were obtained from peripheral blood by venipuncture from Lupus patients or normal subjects. This protocol was approved by the Institutional Review Board (IRB) of the INNCMSZ and the samples were obtained following informed consent. Lupus patients fulfilled at least four 1982 American Rheumatism Association revised criteria for SLE [18]. Clinical disease activity was scored using the SLE Disease Activity Index or SLEDAI [19]. Controls had inactive disease (SLEDAI < 3) and patients with disease indices above 3 were considered as having active disease. cDNA was prepared using the M-MLV reverse transcriptase according to the manufacturer's instructions (Invitrogen, Carlsbad, CA).

### 2.8. Tissue array

Human tissue samples for immunohistochemistry were obtained from autopsies and represent archival samples from the Anatomy and Pathology Service of the University Hospital of the UANL. Tissue arrays were performed on normal human kidney or human lymphoma biopsies, including 6 HL patients, 6 DLBCL patients, and 2 mantle cell lymphoma, following antigen retrieval (demasking) using protease and/or heat treatment as described [20]. Sections were then stained using anti-TSPAN33 antibodies followed by secondary donkey anti-rabbit IgG enzyme conjugates (Abcam).

### 2.9. Statistical analyses

The statistical significance was calculated using the student's *t*-test. Values of  $p < 0.05$  were considered statistically significant. Error bars indicate standard deviation (SD).

## 3. Results

### 3.1. TSPAN33 is highly expressed in activated B cells

We identified TSPAN33 as a B cell activation-specific marker through the analysis of its expression in the BIGE database (Fig. 1). Its expression profile indicates specific and restricted expression, with the highest levels observed in peripheral blood B cells activated with anti-CD40 and IL-4, followed by kidney (Table 1 lists the top ten sites of *Tspan33* expression; the complete list is shown in Supplementary information (SI 1)). The *Tspan33* expression pattern from the BIGE database was confirmed using qRT-PCR on human RNAs SI 2A with low or undetectable expression in most other tissues including bone marrow, thymus and spleen.

**Table 1** Top ten sites of *TSPAN33* expression in humans.

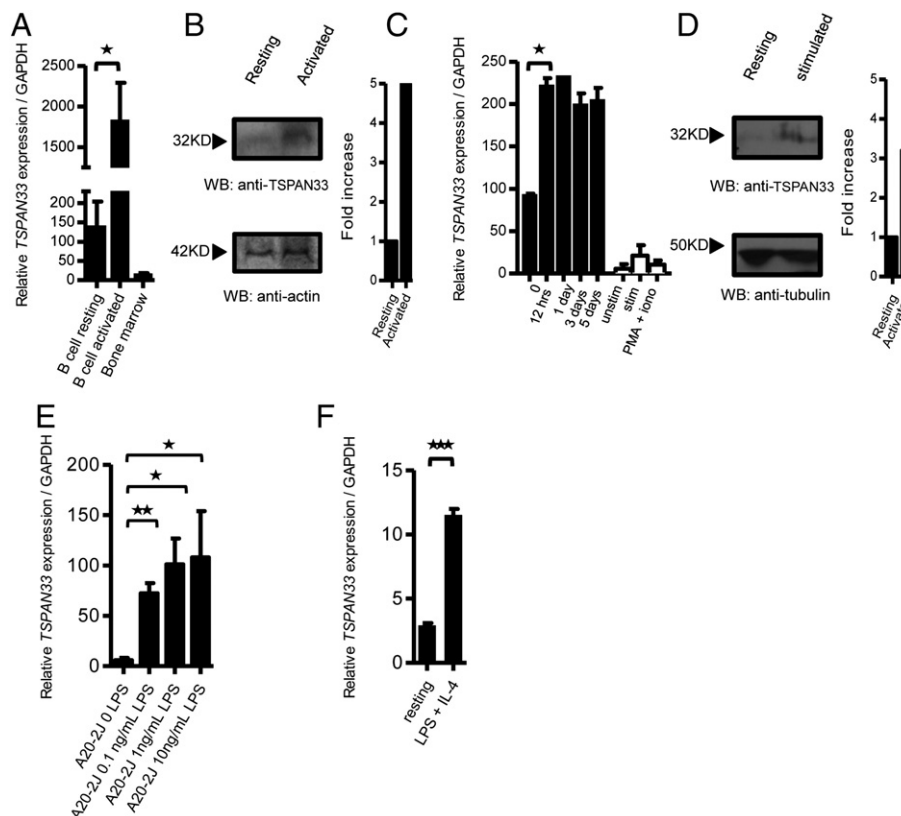
Sample	Average intensity
B cells, activated	985.4
Kidney	526
Kidney medulla	519.1
Kidney cortex	471.3
B cells, resting	305.1
Salivary gland	244.1
Monocytes, activated (LPS + IFN $\gamma$ )	238.5
Tonsil	218
Pituitary gland	189.3

Table shows the top ten sites of *TSPAN33* expression ranking from highest to lowest average intensity. The data is derived from the BIGE database shown in Fig. 1.

To confirm the microarray data, we performed qRT-PCR for *Tspan33* mRNA on human B cells isolated from PBMCs, under resting or activating conditions (anti-CD40 + IL-4) as well as human bone marrow (Fig. 2A). Although *Tspan33* was

initially identified as expressed in a subset of erythrocyte progenitors in mouse bone marrow [14], we did not detect significant *Tspan33* expression in human bone marrow (Fig. 2A). *Tspan33* levels in activated B cells are over 40-fold higher than either resting B cells ( $p = 0.0204$ ) or whole bone marrow. Since *Tspan33* has only recently been studied, there are not many reagents available (including antibodies). However, we obtained an anti-TSPAN33 polyclonal antibody (Abcam) that worked in western blot and immunohistochemistry (IHC) (following epitope retrieval) but not for FACS analyses (data not shown). Using this antibody, we observed a significant increase in TSPAN33 protein expression in activated human PBMCs (Fig. 2B). Densitometric analyses revealed an ~5 fold increase in TSPAN33 protein expression in stimulated versus unstimulated PBMC samples.

The human 2E2 B cell line is a model for inducible B cell activation and differentiation [16]. It expresses IgM and IgD in a non-stimulated state and it readily upregulates activation-induced cytidine deaminase (*Aicda*) to induce class switching to downstream isotypes (a measure of

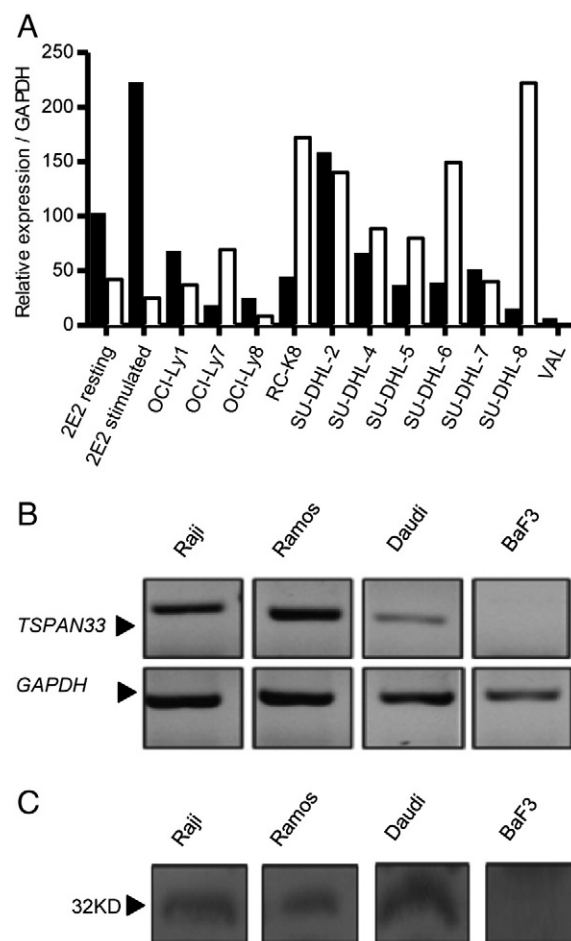


**Figure 2** TSPAN33 expression is restricted to activated B cells in mice and humans. A) qRT-PCR of *TSPAN33* expression in resting and activated (anti-CD40 + IL-4) human B lymphocytes purified from human blood compared to human bone marrow,  $n = 3$ . B) Western blot of PBMCs for TSPAN33 expression under resting and activating conditions with CpG + pokeweed mitogen (PWM) + pansorbin using actin as a loading control. Also shown are densitometric analyses. C) qRT-PCR of *TSPAN33* expression over time in human 2E2 B cells (Black bars) with anti-CD40 mAb + IL-4 stimulation and human Jurkat T cells (white bars) under unstimulated, anti-CD3 + anti-CD28 mAb, or PMA + ionomycin stimulation for 12 h,  $n = 3$ . D) Western blot of TSPAN33 expression in resting vs. activated human 2E2 B cells with anti-CD40 mAb + IL-4. Also shown are densitometric analyses. E) qRT-PCR of *Tspan33* A20-2J B cells under resting and activating conditions with 0.1, 1, or 10 ng/mL of LPS + IL-4. F) qRT-PCR of resting or stimulated B cells enriched from C57BL/6 spleens with 10 ng/mL LPS + IL-4 for 12 h,  $n = 3$ ,  $*p \leq 0.05$ ,  $**p \leq 0.01$  and  $***p \leq 0.001$  indicate statistical significance according to Student's *t* test. Data are representative of three independent experiments. Error bars indicate standard deviation (SD).

activation) [21,22] following stimulation with anti-CD40 mAb + IL-4. Using qRT-PCR we observed a significant increase in *Tspan33* mRNA levels following stimulation with anti-CD40 + IL-4 for 12 h compared with unstimulated 2E2 cells ( $p = 0.013$ ) (Fig. 2C), and the elevated *Tspan33* transcript levels remained high for up to 120 h after stimulation. Conversely, *Tspan33* expression was not detectable in resting, anti-CD3+, anti-CD28 or PMA + ionomycin-stimulated Jurkat cells (human T cell leukemia). The increased expression of *Tspan33* in 2E2 cells was confirmed by western blot, with a >3 fold increase (by densitometry) observed when using a polyclonal anti-*Tspan33* antibody (Fig. 2D). *Tspan33* expression was also measured in mouse tissue using qRT-PCR (SI 2B) and the results confirmed the human expression profile. We also observed a dose-dependent increase in *Tspan33* mRNA expression in the murine B cell line A20-2J upon stimulation with increasing concentrations of LPS + IL-4 and measured by qRT-PCR (Fig. 2E). *Tspan33* transcription increased in A20-2J over 50 fold ( $p = 0.0014$ ) with 0.1 ng/mL LPS + IL-4 stimulation and over 100 fold with 1 ng/mL or 10 ng/mL LPS + IL-4 ( $p = 0.011$  and  $p = 0.045$ ). Additionally, mouse spleen B cells were isolated by Ficoll density gradient separation and enriched by panning with anti-CD3 and anti-CD11c [23]. The enriched B cells were stimulated with 10 ng/mL of LPS + IL-4 for 12 h and analyzed for *Tspan33* expression by qRT-PCR. As shown in Fig. 2F, there was a ~4 fold increase in *Tspan33* transcription following LPS stimulation compared to resting conditions ( $p = 0.00003$ ). We should note that we also performed qRT-PCR on total mouse splenocytes under various stimulation conditions and significant upregulation of *Tspan33* expression was observed when splenocytes were stimulated with CD40L + IL-4 or with anti-IgD + IL-4, but not with anti-CD3 + anti-CD28 (which stimulates T cells) (data not shown). Taken together, these results indicate that TSPAN33 is a novel marker of activated B cells in both mouse and human.

### 3.2. TSPAN33 is expressed by malignant B cells

B cell activation markers are important as diagnostic tools, since elevated levels of some of these molecules, such as serum levels of sCD23, sCD27, sCD30, sCD44, CXCL13, IL-6 and IL-10 [24,25] have been reported to be associated with cancer (for example, NHL). Other known B cell antigens (i.e. CD19 and CD20) are also highly expressed in NHL [26]. We therefore hypothesized that TSPAN33 would also be expressed in human lymphomas. To test this, we performed qRT-PCR for *Tspan33* expression and compared it to *ms4a1* (CD20) in 11 lines including NHL cell lines characterized as DLBCL (OCI-LY1, OCI-LY7, OCI-LY8, RC-K8, SU-DHL-2, SU-DHL4, SU-DHL-5, SU-DHL-6, SU-DHL-7, and SU-DHL-8 and VAL), along with non-stimulated or stimulated (anti-CD40 mAb + IL-4) 2E2 cells (Fig. 3A). DLBCL is the most common type of aggressive NHL and represents a heterogeneous group of lymphomas with a common characteristic of diffuse proliferation of large B cells with nuclei at least twice the size of normal lymphocytes [27]. DLBCL may include centroblast, immunoblast, or anaplastic variants (similar to highly activated Reed Sternberg cells of HL) and have a proliferative index of >90% [28]. *ms4a1*/CD20 mRNA levels were also measured to compare its expression with *Tspan33*



**Figure 3** TSPAN33 is expressed in human Hodgkin's and non-Hodgkin's lymphoma. A) qRT-PCR was performed on several human NHL lines and measured for *TSPAN33* (black bars) vs. *MS4A1*/CD20 (white bars) expression. Samples were normalized to GAPDH. B) RT-PCR expression analysis corresponding to the large extracellular loop 33 (LEL) of *TSPAN33* in human Burkitt's lymphoma lines Raji, Ramos, and Daudi against BaF3 (a mouse pro-B cell line) compared to GAPDH. C) Western blot analysis of *TSPAN33* expression of Raji, Ramos, Daudi, and BaF3 cells using a rabbit anti-*TSPAN33* polyclonal antibody. Data are representative of three independent experiments.

in these lymphoma cell lines. Both *ms4a1*/CD20 and *Tspan33* were detected in all DLBCL lines. In fact, *Tspan33* expression levels were comparable to CD20 in DLBCL.

In contrast to DLBCL, Burkitt's lymphoma (BL) has a germinal center phenotype [21], including a CD10<sup>+</sup>, BCL6<sup>+</sup> and BCL2<sup>+</sup> distinct phenotype with round, medium-sized morphology, with a proliferative index of 100% [29] and may express CD20 [28]. To explore the expression of TSPAN33 in Burkitt's lymphoma, we performed RT-PCR and western blotting on several Burkitt's lymphoma lines including Raji, Ramos, and Daudi, as well as in mouse BaF3 cells (Pro-B cell line) as a control (Figs. 3B and C). TSPAN33 expression was detected at both the mRNA and protein levels in all Burkitt's lymphoma lines, but not in BaF3 cells. We conclude that TSPAN33 is also expressed in human Burkitt's lymphoma.

**Table 2** TSPAN33 expression in human lymphomas.

Case	TSPAN33 positive samples	Pattern of staining
HL	6/6	Localized to Reed Sternberg cells
DLBCL	6/6	uniform
Mantle cell lymphoma	0/2	negative

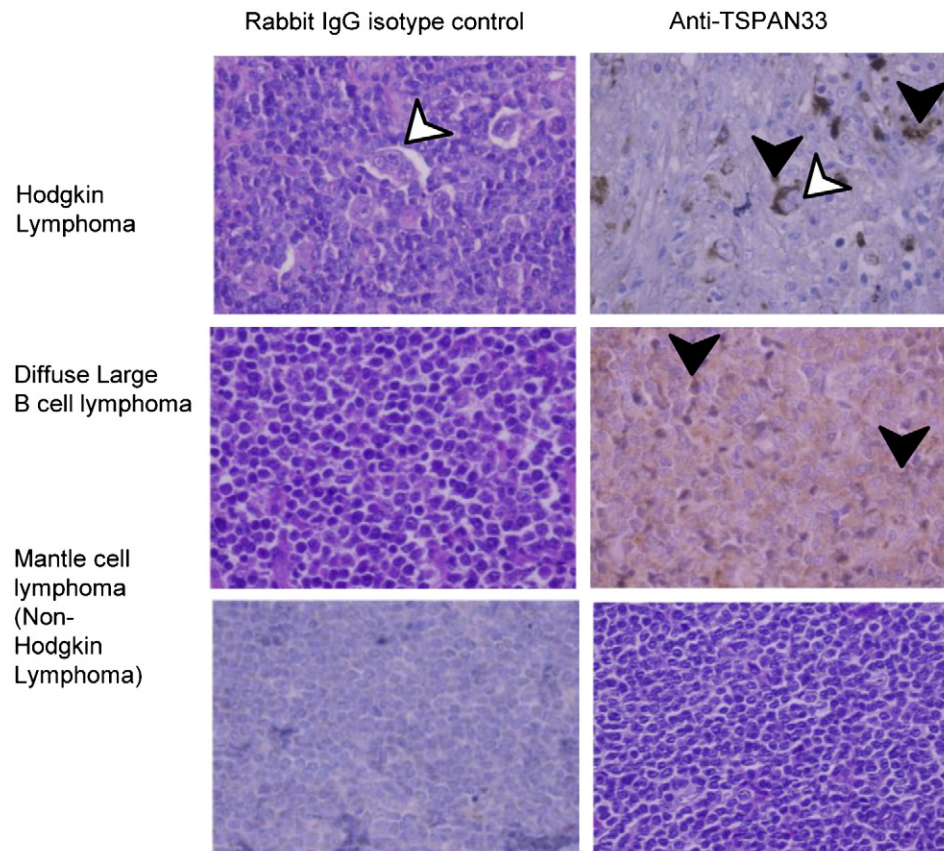
Table shows the results from the IHC staining of TSPAN33 expression on tissue arrays taken from human biopsies from individual patients diagnosed with HL (n = 6), DLBCL (n = 6), and mantle cell lymphoma (n = 2). The total number of patients and staining pattern are also indicated.

To further characterize TSPAN33 expression in other B cell lymphomas, we sought to perform immunohistochemistry (IHC) on tissue arrays prepared from biopsies of patients diagnosed with DLBCL (n = 6), mantle cell lymphoma (another type of NHL, n = 2), and HL (n = 6). Table 2 and Fig. 4 show the representative images of lymph nodes from patients with HL, DLBCL, or mantle cell lymphomas. Tspan33 was highly expressed in Reed-Sternberg cells (a cell characteristic of Hodgkin's Lymphoma) in HL, while DLBCL also stained positive for TSPAN33 uniformly, consistent with the qPCR data shown

in Fig. 3. Mantle cell lymphoma was negative for TSPAN33 staining. Reed-Sternberg cells are thought to be derived from germinal center B cells that have undergone somatic hypermutation and failed to undergo apoptosis, and therefore may represent an activated form of lymphoma [30]. DLBCL has been described above. Mantle cell lymphoma, on the other hand, is a type of mature CD5<sup>+</sup> B cell lymphoma believed to originate from naïve, pre-germinal center lymphocytes, and may represent a form of non-activated B lymphocyte [31]. These differences in TSPAN33 levels may reflect the activation or differentiation state of each B cell lymphoma. On the other hand, the expression of TSPAN33 in each lymphoma suggests that it may represent another biomarker that could reflect the aggressiveness of each lymphoma or could be used as a prognostic factor [32,33].

### 3.3. TSPAN33 is expressed in systemic lupus erythematosus and rheumatoid arthritis lesions

Markers of B cell activation are also associated with certain autoimmune diseases. For example, CD25, HLA-DR, CD38, and BlyS are all elevated and associated with autoantibody production in clinical SLE [34,35]. Serum immunoglobulin levels and the B cell-associated cytokines IL-6, IL-21 and BlyS are all significantly elevated in patients with newly



**Figure 4** TSPAN33 is expressed in human lymphomas. Lymphoma biopsies were sectioned and stained with hematoxylin/eosin and anti-TSPAN33, followed by isotype or anti-rabbit IgG-HRP. White arrows indicate Reed-Sternberg cells and black arrows indicate positive TSPAN33-stained cells. Representative images from biopsies taken from patients diagnosed HL (n = 6), DLBCL (n = 6), and mantle cell lymphoma (n = 2).

diagnosed RA [36–38]. Blocking BlyS reduces disease symptoms in MRL/*fas<sup>lpr/lpr</sup>* mice (soluble TACI) [39] and also provides therapeutic benefit in humans (anti-BlyS mAb: Benlysta) [40]. To address the role of Tspan33 in autoimmune diseases, we measured *Tspan33* mRNA expression in PBMCs from SLE patients, in RA synovial lesions or in a mouse model of SLE.

MRL/*fas<sup>lpr/lpr</sup>* mice develop a spontaneous and progressive systemic autoimmune syndrome sharing many features with human SLE and RA, including dysregulated B cell activation, elevated antibody and autoantibody production, inflammation, and immune complex deposition in the kidney, which results in fatal glomerulonephritis [39,40]. The abnormal activation of B cells in MRL/*fas<sup>lpr/lpr</sup>* mice and human SLE leads to elevated *Aicda* expression, resulting in pathogenic class-switched and hypermutated antibodies, which mediate tissue and organ damage [39,41]. MRL/*fas<sup>lpr/lpr</sup>* mice develop high titers of autoantibodies and severe kidney damage by 16 weeks of age [42]. Thus, B cells play important roles in lupus pathogenesis, through both antibody-dependent and antibody-independent mechanisms [43].

We measured *Tspan33* mRNA expression in splenocytes from MRL/*fas<sup>lpr/lpr</sup>* mice at 9, 24 and 36 weeks of age and normalized it to CD19 in order to explore the B cell contribution (Fig. 5A). We found that 24-week-old MRL/*fas<sup>lpr/lpr</sup>* mice, which already exhibit extensive Lupus symptoms including skin lesions, autoantibodies, and renal pathology, had an ~10-fold increase in *Tspan33* mRNA expression when compared to their 9-week-old counterparts ( $p = 0.016$ ), which did not yet show overt signs of pathology (although some B cells may already be activated at 9 weeks, MRL/*fas<sup>lpr/lpr</sup>* display 90% mortality by 30 weeks of age, with the few surviving mice displaying particularly dysregulated levels of cytokines and chemokines) [42]. *Tspan33* transcript expression in 36-week-old MRL/*fas<sup>lpr/lpr</sup>* mice increased further (compared to 24-week-old mice), although this increase was not statistically significant ( $p = 0.062$ ). Taken together, these observations strongly suggest an important role for TSPAN33 in the pathogenesis of SLE.

As B cells are not exclusively responsible for Lupus pathogenesis, we sought to determine whether TSPAN33 upregulation during Lupus disease in MRL/*fas<sup>lpr/lpr</sup>* mice was associated with plasma cells. To address this, we FACS-sorted splenocytes from 12 week old male and female MRL/*fas<sup>lpr/lpr</sup>* mice for CD19<sup>+</sup> 138<sup>-</sup> B cells and CD19<sup>-</sup> CD138<sup>+</sup> plasma cells and analyzed *Tspan33* expression by qRT-PCR (Fig. 5B). *Tspan33* expression was significantly upregulated in CD19<sup>+</sup> B cells from 12-week-old female MRL/*fas<sup>lpr/lpr</sup>* mice ( $p = 0.004$ ) over their male counterparts (similar to the human disease, females are more prone to lupus-like disease with an earlier onset than males in MRL/*fas<sup>lpr/lpr</sup>* mice). Furthermore, *Tspan33* was not expressed in CD138<sup>+</sup> cells, indicating that its expression is restricted to activated B cells but does not extend to terminally differentiated B cells (plasma cells). Further support for this conclusion comes from the expression of plasma cell specific markers in the BIGE database. For example, B cell maturation antigen (BCMA) is a receptor for BlyS and APRIL expressed by plasma cells [44]. In the BIGE database, BCMA is strongly expressed in human tonsil, bronchus and trachea, indicating that these tissues contain significant numbers of plasma cells (data not shown); in contrast, TSPAN33 expression is low or absent in

these tissues (Fig. 1 and SI 1). We conclude that TSPAN33 is unlikely to be expressed by plasma cells. This is consistent with other markers of B cell activation that decrease upon differentiation into plasma/memory cells [45–47].

To confirm a possible role of TSPAN33 in human SLE, we measured the expression of *Tspan33* mRNA by qRT-PCR in PBMCs from 9 healthy subjects or 9 SLE patients (Fig. 5C). PBMCs from SLE patients had a >3 fold increase in *Tspan33* mRNA expression ( $p = 0.038$ ). These results indicate that TSPAN33 is elevated in human SLE.

We next sought to explore a possible role of activated B cells in RA. To this end, we analyzed *TSPAN33* mRNA expression in a RA microarray database produced from synovial membranes of patients with this disease [48]. Levels of both TSPAN33 ( $p = 0.0019$ ) and CD20 ( $p = 0.0008$ ) transcripts were elevated in RA patients (Fig. 5D). It has been reported that the top genes elevated in the RA synovial joint membranes include multiple markers of B cell activation, including immunoglobulin light and heavy chain genes, as well as genes that target B cells like BlyS and CXCL13 [48]. These observations are consistent with previous reports that have documented the role of activated B cells in RA lesions [49,50] as well as the fact that anti-CD20 (rituxan) is an effective treatment in RA [51,52].

### 3.4. TSPAN33 expression in the kidney

As shown in Fig. 1A and SI 2A, TSPAN33 mRNA is also detectable in the kidney by both microarray and qPCR. Given the important physiologic role of the kidney, we sought to determine the location of TSPAN33 expression within the kidneys. To this end, we performed immunohistochemistry to detect TSPAN33 in normal human kidney sections (Figs. 6A–D) including a section of renal tissue where lymphoid infiltrates are present (Fig. 6A). TSPAN33 staining was detected in the proximal convoluted tubules, distal convoluted tubules and collecting ducts (Figs. 6B–C) but not in infiltrating lymphocytes (a result consistent with previous experiments) or in the glomeruli. Higher magnification revealed that TSPAN33 is expressed at the apical membrane and granules of epithelial brush border cells of the proximal convoluted tubules (Fig. 6D). These results support TSPAN33 as a target for therapeutic antibody development, because these sites are normally not accessible to antibodies.

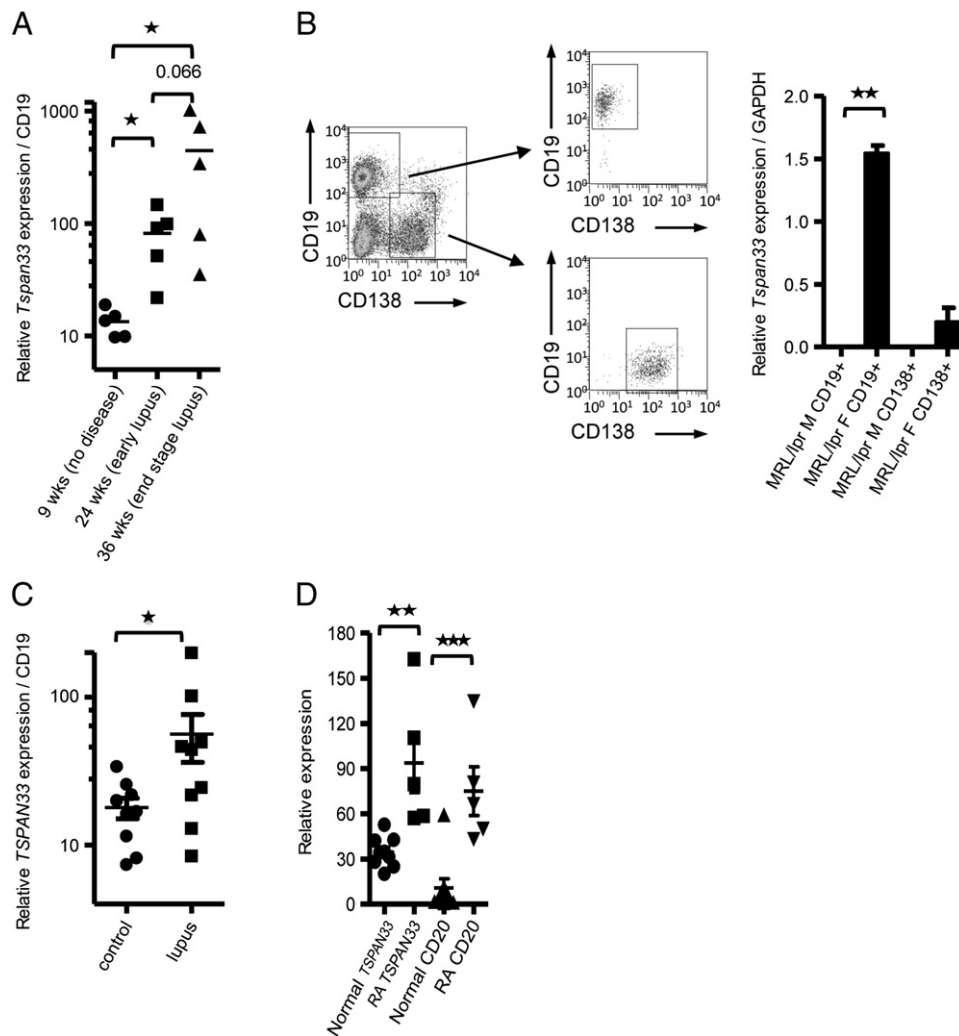
## 4. Discussion

### 4.1. Overview

We have found that a member of the tetraspanin family (TSPAN33) is a B cell activation marker because it is strongly expressed in activated B cells, and is also expressed in several lymphomas and in autoimmune diseases where pathogenic B cells are involved (including SLE and RA).

### 4.2. TSPAN33 as a novel B cell activation biomarker

A number of markers, including CD72, CD20, CD19, and CD24 are currently used to identify and track B cells [53]. Activated germinal center B cells have been reported to



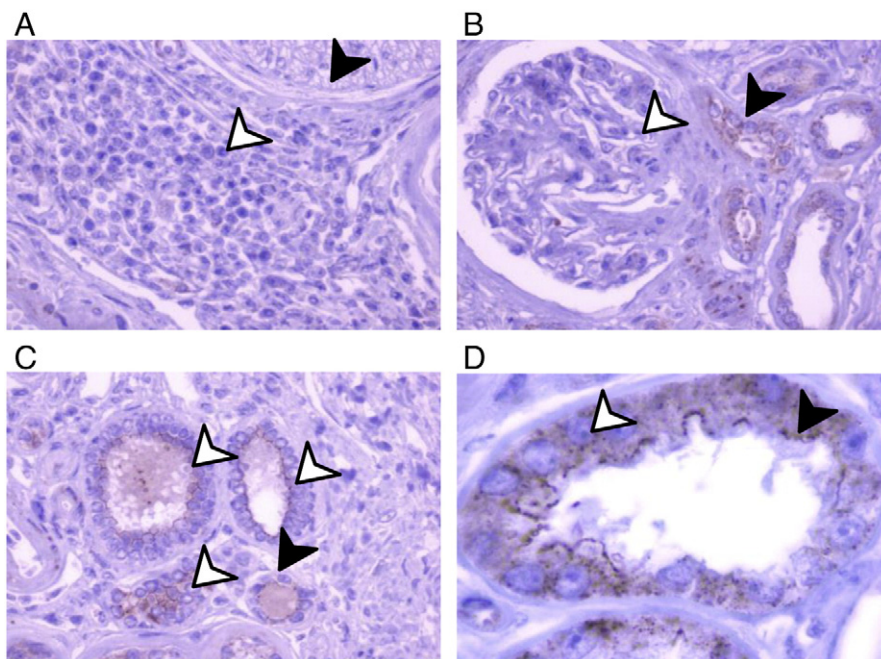
**Figure 5** TSPAN33 is upregulated in B cell-associated autoimmunity. A) qRT-PCR of *Tspan33* expression of total splenocytes taken from MRL/fas<sup>lpr/lpr</sup> mice normalized to CD19 expression. Mice ages 9 weeks old (no detectable pathology), 24 weeks old (lymphadenopathy with or without mild ear lesions) and 36 weeks old (lymphadenopathy with ear and face lesions) were compared for *Tspan33* expression,  $n = 5$ . B) qRT-PCR of *Tspan33* expression in CD19<sup>+</sup>, CD138<sup>-</sup> and CD19<sup>+</sup>CD138<sup>+</sup> splenocytes from 11.5 week old female (lymphadenopathy) and 12.5 week old male (no pathology) MRL/fas<sup>lpr/lpr</sup> mice,  $n = 2$ . C) qRT-PCR of *TSPAN33* expression analysis of PBMCs from human SLE patients or healthy controls,  $n = 9$ . D) Microarray analysis of *TSPAN33* vs *MS4A1/CD20* expression in synovial membranes from healthy and RA patients. Synovial membranes were isolated from controls or RA patients as described [48]. The RNA was isolated from the membranes and analyzed for *MS4A1/CD20* and *TSPAN33* expression using the Affymetrix gene array U133 plus 2.0,  $n = 9$  healthy and  $n = 5$  RA patients,  $*p \leq 0.05$ ,  $**p \leq 0.01$ ,  $***p \leq 0.001$  (Student's *t* test). Data are representative of at least three independent experiments (A–C). Error bars indicate standard deviation (SD).

express a variety of genes, including GL7 [54], CD10 and BCL6 [55]. Other B cell activation markers such as MUM1/IRF4 and FOXP1, as well as CD23, CD69 and the systemic B cell activation markers CXCL13, sCD23, sCD27, sCD30, sCD44 have been used as markers in the diagnosis and risk assessment of NHL and RA [25,32,56]. Importantly, none of these activation markers are exclusively expressed on activated B cells, as they have also been associated with other immune cell types in the periphery. Therefore, TSPAN33 represents a B cell specific activation marker that may be useful as a diagnostic tool for diseases involving B cell activation. The likelihood of using TSPAN33 expression as a potential prognostic biomarker in both lymphoma and autoimmune diseases deserves further study [57–59].

### 4.3. TSPAN33 is a possible target for therapeutic mAbs against malignant B cells

In addition to a possible use of TSPAN33 as a B cell activation marker, TSPAN33 is the 33th member of the tetraspanin family (TSPAN33), and therefore a transmembrane protein. This makes TSPAN33 a suitable candidate for the production of anti-TSPAN33 mAbs for therapeutic purposes. CD20, a closely related protein now assigned to the membrane-spanning 4-domains superfamily (MS4A1), is an example of an important target for the production of therapeutic monoclonal antibodies that have proven effective for the treatment of B cell malignancies such as NHL, chronic lymphocytic leukemia (CLL)





**Figure 6** TSPAN33 is expressed in the proximal, distal convoluted tubules and collecting duct but not in the kidney glomerulus. Kidney biopsies were stained for IHC in a tissue array. Samples were stained with H&E and anti-TSPAN33 or rabbit IgG isotype control, followed by anti-rabbit IgG-HRP. A) 40× magnification showing lymphocytes (black arrows) and nerves (white arrows). B) 40× magnification showing proximal convoluted tubules (black arrows) and kidney glomeruli (white arrows). C) 40× magnification showing distal convoluted tubule (black arrows) and collecting duct (white arrows). D) 100× magnification of proximal convoluted tubule showing the apical surface (black arrow) and granules (white arrows).

and also for certain autoimmune diseases including RA [51,52,60]. However, since CD20 is expressed on both resting and activated B cells, anti-CD20 mAb therapy results in depletion of all B cells in the peripheral blood as well as 70% of B cells in the bone marrow [25,36,61]. Therefore the identification of a B cell marker restricted to activated B cells, such as TSPAN33, could represent an alternative strategy for the development of a “second generation” of mAbs for the treatment of B cell-associated pathologies [32]. Other tetraspanins (CD151) are being explored as possible therapeutic antibody targets [62]. Our data strongly suggest that anti-TSPAN33 therapeutic mAbs would have the important advantage of avoiding depletion of most resting B cells in the treated patients.

#### 4.4. Other sites of TSPAN33 expression

TSPAN33 has been previously reported as Penumbra (Pro Erythroblast nu membrane) because it was originally identified as a molecule expressed in a small erythrocyte progenitor population in the bone marrow [14]. Given this expression pattern, it was described to play a role in hematopoiesis. *Tspan33*<sup>-/-</sup> mice have been described [14] and some of them developed abnormal erythrocytes at 3 months of age. Acquired pure red cell aplasia is a related condition in humans where patients lack erythroblasts and depending on the cause may be self limiting [63]. These observations suggest that temporary inhibition of TSPAN33 in humans may have limited or manageable side effects.

Another possible complication in the use of anti-TSPAN33 mAbs as human therapeutics is its expression in the kidney. Its expression pattern there, however, suggests that this will not represent a significant obstacle because *Tspan33* is not expressed in the glomeruli (Fig. 6B) but is instead expressed by epithelial cells in the proximal and distal convoluted tubules (Figs. 6B–C). Access of antibodies to these sites is normally prevented by size exclusion, since only smaller molecular weight proteins (like albumin ~67 kD or hemoglobin ~68 kD) are permeable through the glomerular barrier [64]. TSPAN33 protein expression was observed in the apical surface and granules of the epithelial cells of the kidney and these cells are involved in the secretion and absorption of small proteins, ions, and organic solutes (glucose and amino acids), suggesting that TSPAN33 may participate in vesicular trafficking and/or signaling during urine filtration [12]. Moreover, kidney epithelial cells have been reported to be refractory to biologically-based cytotoxic agents and kidney cell carcinomas are also resistant to ADCC (antibody dependent cellular cytotoxicity) [65]. Finally, a *Tspan33*<sup>-/-</sup> mouse has been reported to be viable and fertile [14], indicating that absence of *Tspan33* has limited physiological impact in kidney function.

#### 4.5. The function of *Tspan33* in B cell activation

Although the function of *Tspan33* in B cells is currently unknown, the strong induction of *Tspan33* expression upon B cell activation strongly suggests that it may be involved in B

cell signaling/activation (i.e. CD9 and CD81), maturation/survival (i.e. CD37), or antigen presentation (i.e. CD63), since other B cell-expressed tetraspanins are known to participate in these processes [66–69].

#### 4.6. Summary

We conclude that TSPAN33 represents a potentially important biomarker of activated and malignant B cells, as well as a potential target for the development of therapeutic mAbs for the treatment of several types of B cell lymphoma (DLBCL, BL, HL) as well as some autoimmune diseases associated with pathogenic B cells showing an activated B cell phenotype (SLE and RA).

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.clim.2013.08.005>.

#### Conflict of interest statement

The author(s) declare that there are no conflicts of interest.

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