

Pivotal Advance: CD45RB glycosylation is specifically regulated during human peripheral B cell differentiation

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ABSTRACT

A screen of cell surface markers differentially expressed during peripheral B cell differentiation identified that the CD45RB epitope detected by the mAb MEM-55 was highly expressed on CD27⁺ memory B cells and absent on CD27⁻ naïve B cells. IgG⁺CD27⁻ memory and a previously unacknowledged CD27⁺ population in blood also expressed high levels of CD45RB^{MEM55}. Naïve and memory B cells from tonsils followed the pattern observed in blood, and CD38^{high} B cells had a bimodal expression pattern when analyzed using flow cytometry. No CD38^{high} GC B cells, however, expressed the CD45RB^{MEM55} epitope when assayed using immunohistochemistry. Rather, CD38^{high}CD45RB^{MEM55}^{high} B cells had a distinct cellular phenotype and were localized outside of GCs. CD45RB epitopes, detected by other antibody clones, were expressed at high levels through B cell differentiation, and no changes in splicing of the CD45RB exon were observed during B cell differentiation. Instead, B cells regulated their expression of the CD45RB^{MEM55} epitope through site-specific modifications of an O-linked glycochain. CD4⁺ T cells differentially spliced CD45 but did not vary the glycosylation of the CD45RB^{MEM55} epitope, and CD8⁺ cells modified CD45RB^{MEM55} expression in a similar manner as B cells. Monocytes expressed the CD45RB exon but not the CD45RB^{MEM55} epitope. As CD45 is a highly expressed tyrosine phosphatase that regulates antigen receptor signaling strength in lymphocytes, we conclude that regulated O-linked glycosylation of CD45RB can be used to follow B cell differentiation and that this regulation may be involved in fine-tuning antigen signaling in the cell. *J. Leukoc. Biol.* 90: 5–19; 2011.

Abbreviations: ABCB-1=ATP-binding cassette sub-family B member 1, AID=activation-induced cytosine deaminase, APC=allophycocyanin, FCRL4=FcR-like protein 4, FM=follicular mantle, FSC=forward-scatter, GalNAc=N-acetylgalactosamine, GC=germinal center, ppGalNAcT, polypeptide N-acetylgalactosamine transferases, SSC=side-scatter, UCHL1=ubiquitin carboxyl-terminal esterase L1

The online version of this paper, found at www.jleukbio.org, includes supplemental information.

Introduction

The transmembrane protein CD45 is expressed at high levels on nucleated hematopoietic cells. Its intracellular cytoplasmic tail is composed of two tyrosine phosphatase domains, and extracellularly, it has three fibronectin type III domains, a cysteine-rich domain, and an extended region with several N- and O-linked glycosylation sites [1, 2]. It is a regulator of antigen receptor signals, probably through dephosphorylation of Src kinases, but also modifies the response to other extracellular signals. Functionally, it overlaps with distinct phosphatases, i.e., CD148 [3], but at least some of its functions are nonredundant; mice and humans that lack expression of functional CD45 have severe combined immunodeficiencies [4, 5]. Extracellular interactions can modify the activity of CD45, probably as a result of regulation of homodimerization [6–10]. Several lectins, including endogenous galectins, are able to interact with sugar chains attached to CD45, but these interactions are not CD45-specific, and no specific ligands have been identified so far [1, 11].

Despite being a ubiquitously expressed marker for hematopoietic cells, CD45 is used as a differentiation marker for T lymphocytes and within myeloid lineages [1, 2]. This is possible as a result of differential splicing of the CD45 mRNA because of a changed expression of the RNA-binding protein heterogeneous nuclear ribonucleoprotein L-like [12, 13]. The outcome is expression of transcripts containing different combinations of exons 4–6, resulting in CD45 proteins containing the RA, RB, and RC parts. In addition, N- and O-linked glycosylation patterns change during T cell differentiation [11, 14, 15]. Together, these modifications result in large numbers of CD45 isoforms expressed with molecular weights ranging from 180 kDa (the CD45R0 form lacking any of the exons) to 240

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kDa (CD45RABC containing all three exons). In T cells, regulated expression of these influences activation, differentiation, and susceptibility to cell death, although the molecular mechanisms are still debated [14–18].

The expression of CD45 isoforms during human B cell differentiation has been less studied. In human tonsillar GCs, CD45R0^{high} cells carry more mutations and express less AID and higher levels of the activation marker CD69 than CD45R0^{low/-} cells [19]. It was subsequently reported that the expression of the RB exon of CD45 is also regulated during tonsillar GC B cell differentiation and in this case, in a distinct manner compared with CD45R0 [20]. Although expression of the activation marker CD69 and low levels of AID were associated with high RB expression, the mutational load of Ig V regions decreased with increased RB expression. A more detailed analysis of V region mutation levels and the expression of B cell surface markers led to the proposal of a complex mode for expression of the RB exon in tonsils: low levels on naïve cells, up-regulation when these are activated to become pro-GC cells, then down-regulation in GC centroblasts, followed by increased expression in centrocytes, and finally, a gradual disappearance of RB as cells differentiated toward a memory cell phenotype [20]. Based on this model, naïve and memory B cells circulating in blood would be expected to express low levels of the RB exon. The expression of RB-containing splice forms on human B cells outside of the tonsils was, however, not determined, and the mechanisms involved in regulating B cell expression of CD45 were not studied.

The expression of different CD45 splice forms is in general determined using exon-specific mAb. At least nine distinct antibody clones have been confirmed to detect CD45 protein translated from splice forms containing the RB exon, but most studies rely on two clones: MEM-55 or MT4. The MEM-55 clone has been described to detect a glycosylation-dependent epitope, and the epitope detected by the MT4 clone has not, to our knowledge, been mapped extensively [21]. A third, less used, CD45RB-reactive clone (MEM-143) detects the protein backbone of CD45RB. No systematic comparison has been made with regard to the staining patterns between different antibody clones and thus, it is unsure whether all described changes in RB-staining patterns in human cells can be accredited to differential splicing of the RB exon or if post-translational mechanisms also play a role.

Here, we describe an unbiased screen to detect cell surface molecules that are differentially expressed on the human blood peripheral B cell during differentiation, and we show that the CD45RB-reactive antibody MEM-55 [21] detects an epitope that is highly expressed on memory B cells and plasmablasts but not naïve cells. Among CD27⁺ B cells in blood, we find that IgG⁺CD27⁺ memory cells and a novel population of immature B cells are also CD45RB^{MEM55high}. In tonsils, a proportion of CD38^{high} B cells expresses CD45RB^{MEM55}, but when assayed using immunohistochemistry, few, if any, B cells within GCs express the epitope. Thus, the immunohistochemical expression pattern of CD45RB^{MEM55} contrasts with the distribution proposed by Jackson et al. [20], most likely as CD38^{high}CD45RB^{MEM55high} B cells in tonsils are located outside of GCs. We further show that the expression of the

CD45RB^{MEM55} epitope is not regulated through differential splicing but as a result of changes in site-specific glycosylation during B cell differentiation. Finally, we demonstrate that other hematopoietic cell lineages also differentially glycosylate the RB exon of CD45. Thus, the expression of CD45RB is modified through a previously undescribed mechanism of regulated, site-specific glycosylation during B cell differentiation, as well as between different hematopoietic lineages.

MATERIALS AND METHODS

Blood collection and preparation of tonsils for flow cytometry

Blood was collected by venipuncture into EDTA tubes (BD Biosciences, San Jose, CA, USA), and mononuclear cells were isolated using Ficoll-Paque (GE Healthcare, Uppsala, Sweden). Tonsillar mononuclear cells were prepared by dissecting tonsillar tissue into smaller pieces and passing it through a mesh before removing any RBCs on Ficoll-Paque. Cells (1×10^6) were used in each staining reaction. Samples were collected after informed consent and had been approved by the relevant ethical committees.

Antibodies, flow cytometry, and cell sorting

For screening, cells were stained with PE-labeled anti-CD27, APC-labeled anti-IgM, and Alexa Fluor 700-labeled anti-CD19 (BD Biosciences), together with the FITC-labeled antibodies in IT-box-167 (ImmunoTools, Friesoythe, Germany), complemented with additional antibodies. Each FITC-labeled antibody (2 μ l) was used. Details of the antibodies used for screening and further analysis and cell sorting are found in Supplemental Material. In assays to determine whether the CD45RB epitopes were dependent on terminal sialic acids, the cells were preincubated in sialidase buffer with or without *V. Cholerae* sialidase (Sigma-Aldrich, St. Louis, MO, USA) before analysis. Analysis of Rhodamine-stained cells was performed exactly as described by Wirths and Lanzavecchia [22]. Flow cytometric analysis was performed on an LSR II flow cytometer and cell sorting on a FACSARIA cell sorter (BD Biosciences). Cells were collected using FACSDiva software (BD Biosciences), and data files were analyzed using the FlowJo software package (Tree Star Inc., Ashland, OR, USA).

Cell stimulations

Cells (1×10^6) sorted based on CD19 and CD27 expression (blood) or CD20, CD38, and CD45RB^{MEM55} (tonsils) were stained in 1 ml PBS with 10 mM CFSE at 37°C for 15 min. The cells were then re-pelleted and incubated in normal medium at 37°C for 30 min before they were washed. Cells (50,000)/well of a CFSE-labeled or unlabeled cell were then incubated in growth media (RPMI with 10% FCS) in round-well, 96-well plates in media alone (unstimulated) or in media containing 2.5 μ g/ml goat anti-human Ig H⁺L (Southern Biotech, Birmingham, AL, USA), 10 ng/ml IL-2 (Invitrogen, Carlsbad, CA, USA), and 2.5 μ g/ml CpG (Invivogen, San Diego, CA, USA) at 37°C for 1 or 4 days before staining with MEM-55 antibodies labeled with FITC or APC and analyzed using flow cytometry.

Analysis of CD45-exon expression

RNA was prepared from sorted cells using a Micro RNA kit (Qiagen, Hilden, Germany), and cDNA was prepared using Superscript III (Invitrogen). PCR reactions were performed over the exon 4–6 region that is differentially spliced as described [23] except for using Phusion enzyme (Finnzyme, Espoo, Finland) instead of Taq. The PCR reactions were separated on a 1.5% agarose TBE gel, and ethidium bromide-stained gels were visualized using a Chemidoc XLR system (BioRad, Hercules, CA, USA). Gels were subsequently alkali-blotted onto Hybond XL membranes (GE Healthcare) and were incubated with ³²P-labeled probes as described [23].

Membranes were developed using storage phosphor screens and a PharosFX Plus system and were analyzed using the Quantity One software package (BioRad).

Immunoprecipitation and Western blotting

Mononuclear cells were isolated from a healthy donor using Ficoll-Paque (GE Healthcare), or Hmy2 cells, grown in RPMI, complemented with 10% FCS (Invitrogen), were harvested. Cell extracts were then prepared in RIPA buffer according to standard procedures or were sorted directly into 4× SDS load buffer (Invitrogen). In some cases, the extracts were then treated with sialdiase (Sigma-Aldrich). Immunoprecipitation was performed using protein-G Dynabeads as described by the manufacturer (Invitrogen) using a polyclonal anti-CD45 antibody (H-230; Santa Cruz Biotechnology, Santa Cruz, CA, USA). Extracts or proteins eluted after immunoprecipitation were separated on Novex 3–8% Tris-acetate gels (Invitrogen) and were transferred to ECL membranes (GE Healthcare) according to the manufacturer's instructions. After labeling with antibodies (Supplemental Material), the blots were developed using ECL Advance reagent (GE Healthcare).

Immunohistochemistry

Expression of CD45RB^{MEM55}, CD45RB^{MEM143}, and CD3 was visualized in 3 μ m paraffin sections of three different specimens of human tonsils and two each of terminal ileum, colon, and appendix using mouse antihuman mAb (Supplemental Material) after antigen retrieval using antigen retrieval solution (Dako, Glostrup, Denmark). Staining controls were species- and concentration-matched antibodies with other specificities. Antibody reactivity was visualized with the EnVision kit (Dako) and developed with DAB Plus (Dako). Double-stained slides were visualized with a double-staining EnVision system (Dako). For immunofluorescent staining of cells on microscope slides, sorted cells were cytopinned onto slides, acetone-fixed, air-dried, and rehydrated in PBS before they were incubated with the indicated FITC-conjugated antibodies and photographed using a fluorescent microscope (Leica, Wetzlar, Germany).

RESULTS

The CD45RB-specific MEM-55 antibody, but not other CD45RB-reactive antibodies, defines human B cell subsets in peripheral blood

In an effort to identify novel B cell subset markers, mononuclear cells were isolated from human peripheral blood and stained with anti-CD19, anti-CD27, and anti-IgM antibodies, together with mAb clones against other cell surface antigens, to determine their respective expression on CD27[−] naïve B cells, CD27⁺IgM[−] class-switched memory B cells, and CD27⁺IgM⁺ marginal zone-like memory B cells using flow cytometry (Supplemental Material). A majority of the more than 75 antibodies tested did not show any labeling differences during B cell differentiation, as their respective epitopes were absent or uniformly expressed through B cell differentiation. Others divided one or more B cell subset into subpopulations or gave minor differences between memory and naïve cells. One, the MEM-55 clone that binds to the RB exon of CD45 [21], however, brightly stained CD27⁺ memory B cells, and naïve CD27[−] cells were not stained (Fig. 1A). Antibodies reactive to other CD45 splice forms did not show any major differences in expression, with the exception of an antibody against the mouse B220 epitope (Fig. 1B). As described previously, this antibody detected a cross-reactive epitope in humans that was expressed on naïve B cells and a proportion of CD27⁺

memory cells [24]. Unexpectedly, two other antibody clones described to bind to the RB exon of CD45, MT4, and MEM-143, did not show any major differential staining between CD27⁺ and CD27[−] B cells (Fig. 1C). When the expression levels detected with the MEM-55, MEM-143, and MT4 antibody clones were compared in five healthy volunteers, the geometrical mean of expression, as determined by flow cytometric analysis, was 10.5 (range 7.7–13.8) times higher in CD27⁺ compared with CD27[−] B cells using the MEM-55 clone but only 1.2 (range 1.1–1.3) and 1.8 (range 1.7–2.0) using the MEM-143 or MT4 clones, respectively. As a consequence, when the expressions of these CD45RB epitopes on B cells were plotted against that of the MEM-55 epitope, their expression did not correlate (Fig. 1C). Thus, one, but not all, epitope on the RB exon of CD45 was differentially expressed during B cell development.

Another B cell subset circulating in blood is CD19⁺CD27^{high}CD38^{bright}CD20[−] plasmablasts [25]. Similarly with memory cells, these late differentiation-stage B cells expressed high levels of the CD45RB^{MEM55} epitope (Fig. 2A). Furthermore, in addition to the major CD27[−]CD45RB^{MEM55low} and CD27⁺CD45RB^{MEM55high} populations, on average, 7.6% of the blood B cells was CD27[−] and still was CD45RB^{MEM55high} (Fig. 1A). To confirm the presence of cells with this phenotype, B cells were sorted into highly purified CD27⁺ and CD27[−] populations and were fixed onto microscope slides before they were stained with the RB exon-reactive clones MEM-55 and MEM-143 (Fig. 2B and C). Both B cell populations expressed high levels of the CD45RB^{MEM143} epitope and CD27⁺ B cells also of the CD45RB^{MEM55} epitope. In line with the flow cytometric analysis, the vast majority of CD27[−] B cells was negative for the CD45RB^{MEM55} epitope, and a minority of them expressed it. Different RB exon-recognizing antibody clones differentially detected CD45 from CD27⁺ and CD27[−] B cells in Western blots (Fig. 2D). Although all antibody clones detected one major band, corresponding to the long CD45RABC form in size, in extracts prepared from sorted CD27⁺ as well as CD27[−] B cells, the amount of the MEM-55 epitope was much lower in CD27[−] versus CD27⁺ cells, whereas similar amounts were detected with the MT4 and MEM-143 clones. Similar expression of CD45 during B cell differentiation was also detected using a pan-CD45 or a CD45RA-recognizing clones. When CD27[−]CD45RB^{MEM55high} B cells were analyzed further using flow cytometry, we found that CD27[−] IgG-expressing memory cells made up ~10% of the CD27[−] CD45RB^{MEM55high} peripheral blood B cells (Fig. 2E) [22, 26]. Most of them did, however, not express switched antibody classes but instead, high levels of IgM and IgD, which differentiated them from CD27[−]CD45RB^{low} naïve B cells that were predominantly IgM^{low}IgD^{high} (Fig. 2E). The class-switched CD27[−] CD45RB^{MEM55high} cells did not seem to correspond to FCRL4-expressing memory cells [27], as only a minor fraction of the IgG-switched cells expressed the marker (Fig. 2F). Based on expression of CD27, IgM, and IgD, the CD27[−] CD45RB^{MEM55high}IgM^{high} B cells were similar to transitional B cells [28, 29]. Similar to what has been described for human memory and transitional B cells [22], the CD27[−] CD45RB^{MEM55high} cells lacked expression of the ABCB-1 transporter protein, as judged by their inability to extrude the dye

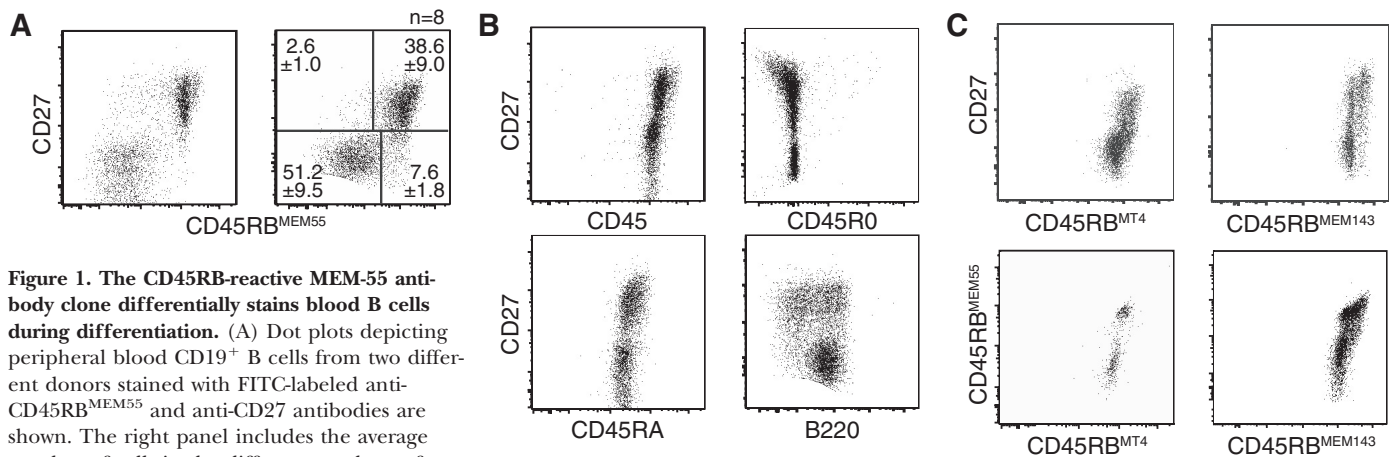


Figure 1. The CD45RB-reactive MEM-55 antibody clone differentially stains blood B cells during differentiation. (A) Dot plots depicting peripheral blood CD19⁺ B cells from two different donors stained with FITC-labeled anti-CD45RB^{MEM55} and anti-CD27 antibodies are shown. The right panel includes the average number of cells in the different quadrants from eight healthy donors with standard deviations indicated. (B) Dot plots depicting CD19⁺ cells stained with four distinct, CD45-reactive antibodies and anti-CD27. (C) The upper panels show dot plots depicting the staining patterns for CD19⁺ B cells using two different CD45RB-reactive antibody clones (MT4 and MEM-143) plotted against CD27, and the lower panels show dot plots depicting the expression of different CD45RB-reactive clones against each other. (B and C) Dot plots show typical results from at least three different donors.

Rhodamine 123 (Fig. 2G). The CD27⁺CD45RB^{MEM55high} cells, however, lacked or had low expression of the transitional B cell markers CD10 and CD38 (Fig. 2H). We have found a population with these characteristics that is enlarged in young children and after bone marrow transplantation, and it seems to represent a previously uncharacterized population of immature, circulating, peripheral B cells or IgM-expressing memory cells (Mellgren, Friskopp, and Bemark, unpublished results).

CD45RB^{MEM55} expression on B cells is regulated during tonsillar differentiation

Jackson et al. [20] recently described that CD45RB is differentially expressed during tonsillar B cell differentiation. To verify that this was the case, we determined the expression patterns of the MEM-55 epitope on tonsillar B cells. B cell subtypes, identified according to the BM1–BM5 system [30], indeed differentially expressed the CD45RB^{MEM55} epitope during tonsillar differentiation, in accordance with what we observed in blood. Essentially, all cells within the BM5 gate (mainly class-switched memory B cells) expressed high levels of the CD45RB^{MEM55} epitope but not the GC/transitional marker CD10, and BM1 and BM2 cells (which are mostly naïve B cells but also some CD27⁺IgM⁺ memory cells) did not express CD10 and only rarely high levels of CD45RB^{MEM55} (Fig. 3A). The majority of BM3 and BM4 cells (GC and transitional B cells) expressed the GC/transitional marker CD10, but some of them (10–20%) expressed CD45RB^{MEM55}.

The majority of CD27⁺ B cells in tonsils is highly mutated cells with a memory phenotype, whereas CD27⁻ tonsillar B cells are unmutated, naïve cells or mutated cells with a GC phenotype [31]. When the expression of CD27 and CD45RB^{MEM55} was compared with each other, it divided the tonsillar B cells into similar subpopulations to those identified in blood, and the majority of the cells was CD45RB^{MEM55low}CD27⁺ or CD45RB^{MEM55low}CD27⁻ (Fig. 3B). A population of CD27⁻CD45RB^{MEM55high} B cells similar to that in blood was also detected in tonsils. B cells that expressed CD27 in the absence of

CD45RB^{MEM55} were, however, much more common in tonsils than in blood. Approximately half of these expressed high levels of the GC/transitional B cell marker CD10, indicating that these may be situated within GCs (Fig. 3B). As in blood, neither MT4 nor MEM-143 differentially stained tonsillar B cells, showing that the only CD45RB epitope that was regulated during peripheral B cell differentiation was MEM-55 (Fig. 3C). Plasmablasts identified based on their CD19⁺CD27^{high}CD38^{bright}CD20⁻ phenotype expressed high levels of CD45RB^{MEM55} (Fig. 3D), and in tonsils, FCRL4-expressing memory B cells were enriched among CD45RB^{MEM55high} B cells (Fig. 3E).

The gate for CD38^{high} GC cells in Fig. 3A includes several distinct differentiation stages, including BM2⁺, BM3, and BM4 cells. To better elucidate during which stages CD45RB^{MEM55} was expressed, a phenotypical characterization was made of CD38^{high}CD45RB^{MEM55high} tonsillar B cells. Based on FSC analysis, the cells were as large as GC B cells, and based on SSC analysis, their cytoplasm was more complex than any other of the B cell subtypes (Fig. 3F). Although high expression of CD10 and CD95 and low expression of CD21 and CD23 also correlated with that found on CD38^{high}CD45RB^{MEM55low} cells, the CD38^{high}CD45RB^{MEM55high} cells could be clearly differentiated from these based on their expression of CD44 and IgM. Notably, several markers, including IgD, Ki67, CD5, CD24, and CD27, showed bimodal expression patterns on CD38^{high}CD45RB^{MEM55high} cells, indicating that the population was not homogenous.

As the expression of the CD45RB^{MEM55} epitope was complex on tonsillar B cells, and it is impossible to elucidate the localization of cells using flow cytometry, we determined the organization of CD45RB^{MEM55}-expressing cells in tonsil tissue sections using immunohistochemistry. Within tonsillar GC, few cells (<5%) expressed any CD45RB^{MEM55}, and these rare cells localized preferentially to the light zones, where centrocytes and T cells are concentrated (Fig. 4A and B). Surrounding the GCs, at the outer border of the FM, was a ring of cells expressing high levels of the CD45RB^{MEM55} epitope, and a lower proportion of cells expressed the antigen in the FM closer to the

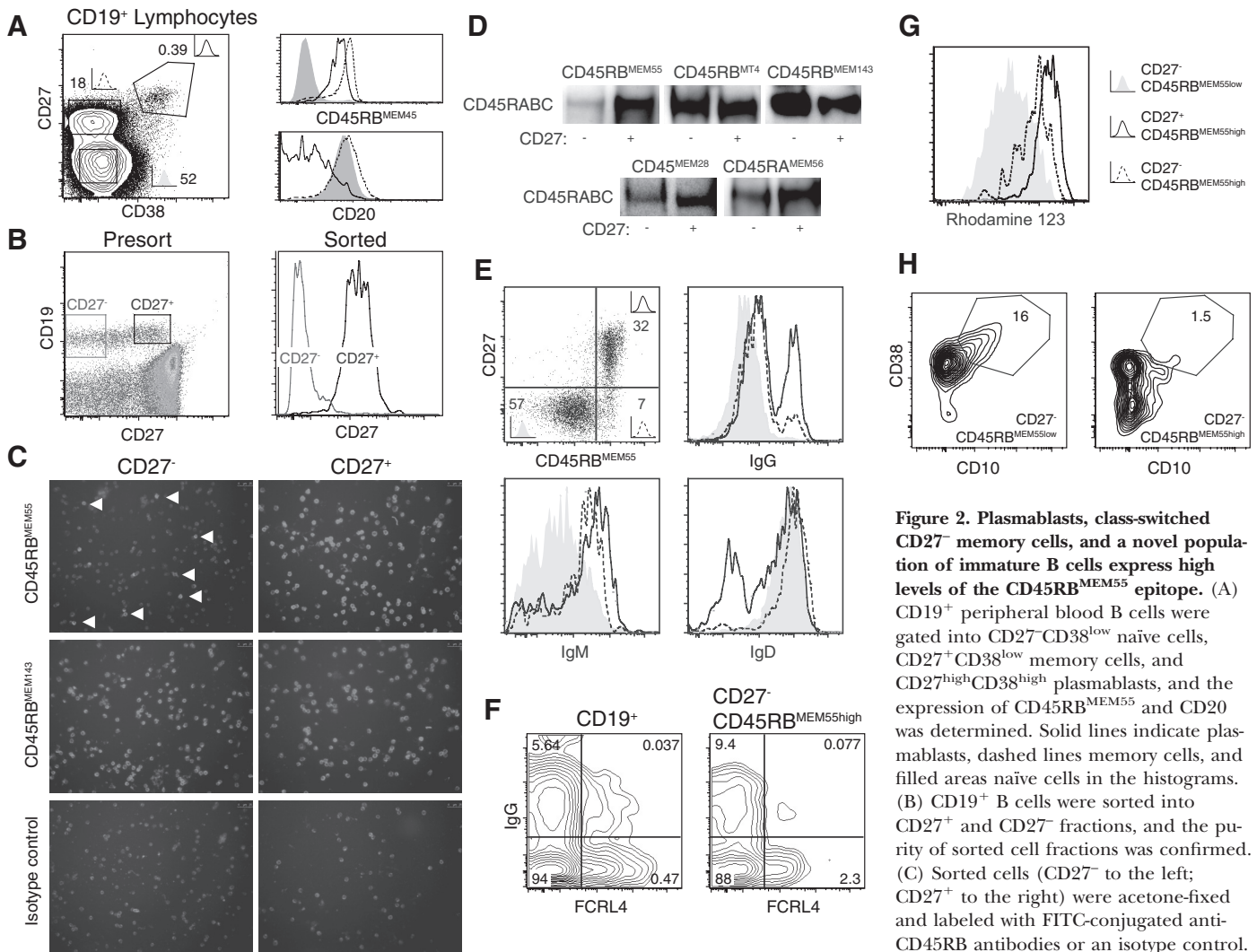


Figure 2. Plasmablasts, class-switched CD27⁻ memory cells, and a novel population of immature B cells express high levels of the CD45RB^{MEM55} epitope. (A) CD19⁺ peripheral blood B cells were gated into CD27⁻CD38^{low} naive cells, CD27⁺CD38^{low} memory cells, and CD27^{high}CD38^{high} plasmablasts, and the expression of CD45RB^{MEM55} and CD20 was determined. Solid lines indicate plasmablasts, dashed lines memory cells, and filled areas naive cells in the histograms. (B) CD19⁺ B cells were sorted into CD27⁺ and CD27⁻ fractions, and the purity of sorted cell fractions was confirmed. (C) Sorted cells (CD27⁻ to the left; CD27⁺ to the right) were acetone-fixed and labeled with FITC-conjugated anti-CD45RB antibodies or an isotype control. White arrowheads indicate MEM-55-binding. (D) The expression patterns of different CD45RB epitopes were determined in sorted CD27⁺ and CD27⁻ peripheral blood B cells using Western blot analysis with the indicated primary antibodies against the RB exon or other CD45 epitopes. The CD27⁺ and CD27⁻ B cells were sorted directly into 4× SDS loading buffer to a final concentration approximately 7.5 × 10⁵ cells/ml, and the same amounts of the extract were loaded into each well. The band corresponded to CD45RABC in size. (E) CD19⁺ PBMC B cells were gated based on expression of CD45RB^{MEM55} and CD27 (upper left panel) and were analyzed for expression of IgG (upper right panel), IgM (lower left panel), and IgD (lower right panel). The antibody class expression in CD45RB^{MEM55low}CD27⁻ naive (filled), CD45RB^{MEM55high}CD27⁺ memory (solid line), and CD45RB^{MEM55high}CD27⁻ B cells (dashed line) is indicated in the histograms. (F) The expression of IgG and FCRL4 was determined on CD19⁺ B cells (left) or CD19⁺CD27⁻CD45RB^{MEM55high} B cells (right). The percentages of cells falling into the different quadrants are indicated. (G) The ability to extrude the dye Rhodamine 123 (a sign of expression of the ABCB-1 transporter) was determined in populations defined in E. (H) The expression levels of CD38 and CD10 were determined on cells from the two CD27⁻ quadrants in E. The percentages of CD38^{high}CD10⁺ transitional cells are indicated in each plot.

ing CD27⁻ cells. Cells sorted from two different donors gave similar results. (D) The expression patterns of different CD45RB epitopes were determined in sorted CD27⁺ and CD27⁻ peripheral blood B cells using Western blot analysis with the indicated primary antibodies against the RB exon or other CD45 epitopes. The CD27⁺ and CD27⁻ B cells were sorted directly into 4× SDS loading buffer to a final concentration approximately 7.5 × 10⁵ cells/ml, and the same amounts of the extract were loaded into each well. The band corresponded to CD45RABC in size. (E) CD19⁺ PBMC B cells were gated based on expression of CD45RB^{MEM55} and CD27 (upper left panel) and were analyzed for expression of IgG (upper right panel), IgM (lower left panel), and IgD (lower right panel). The antibody class expression in CD45RB^{MEM55low}CD27⁻ naive (filled), CD45RB^{MEM55high}CD27⁺ memory (solid line), and CD45RB^{MEM55high}CD27⁻ B cells (dashed line) is indicated in the histograms. (F) The expression of IgG and FCRL4 was determined on CD19⁺ B cells (left) or CD19⁺CD27⁻CD45RB^{MEM55high} B cells (right). The percentages of cells falling into the different quadrants are indicated. (G) The ability to extrude the dye Rhodamine 123 (a sign of expression of the ABCB-1 transporter) was determined in populations defined in E. (H) The expression levels of CD38 and CD10 were determined on cells from the two CD27⁻ quadrants in E. The percentages of CD38^{high}CD10⁺ transitional cells are indicated in each plot.

GCs (Fig. 4A and B). Cells in the T cell zones expressed high levels of the CD45RB^{MEM55} epitope. A similar staining pattern was observed within GALT GCs from appendix, ileum, and colon, and scarce CD45RB^{MEM55}-expressing GC cells primarily within the light zones (Fig. 4C). However, in GALT, a larger proportion of FM cells expressed high levels than in tonsils. Thus, B cells expressing different levels of CD45RB^{MEM55} are differentially located within lymphoid tissue. Notably, the staining patterns observed, in tonsils and GALT, are in very good agreement with what has been described for CD27 [32].

The scattered expression of CD45RB^{MEM55} on rare cells within the light zones of the GCs suggested that CD45RB^{MEM55high} cells in GCs could be centrocytes or T cells. Costaining with an anti-CD3 antibody showed that CD45RB^{MEM55}-expressing GC cells were almost exclusively T cells. In addition, some CD3-expressing T cells that did not express CD45RB^{MEM55} were observed within the GCs, whereas no CD45RB^{MEM55}-expressing cells that did not express CD3 were present (Fig. 4D). In line with what had been observed in blood and tonsils using flow cytometry, the MEM-143 CD45RB-reactive antibody stained essentially all cells in the FM

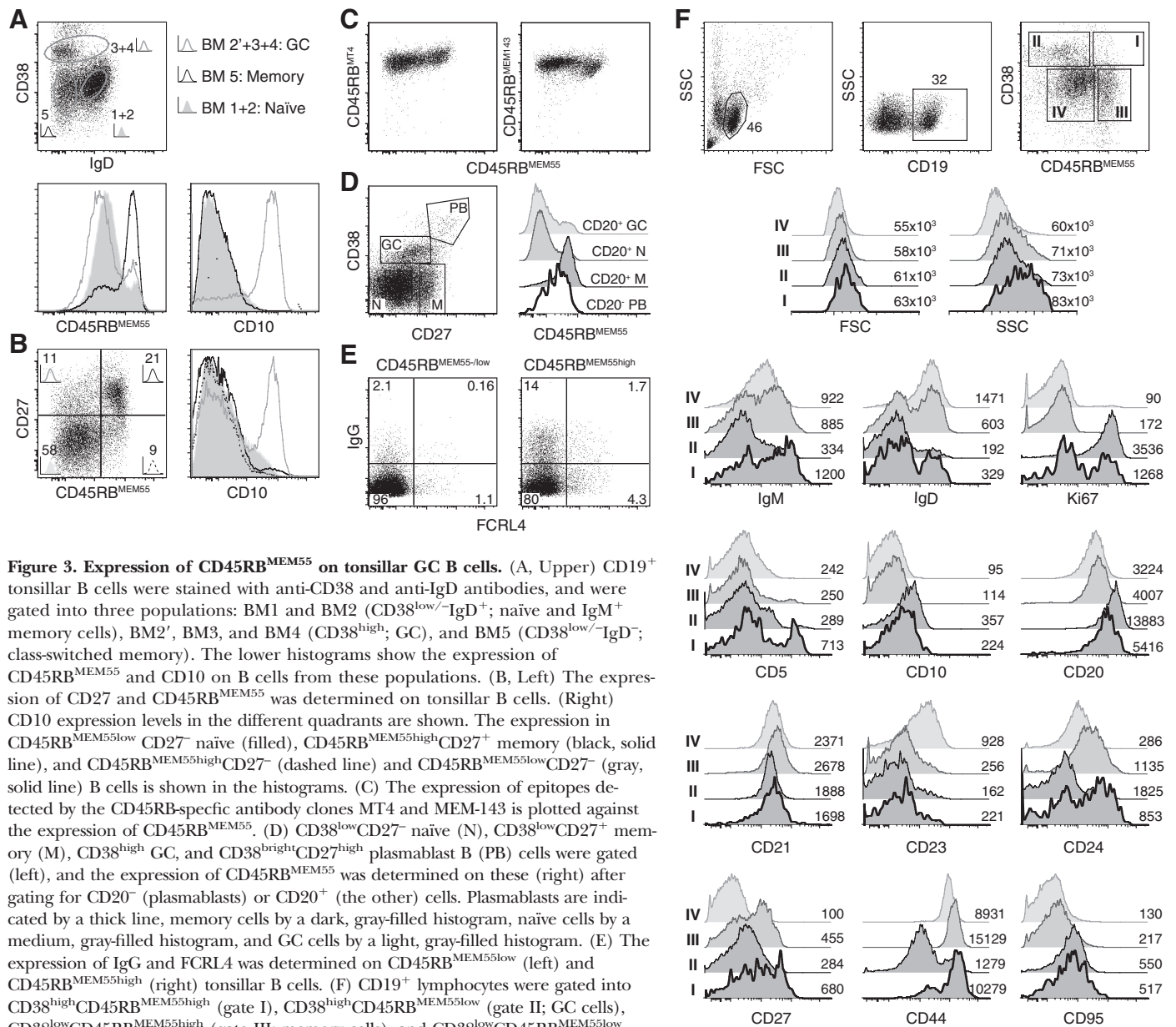


Figure 3. Expression of CD45RB^{MEM55} on tonsillar GC B cells. (A, Upper) CD19⁺ tonsillar B cells were stained with anti-CD38 and anti-IgD antibodies, and were gated into three populations: BM1 and BM2 (CD38^{low/-}IgD⁺; naïve and IgM⁺ memory cells), BM2', BM3, and BM4 (CD38^{high}, GC), and BM5 (CD38^{low/-}IgD⁻; class-switched memory). The lower histograms show the expression of CD45RB^{MEM55} and CD10 on B cells from these populations. (B, Left) The expression of CD27 and CD45RB^{MEM55} was determined on tonsillar B cells. (Right) CD10 expression levels in the different quadrants are shown. The expression in CD45RB^{MEM55low} CD27⁻ naïve (filled), CD45RB^{MEM55high} CD27⁺ memory (black, solid line), and CD45RB^{MEM55high} CD27⁻ (dashed line) and CD45RB^{MEM55low} CD27⁻ (gray, solid line) B cells is shown in the histograms. (C) The expression of epitopes detected by the CD45RB-specific antibody clones MT4 and MEM-143 is plotted against the expression of CD45RB^{MEM55}. (D) CD38^{low}CD27⁻ naïve (N), CD38^{low}CD27⁺ memory (M), CD38^{high} GC, and CD38^{bright}CD27^{high} plasmablast B (PB) cells were gated (left), and the expression of CD45RB^{MEM55} was determined on these (right) after gating for CD20⁻ (plasmablasts) or CD20⁺ (the other) cells. Plasmablasts are indicated by a thick line, memory cells by a dark, gray-filled histogram, naïve cells by a medium, gray-filled histogram, and GC cells by a light, gray-filled histogram. (E) The expression of IgG and FCRL4 was determined on CD45RB^{MEM55low} (left) and CD45RB^{MEM55high} (right) tonsillar B cells. (F) CD19⁺ lymphocytes were gated into CD38^{high}CD45RB^{MEM55high} (gate I), CD38^{high}CD45RB^{MEM55low} (gate II; GC cells), CD38^{low}CD45RB^{MEM55high} (gate III; memory cells), and CD38^{low}CD45RB^{MEM55low} (gate IV; naïve cells) cells. The FSC, SSC, and fluorescent profiles within the gates are shown and the mean fluorescent intensity (FSC/SSC) and mean geometrical mean (the other) indicated. In the case of the Ki67 staining, the cells were fixed and permeabilized before staining with the Ki67 antibody. All panels show one representative experiment out of three.

and the GC, thus resulting in a distinct staining pattern compared with the MEM-55 antibody (Fig. 4E). Costaining of cells using both antibodies together confirmed that many cells indeed were stained with the MEM-143 but not MEM-55 antibody clone (Fig. 4F). These data indicated that CD38^{high}CD45RB^{MEM55} B cells identified using flow cytometry (Fig. 3) were not located primarily within the GC. In an effort to identify their localization, tonsil sections were stained with anti-CD38 antibodies and were developed to be able to distinguish between CD38 high and low cells as positive and negative cells, respectively, together with the MEM-55 antibody clone. As expected, CD38^{high} B cells within the GC were positive for CD38 (Fig. 4G). This was also true for cells within the FM, in particular within the outer part. No costaining

was, however, observed within GC. Rather, cells at the border between the GC and FM (Fig. 4H) or at the outer FM (Fig. 4I) co-expressed the markers. Thus, our immunohistochemistry data did not find any evidence for CD45RB^{MEM55} expression by GC B cells, despite that some CD38^{high} B cells expressed it using flow cytometry, and they also confirmed that only the MEM-55 RB epitope on CD45 was differentially expressed on human B cells.

The CD45RB^{MEM55} epitope is not regulated during TLR9-mediated activation of human B cells

The expression of the MEM-55 CD45RB epitope did not seem to correlate directly with activation or proliferation of B cells.

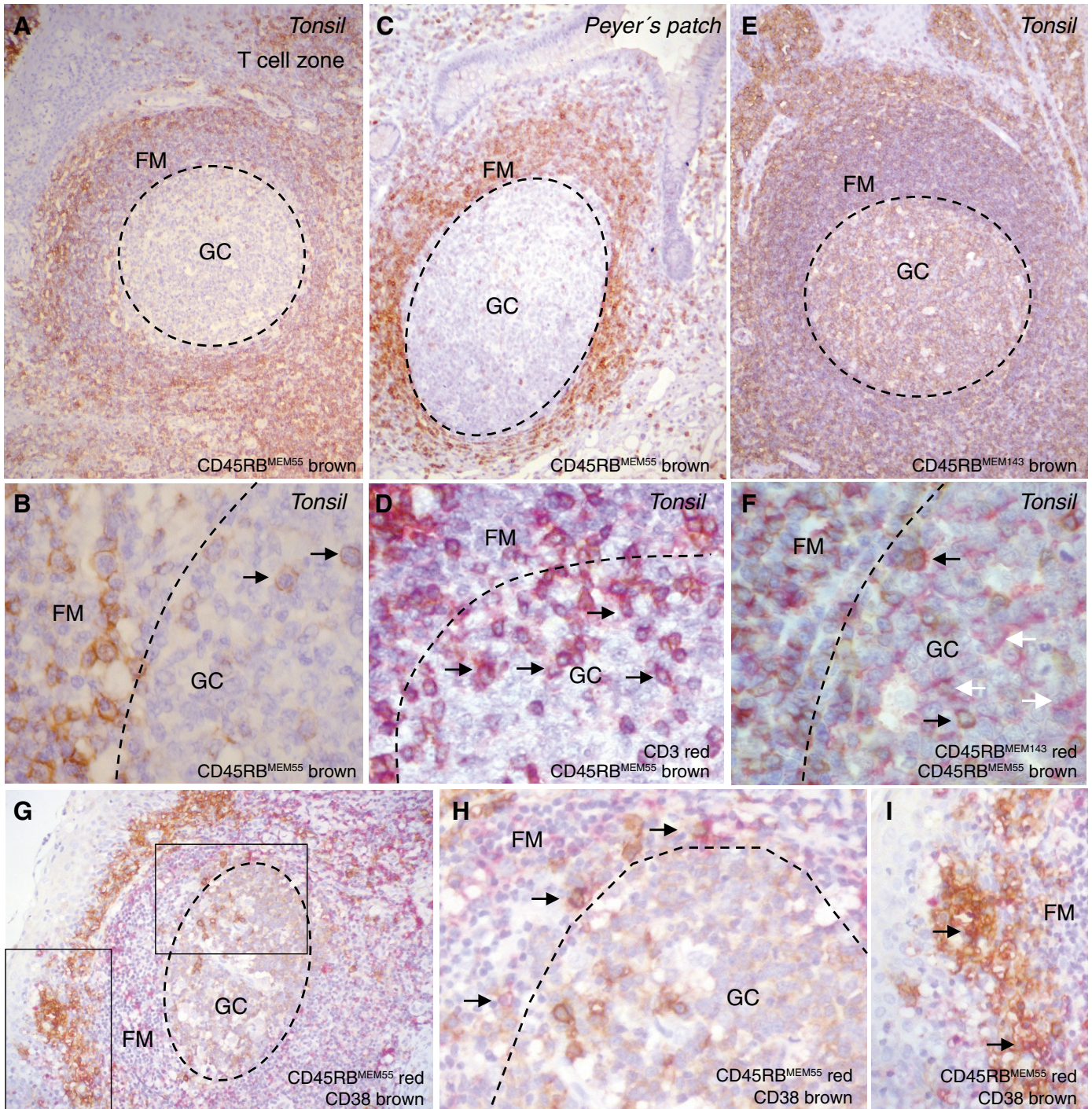


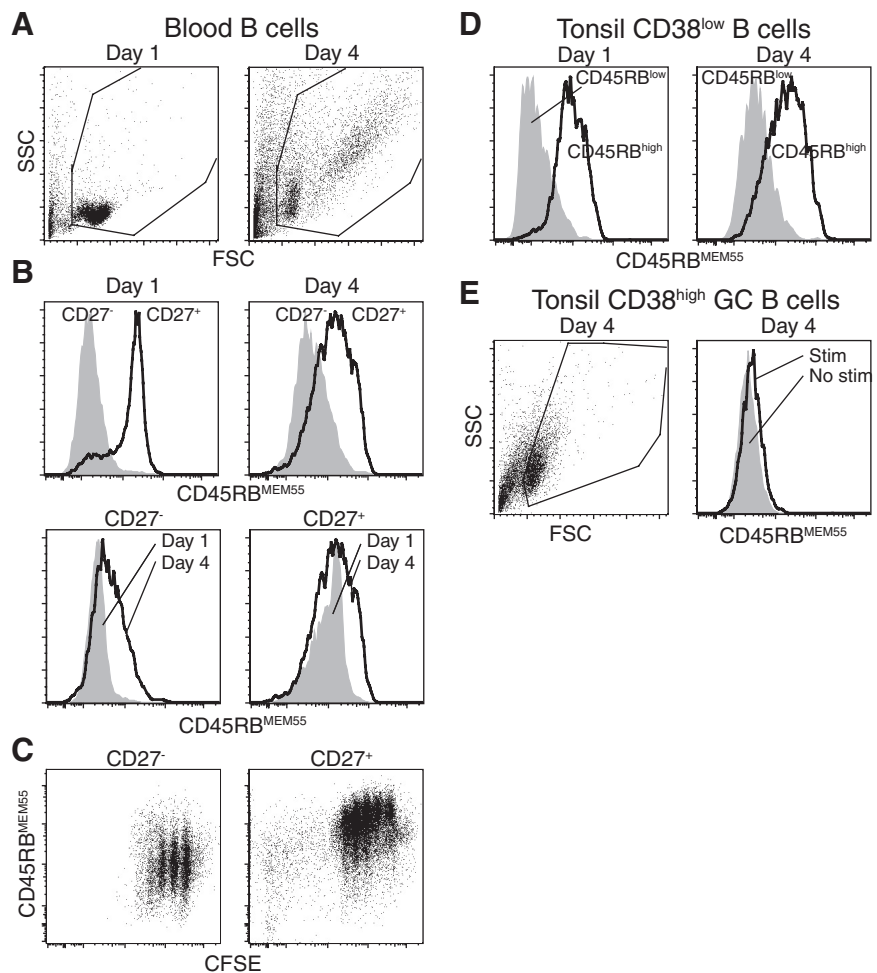
Figure 4. Organization of CD45RB-expressing cells in lymphoid tissue. (A) A representative tonsil section analyzed for expression of CD45RB^{MEM55} using immunohistochemistry. Expression is observed in the FM zone, and more cells express it far from the GC than close to it. Inside GCs, rare cells within the light zone expressed the marker. (B) A close-up with CD45RB^{MEM55}-expressing GC cells marked with arrows. (C) A representative ileal Peyer's patch section analyzed for expression of CD45RB^{MEM55}. (D) A tonsillar section costained for expression of CD3 (red) and CD45RB^{MEM55} (brown). The arrows point at GC cells coexpressing the markers. No CD45RB^{MEM55}-only-expressing cells could be identified. (E) A tonsil section stained with the CD45RB-recognizing MEM-143 antibody clone. (F) A close-up of a section costained with the two CD45RB-recognizing clones (MEM-143 in red; MEM-55 in brown). Black arrows point at cells that coexpress the two epitopes and white arrows at cells that only express the MEM-143 epitope. (G) A tonsil section stained with anti-CD38 (brown) and anti-CD45RB^{MEM55} (red). Two areas marked with squares are shown at a higher magnification in H and I. In these, cells that coexpress CD38 and CD45RB^{MEM55} are indicated with black arrows. The sections represent typical results from three tonsils and six GALT tissues examined.

It was, however, still possible that activation of cells would influence its expression. To test this, CD27⁺ and CD27⁻ B cells were sorted from peripheral blood and were stimulated with anti-Ig, CpG, and IL-2. This resulted in that most of the B cells blasted within 4 days, from CD27⁻ and CD27⁺ cultures (Fig. 5A). As expected, most CD27⁻ B cells were CD45RB^{MEM55low} after 1 day of stimulation, and CD27⁺ B cells were CD45RB^{MEM55high} (Fig. 5B, upper left). These differences were maintained after 4 days (Fig. 5B, upper right). A slight up-regulation of the levels of the CD45RB^{MEM55} epitope was observed on CD27⁻ cells when Days 1 and 4 were compared, and a slight widening of the peak was observed on CD27⁺ cells (Fig. 5B, lower). Notably, neither population showed any major differences in CD45RB expression, depending on the number of cell cycles that the cells had completed (Fig. 5C). Using identical stimulation conditions, sorted CD38^{low}CD45RB^{MEM55low} and CD38^{low}CD45RB^{MEM55high} tonsillar B cells showed similar blasting responses and expressions of CD45RB^{MEM55} as peripheral blood B cells (Fig. 5D, and data not shown). CD38^{high}CD45RB^{MEM55low} GC cells did not blast using these stimulatory conditions and also did not change their expression of CD45RB depending on stimulation (Fig. 5E).

Differential CD45RB^{MEM55} expression on human B cells is a result of post-translational modifications of CD45

The different staining patterns observed between antibody clones reactive to CD45RB suggested that the expression of the MEM-55 epitope was regulated through a post-translational mechanism rather than through differential splicing. To further confirm this, RNA samples were prepared from peripheral blood B cells sorted based on CD27 and CD45RB^{MEM55} expression to determine if there were any differences in CD45 splice forms expressed as detected by RT-PCR (Fig. 6A). A bit unexpectedly, given that only a single band was detected in Western blots with extracts from CD27⁺ and CD27⁻ B cells (Fig. 2D), several CD45 splice forms were detected in peripheral blood B cells (Fig. 6B and C). Notably, these did not change whether cells expressed high and low levels of the CD45RB^{MEM55} epitope. The RB exon was, however, present in all major CD45 transcripts in B cells, except for the shortest form coding for CD45R0 (Fig. 6D). Thus, although only a single CD45 protein can be detected in peripheral blood B cells using Western blots, they seem to express several splice forms at the RNA levels.

Figure 5. The expression of the CD45RB^{MEM55} epitope does not change during activation and proliferation of B cells. (A) The FSC/SSC profile was analyzed for blasting of CD27⁻ cells that had been stimulated with anti-Ig, IL-2, and CpG for 1 (left) or 4 (right) days. Similar results were achieved after stimulation of CD27⁺ cells (data not shown). (B) The expression of CD45RB^{MEM55} was determined on B cells sorted based on CD27 expression. (Upper) The expression was compared between CD27⁺ and CD27⁻ cells stimulated for 1 day (left) or 4 days (right). (Lower) The expression of CD45RB^{MEM55} at 1 day and 4 days was compared in CD27⁻ (left) and CD27⁺ (right) cells. (C) The expression of CD45RB^{MEM55} in B cells that had gone through different numbers of cell cycles was determined in cells labeled with CFSE. (D) CD38^{low} B cells isolated from tonsils were sorted into CD45RB^{MEM55high} and CD45RB^{MEM55low} populations, and the expression of CD45RB^{MEM55} was determined at Days 1 and 4. At Day 4, cells within the two populations had blasted (data not shown). CD38^{high} cells did not blast even after 4 days (E, left), and CD38^{high} cells that had been stimulated did not show any difference in their expression of CD45RB^{MEM55} (Stim) compared with nonsimulated (No stim) cells (right). All panels show typical results when the experiments were repeated three times using three different donors.



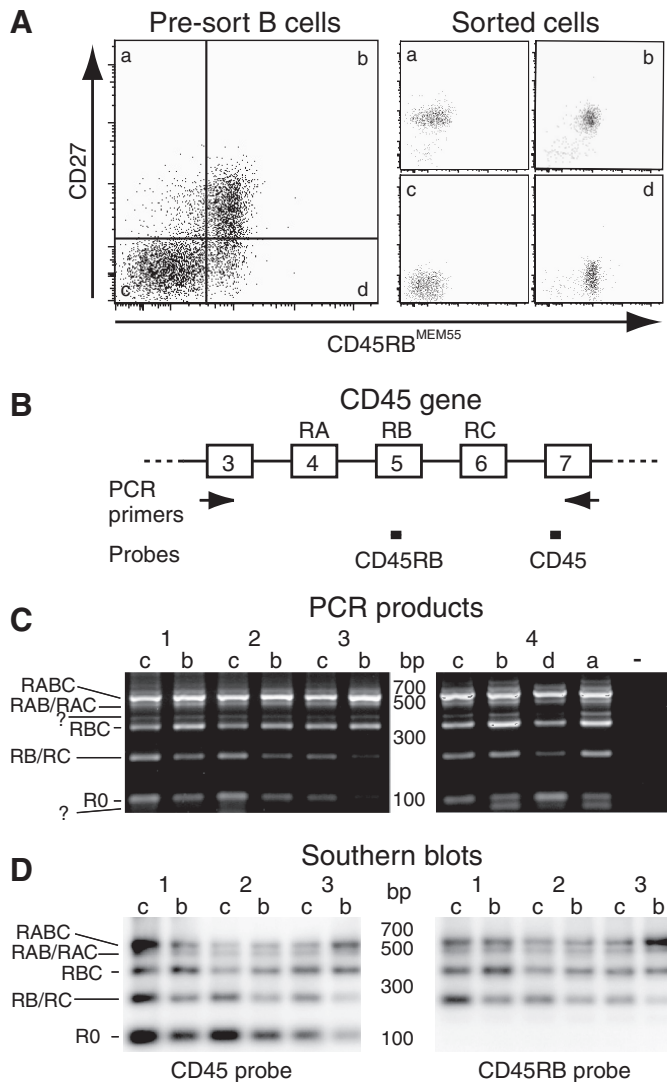


Figure 6. The expression of different CD45 splice forms does not change during peripheral B cell differentiation. (A) Cell sorting of peripheral blood CD19⁺ B cells into four populations: CD27⁺CD45RB^{MEM55}^{low} (a), CD27⁺CD45RB^{MEM55}^{high} (b), CD27⁻CD45RB^{MEM55}^{low} (c), and CD27⁻CD45RB^{MEM55}^{high} (d). The left dot plot shows presorted cells and the panels to the right the sorted populations. (B) Schematically differentially spliced exons in CD45 and PCR primers and Southern blot probes used. (C) Ethidium bromide-stained agarose gels on which RT-PCR reactions spanning the differentially spliced region have been separated. To the left, cDNA prepared from CD27⁺CD45RB^{MEM55}^{high} (b) and CD27⁻CD45RB^{MEM55}^{low} (c) cells isolated from three healthy individuals is shown and to the right, an experiment where all four quadrants were analyzed. –, A negative PCR control lane without any cDNA added. Two bands in the primary PCR—one below the R0 band and one between RBC and RAB/RAC—did not hybridize with the pan-CD45 probe and are indicated by question marks. (D) The left gel in C was analyzed using Southern blotting with a probe binding to all CD45 splice forms (left) or a probe specific for CD45RB (right). The predicted mobilities of the different splice forms (R0, 95 bp; RB, 236 bp; RC, 239 bp; RA, 293 bp; RBC, 380 bp; RAB, 434 bp; RAC, 437 bp; RABC, 577 bp) and the binding of the probes were used to determine the relative mobilities of these splice forms.

Differential expression of CD45RB^{MEM55} during B cell development is a result of changes in O-linked glycosylation patterns

The post-translational modification most likely to change during B cell differentiation was glycosylation. As an initial test to determine if this may be the case, terminal sialic acids were removed from glycochains on PBMCs using sialidase treatment, before the expression of the MEM-55-detected epitope was determined on gated B cells using a flow cytometer. Sialidase treatment of the cells abolished binding of the MEM-55 antibody, showing that the binding was dependent on the presence of terminal sialic acids on glycochains (Fig. 7A, left). Interestingly, this was also the case for the MT4 antibody clone, which detected an epitope that was not differentially expressed during B cell differentiation (Fig. 7A, middle). The binding of MEM-143, which has been described to bind the protein backbone of CD45RB, was, as expected, not affected by sialidase treatment (Fig. 7A, right). Western blot analysis using the MEM-55 antibody clone on extracts prepared from a human myeloma cell line (Hmy-2), which similar to blood B lymphocytes, expresses a single, RB-containing CD45 isoform, confirmed that the MEM-55 antibody was specific for a single protein and not unspecifically bound to glycosylated proteins, as a single band of the same mobility as that detected by a pan-CD45-reactive antibody MEM-28 was observed (Fig. 7B). The MEM-55-binding protein was immunoprecipitated with a pan-CD45-reactive polyclonal rabbit antibody but not by empty precipitation beads, confirming that the detected band was CD45 (Fig. 7B). When human PBMCs were used instead as a source for protein, three reactive bands, representing CD45RB, CD45RAB/CD45RBC (only differing 1 aa in size), and CD45RABC forms, were detected in Western blots probed using the MEM-55 or MEM-143 CD45RB-reactive antibody clones (Fig. 7C). A similar staining pattern was observed using the pan-CD45 mAb MEM-28, but this clone also detected a faster migrating CD45R0 band that had the same mobility as that detected by the CD45R0-specific antibody UCHL1 (Fig. 7C). Similarly as when cells were analyzed by flow cytometry, sialidase treatment of extracts prevented binding of the MEM-55 antibody in Western blots, and it did not influence the pan-CD45-reactive MEM-28 antibody (Fig. 7C, right).

Glycosylation can occur at asparagine residues in the amino acid context Asn-X-Ser/Thr, and X is anything but proline (N-linked glycosylation) or at serine and threonine residues with less-defined acceptor sites (O-linked glycosylation). The CD45RB exon does not contain any sites for N-linked glycosylation but several serine and threonine residues with less-defined acceptor sites (O-linked glycosylation). Four of the threonines were predicted as O-linked glycosylation sites based on comparisons with known glycosylation sites using the NetOGlyc 3.1 program [33], although this does not exclude that other serines and threonines may also be glycosylated (Fig. 7D). Thus, the binding of the MEM-55 antibody clone to CD45RB is dependent on the presence of terminal sialic acids on glycochains, and these glycochains must be attached through O-linked glycosylation.

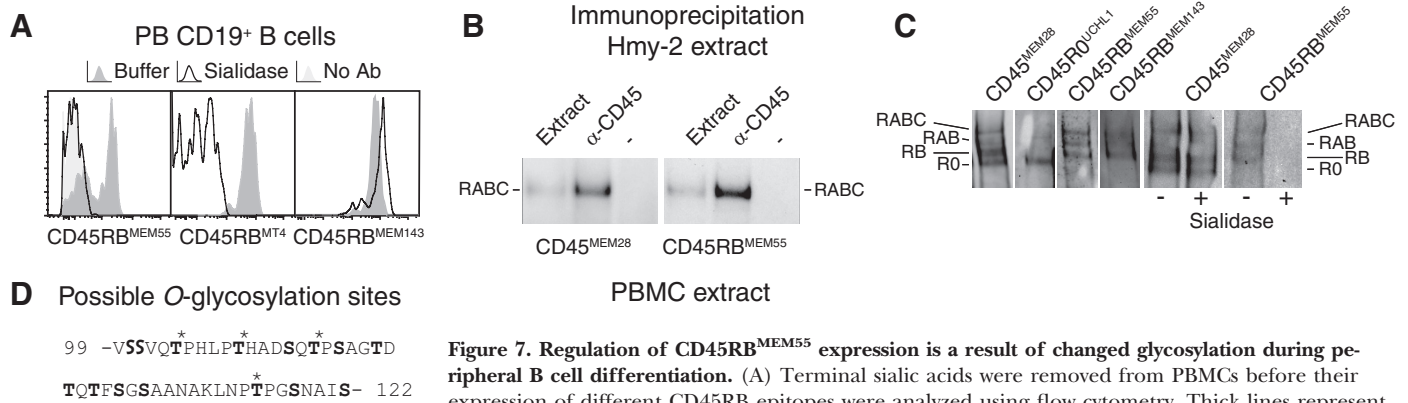


Figure 7. Regulation of CD45RB^{MEM55} expression is a result of changed glycosylation during peripheral B cell differentiation. (A) Terminal sialic acids were removed from PBMCs before their expression of different CD45RB epitopes were analyzed using flow cytometry. Thick lines represent cells treated with sialidase, and dark-gray histograms mock samples incubated in buffer. The light-gray histogram for the MEM-55 antibody represents unstained cells. (B) A representative of Western blots labeled with the pan-CD45 MEM-28 mAb (left) or the CD45RB-reactive MEM-55 antibody (right) using extracts from the human Hmy-2 myeloma cell line. The first lane contains cellular extract and the second and third proteins immunoprecipitated using a rabbit polyclonal pan-CD45 antibody (α -CD45) or empty beads. (C) Representative Western blots using PBMC extracts and the indicated mAb clones for detection to the left. The right lanes show extracts that had been preincubated with sialidase or not before gel electrophoresis. Deduced mobilities of different CD45 splice forms based on the antibody-staining patterns presented here and after sorting of CD4⁺ T cells (see Fig. 8) are indicated. (D) The full amino acid sequence of the human CD45RB exon is shown with serines and threonines in bold. *, Putative mucin type GalNAc O-glycosylation sites.

The expression of CD45RB is regulated through glycosylation and splicing in other hematopoietic cell lineages

The presence of CD45RB^{MEM55}-expressing and -nonexpressing T cells within GCs suggested that the expression of CD45RB was also regulated in T cells. Changes of CD45 expression in T cell have been attributed earlier to differential splicing of the CD45 transcript [34]. This does, however, not exclude the possibility that T cells may also regulate the glycosylation pattern on CD45RB. Using the antibody clones that detect different CD45RB epitopes (MEM-55, MT4, and MEM-143), we addressed whether CD45RB^{MEM55} glycosylation was regulated in T cells as in B cells. This showed that most CD8⁺ T cells expressed high levels of CD45RB but that some, akin to B cells, regulated their expression of CD45RB^{MEM55} while they expressed high levels of other CD45RB epitopes (Fig. 8A). Most CD4⁺ T cells from blood and tonsils also expressed high levels of all CD45RB epitopes, but a minor population expressed lowered levels of all three CD45RB epitopes and maintained high expression of CD45 (Fig. 8A, and data not shown), indicating that these changes were a result of differential splicing.

To quantify the number of cells that regulated glycosylation or differentially spliced CD45RB, the expression levels of CD45RB^{MEM55} and CD45RB^{MEM143} on lymphocytes and monocytes were analyzed in detail in blood samples from healthy donors using flow cytometry. After gating for the respective cell types, secondary gates were set to indicate high, intermediate, or low expression of the CD45RB^{MEM55} epitope in cells expressing high levels of the CD45RB exon or of lowered CD45RB expression as a result of differential splicing (Fig. 8B). As observed previously, B cells were CD45RB^{MEM55high} or CD45RB^{MEM55low} and expressed high levels of the RB exon (Fig. 8C). Most CD4 and CD8 T cells showed high expression of both CD45RB epitopes. A minor proportion of the CD8⁺ cells, however, down-regulated their CD45RB^{MEM55} expression as a result of differential glycosylation, and some CD4⁺ T cells

changed their splicing patterns (Fig. 8C). The changed splicing patterns could be observed in CD4⁺ T cells at the protein level and were confirmed using Western blot analysis of T cells sorted based on expression of CD45R0, CD45RA, and CD45RB^{MT4} (Fig. 8D). Although CD45RA⁺CD45RB^{MT4low}CD4⁺ T cells essentially only expressed the R0 exon, CD45RA⁺CD45RB^{MT4+}CD4⁺ T cells also expressed the RB exon. CD45RA⁺CD45RB^{MT4+}CD4⁺ T cells expressed CD45RAB and CD45RABC forms. Notably, all RB exon-recognizing antibody clones gave identical staining patterns, showing that the MEM-55 epitope can be present on all RB-containing splice forms regardless of surrounding exons. Interestingly, the vast majority of monocytes expressed high levels of the CD45RB exon but did not express the CD45RB^{MEM55} epitope at all (Fig. 8C). Notably, however, rare CD14^{int}/CD16⁺ proinflammatory monocytes expressed intermediate levels of the MEM-55 epitope, raising the possibility that activation of monocytes resulted in increased expression (Fig. 8E). Intermediate expression of CD45RB^{MEM55} was also found in CD16⁺ NK cells, and granulocytes expressed low levels of all CD45RB epitopes and also had a lowered CD45 expression compared with other hematopoietic cell lineages.

DISCUSSION

Here, we show that the expression of a CD45RB-attached, O-linked glycoepitope is strictly regulated during human peripheral B cell development with low expression in naïve and high expression in CD27⁺ and CD27⁻ memory peripheral blood B cells (Fig. 9). The epitope also defines a novel population of IgM^{high} CD27⁻ B cells, which may represent a subtype of transitional or atypical memory B cells that is enlarged in children and after bone marrow transplantation (Mellgren et al., unpublished results). The expression on B cells in secondary lymphoid organs mimicked that in blood, and naïve B cells were CD45RB^{MEM55low} and memory cells CD45RB^{MEM55high}. In addition, we found that plasmablasts were CD45RB^{MEM55high}. Thus, with the exception of the novel cell type, the expression

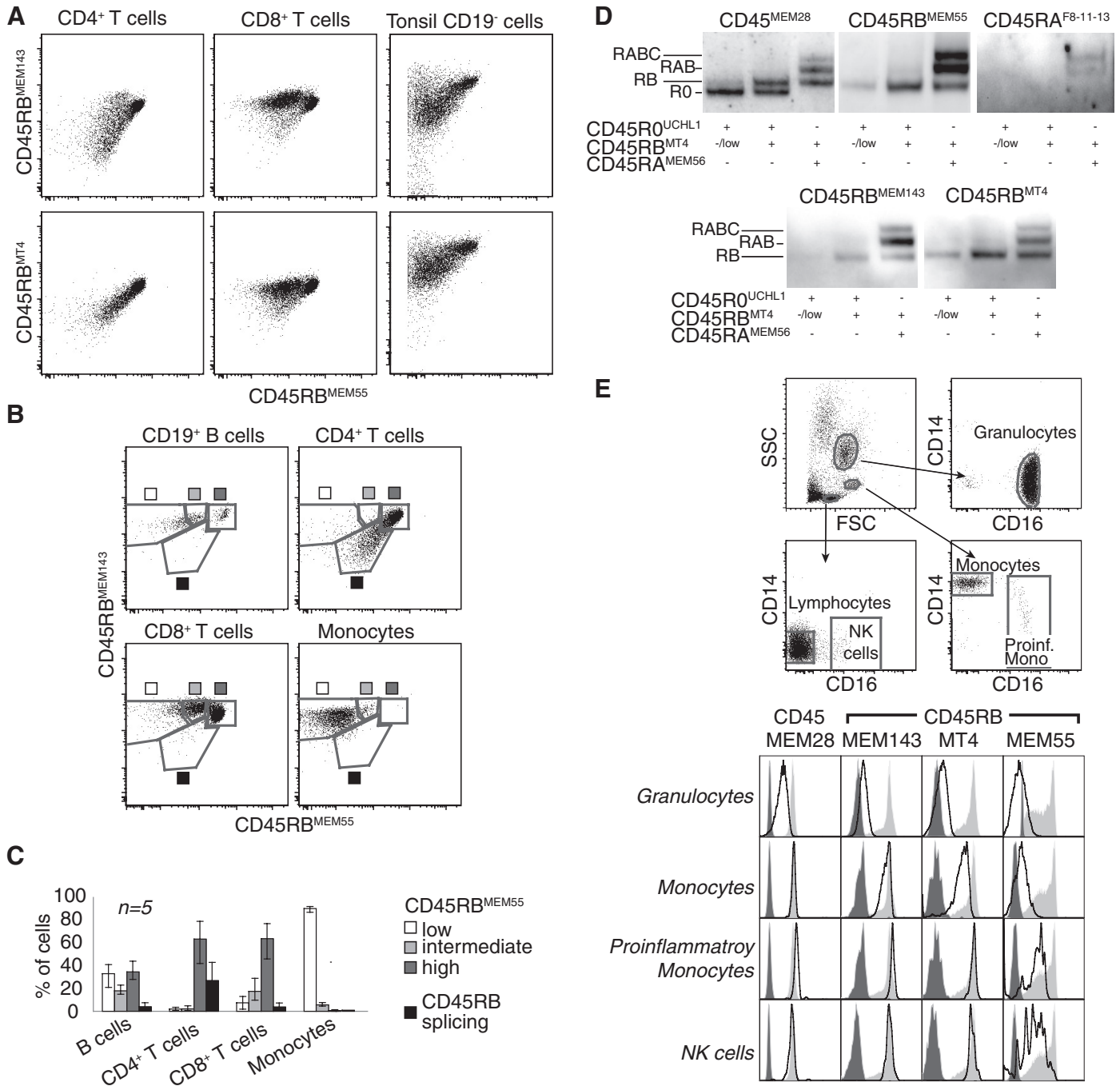


Figure 8. Expression of the CD45RB^{MEM55} epitope on other hematopoietic cell lineages. (A) The expression of different CD45RB epitopes on peripheral blood CD4⁺ and CD8⁺ T cells and tonsil CD19⁺ cells. (B) The mechanisms regulating CD45RB expression in different cell types were analyzed. The indicated gates identified cells expressing high levels of the CD45RB exon (as detected by MEM-143), and low (white), intermediate (light gray), or high (dark gray) levels of the CD45RB^{MEM55} epitope or were CD45RB^{MEM143low}CD45RB^{MEM55low} (black) as a result of differential splicing. (C) The relative proportion of cells having different CD45RB expression patterns in five healthy donors. Standard bars indicate the maximum and minimum among the donors. (D) CD4⁺ T cells were sorted into CD45R0⁺CD45RB^{MT4-/low}CD45RA⁻, CD45R0⁺CD45RB^{MT4+}CD45RA⁻, and CD45R0⁻CD45RB^{MT4+}CD45RA⁺ populations, and whole cell extracts were prepared. The extracts were separated using SDS-gel electrophoresis and proteins were detected by the indicated antibodies in Western blots. Predicted splice forms, as determined by staining patterns and mobilities, are indicated. (E) Peripheral blood cells were divided into granulocytes, monocytes, proinflammatory monocytes, NK cells, and lymphocytes using FSC/SSC and CD14/CD16 gating as indicated, and the expression of CD45 (MEM-28) and different CD45RB epitopes on these cell types was determined. As comparisons, unstained cells are shown as negative controls (dark, gray-filled histograms), and lymphocytes are shown as a positive control (light, gray-filled histograms) in each diagram.

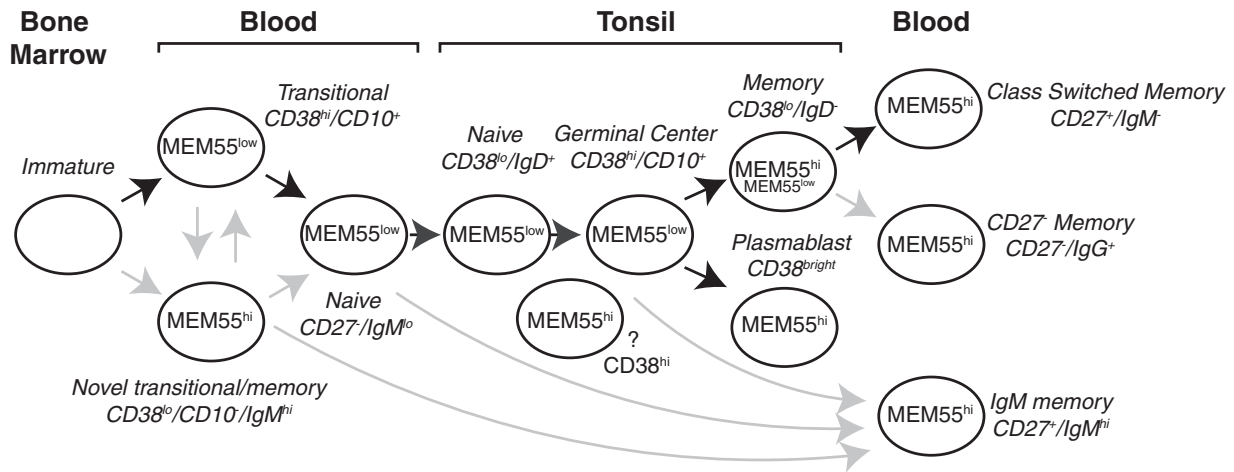


Figure 9. Expression of the CD45RB^{MEM55} epitope during B cell differentiation. The diagram shows different CD19⁺ B cell subtypes identified in Figs. 1–4 and the expression of CD45RB^{MEM55} glycoepitope on them. For each cell type, surface markers used to identify the cell type are shown in oblique bent next to it, and arrows indicate differentiation pathways, which when well-established are in black, and less-established pathways are in gray.

goes from CD45RB^{MEM55-low} in a recent emigrant from the bone marrow and naïve B cells to high at the memory and effector stages in B cells (Fig. 9).

This mode of regulated expression suggests that there is a switch from low to high expression of CD45RB^{MEM55} in connection to the GC reaction, immediately before, during, or just after a B cell enters a follicle. A recent publication from Jackson et al. [20] suggested that CD45RB expression was regulated during the GC reaction. Their proposed model, however, required that the expression changed several times during the GC reaction, as naïve B cells, GC centroblasts, as well as late memory B cells were CD45RB^{low}, and pro-GC B cells and GC centrocytes were CD45RB^{high}. Notably, they used the same CD45RB-reactive antibody clone, MEM-55, which we find to be regulated during B cell differentiation here. Similarly with their observations, we find that few CD38^{hi}/IgD⁺ naïve and CD38^{high} GC B cells express high levels of CD45RB^{MEM55}, and a majority of CD38^{low}/IgD⁻ memory cells is CD45RB^{MEM55-high}. Our immunohistochemistry data, however, question their model for expression during the GC reaction, as we show that few, if any, B cells actually express CD45RB^{MEM55} within GC. Thus, although some CD38^{high} B cells express high levels of CD45RB^{MEM55} when assayed using flow cytometry, CD45^{MEM55} expression was restricted to follicular Th cells within the GC. Consequently, CD38^{high}CD45RB^{high} B cells, detected by flow cytometry, are not situated within GCs in tonsils but rather, represent a distinct population of CD38^{high} B cells, a suggestion that gets some support, as they have a unique flow cytometric phenotype compared with other B cell subtypes within tonsils. We do indeed find cells outside of the GC—at the border between the GC and the FM, as well as in the outer FM—which express high levels of CD38 and CD45RB^{MEM55}. These could, for example, represent transitional B cells or B cells undergoing T-independent class-switch recombination outside of GC. T-independent IgA class-switch recombination in murine Peyer's patches indeed occurs in a population of cells that are similar but not identical to GC B cells, which are

formed outside of GC in a CD40-independent manner [35]. Notably, the mouse cells have low levels of mutations in their antibody genes and have a lowered AID expression compared with GC B cells. If CD38^{high}CD45RB^{high} B cells represent a similar population in humans, it could explain some of the observations made by Jackson et al. [20], as they found CD38^{high} CD45RB^{MEM55-high} tonsil B cells to be less mutated and to express less AID than CD38^{high}CD45RB^{MEM55-low} B cells.

There is a good correlation between CD27 and CD45RB^{MEM55} expression on B cells, and CD27⁺ cells are CD45RB^{MEM55-high} and CD27⁻ cells CD45RB^{MEM55-low}. This is evident using flow cytometry but also in that the organization of CD27⁺ B cells in solid tissue is more or less identical to the one we find here [32]. One discrepancy between the expression of the two markers is that there is a population of CD27⁻CD45RB^{MEM55-high} B cells in blood. Two types of B cells can be identified among these cells: class-switched CD27⁻ memory B cells and a novel population of B cells enlarged in children and after bone marrow transplantation. We are currently defining this population further. Preliminary data suggest that it is similar to the novel, transitional population described recently by Palanichamy et al. [36], as it lacks many transitional markers, in particular, CD5, CD10, and CD38, but still differs from naïve cells in that it does not express the ABCB-1 transporter. The class-switched CD27⁻CD45RB^{MEM55} B cells in blood do not express FCRL4⁺, whereas many CD45RB^{MEM55-high} B cells in tonsils do. As CD27⁺FCRL4⁺-switched memory cells are rare in blood from healthy individuals in industrialized countries [37, 38], it is, however, hard to predict whether CD45RB^{MEM55} will be coexpressed on FCRL4⁺ class-switched cells in conditions where they are increased in blood, such as HIV infections. Nevertheless, using the MEM-55 antibody clone, alone or together with CD27 when defining naïve B cells, may prove more stringent than using CD27 alone and may be advantageous when purifying naïve cells. Analysis using different staining conditions shows that the antibody clone is well-suited for clinical analysis,

as the measured expression does not change significantly as a consequence of using different staining conditions or incubating cells overnight before analysis (Supplemental Material). Notably, changed expression of the CD45RB epitope does not seem to relate to activation of B cells, at least not when mediated by TLR9 stimulation, as B cells did not change expression after blasting and proliferation, and the most prominent populations expressing low and high levels, naïve and memory cells, are both quiescent cells. Rather, it seems that CD45RB^{MEM55} expression changes at stages when the response to BCR signals is altered, i.e., in an immature stage when B cells are selected for further survival and when passing into the memory cell stage when cells should be able to respond rapidly to antigenic stimulation. That cross-linking of CD45 may lead to intracellular changes in lymphocytes has been known for some time [39], and more recently, cross-linking of the RB exon was demonstrated to activate the intracellular phosphatase activity of CD45 in T cells directly and change intracellular kinase activities [10]. The specific changes of CD45RB glycosylation and modifications of other O-linked glycosylation sites by the same mechanism may consequently regulate the activity of CD45 and other surface proteins to fine-tune the response to antigen signals during B cell differentiation.

We find a single reactive band in Western blots using extracts prepared from CD27⁺ and CD27⁻ peripheral blood B cells that correspond to CD45RABC in size using antibodies reactive to conserved CD45 regions or the RA and RB exons. Despite this, mRNA encoding smaller splice forms is detected using RT-PCR. We do not know the reason for this discrepancy but think that it may be a result of translational control in B cells. Nevertheless, the assays clearly demonstrate that the RB exon is expressed in CD27⁻ and CD27⁺ B cells in blood and that regulated MEM-55 expression is a result of a post-

translational regulation. The specificity of the MEM-55 antibody clone for the RB exon and its sensitivity to sialidase treatment are most likely a result of simultaneous interactions with the protein backbone of the RB exon and a glycochain attached to it. As no attachment sites exist for N-linked glycans within the RB exon, it is highly likely to be an O-linked glycosylation. Several types of glycosylation changes affecting the binding specificity of MEM-55 can be envisioned. Possible mechanisms include that the overall level of sialylation in B cells is changed, that sialic acids are specifically modified, that there is a shift in core glycosylation, or that a specific serine/threonine on CD45RB is O-glycosylated or not. The first two of these possible mechanisms have been shown to occur as a consequence of differential expression of sialyltransferases and sialic acid modifiers during B cell development and to modify BCR signal strength [40–43]. These types of glycosylation changes have also been observed on T lymphocytes, and T cells also have a shift between core 1 and core 2 O-glycans during differentiation [44–47]. We do, however, think that these mechanisms are unlikely to be involved in modifying the binding of the MEM-55 clone to the RB exon of CD45. We, for example, find that the expression of two different, closely situated, sialidase-sensitive, RB-attached epitopes, detected by the mAb MT4 and MEM-55, is regulated in a different manner during human B cell differentiation. The changes discussed above are global changes that would simultaneously affect most glycosylated epitopes and would thus be likely to influence the MEM-55 and MT4 epitopes. Furthermore, changed expression of CD45RB^{MEM55} occurs at differentiation stages that do not correlate with the changes described previously, discussed above. Thus, it would rather seem that regulated expression of CD45RB^{MEM55} is dependent on a different mecha-

TABLE 1. Alignment of the CD45RB Sequence

Primates	
<i>Homo sapiens</i> (15)	VSSVQTPHLPTHADSQTTPSA-GTDTQTTFSGSAANAKLNPTP-GSNAIS
<i>Pan troglodytes</i> (15)	VSSVQTPHLPTHADSQTTPSA-GTDTQTTFSGSAANAKLNPTP-GSNAIS
<i>Gorilla gorilla</i> (15)	VSSVQTPHLPTHADSQTTPSA-GTDTQTTFSGSAANAKLNPTP-GSNAIS
<i>Pongo pygmeus</i> (15)	VSSVQTPHLPTHADSQTTPSA-GTDTQTTFSGSAANAKLNPTP-GSNAIS
<i>Hylobates muelleri</i> (14)	VSSVQTPHLPTHADSQTTPA-GTDTQTTFSGSAANAKLNPTP-GSNAIS
<i>Colobus guereza</i> (17/1)	VSSAQTPHLPTHADSQTTPS T-GTDTQT/LSGSAANATLNPTP-GSNDIS
<i>Cercopithecus neglectus</i> (17)	VSSAQTPHLPTHADSQTTPS T-GTDTQTALSGSAANTALSPTP-GSNDIS
<i>Macaca nemestrina</i> (18/1)	VSSVLTPLPHPTHADSQTTPST-GTDTQT/SGSAAN/TTLNPTP-RSNDIS
<i>Aotus nigriceps</i> (12)	VPSEQPPLPHPTHADSQTTPSA-GTDTQTALSGLAHATLSPAP-GSNDAS
<i>Aotus vociferans</i> (12)	VPSEQPPLHPTQADSQTTPSA-GTDTQTALSGLAHATLSPAP-GSNDAS
<i>Aotus nancymae</i> (12)	VPSEQPPLHPTQADSQTTPSA-GTDTQTALSGLAHATLSPAP-GSNDAS
Other mammals	
<i>Oryctolagus cuniculus</i> (13)	VPPAPTPLPHPTHADSQTTPSA-GADTQT/LSSNADLTALTPAP-GSNDTE
<i>Equus ferus caballus</i> (15)	VAPTPTSHLPHPTHADSQTTPSA-GSDTQT/SSQGGPLTKTPAP-GRNDTS
<i>Canis lupus familiaris</i> (11/1)	VKGVTQPPLTH TGSQASSA-GPDQT/PNSTVALLARTPAP-GRNDPS
<i>Monodelphis domestica</i> (15/2)	APPTKSTHL TTHA VSQAPSD-GSVNQT/LSSSPA VLTTLIAD-RANKST
<i>Mus musculus</i> (13)	VLSTLLPHLSPQPDSTTPSA GGADTQTTFSSQADNPTLTPAPGGGTDP
<i>Rattus norvegicus</i> (16)	FSSTLMPHLTPQPDSTTPSARGADTQT/LSSQADLTTLTAAPSGETDPP

The amino acid sequences for CD45RB are shown for the indicated species divided into primates and other mammals. After each species name is shown the number of O-linked glycosylation/N-linked glycosylation sites within parentheses. Amino acids identical to the human sequence are in normal font, and amino acids that differ from the human sequence are in italics. Potential sites for O-linked glycosylation are bold, and potential sites for N-linked glycosylation are underlined.

nism, a likely candidate being regulated targeting of serine/threonine residues for O-linked glycosylation.

Although CD45 seems to have been more highly evolutionary conserved intracellularly than extracellularly, we find that some potential O-linked glycosylation sites have been rather well-preserved within the RB exon during mammalian evolution (Table 1). Furthermore, multiple O-glycosylation sites with no or few N-linked glycosylation sites are common features of mammalian RB exons. It is, however, currently impossible to predict which of these are actually glycosylated in a given cell type, as there is a family of 20 different ppGalNAcT with distinct target specificities that mediate the initial GalNAc addition to the serine/threonine [48]. The expression of ppGalNAcTs, however, differs during embryonic development and between different adult tissues [49]. Regulated expression of ppGalNAcTs during B cell development may differentially glycosylate membrane proteins during differentiation accordingly, e.g., to fine-tune the response to extracellular interactions. Such changes may, for example, influence the ability of CD45 to homodimerize or to interact with ligands and may simultaneously change the glycosylation of other transmembrane proteins, thereby influencing signal transmission into the cell through several pathways simultaneously. That changed expression of a single GalNAcT can have a strong influence on B cell function was demonstrated recently in mice that lacked ppGalNAcT-1, as they exhibited diminished antibody responses and disturbed GC reactions [50]. Thus, it is possible that regulated expression of ppGalNAcTs is used to fine-tune B cell responses and that this may, at least in part, be mediated through changes in the glycosylation of CD45.

Thus, in conclusion, we show that O-linked glycosylation of CD45RB is regulated specifically during B cell differentiation, and we suggest that this mechanism may regulate the response to extracellular signals through modifications of CD45 as well as other cell surface proteins. Changed expression of a ppGalNAcT targeting a specific O-glycosylation site in the CD45RB exon is an attractive hypothesis to explain the findings, but its confirmation has to await identification of the glycosylation site and enzyme involved.

AUTHORSHIP

J.S. and M.B. designed the study; S. Koethe, L.Z., S. Köster, A.A., J.S., and M.B. performed experiments and analyzed data; A.E. and J.S. collected and provided clinical samples; M.B. wrote the manuscript; and J.S. critically reviewed the manuscript.

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