Differential surface expression and possible function of 9-*O*- and 7-*O*-acetylated GD3 (CD60 b and c) during activation and apoptosis of human tonsillar B and T lymphocytes

Michael Erdmann · Dirk Wipfler · Anette Merling · Yi Cao · Christine Claus · Bernhard Kniep · Haneen Sadick · Wolfgang Bergler · Reinhard Vlasak · Reinhard Schwartz-Albiez

Abstract The disialoganglioside GD3 (CD60 a) and its *O*-acetylated variants have previously been described as surface molecules of human T lymphocytes of the peripheral blood system. Here we report the expression of the 9-*O*-, and 7-*O*-acetylated disialoglycans of GD3 (CD60 b and CD60 c respectively) on human tonsillar lymphocytes. CD60 b and c are surface-expressed on activated germinal centre B cells and colocalize in raft-like structures on the cell surface together with the cytoplasmic tyrosine kinase Lyn and Syk. Addition of CD60 b and c mAb together with anti-IgM/IL-4 to *in vitro* cultivated tonsillar B cells resulted in a costimulatory effect. During spontaneous and staurosporine-induced apoptosis a distinct population of activated annexin V+/CD60 b+/CD60 c- B cells was observed. CD60 b and c are also found on cells of the extrafollicular T cell area. On tonsillar T cells, CD60 b mAb had a costimulatory effect together with PHA while CD60 c mAb alone was sufficient to induce proliferation. In further contrast to B cells, during apoptosis a distinct CD60 b+ T cell subpopulation was not observed. Together, surface-expressed CD60 b and c are differently expressed on tonsillar B and T cells and may be involved in the regulation of activation and apoptosis of lymphocytes in secondary lymphatic tissue.

Keywords Ganglioside · Germinal center · Lipid rafts · *O*-acetylated sialic acid · Signal transduction

M. Erdmann · D. Wipfler · A. Merling ·
Y. Cao · R. Schwartz-Albiez
Tumor Immunology Program, German Cancer Research Center,
D-69120 Heidelberg, Germany

C. Claus
Department of Cardiothoracic and Vascular Surgery,
University Hospital Mainz,
Mainz, Germany

B. Kniep
Institute of Immunology, Technical University of Dresden,
D-01307 Dresden, Germany

H. Sadick
Department of Oto-Rhino-Laryngology, University of Mannheim,
Mannheim, Germany

W. Bergler
St. Joseph Hospital,
D-28209 Bremen, Germany

R. Vlasak
Applied Biotechnology,
Department of Cell Biology, University Salzburg,
A-5020 Salzburg, Austria

R. Schwartz-Albiez (✉)
German Cancer Research Center,
D010, Im Neuenheimer Feld 280,
D-69120 Heidelberg, Germany
e-mail: r.s-albiez@dkfz-heidelberg.de

Abbreviations

BSA	bovine serum albumin
CTB	cholera toxin subunit B
ECL	enhanced chemoluminescence
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
LSM	laser scan microscopy
mAb	monoclonal antibody(ies)
OSGE	*O*-sialoendoglycoprotein endopeptidase
PBS	phosphate buffered saline
PE	phycoerythrin
PHA	phytohemagglutinin
VCN	*Vibrio cholerae* sialidase

Introduction

O-acetylation is a modification of sialic acids which significantly alters the biological properties of the parent molecule [for review see [1, 2]. In particular, terminal 9-O-acetylated sialic acid as constituent of gangliosides and possibly glycoproteins has been implicated with functions such as migration of developing neurons [3, 4] and masking of the B lymphocyte lectin CD22 recognizing $\alpha 2,6$ sialylated lactosaminyl carbohydrate sequences [5]. Further, 9-O-acetylated terminal sialic acid is recognized by influenza C virus and human corona virus OC43 for specific attachment [6, 7].

The disialoglycan sequence of the ganglioside GD3 and its 9-O- and 7-O-acetylated variants are surface-expressed on various subpopulations of peripheral human T lymphocytes [8–11]. Antibodies differentiating between the three subtypes of GD3 influence proliferation of peripheral T lymphocytes in two ways: 9-O-acetylated GD3-specific mAb transfer costimulatory signals [12] whereas mAb U5 against 7-O-acetylated GD3 or GD3-reactive mAb R24 alone are sufficient to induce enhanced proliferation of T cells [13, 14]. In order to distinguish cellular expression and possible different functions of the GD3 carbohydrate sequence and its O-acetylated variants, the three subtypes recognized by specific monoclonal antibodies have been clustered in CD60 a (GD3), CD60 b (9-O-acetylated GD3) and CD60 c (7-O-acetylated GD3) during the Human Leucocyte Differention Antigen Conference (HLDA VII, Harrogate 2000) [15], the CD (cluster defined) nomenclature for these carbohydrate sequences will be used throughout this study where antibody reactions are concerned.

The molecular mechanisms underlying the stimulatory effect on T cell proliferation by CD60 mAb are not known. In general, GD3 gangliosides have been implicated with two major cellular events: 1) gangliosides drive lipid raft formation on lymphocyte surfaces in which key receptors triggering signal cascades are concentrated. In fact, it had been shown that TAG-1, a GPI-anchored neuronal cell adhesion molecule is located in a complex with GD3 and the Src family kinase Lyn within lipid rafts [16]. Whether O-acetylated variants of GD3 direct a different composition of rafts as compared to GD3 itself remains to be settled. 2) It became evident that CD95- or ceramide-initiated apoptosis cascade includes increased formation of intracellular GD3 which in turn induces mitochondrial permeability transition and cytochrome C release [17]. Thus intracellular GD3 propagates lipid-mediated apoptosis while 9-O-acetylation of GD3 suppresses its pro-apoptotic effect [18].

In a previous report we described expression of CD60 b on in vitro cultivated human B lymphocytes [19]. At this point it was unclear whether other CD60 variants are expressed and may participate in functions of B lympho-cytes in comparison to T lymphocytes during the activation process within lymphatic organs. In this study we describe the surface expression and possible roles of CD60 b and c on activated and apoptotic native tonsillar B and T cells.

Materials and methods

Materials

Immune reagents and enzymes

The following immune reagents were used in this study: CD60 a mAb R24 and CD60 c mAb U5 (both of IgG3 isotype) were provided by one of the authors (CC). Both mAb were used either for staining experiments (antibody preparations containing 0.01% NaN_3) or for functional assays in sterile preparations (without NaN_3), CD60 b mAb UM4D4 unconjugated and biotin- and FITC-conjugated was purchased from Ancell, Bayport, MN; CD60 b mAb M-T6004 was provided by one of the authors (BK). Both CD60 b antibodies used were of IgM isotype and had identical reactivity patterns specific for 9-O-acetyl GD3 [20]. The CD60 b mAb M-T6004 was used for functional studies in proliferation assays in sterile preparations without NaN_3 whereas the commercial CD60 b mAb containing NaN_3 was used for staining experiments. CD75s mAb HH2 (IgM) was kindly donated by Dr. S. Funderud, Radium Hospital, Oslo, Norway; CD19mAb HD37 FITC-conjugated was kindly provided by Dr. G. Moldenhauer, German Cancer Research Center; CD19-CY-Chrome-conjugated was from BD Biosciences, Heidelberg, Germany; CD19 (mAb J4.119) PE-conjugated, CD45RA PE-conjugated, CD3 Cy3-, PE- and FITC-conjugated, apo2.7-PE-conjugated were purchased from Immunotech, Marseille, France. The anti-Syk mAb was purchased from Dunn Laboratories, Asbach, Germany, the anti-Lyn mAb from BD Transduction, Heidelberg, Germany. As secondary antibodies we used goat anti-mouse IgM F(ab') fragment Cy3-conjugated, goat anti-mouse IgG+IgM F(ab') fragment FITC-conjugated, goat anti-mouse IgG+IgM polyclonal antibody peroxidase-conjugated, all purchased from Dianova, Hamburg, Germany. As isotype controls IgG1κ, IgG2aκ, IgG2bκ, IgG3κ (BD Biosciences), IgM (Dr. Moldenhauer), IgM FITC-conjugated (Cymbus Biotechnology, UK) were applied. All isotype control antibodies preparations were NaN_3 free and were directed against epitopes irrelevant for human lymphocytes.

Streptavidin conjugated to Alexa Fluor 488 and cholera toxin subunit B (CTB) conjugated to Alexa Fluor 488 were from MOBiTec, Göttingen, Germany. Annexin V PE-conjugated and annexin V binding buffer, Via probe from BD Biosciences for detection of dead cells, streptavidin Cy3-conjugated and streptavidin-peroxidase conjugated,

human immunoglobulin (Endoglobin S/D) from Dianova. For induction of lymphocyte activation we used anti-human IgM antibody (Dianova), recombinant human IL-2 and IL-2 from Strathmann Biotech, Hannover, Germany, Pansorbin cells from Calbiochem, Schwabach, Germany, phytohemagglutinin (PHA) from Sigma (St Louis, MO), and the respective CD60 mAb as outlined above, and for apoptosis induction staurosporine derived from *Streptomyces sp* (Roche, Mannheim, Germany).

For degradation of carbohydrates and glycoproteins the following enzymes were applied: sialidase from *Vibrio cholerae* (VCN, EC 3.2.1.18, Roche), recombinant 9-*O*-acetyl-esterase of Influenza C virus (EC 3.4.24.57) produced by one of the authors (RV) [21], *O*-sialoendoglycoprotein endopeptidase (OSGE; EC 3.4.24.57) from *Mannheimia haemolytica* (Biozol, Munich, Germany).

Methods

Isolation of tonsillar lymphocytes and cell culture

Non-inflammatory tonsils were obtained from children undergoing tonsillectomy because of tonsillar hyperplasia after having received informed consent of the parents according to the guidelines and after approval of the ethical committee on the use of human tissue in research at the University Heidelberg-Mannheim in compliance with the Helsinki protocol. Tonsillar cell suspensions were prepared from minced tonsils as described earlier [22]. Lymphocytes were enriched by Ficoll separation, B lymphocytes were isolated by two cycles of magneto-bead separation using Dynabeads M450 of 4.5 μm diameter coupled to a CD19 mAb (Dynal, Norway). After isolation lymphocytes were detached from beads by using DetachaBEADS (Dynal). Thus selected B lymphocytes were to >95% positive for CD19 as evaluated by flow cytometry. Two times CD19 depleted cells were to >90% positive for CD3 and were used as tonsillar T cells.

CD19+ B lymphocytes were further fractionated by Percoll (Pharmacia, Freiburg, Germany) density gradient centrifugation as described [22].

Flow cytometry

CD60 expression on tonsillar lymphocytes was measured with four antibodies which had previously been described [15]: CD60 a mAb R24 reacted specifically with the GD3 standard [10], CD60 b mAb UM4D4 and M-T6004 reacted exclusively with the 9-*O* acetylated GD3 [15] and CD60 c mAb U5 showed a strong reaction with 7-*O*-GD3 and a considerably weaker reaction with GD3 [13].

Prior to staining with specific mAb, cells were incubated with 5% (v/v) Endoglobin S/D diluted in phosphate buffered

saline (PBS) containing 1% BSA and 0.01% NaN$_3$ ("cytometry buffer") for 30 min on ice. Lymphocytes (1×10^6 cells/sample) were incubated with mAb conjugated to fluorescence dye in a volume of 100 μl cytometry buffer for 20 min on ice. Incubation with non-conjugated mAb was performed in the same fashion, followed by incubation with the respective secondary antibody for 20 min on ice. Prior to further mAb stainings, cells were incubated with 5 μl of mouse serum in 95 μl cytometry buffer in order to block unspecific binding sites of anti-mouse secondary antibodies. For multi color analysis cells were first incubated with non-conjugated antibodies followed by conjugated antibodies, between all incubation cells were extensively washed with cytometry buffer. Shortly before cytometric analysis 10 μl of Via-probe were added to the cells to discriminate between live and dead cells. AnnexinV was applied according to the manufacturer's protocol. For controls, staining with irrelevant isotype-matched mouse mAb was performed to calibrate to settings for flow cytometric analysis.

For intracellular staining with mAb apo2.7 after cell surface staining, cell membranes were perforated by treatment with 100 μg/ml digitonin in PBS containing 2.5% fetal calf serum (FCS), 0.01% NaN$_3$ for 20 min at room temperature.

For flow cytometry a FACScan (Becton & Dickinson, Heidelberg, Germany) was used.

For induction of apoptosis cells were treated with staurosporine at a concentration of 5 μM for 4 to 18 h before flow cytometric analysis.

Immunohistology and confocal laser scan microscopy

Immunohistological studies were performed with fresh tissue quick-frozen in liquid nitrogen, frozen sections of 4-μm thickness were cut, air-dried overnight, fixed in acetone for 20 min at −20°C and subsequently treated with 3% H$_2$O$_2$ for 30 min to block endogenous peroxidases. Immunostaining with respective mAb was done for 1 h followed by incubation with anti-mouse peroxidase-conjugated secondary antibody for 30 min at room temperature. Cells were washed between each incubation step. Immunoreaction was visualized by reaction with diaminobenzidine and counterstaining with hematoxylin.

In order to determine carriers of cell surface-expressed carbohydrates by immunochemical methods we cultivated separated tonsillar B and T cells on Teflon-coated slides for 2 days. Medium was removed and slides air-dried and stored at −80°C until further use. Then samples were fixed in acetone for 20 min at −20°C and further treated with methanol for 30 min at room temperature before staining with CD60 mAb.

For confocal laser scan microscopy (LSM), cells were stained with antibodies as described for flow cytometry

with the exception that Alexa Fluor 488 instead of FITC and Cy3 instead of Cy-Chrome was used in order to prevent bleaching. Before microscopic analysis cells were fixed with 3% paraformaldehyde. Slides were coated with poly-L-lysine to increase adhesion and prevent cell motility during microscopy. Cells were incubated with CTB coupled to Alexa Fluor 488 nm and CD60 mAbs recognized by anti-mouse IgG/IgM coupled to Cy3. For double stainings of CD60 b and c cells were initially treated with anti-CD60 c mAb, anti-mouse IgG/IgM coupled to Cy3, followed by biotin-labeled anti-CD60 b mAb which was then recognized by Alexa-Fluor-488 nm-streptavidin. Confocal LSM was performed using a Zeiss LSM 510 UV microscope operating with an argon ion laser (488 nm) and an HeNe laser (543 nm) (Zeiss, Jena and Oberkochen, Germany).

Enzymatic treatment of cells

In order to examine glycan specific reactivity of CD60 mAb on live cells, cells were pretreated before immunostaining with the following enzymes: VCN (15 μl/1×10^6 cells in 100-μl PBS+1% BSA for 45 min at 37°C), recombinant 9-O-acetyl-esterase (stock solution 42 mU/100 μl; 3 μl/1×10^6 cells in 100 μl PBS+1% bovine serum albumin (BSA) for 45 min at room temperature), OSGE (10 μl/1×10^6 cells in 100 μl PBS+1% BSA for 45 min at 37°C).

In vitro stimulation of lymphocytes

B cells were stimulated with anti-IgM (7.5 μg/ml) and IL-4 (0.25 ng/ml) or Pansorbin diluted 1:200,000 and the respective CD60 mAb in a concentration of 50 μg/ml. For CD60 b stimulation we used our mAb M-T6004. This mAb has identical reactivity as the CD60 b mAb UM4D4 and was applied because NaN₃-free mAb UM4D4 was not available. T cells were stimulated by PHA (0.2 μg/ml) and IL-2 (100 ng/ml) and the respective CD60 mAb as indicated above. In order to exclude non-specific stimulatory effects of mAb-induced proliferation we also applied irrelevant isotype-matched IgM and IgG3 controls in proliferation assays as shown in Fig. 5. Cells were incubated in 96 well microtiter plates (2×10^5 cells/well in 200 μl AIM V medium) (Invitrogen, Karlsruhe, Germany) for 24, 48, 72 and 96 h at 37°C in a 5% CO₂ atmosphere.

Proliferative activity of cells was determined by ³H-thymidine incorporation (Amersham, Braunschweig, Germany). Cells were pulsed with ³H-thymidine (1 μCi ³H-thymidine/well of a 96 well flat bottom microtiter plate, in triplicates) for 6 h before harvesting at each time point indicated above. Incorporated radioactivity was determined by standard liquid scintillation counting. In order to detach sessile cells, shortly before harvesting 10 μl of a 2.5% EDTA solution were added to the cultures.

Immunoprecipitation and Western blotting

For coprecipitation experiments 1×10^7 cells were lysed in PBS containing 0.5% Triton X-100 and protease inhibitors ("complete protease inhibitor cocktail", Roche, Mannheim, Germany) for 20 min on ice. The lysates were centrifuged in an Eppendorf centrifuge at 16,000×g for 10 min and the supernatants were harvested. For immunoprecipitation the Seize TM primary immunoprecipitation kit (Perbio, Bonn, Germany) was applied according to the manufacturers' protocol using CD60 b and CD60 c specific mAb as well as isotype-matched control mAb. The eluted precipitated proteins were further separated by SDS-PAGE, transferred to Western blots and subsequently incubated for detection of coprecipitated kinases with mAb for Syk and Lyn respectively followed by peroxidase-conjugated secondary antibody. The precipitated proteins were then visualized on Western blots by "Enhanced Chemoluminescence" (ECL) technology.

Results

Carriers of the CD60 epitopes

In order to examine the reactivity in particular of the CD60 b and c antibodies with cell surface-expressed glycans on live cells we subjected tonsillar lymphocytes to various enzymatic treatments prior to antibody reactions. A significantly decreased reactivity of CD60 b and c mAb was observed after pretreatment of tonsillar B and T cells with VCN as indicated by strong reduction of mean fluorescence intensity (Fig. 1a). Although 9-O-acetylation of terminal sialic acid may impede the degradation by sialidases [23] degradation of the O-acetylated GD3 was possibly effective because of high VCN concentrations applied.

The enzyme 9-O-acetyl-esterase of influenza C virus removes the 9-O-linked acetyl group of 9-O-sialic acid and thus reduced binding capacity of CD60 b mAb but not that of CD60 c mAb (Fig. 1b). A direct proof of 7-O-acetylated sialic acid specificity of CD60 c mAb U5 by enzymatic degradation on live cells is not possible because a 7-O-acetyl esterase is not available. Evidence for 7-O-acetyl sialic acid specificity of mAb U5 is therefore based on chromatographical methods and comparative staining of cells as shown below.

In previous publications it has been reported that O-acetyl ester groups located at the 7-O-position can spontaneously migrate to the 9-O-position provided this position is not already substituted [24]. In another publication a "migrase" was postulated for the shift of the O-acetyl group from C-7 to C-9 [25]. In order to exclude a possible

pH-dependent shift of *O*-acetyl groups on cell surface expressed sialoglycans in particular during tissue and cell preparations we repeatedly incubated tonsillar B lymphocytes for 10 min in culture medium of various pH values (pH 7.3, 8.0, 9.0). In these experiments we could not observe a shift in binding of CD60 b and c mAb with regard to mean fluorescence intensity and percentage of stained live cells (data not shown). We therefore conclude that a shift of *O*-acetyl groups from C-7 to C-9 is at least unlikely to occur rapidly on cell surface-expressed CD60 b and c epitopes and that our immunostaining results with the CD60 mAb reflect the natural situation of cell surface-expressed 7-*O*- and 9-*O*-acetylated sialoglycans on tonsillar lymphocytes.

The expression of the CD60 b sequence on T cells has been described as part of glycosphingolipids [10] and glycoproteins [8]. Only few information was available for the carrier of CD60 b and c on B lymphocytes. In cellular extracts of human B lymphocytes the 9-*O*-acetyl-GD3
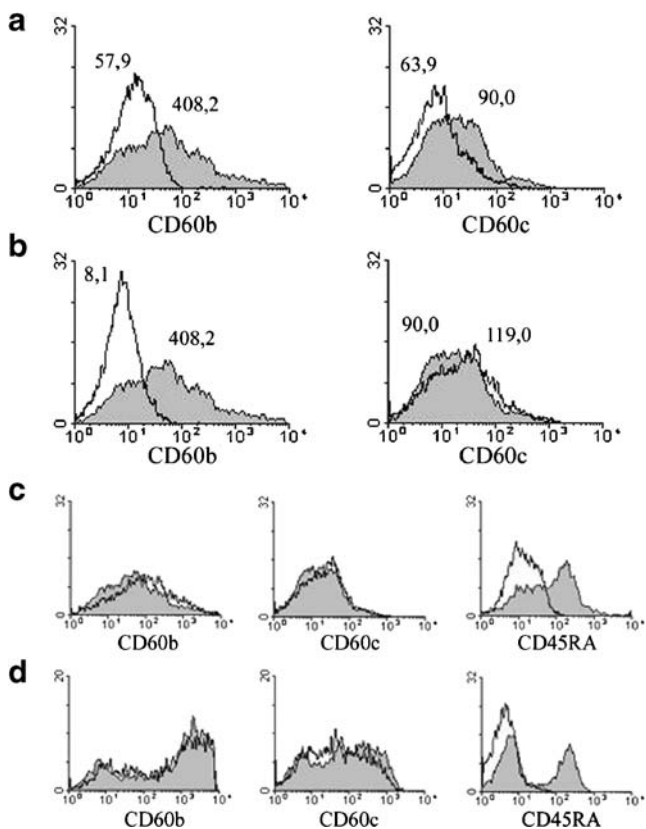

Fig. 1 *Biochemical characterization of cell surface expressed CD60 b and CD60 c on live tonsillar B and T lymphocytes. Shaded curves* depict untreated cells, *blank curves* enzyme-treated cells throughout the figure. (a) B cells treated with *Vibrio cholerae* sialidase, (b) B cells treated with 9-*O*-acetyl-esterase, (c) B cells treated with OSGE, (d) T cells treated with OSGE. For (a) and (b) numbers indicate mean intensity of fluorescence of cells. Control stainings with IgM- and IgG3 isotype-matched irrelevant antibodies were always in the first decade with mean fluorescence values of 4 to 10 (not shown in figure)
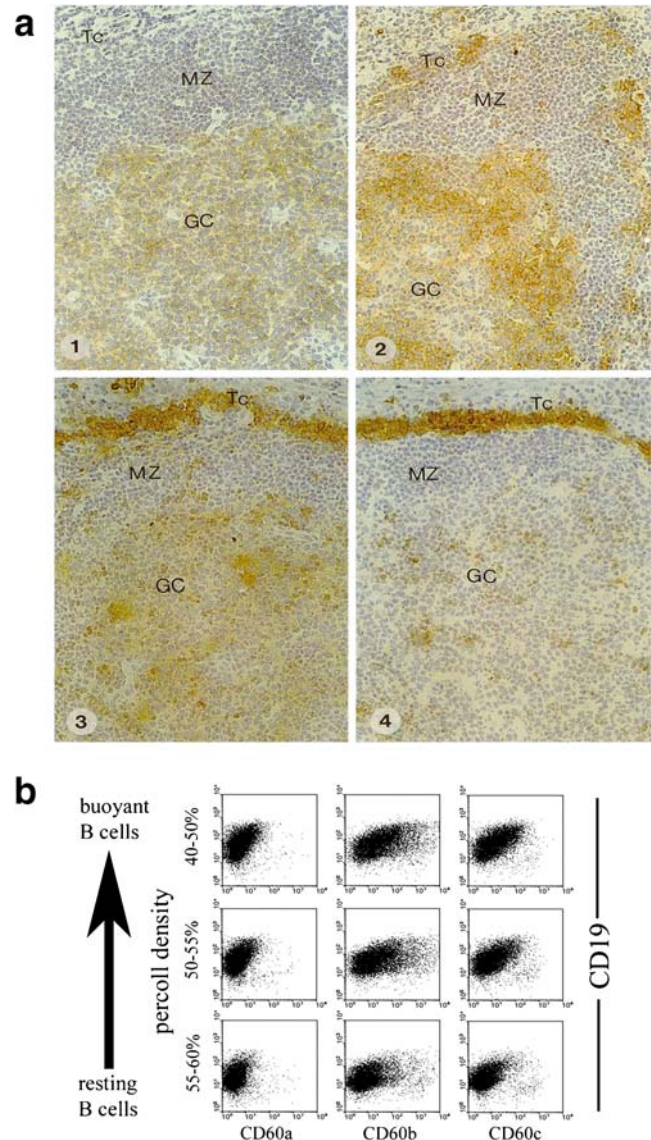

Fig. 2 *Expression of CD60 sequences on native tonsillar B and T lymphocytes.* (a) Immunohistochemistry of tonsillar cryosections. Germinal center (GC), mantle zone (MZ) and T cell area (TC). (*1*) staining with CD75s (α2,6 sialylated lactosamines) specific mAb HH2 which characterizes germinal center B cells, (*2*) staining with CD60 b mAb UM4D4, (*3*) CD60 c mAb U5, and (*4*) CD60 a mAb R24. (b) Differential expression of CD60 sequences on tonsillar B cells isolated by CD19 magnetobeads and subsequently separated by Percoll gradient. Buoyant B cells represent cells in advanced activation stage in comparison to small resting B cells. In this experiment for CD60 a mean fluorescence values raised slightly from 6 (55–60% Percoll), to 9 (50–55% Percoll) and to 11 (40–50% Percoll), for CD60 b from 71 (55–60% Percoll), to 167 (50–55% Percoll) and to 176 (40–50% Percoll), for CD60 c from 17 (55–60% Percoll), to 27 (50–55% Percoll) and to 34 (40–50% Percoll). The *histogram* depicts representative results from one of three independent experiments

ganglioside was identified [10]. In order to examine whether CD60 b and c sequences are expressed on glycoproteins of tonsillar lymphocytes we applied the enzyme OSGE on live tonsillar T and B cells which cleaves specifically *O*-linked sialoglycoproteins [26]. Although OSGE considerably
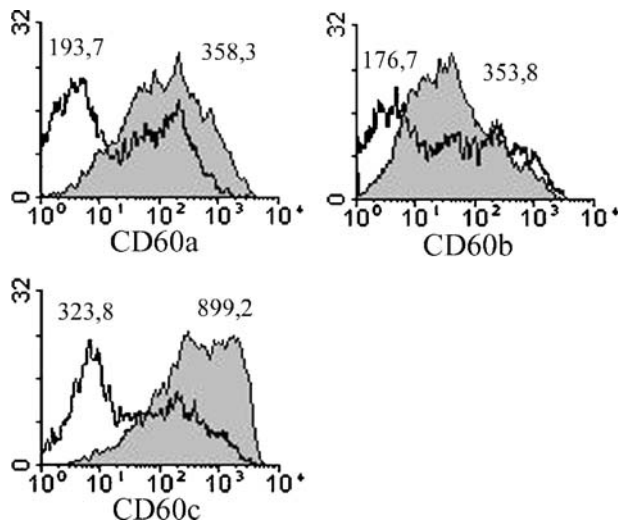
Fig. 3 *In vitro activation of tonsillar T lymphocytes*. Expression of CD60 sequences on PHA-stimulated tonsillar T cells after 48-h *in vitro* cultivation (*shaded*) versus *in vitro* cultivated, non-treated cells (*blank*). Numbers represent mean intensity of fluorescence for each cell population. Isotype control stainings for IgM and IgG3 mAbs resulted in mean fluorescence values of 5–20 in this experiment (not shown in the figure)

reduced expression of the CD45RA sialoglycoprotein on both cell types no influence of OSGE treatment on expression of CD60 b and c was observed (Fig. 1c,d). In another approach we removed glycosphingolipids from membranes of fixed cells by methanol treatment. This treatment abolished expression of all CD60 variants on B cells whereas on T cells expression of CD60 c was totally abolished and that of CD60 a and b was reduced.

Taken together, we tentatively conclude from these results that CD60 carbohydrate sequences are to the largest extent expressed as glycosphingolipids on tonsillar lymphocytes. However the existence of a minor proportion of glycoprotein-expressed CD60 carbohydrates on B cells cannot be excluded by the methods used in this study.

Expression of CD60 carbohydrates on tonsillar lymphocytes

The *in vivo* distribution of the CD60 glycotopes on tonsillar lymphocytes was investigated in immunohistology of cry-osections. As positive control for staining of B lymphocytes in the germinal center of secondary follicles we used mAb HH2 of CD75s which recognizes α2,6 sialylated lactosa-minyl structures on germinal center activated B cells [15] (Fig. 2a.1). CD60 a was strongly expressed on cells within the T cell zone at the border region of the mantle zone and on scattered single cells within the germinal center (Fig. 2a.4). CD60 b was positive on cells in the T cell region, strongly positive on germinal center lymphocytes and
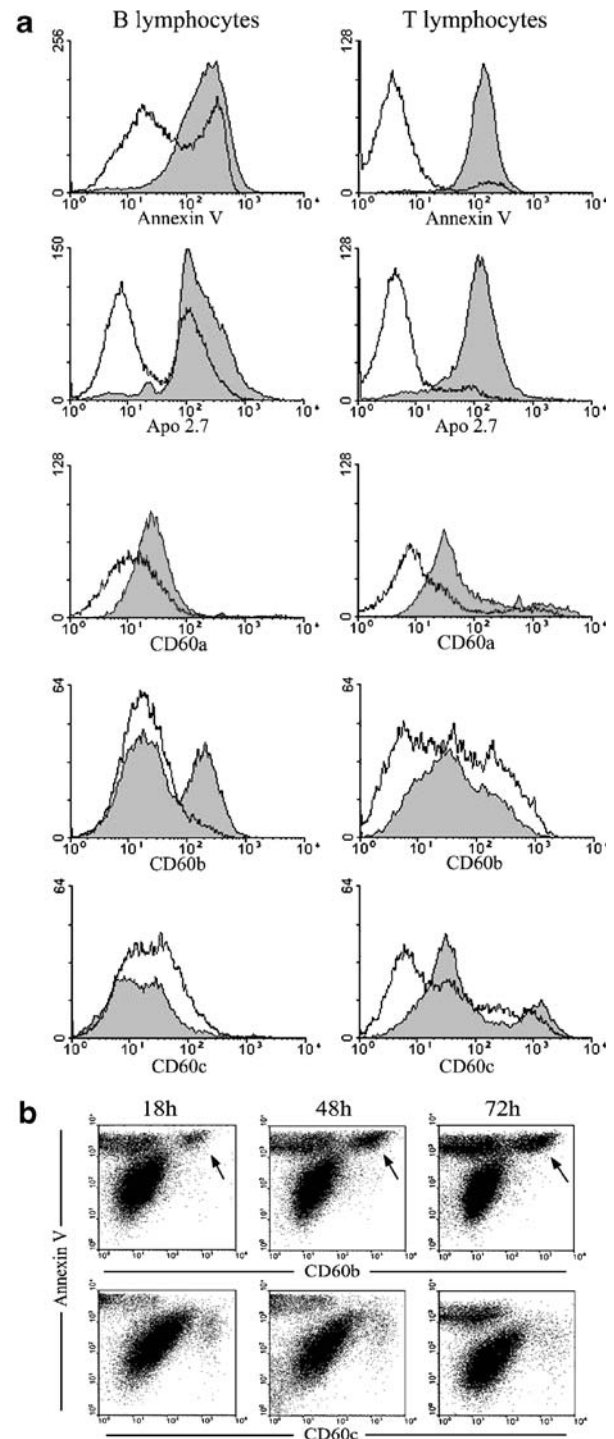

Fig. 4 *Expression of CD60 b and c during apoptosis of tonsillar lymphocytes*. (a) Apoptosis induction of freshly isolated tonsillar T and B cells by 18 h staurosporine treatment and its effect on CD60 expression. Apoptosis was measured by annexin V and intracellular mAb apo2.7 bind-ing of live (Via negative) cells in separate preparations before (*blank*) and after (*shaded*) induction of apoptosis. The experiment was performed four times with the same results as shown here. (b) Expression of CD60 b and c during *in vitro* culture of live (Via-negative) tonsillar B cells and concom-itant determination of apoptotic cells (annexin V). The CD60 b+/annexinV+ B cell subpopulation is indicated by an *arrow*. Expression of CD60 b and c during spontaneous apoptosis of *in vitro* cultured tonsillar lymphocytes as shown here was observed in three independent experiments

Fig. 5 *Stimulation of tonsillar B and T cells by CD60 a,b,c mAb*. Proliferation of T and B cells after activation with PHA or anti-IgM/IL-4 respectively, additionally with or without CD60 a,b,c mAb or CD60 mAb alone was determined by incorporation of ^{3}H-thymidine. Isotype controls, IgM (for mAb UM4D4) and IgG3 (for mAb R24 and U5), were applied in separate sets of the experiment. Assays were performed in triplicates, standard error of the mean (SEM) is indicated. The results are shown for one representative of at least three independently performed experiments
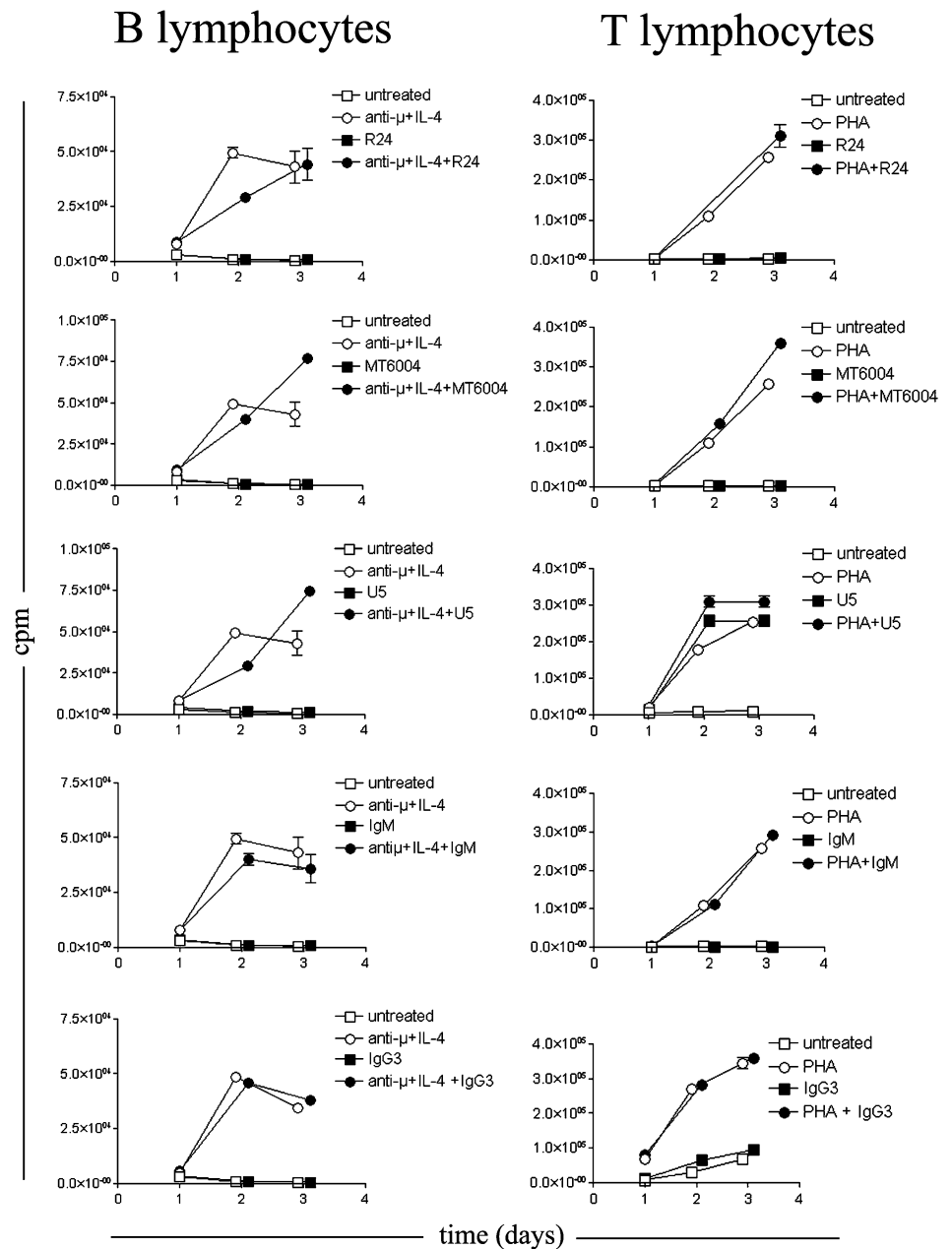
only weakly positive in cells of the mantle zone (Fig. 2a.2). The CD60 c mAb showed strong reactivity with cells of the T cell zone comparable with that of CD60 a mAb and staining of fewer germinal center lymphocytes in patchy formation as compared to CD60 b reactivity (Fig. 2a.3). When freshly isolated live tonsillar CD19+ B lymphocytes were separated on a Percoll density gradient it became evident that CD60 a expression on these cells was weak to absent. CD60 b expression increased from high density to buoyant cells (Fig. 2b). Since buoyant cells represent lymphoblasts it is concluded that expression of CD60 b and c increases during B cell activation. This observation was in accordance

with the strong *in vivo* expression of CD60 b and c in germinal center lymphocytes.

In contrast CD19+ tonsillar B cells stimulated *in vitro* for 24 and 48 h with either anti-IgM antibody/IL-4 or Pansorbin did not display an enhanced expression of CD60 b and c as compared to untreated cells. Also CD60 a expression was not induced in *in vitro* activated B cells (data not shown). In CD3+ tonsillar T cells, expression of all CD60 glycotopes increased over a cultivation time of 48 h without stimulation and even stronger after PHA-induced activation as indicated by increase of mean fluorescence intensity (Fig. 3).

Fig. 6 *Confocal LSM of CTB, CD60 b and CD60 c expression on tonsillar B and T cells.* (a) CD60 b (*red*) versus CTB (*green*). (b) CD60 c (*red*) versus CTB (*green*). C CD60 c (*red*) versus CD60 b (*green*). *Left upper quadrant* of each set always *red fluorescence, right upper quadrant green fluorescence, left lower quadrant* phase contrast microscopy, *right lower quadrant overlay of red and green fluorescence* (colocalization of both fluorescences results in *yellow color*)
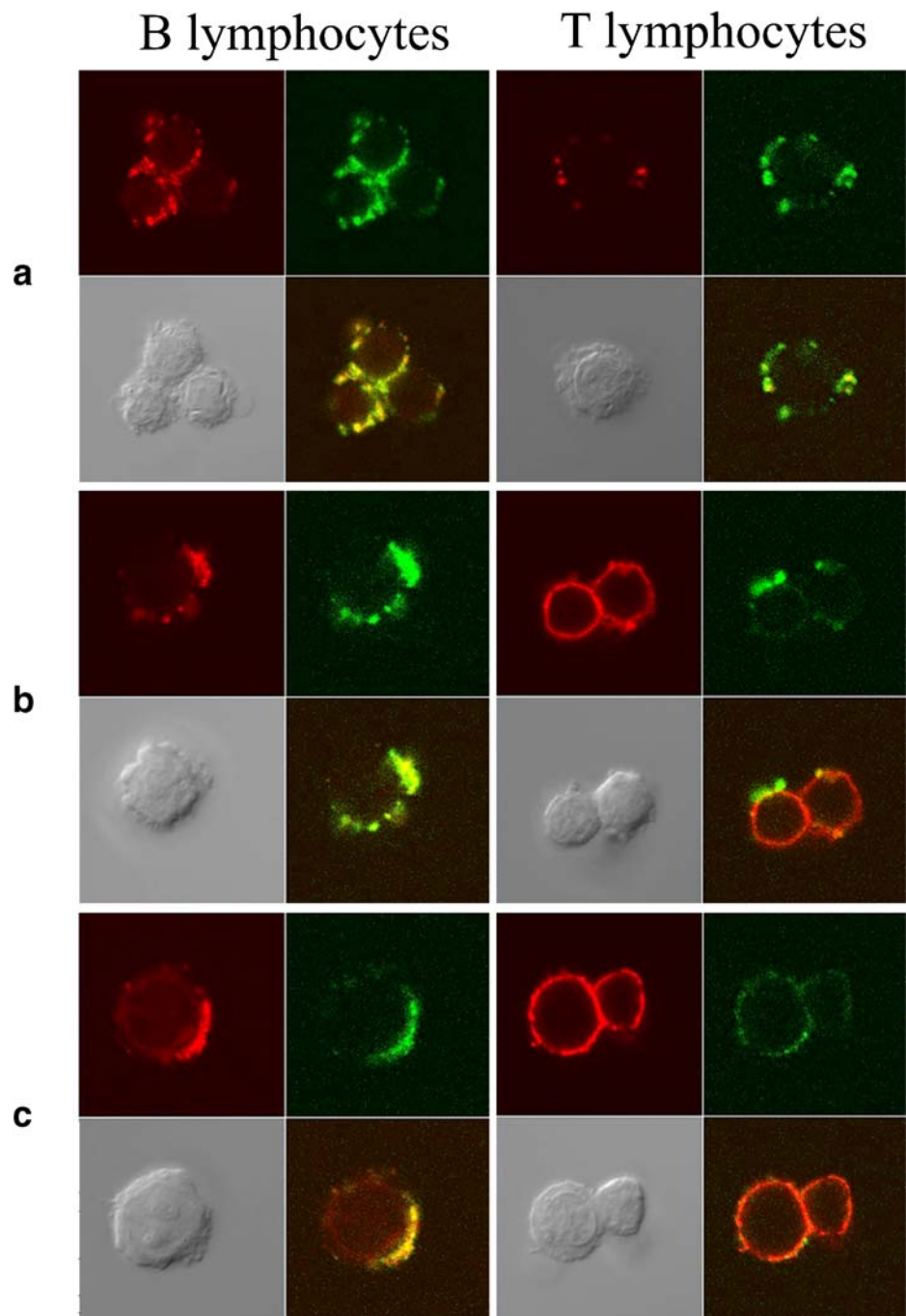
Expression of CD60 b and c during apoptosis

GD3 has been shown to be an inducer of apoptosis whereas 9-*O*-acetylated GD3 lacks a pro-apoptotic function. We measured the presence of cell surface-expressed CD60 variants during apoptosis of *in vitro* cultivated tonsillar B lymphocytes. It should be noted that non-apoptotic B cells in contrast to T and other cells bind annexin V to a certain extent [27]. In order to distinguish apoptotic from non-apoptotic B cells we raised in flow cytometric measure-

ments the cut-off for annexin V positivity accordingly and used apo2.7 as an additional marker for apoptosis.

Two tonsillar B lymphocyte populations could be distinguished: one of annexin V high/CD19 low (late apoptotic cells) and one of annexin V low/CD19 high (early apoptotic cells), the same applied for apo2.7 recognizing the mitochondrial protein 7A6 [28]. During *in vitro* cultivation a large proportion of the annexin V low/CD19 high population shifted to stronger annexin V binding. Three color fluorescence of live (via probe
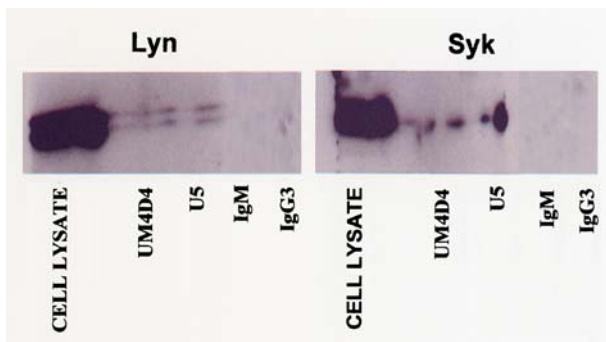
Fig. 7 *Colocalization of CD60 b and c with intracellular protein kinases*. Coimmunoprecipitations performed with CD60 b and c mAb from B cell membrane. Cell lysates were first incubated with CD60 b and c mAb and IgM and IgG3 isotype controls, then coimmunoprecipitated proteins were visualized after subsequent Western blotting using mAb specific for protein kinases Lyn and Syk as described in detail in the Materials and methods section. No bands were visible after immunoprecipitation with irrelevant control antibodies at the respective positions of precipitated kinases

negative) CD19+ B cells with annexin V/CD60 b mAb and annexinV/CD60 c mAb revealed during *in vitro* cultivation from 0 to 72 h an increasing, distinct population of annexinV high/CD60 b high cells as indicated by an arrow (18 h: 5%, 48 h: 16%, 72 h: 19%). A similar population was not observed with CD60 c (Fig. 4b). When apoptosis was induced in freshly harvested tonsillar B cells by staurosporine over a period of 18 h a distinct apoptotic cell population with high CD60 b expression was observed (Fig. 4a). Interestingly, this subpopulation was not seen in untreated annexinV/apo2.7 positive B cells. Stronger expression of CD60 b during the apoptotic process may therefore follow those events which are marked by annexin V binding and staining with mAb apo2.7. Only small changes in CD60 a and CD60 c surface expression on B cells were seen (Fig. 4a). Thus it seems that both CD60 variants have different functions during the apoptotic process of tonsillar B cells. During apoptosis of tonsillar T cells a small population with increased CD60 c and slightly increased CD60 a expression appeared whereas no significant changes of CD60 b expression were visible after staurosporine treatment of CD3+ T cells (Fig. 4a).

Influence of CD60 b and c on the proliferation of tonsillar lymphocytes

Antibodies against CD60 b (UM4D4, M-T6004) and CD60 c (U5) have been reported to induce proliferation in T lymphocytes. In particular, addition of CD60 b mAb to *in vitro* cultivated T cells from synovial fluid resulted in a costimulatory effect together with PMA or IL-2 [12] whereas CD60 c mAb U5 induced proliferation of peripheral blood T cells without further exogenous signals [13]. We compar-

atively investigated stimulatory effects on tonsillar CD19+ B and CD3+ T cells.

We found that both CD60 b and c mAb had a costimulatory effect on B cells together with anti-IgM/IL-4 treatment, as demonstrated by enhanced proliferation rates. Antibody treatment alone did not induce proliferation in B cells. No positive effect of CD60 a mAb R24 on B cell proliferation was seen (Fig. 5). Although a stimulatory effect of mAb R24 on proliferation of peripheral T cells had been described earlier [14] we could not observe this effect with tonsillar T cells. Tonsillar T cells were induced to proliferation by CD60 c mAb alone with a slightly enhancing effect to PHA-activation, CD60 b mAb gave only a costimulatory signal together with PHA.

Considering the different expression of CD60 b and c during tonsillar lymphocyte apoptosis we also measured the percentage of apoptotic cells after addition of CD60 a, b and c mAb to the cells by annexin V binding and apo2.7 expression. No effect on apoptosis induction of the CD60 antibodies could be observed as compared to untreated cells (data not shown).

Involvement of CD60 b and c in raft formation

Lymphocyte activation coincides with raft formation which concentrates relevant receptors in defined cell surface domains [29, 30]. Glycosphingolipids and GPI-anchored glycoproteins are highly enriched in rafts, possibly involved in the contraction process [30]. We investigated localization of CD60 b and c on tonsillar B and T cells by confocal LSM. As specific marker for glycolipid-rich raft domains we used CTB which binds to GM1.

GM1 was located in concentrated raft-like areas both of B and T cells (Fig. 6a,b). CD60 b showed a distribution pattern on B and T cells which was identical to that of GM1 as demonstrated with double fluorescence (Fig. 6a). Also CD60 c could be colocalized with GM1 in clustered rafts of B cells whereas on T cells CD60 c showed a more homogenous distribution on the cells surface with only a partial overlapping binding pattern with CTB (Fig. 6b). Double fluorescence of CD60 b and c of B cells revealed a colocalization of these carbohydrate structures in the same rafts to the largest extent, on T cells as expected no overall colocalization was observed (Fig. 6c).

Tyrosin kinases Lyn and Syk are involved in the intracellular signalling cascade during B cell activation [31] and are enriched in raft domains [30]. In order to investigate whether CD60 b and c containing surface domains are possibly involved in these kinase-mediated signalling pathways we performed immunoprecipitation using CD60 b and c mAb and subsequent Western blot staining with mAb against Syk and Lyn.

As shown in Fig. 7, both kinases were coprecipitated together with CD60 b and c which underlines the possible

involvement of the CD60 b and c structures in surface domains of activation receptors.

Discussion

In this paper we show that CD60 b and c mAb define different populations of tonsillar T and B lymphocytes during the process of activation and apoptosis.

The different staining patterns of cells within the secondary follicles of tonsils points to the expression of CD60 b and c on different subpopulations in particular of activated germinal center B cells. CD60 b and even stronger CD60 c are expressed on cells within the extrafollicular T cell area and are markers for tonsillar T cells as shown on isolated CD3+ T cells. The extrafollicular T cell area is primarily colonized by CD4+>CD8+ lymphocytes and to a minor proportion by interdigitating dendritic cells, some macrophages and fewer B cells [32, 33]. With regard to CD60 a,b,c expression no major differences were seen between tonsillar CD4+ and CD8+ T cells (data not shown). Interestingly, in contrast to lymphocytes of the T cell zone, tonsillar B cells showed no significant surface expression of CD60 a as demonstrated by missing or weak mAb R24 reactivity and as observed both on native and in vitro cultivated tonsillar B cells. Spotted R24 staining in tonsillar immunohistology in germinal centers may be due to reactivity with T cells in this area. Thus it appears that in B cells mechanisms exist which regulate the controlled and almost exclusive transport of O-acetylated GD3 to the plasma membrane.

As another difference between T and B cells, in vitro stimulation of tonsillar B cells by either anti-IgM/IL-4 or pansorbin did not significantly alter expression of CD60 b and c whereas PHA stimulation induced enhanced expression of all CD60 subgroups in T cells. Therefore it seems that also the density of surface expressed CD60 structures is differentially regulated in tonsillar B and T cells. In this study we could not clarify which intrinsic or exogenous factors regulate surface expression of CD60 b and c during the in vivo activation and differentiation process of tonsillar B cells.

Nonetheless both O-acetylated CD60 structures are involved in the activation of B cells since CD60 b and c mAb could provide a costimulatory signal for enhanced proliferation of in vitro anti-μ/IL-4 treated B cells. This again is in contrast to T cells where CD60 c mAb alone was able to provide a mitogenic signal for T cell proliferation, CD60 b mAb was costimulatory together with PHA. It should be noted that in our experiments CD60 a mAb R24 despite its strong staining of tonsillar T cells and in contrast to CD60 c mAb U5 gave no mitogenic signal but like CD60 b mAb MT6004 a costimulatory signal. Our experi-

ments confirmed earlier observations that cell surface-expressed 9-O-acetyl- and 7-O-acetyl-GD3 variants are involved in activation of T lymphocytes derived from peripheral blood or synovial fluid [12, 13].

In order to answer the question as to which carrier molecule CD60 sequences are associated at the cell surface we followed three independent approaches: 1) treatment of the cells with OSGE did not result in a decrease of binding both of CD60 b and c mAb on tonsillar B and T cells, 2) extraction of glycosphingolipids from membranes by methanol treatment abolished binding of CD60 mAb to B cells and significantly reduced the binding to T cells. 3) immunoprecipitation of lysates from surface biotinylated T and B cells only revealed a distinct though weak band of approximately 65 kDa in CD60 b precipitates of T cells (data not shown). Such a band was earlier reported on other T cell preparations [8, 34]. In summary of these results we tentatively propose that on tonsillar B cells CD60 b and c carbohydrate moieties are exclusively expressed on gangliosides whereas on T cells a minor proportion may also be expressed on a distinct glycoprotein.

During apoptosis of B and T cells CD60 b and c were differently expressed. Apoptosis is a key regulator system of tonsillar lymphatic development by elimination of negatively selected germinal center B cells [35]. After in vitro staurosporine induction of apoptosis a distinct sub-population of annexin V positive/Via negative B cells were highly positive for CD60 b but negative for CD60 c. The same was observed with in vitro cultivated B cells undergoing spontaneous apoptosis. How can these results be explained in view of the aforementioned observation that 9-O-acetylated GD3 (CD60 b) suppresses GD3 (CD60 a) induced mitochondrial permeability transduction thus preventing apoptosis [18]? Recently it has been shown that CD60 b was induced in Jurkat cells after apoptosis induction with GD3 [36]. In our study we solely describe cell surface phenomena. It may well be that O-acetylated gangliosides fulfill different tasks at the cell surface and within the cell and that surface transport is differentially regulated for GD3 and O-acetylated variants thereof. In first experiments we could observe by flow cytometric analysis of antibody stainings that during staurosporine-induced apoptosis of tonsillar B lymphocytes the cytoplasmic amount of GD3 but not that of 9-O-acetyl- and 7-O-acetyl GD3 is increasing while surface expression of GD3 remains weak (unpublished results).

Our results point to the possibility that CD60 b+/annexinV+germinal center B cells are programmed for apoptosis whereas CD60 c+ B cell subpopulations may represent positively selected survivors. In T cells no distinct subsets with regard to CD60 b and c expression in relation to apoptosis induction were observed. For T cells it has previously been shown that GD3 contributes to FAS-mediated apoptosis [17, 37].

Since lipid raft plasma microdomains serve as platform for coordinated B cell receptor signaling [29, 30] and given the fact that glycolipids such as CD77/Gb3 on B cells [38] and GM1 and GD3 in neuronal cells [16] have been described as supportive elements for raft formation we investigated whether CD60 b and c gangliosides are present in rafts of tonsillar B cells. It became evident that CD60 b and c are constituents of raft-like structure on plasma membranes of B cells together with GM1 as demonstrated by CTB-binding. In T cells CD60 b appears together with GM1 in rafts whereas CD60 c shows a more homogenous plasma membrane expression. The coincidence of CD60 c surface distribution and mitogenic properties of CD60 c mAb in T cells is a phenomenon which deserves further investigation. It should be noted that mitogenic reagents as for instance PHA or pokeweed mitogen (PWM) lectins although possibly inducing raft formation themselves may not require T- or B-cell receptor enriched rafts for inducing signal transduction during activation. It may be that in T cells, CD60 c is linked to a different activation mechanism as compared to costimulatory signals transferred by CD60 b both in T and B cells and CD60 c in B cells. In corroboration of the contribution of CD60 b and c in B cell activation we found that both ganglioside structures are apparently in close physical proximity to Src family tyrosine kinases Syk and Lyn within raft surface domains of tonsillar B cells. Syk and Lyn play a dominant role in the signalling cascade during B cell activation [31]. Together, we postulate from our observations that CD60 b and c have a regulatory role in raft formation for coordinated receptor complexing during B cell activation and provide selection markers for germinal center apoptosis of B cells.

References

1. Schauer, R.: Sialic acids: Fascinating sugars in higher animals and man. Zoology **107**, 49–64 (2004)
2. Tiralongo, J., Schauer, R.: The enigma of enzymatic sialic acid O-acetylation. Trends Glycosci. Glycotechnol. **16**, 1–15 (2004)
3. Blum, A.S., Barnstable, C.J.: O-acetylation of a cell-surface carbohydrate creates discrete molecular patterns during neuronal development. Proc. Natl. Acad. Sci. USA **84**, 8716–8720 (1987)
4. Santiago, M.F., Costa, M.R., Mendez-Otero, R.: Immunoblockage of 9-O-acetyl GD3 ganglioside arrests the *in vivo* migration of cerebellar granule neurons. J. Neurosci. **24**, 474–478 (2004)
5. Shi, W.X., Chammas, R., Varki, N.M., Powell, L., Varki, A.: Sialic acid 9-O-acetylation on murine erythroleukemia cells affects complement activation, binding to I-type lectins, and tissue homing. J. Biol. Chem. **271**, 31526–31532 (1996)
6. Rogers, G.N., Herrler, G., Paulson, G.C., Klenk, H.D.: Influenza C virus uses 9-O-acetyl-N-acetylneuraminic acid as a high affinity receptor determinant for attachment to cells. J. Biol. Chem. **261**, 5947–5951 (1986)
7. Vlasak, R., Luytjes, W., Spaan, W., Palese, P.: Human and bovine coronaviruses recognize sialic acid containing receptors similar to those of influenza C viruses. Proc. Natl. Acad. Sci. USA **85**, 4526–4529 (1988)
8. Fox, D.A., He, X., Abe, A., Hollander, T., Li, L.L., Kann, L., Friedman, A.W., Shimizu, Y., Shayman, J.A., Kozarsky, K.: The T lymphocyte structure CD60 contains a sialylated carbohydrate epitope that is expressed on both gangliosides and glycoproteins. Immunol. Invest. **30**, 67–85 (2001)
9. Kniep, B., Peter-Katalinic, J., Flegel, W.A., Northoff, H., Rieber, E.P.: CDw60 antibodies bind to acetylated forms of ganglioside GD3. Biochem. Biophys. Res. Commun. **187**, 1343–1349 (1992)
10. Kniep, B., Flegel, W.A., Northoff, H., Rieber, E.P.: CDw60 glycolipid antigens of human leukocytes: Structural characterization and cellular distribution. Blood **82**, 1776–1786 (1993)
11. Rieber, E.P., Rank, G.: CDw60: A marker for human CD8+ T helper cells. J. Exp. Med. **179**, 1385–1390 (1994)
12. Fox, D.A., Millard, J.A., Kan, L., Zeldes, W.S., Davis, W., Higgs, J., Emmrich, F., Kinne, R.W.: Activation pathways of synovial T lymphocytes. Expression and function of the UM4D4/CDw60 antigen. J. Clin. Invest. **86**, 1124–1136 (1990)
13. Kniep, B., Claus, C., Peter-Katalinic, J., Monner, D.A., Dippold, W., Nimtz, M.: 7-O-acetyl-GD3 in human T lymphocytes is detected by a specific T-cell-activating monoclonal antibody. J. Biol. Chem. **270**, 30173–30180 (1995)
14. Welte, K., Miller, G., Chapman, B.P., Yuasa, H., Natoli, H., Kunicka, J.E., Cordon-Cardo, C., Buhrer, C., Old, L.J., Houghton, A.N.: Stimulation of T lymphocyte proliferation by monoclonal antibodies against GD3 ganglioside. J. Immunol. **139**, 1763–1771 (1987)
15. Schwartz-Albiez, R.: Carbohydrates and lectin: Section report. In: Mason, D. (ed.) Leucocyte Typing VII, pp. 149–164. Oxford University Press, Oxford (2002)
16. Kasahara, K., Watanabe, K., Takeuchi, K., Kaneko, H., Oohira, A., Yamamoto, T., Sanai, Y.: Involvement of gangliosides in glycosylphosphatidylinositol-anchored neuronal cell adhesion molecule TAG-1 signaling in lipid rafts. J. Biol. Chem. **275**, 34701–34709 (2000)
17. DeMaria, R., Lenti, L., Malisan, F., d'Agostino, F., Tomassini, B., Zeuner, A., Rippo, M.R., Testi, R.: Requirement for GD3 ganglioside in CD95- and ceramide-induced apoptosis. Science **277**, 1652–1655 (1997)
18. Malisan, F., Franchi, L., Tomassini, B., Ventura, N., Condò, I., Rippo, M.R., Rufini, A., Liberati, L., Nachtigall, C., Kniep, B., Testi, R.: Acetylation suppresses the proapoptotic activity of GD3 ganglioside. J. Exp. Med. **196**, 1535–1541 (2002)
19. Vater, M., Kniep, B., Gross, H.J., Claus, C., Dippold, W., Schwartz-Albiez, R.: The 9-O-acetylated disialosyl carbohydrate sequence of CDw60 is a marker on activated human B lymphocytes. Immunol. Letters **59**, 151–157 (1997)
20. Claus, C., Gocht, A., Schwartz-Albiez, R., Lünsdorf, H., Kniep, B.: CD60: Specificity of the antibodies, distribution of the antigens, and functional aspects. In: Mason, D. (ed.) Leucocyte Typing VII, pp. 187–188. Oxford University Press, Oxford (2002)
21. Strasser, P., Unger, U., Strobl, B., Vilas, U., Vlasak, R.: Recombinant viral sialate-O-acetylesterases. Glycoconj. J. **20**, 551–561 (2004)
22. Schwartz-Albiez, R., Dörken, B., Möller, P., Brodin, N.T., Monner, D.A., Kniep, B.: Neutral glycosphingolipids of the globo-series characterize activation stages corresponding to germinal center B cells. Int. Immunol. **2**, 929–936 (1990)

23. Corfield, A.P., Wagner, S.A., O'Donell, L.J., Durdey, P., Mountford, R.A., Clamp, J.R.: The roles of enteric bacterial sialidases, sialate *O*-acetyl esterase and glycosyltransferase in the degradation of human colonic mucin. Glycoconj. J. **10**, 72–81 (1993)

24. Kamerling, J.P., Schauer, R., Shukla, A.K., Stoll, S., van Halbeek, H., Vliegenhart, J.F.: Migration of *O*-acetyl groups in *N,O*-acetylneuraminic acids. Eur. J. Biochem. **162**, 601–607 (1987)

25. Vandamme-Feldhaus, V., Schauer, R.: Characterization of the enzymatic 7-*O*-acetylation of sialic acids and evidence for enzymatic *O*-acetyl migration from C-7 to C-9 in bovine submandibular gland. J. Biochem. **124**, 111–121 (1998)

26. Sutherland, D.R., Abdullah, K.M., Cyopick, P., Mellors, A.: Cleavage of the cell-surface *O*-sialoglycoproteins CD34, CD43, CD44, CD45 by a novel glycoprotease from *P.haemolytica*. J. Immunol. Meth. **148**, 1458–1464 (1992)

27. Dillon, S.R., Mancini, M., Rosen, A., Schlissel, M.S.: Annexin V binds to viable B cells and colocalizes with a marker of lipid rafts upon B cell receptor activation. J. Immunol. **164**, 1322–1332, (2001)

28. Carthy, C.M., Granville, D.J., Jiang, H., Levy, J.G., Rudin, C.M., Thompson, C.B., McManus, B.M., Hunt, D.W.: Early release of mitochondrial cytochrome c and expression of mitochondrial epitope 7A6 with a porphyrin-derived photosensitizer: Bcl-2 and Bcl-xL overexpression do not prevent early mitochondrial events but still depress caspase activity. Lab. Invest. **79**, 953–965 (1999)

29. Cheng, P.C., Cherukuri, A., Dykstra, M., Malapati, S., Sproul, T., Chen, M.R., Pierce, S.K.: Floating the raft hypothesis: The roles of lipid rafts in B cell antigen receptor function. Sem. Immunol. **13**, 107–114 (2001)

30. Pierce, S.K.: Lipid rafts and B cell activation. Nat. Rev. Immunol. **2**, 96–105 (2002)

31. Hsueh, R.C., Scheuermann, R.H.: Tyrosine kinase activation in the decision between growth, differentiation, and death responses initiated from the B cell antigen receptor. Adv. Immunol. **75**, 283–316 (2000)

32. Nave, H., Gerbert, A., Pabst, R.: Morphology and immunology of the human palatine tonsil. Anat. Embryol. **204**, 367–373 (2001)

33. Bergler, W., Adam, S., Gross, H.J., Hörmann, K., Schwartz-Albiez, R.: Age-dependent altered proportions in subpopulations of tonsillar lymphocytes. Clin. Exp. Immunol. **116**, 9–18 (1999)

34. Rieber, E.P., Kniep, B., Rank, G.: A membrane-protein-associated oligosaccharide defining functional T-cell subsets. In: Knapp, W. *et al.* (eds.) Leucocyte Typing IV, pp. 366–368. Oxford University Press, Oxford (1989)

35. Defrance, T., Casamayor-Palleja, M., Krammer, P.H.: The life and death of a B cell. Adv. Cancer Res. **86**, 195–225 (2002)

36. Kniep, B., Kniep, E., Özkucur, N., Barz, S., Bachmann, M., Malisan, F., Testi, R., Rieber, E.P.: 9-*O*-acetyl GD3 protects tumor cells from apoptosis. Int. J. Cancer, **119**(1), 67–73 (2006)

37. Giammarioloi, A.M., Garofano, T., Sorice, M., Misasi, R., Gambardella, L., Gradini, R., Fais, S., Pavan, A., Malori, W.: GD3 glycosphingolipid contributes to Fas-mediated apoptosis via association with ezrin cytoskeletal protein. FEBS Lett. **506**, 45–50 (2001)

38. Mori, T., Kiyokawa, N., Katagiri, Y.U., Taguchi, T., Suzuki, T., Sekino, T., Sato, N., Ohmi, K., Nakajima, H., Takeda, T., Fujimoto, J.: Globotriaosyl ceramide (CD77/Gb3) in the glyco-lipid-enriched membrane domain participates in B-cell receptor-mediated apoptosis by regulating lyn kinase activity in human B cells. Exp. Hematol. **28**, 1260–1268 (2000)