Glycoconj J (2007) 24:17–24
DOI 10.1007/s10719-006-9007-y

O-acetylation of sialic acids is required for the survival of lymphoblasts in childhood acute lymphoblastic leukemia (ALL)

Shyamasree Ghosh · Suman Bandyopadhyay · Kankana Mukherjee · Asish Mallick · Santanu Pal · Chhabinath Mandal · Dilip K. Bhattacharya · Chitra Mandal

Published online: 5 December 2006
© Springer Science + Business Media, LLC 2006

Abstract Exploiting the selective affinity of Achatinin-H towards 9-*O*-acetylneuraminic acid(α2-6)GalNAc, we have demonstrated the presence of 9-*O*-acetylated sialoglycoproteins (Neu5,9Ac$_2$-GPs) on hematopoietic cells of children suffering from acute lymphoblastic leukemia (ALL), indicative of defective sialylation associated with this disease. The carbohydrate epitope of Neu5,9Ac$_2$-GPs$_{ALL}$ was confirmed by using several synthetic sialic acid analogues. They are functionally active signaling molecules as demonstrated by their role in mediating lymphoproliferative responses and consequential increased production of IFN-γ due to specific stimulation of Neu5,9Ac$_2$-GPs on PBMC$_{ALL}$ with Achatinin-H. Cells devoid of 9-*O*-acetylations (9-*O*-AcSA$^-$) revealed decreased nitric oxide production as compared to 9-*O*-AcSA$^+$ cells on exposure to IFN-γ. Under this condition, a decrease in viability of 9-*O*-AcSA$^-$ cells as compared to 9-*O*-AcSA$^+$ cells was also observed which was reflected from increased caspase 3 activity and apoptosis suggesting the protective role of this glycotope. These Neu5,9Ac$_2$-GPs are also capable of inducing disease-specific anti-Neu5,9Ac$_2$-GPs antibodies in ALL children. Additionally, we have observed that disease-specific anti-Neu5,9Ac$_2$-GPs have altered glycosylation profile, and they are incapable of exerting a few Fc-glycosylation-sensitive effector functions. These observations hint toward a disbalanced homeostasis, thereby enabling the cancer cells to escape host defense. Taken together, it may be hypothesized that Neu5,9Ac$_2$-GPs and their antibodies play a prominent role in promoting the survival of lymphoblasts in ALL.

Keywords Acute lymphoblastic leukemia · Achatinin-H: an *O*-acetylated sialic acid binding lectin · 9-*O*-acetylated sialoglycoconjugates · IFN-γ · Caspase-3-like activity

Abbreviations

ALL	Acute Lymphoblastic Leukemia
IFN-γ	interferon gamma
9-*O*-AcSA	9-*O*-acetylated sialic acids
9-*O*-acetylated sialoglycoproteins	Neu5,9Ac$_2$-GPs
de-*O*-acetylated cells	9-*O*-AcSA$^-$
PBMC$_{ALL}$	Peripheral blood mononuclear cells from ALL patients
NO	nitric oxide

S. Ghosh · S. Bandyopadhyay · K. Mukherjee · A. Mallick · S. Pal · C. Mandal (✉)
Immunobiology Division, Indian Institute of Chemical Biology,
4 Raja S.C. Mullick Road,
Kolkata 700 032, India
e-mail: cmandal@iicb.res.in

D. K. Bhattacharya
Vivekananda Institute of Medical Sciences,
Kolkata 700045, India

C. Mandal
Drug Design Development and Molecular Modelling,
Indian Institute of Chemical Biology,
4 Raja S.C. Mullick Road,
Kolkata 700 032, India

Introduction

Sialic acids, commonly referred to as *N*-acetylneuraminic acid (Neu5Ac), are a family of 9-carbon carboxylated monosaccharides. Amongst 50 known derivatives of sialic acids, 7, 8, and 9-*O*-acetylated derivatives (*O*-AcSA) are important constituents of the cell membrane and known to influence many physiological and pathological processes

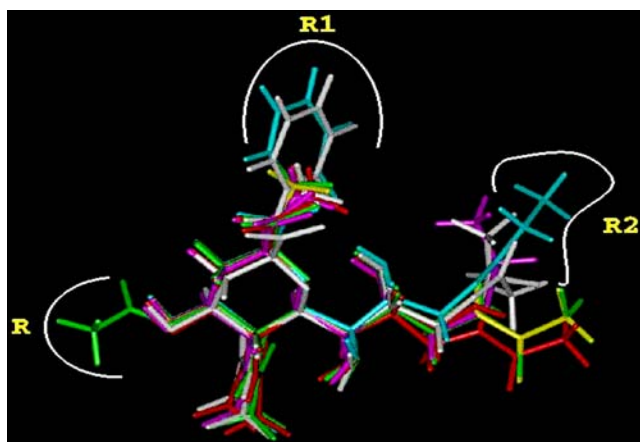

Fig. 1 Glycotope of Neu5,9Ac$_2$-GPs. Superimposed 3D structures of all the sialic acid derivatives which were used as inhibitors of the binding of Neu5,9Ac$_2$-GPs$_{ALL}$ to Achatinin-H as described in Table 1

[1, 2] including cell–cell adhesion, signaling, differentiation, and metastasis [3–6]. Sialic acids and derivatives have been reported to be biomarkers for various types of cells including 9-*O*-AcGD3 as an oncofetal marker for human melanoma [7], cdw60 on activated B lymphocytes [8]. Basal level expression of *O*-acetylated sialic acids is also reported on B lymphocytes [9].

Acute Lymphoblastic Leukemia (ALL) is a malignant transformation of lymphoblasts and represents the single most common type of cancer in the pediatric population. With the advent of modern chemotherapy, virtually all patients achieve remission and approximately 80% are cured. The risk of relapse remains at 20% as patients in remission may harbor residual leukemic blasts referred to as minimal residual disease (MRD), and prediction of this still remains a major challenge in leukemia research [10]. In our search for alternate and better markers to the disease, we demonstrated the over-expression of 9-*O*-acetylated sialoglycoproteins (Neu5,9Ac$_2$-GPs) on lymphoblasts of ALL patients [11, 12]. Subsequently, we reported an enhanced level of anti-Neu5,9Ac$_2$-GPs in the sera of these patients [13, 14] and employed both Neu5,9Ac$_2$-GPs and anti-Neu5,9Ac$_2$-GPs in the successful detection and monitoring of the disease status [15–20] of patients with childhood ALL.

Although evidences on the role of gangliosides in controlling cell death are increasing [21, 22], the role of 9-*O*-acetylated sialic acids in regulating cellular features remains yet to be studied in details and assessment of Neu5,9Ac$_2$-GPs in ALL is a relatively new domain. Thus, in successive studies we tried to understand (a) the structure of the glycotope, (b) their occurrence on different types of hematopoietic cells in these patients, (c) their biochemical and immunological characterization, and (d) their detailed functional significance, especially their role in modulating cellular behavior in the disease.

Neu5,9Ac$_2$-GPs over-expressed on hematopoietic cells of ALL patients

Over-expression of disease-specific Neu5,9Ac$_2$-GPs on peripheral blood mononuclear cells of childhood ALL patients (PBMC$_{ALL}$, 11–12, 15–19) was observed by exploiting the selective binding affinity of a lectin, Achatinin-H, toward Neu5,9Ac$_2$(α2-6)GalNAc [23, 24]. We have further demonstrated the presence of Neu5,9Ac$_2$ on these lymphoblasts by Fluorimetric HPLC and corroborated by Flow cytometric analysis using FITC-Achatinin-H [19]. The nature of the glycotope (Neu5,9Ac$_2$(α2-6) GalNAc) of Neu5,9Ac$_2$-GPs$_{ALL}$ (Fig. 1, Table 1, 19) was successfully established by Inhibition–ELISA experiments using several synthetic sialic acid analogues serving as

Table 1 Inhibitory potency of sialic acid analogues for the binding of Neu5,9Ac$_2$-GPs$_{ALL}$

	Sialic acids	R1	R2	R	Relative inhibition (RI) (%)
1	Me-α-Neu5,9Ac$_2$	CH_3	O•CO•CH_3	OH	100
2	Me-α-Neu 5Ac	CH_3	OH	OH	28
3	Me-α-Neu5Ac,9-SAc	CH_3	S•CO•CH_3	OH	20
4	Me-α-Neu5Ac-9-NHAc	CH_3	NH•CO•CH_3	OH	13
5	Me-α-Neu5Ac9-NHFAc	CH_3	NH•CO•CH_2F	OH	2.8
6	Benzyl-α-Neu4,5,9Ac$_3$	$C_6H_5CH_2$	O•CO•CH_3	O•CO•CH_3	71
7	Benzyl-α-Neu5Ac-9-*O*-Propionyl	$C_6H_5CH_2$	O•CO•CH_2•CH_3	OH	2
8	Neu5 Ac	H	OH	OH	NI
9	Neu4,5Ac$_2$	H	OH	O•CO•CH_3	NI

Purified Neu5,9Ac$_2$-GPs$_{ALL}$ in absence or presence of fixed concentration of analogue (68 mM) in the presence of Ca^{2+} (30 mM) was added to Achatinin-H coated well and incubated at 4°C for 30 min. The binding of Neu5,9Ac$_2$-GPs$_{ALL}$ to Achatinin-H was detected by incubating a fixed amount of purified anti-Neu5,9Ac$_2$-GPs$_{ALL}$ antibodies for overnight at 4°C and followed by HRP-Protein A.
The % inhibition (PI) for a particular inhibitor = 100 – [100 × (O.D. in presence of inhibitor / O.D. in absence of inhibitor)].
RI $= 100 \times$ (PI of an analogue at 68 mM)/(PI of Me -α-Neu5, 9Ac$_2$ at 68 nM).
H = hydrogen, Ac = Acetyl group (−$COCH_3$), S = sulphur, N = Nitrogen, F = Fluorine, NI: not inhibited. The data was taken from [19].

Table 2 Expression of 9-*O*-AcSAs on hematopoetic cells of ALL patients

9-*O*-AcSA positive cells (%)					
	Cells				
	B	T	Monocytes	Myeloid	Erythrocytes
$PBMC_{ALL}$	80 ± 10	90 ± 8	14 ± 4	10 ± 5	60 ± 10
$PBMC_N$	3 ± 1	5 ± 4	2 ± 1	3 ± 2	1 ± 0.5

inhibitors of the binding between Neu5,9Ac_2-GPs_{ALL} and Achatinin-H [19].

Further in-depth study revealed that, although population of myeloid and monocyte cells were lower than that of B or T cells among the entire population of $PBMC_{ALL}$, Neu5,9Ac_2-GPs were over-expressed on all the hematopoietic cells including B, T, myeloid, monocytic cells in these patients (Neu5,9Ac_2-GPs_{ALL}, Table 2). Interestingly, a high level of Neu5,9Ac_2-GPs was also induced on erythrocytes of these patients (Table 2) [25] while only basal level expression was observed on hematopoietic cells of normal individuals (Neu5,9Ac_2-GPs_N). These results were suggestive of a major defect in the glycosylation pattern on hematopoietic cells in patients suffering from this disease.

Neu5,9Ac_2-GPs_{ALL} are signaling molecules

To understand the functional implications of these molecules on the hematopoietic cells in these patients, we activated the newly induced Neu5,9Ac_2(α2-6)GalNAc glycotopes on $PBMC_{ALL}$ with Achatinin-H (0.1 μg/10^5 cells, Fig. 2a and b). This specific interaction led to a significant lymphoproliferative response as reflected by the increased proliferation (Fig. 2a) suggesting the functionally active nature of the disease-specific Neu5,9Ac_2-GPs_{ALL}. In contrast, lack of proliferation of PBMC isolated from patients with cross reactive diseases like acute myelogeneous leukemia (AML) and chronic lymphocytic leukemia (CLL) patients and normal individuals ($PBMC_N$) under similar conditions suggested disease-specific stimulation via interaction with Neu5,9Ac_2-GPs_{ALL} (Fig. 2a).

Absence of lymphoproliferation of $PBMC_{ALL}$ by Achatinin-H was observed when *O*-acetylation was ablated from $PBMC_{ALL}$, by treatment with recombinant 9-*O*-acetyl haemagglutinin esterase of influenza C virus [26]. This reconfirmed the involvement of the *O*-acetyl group in this stimulation process (Fig. 2b).

We have also observed that interaction of Neu5,9Ac_2-GPs_{ALL} on $PBMC_{ALL}$ with Achatinin-H led to the release of a high amount of IFN-γ (Fig. 3a) *in vitro* in the culture supernatant together with an increased mRNA level (Fig. 3b) indicating the potential of Neu5,9Ac_2-GPs_{ALL} in mediating signaling responses [28].

Since a normal B cell has been reported to be incapable of IFN-γ production [27] and as the study population included patients with around 80 to 90% lymphoblasts in the peripheral blood, it may be envisaged that the release of IFN-γ is solely mediated by leukemic lymphoblasts. However, as a small population of other cells also express Neu5,9Ac_2-GPs_{ALL} their collective interplay may also be responsible for the observed effects.

In addition, our observation of a three to four fold increase in serum concentration of IFN-γ in these patients as compared to the normal individuals (652–946 *vs.* 171–235 pg/ml) probably indicates an ongoing signaling process *in vivo* [28]. The presence of anti-Neu5,9Ac_2-GPs_{ALL} in sera of these children [13, 14, 20] may act as ligand for newly induced Neu5,9Ac_2-GPs_{ALL} on $PBMC_{ALL}$ leading to

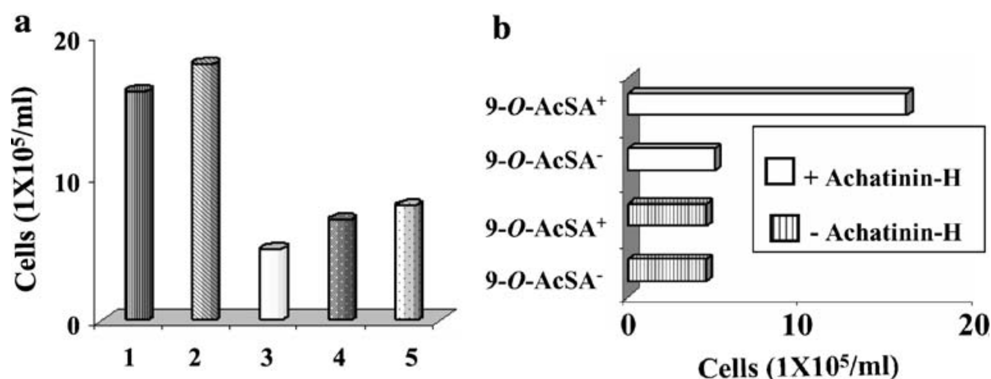

Fig. 2 Achatinin-H induces lymphoproliferation to $PBMC_{ALL}$. (a) PBMC (1×10^5/ml) from B ALL (1), T ALL (2), normal (3), AML (4), CML (5) individuals, are cultured in the presence of Achatinin-H (0.1 μg) for 48 h. Cells were counted microscopically and results are expressed as live cell count after staining with trypan blue. Data is a representative profile of 10 independent experiments after subtracting from cells cultured in the absence of Achatinin-H. (b) Lymphoproliferation needs interaction between Achatinin-H and 9-*O*-AcSAs. Cells, ablated of *O*-acetylations (9-*O*-AcSA$^-$), cultured in presence of Achatinin-H did not show any significant proliferation. Cells cultured in absence of Achatinin-H served as controls [28]

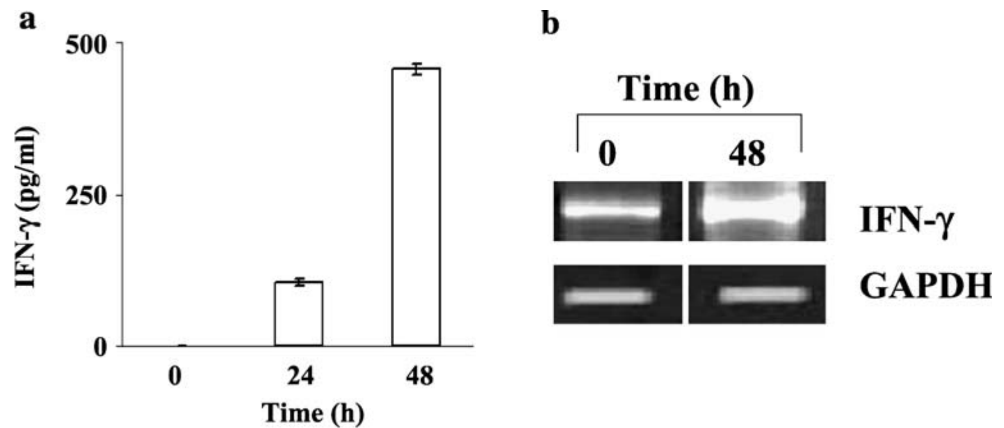

Fig. 3 9-*O*-AcSAs are signaling molecules. (a) $PMBC_{ALL}$ cultured in the presence of Achtatinin-H (0.1 μg) for 48 h and the concentration of released IFN-γ was detected by ELISA. Unstimulated cells served as the control. (b) Total RNA from Achatinin-H (0.1 μg) stimulated $PBMC_{ALL}$ was isolated and the expression of IFN-γ was analyzed by RT–PCR. GAPDH was housekeeping gene. Unstimulated cell served as the control

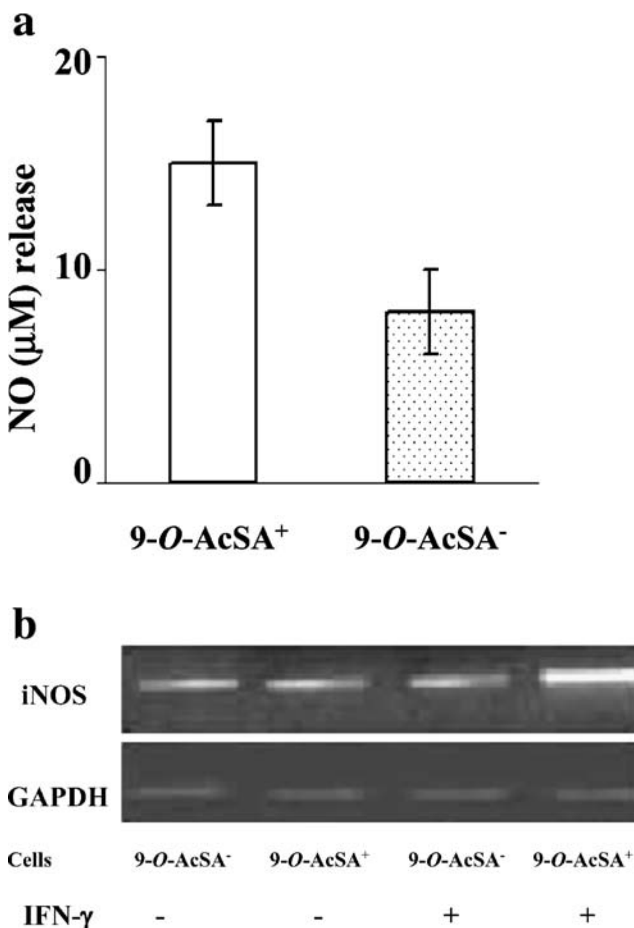

Fig. 4 9-*O*-AcSA$^+$ reveal differential production of NO and iNOS when exposed to IFN-γ (a) $PBMC_{ALL}$ was exposed to IFN-γ (0.1 ng) for 6 h and the release of nitric oxide in the culture supernatant was determined spectrophotometrically by Griess Reaction. Cells in absence of IFN-γ served as the controls. (b) Total RNA was isolated from 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells exposed to IFN-γ (+). Levels of iNOS and a housekeeping gene, GAPDH were assayed by RT–PCR. Cells in absence of IFN-γ (−) served as control [29]

an increased level of IFN-γ in these patients. Thus it may be aptly concluded that Neu5,9Ac_2-GPs_{ALL} can mediate the signaling process.

Neu5,9Ac_2-GPs on $PBMC_{ALL}$ regulates differential cellular behaviour

The major question yet to be answered is whether these Neu5,9Ac_2-GPs_{ALL} had any significant role in regulating the survival of $PBMC_{ALL}$. Accordingly, 9-*O*-AcSA$^-$ cells were generated by treating $PBMC_{ALL}$ (1×10^6) with recombinant 9-*O*-acetyl esterase for 1 h at 37°C [26]. The viability of esterase-treated (9-*O*-AcSA$^-$) cells was checked by trypan blue exclusion. Esterase-treated $PBMC_N$ served as control.

Since *O*-acetylation re-appeared after 3 h on a de-*O*-acetylated cell (9-*O*-AcSA$^-$), the complete de-*O*-acetylated status of $PBMC_{ALL}$ was maintained by replenishing the culture media with *O*-acetyl esterase after every 2 h. As the serum of ALL patients is a repository of IFN-γ, the behavior of $PBMC_{ALL}$ (both, 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$) was detected, keeping them in the presence of IFN-γ (0 to 10 ng) for 6 h, and the release of NO was measured by Griess Reaction. The release of NO_2^- were almost 50% lower in 9-*O*-AcSA$^-$ cells as compared to 9-*O*-AcSA$^+$ cells for all doses of IFN-γ. Exposure of cells (9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$) with IFN-γ (0.10 ng) for 6 h led to the release of NO (15 ± 2 μM *vs.* 8 ± 2 μM) respectively (Fig. 4a). Both 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells in the absence of IFN-γ served as the negative controls (data not shown). Enhanced release of NO_2^- by the 9-*O*-AcSA$^+$ cells correlated well with their status of iNOS mRNA expression (Fig. 4b), further indicative of the fact that Neu5,9Ac_2-GPs_{ALL} regulates cellular behaviour [29].

Neu5,9Ac_2-GPs_{ALL} control cell survival in ALL

To detect whether viability of $PBMC_{ALL}$ was affected by the presence of 9-*O*-acetylations both 9-*O*-AcSA$^+$ and 9-*O*-

Table 3 Increased caspase-3 like activity in 9-*O*-AcSA$^+$ $PBMC_{ALL}$

Absorbance 405 nm		
	9-*O*-AcSA$^+$	9-*O*-AcSA$^-$
IFN-γ (0.1 ng/ml)	0.15 ± 0.08	0.30 ± 0.10
IFN-γ (0.1 ng/ml) + LNAME (10 mM)	0.29 ± 0.09	1.50 ± 0.10

Table 4 Increased annexin-V positivity in 9-*O*-AcSA$^+$ $PBMC_{ALL}$

Annexin-V positive (%)		
	9-*O*-AcSA$^+$	9-*O*-AcSA$^-$
Medium	0.13 ± 0.05%	0.12 ± 0.03%
IFN-γ (0.1 ng/ml)+LNAME (10 mM)	20.00 ± 2.00%	2.00 ± 0.02%

AcSA$^-$ cells were exposed to IFN-γ (0.1 ng) for 6 h and assessed for (a) caspase-3-like activity and (b) Annexin V positivity [29]. The caspase-3-like activity was found to be two-fold higher in 9-*O*-AcSA$^-$ than the 9-*O*-AcSA$^+$ cells, OD 405_{nm} being 0.30 ± 0.10 *vs.* 0.15 ± 0.08 (Table 3). The 9-*O*-AcSA$^-$ cells showed another five-fold increase in caspase-3-like activity, when NO_2^- production was lowered using *N*-omega-nitro-L-arginine methyl ester (LNAME, 10 mM) in IFN-γ containing culture medium as compared to 9-*O*-AcSA$^+$ cells OD 405_{nm} being 1.50 ± 0.10 *vs.* 0.29 ± 0.09 (Table 3). IFN-γ is known to influence the production of nitric oxide in certain tumors and may therefore be considered as a bioregulator of apoptosis. If NO_2^- production could be diminished by LNAME, cells would be more prone to apoptosis.

Western blot analysis of the cytosol of IFN-γ-exposed (9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$) cells with anti-caspase 3 revealed a 20-kDa band indicative of active caspase only in

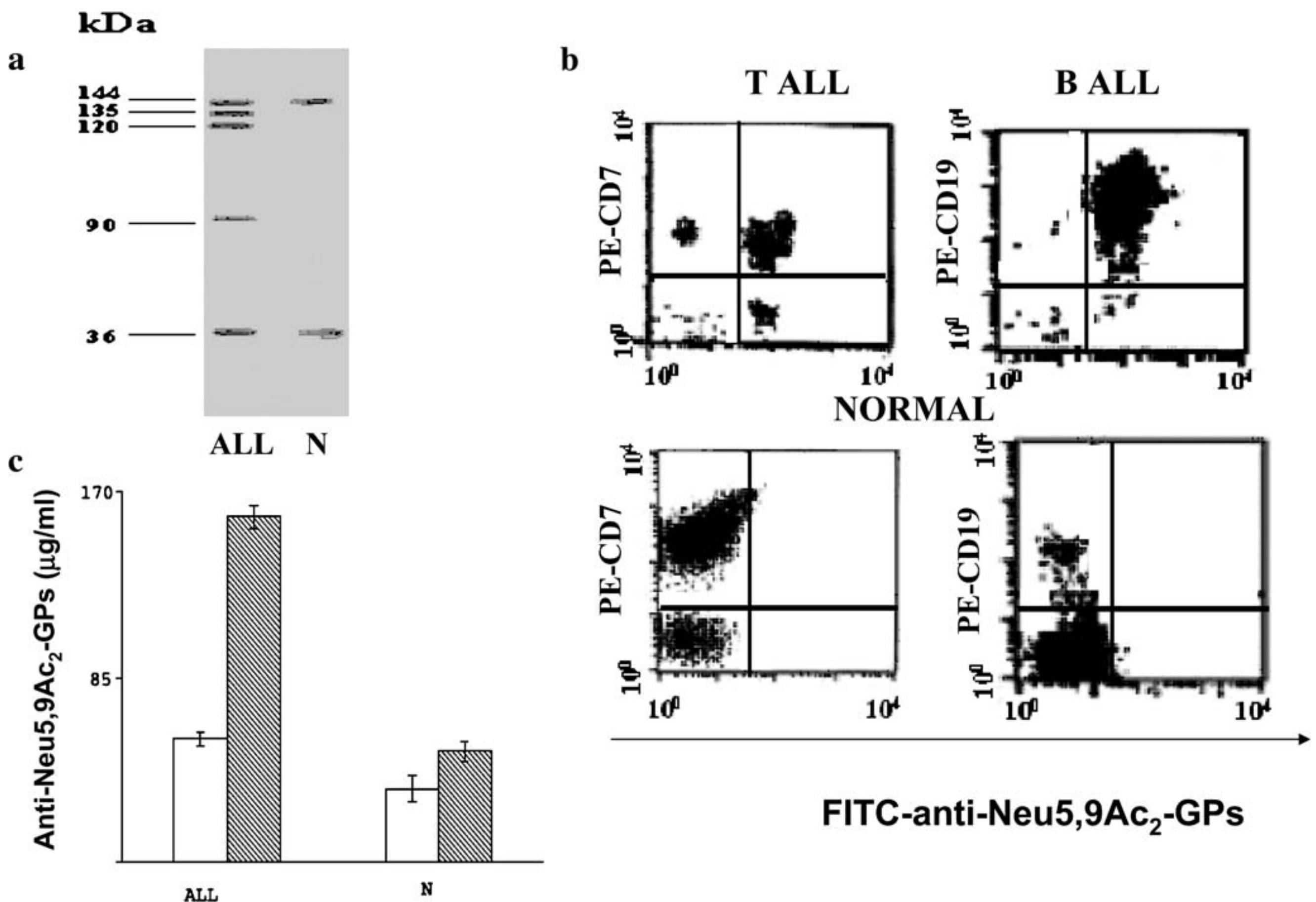

Fig. 5 $Neu5,9Ac_2$-GPs are potentially immunogenic in nature. (a) Western blot analysis of $Neu5,9Ac_2$-GPs present on cell surface of an ALL patient with serum of this patient containing anti $Neu5,9Ac_2$-GPs. (b) Double colour florescence dot plot demonstrating the binding of anti $Neu5,9Ac_2$-GPs to PBMC isolated from ALL (both T and B) patients. Briefly cells (2×10^6) were co incubated with PE-anti-CD7/CD-19 Monoclonal antibody and FITC-purified anti-$Neu5,9Ac_2$-GPs and processed for flow cytometry [14] (c) Quantification of the levels of anti $Neu5,9Ac_2$-GPs. IgG1 (□), IgG2 (▨) in sera of ALL patients at disease presentation along with normal donors (N) by an ELISA using bovine submaxillary mucin as coating antigen [20, 30]

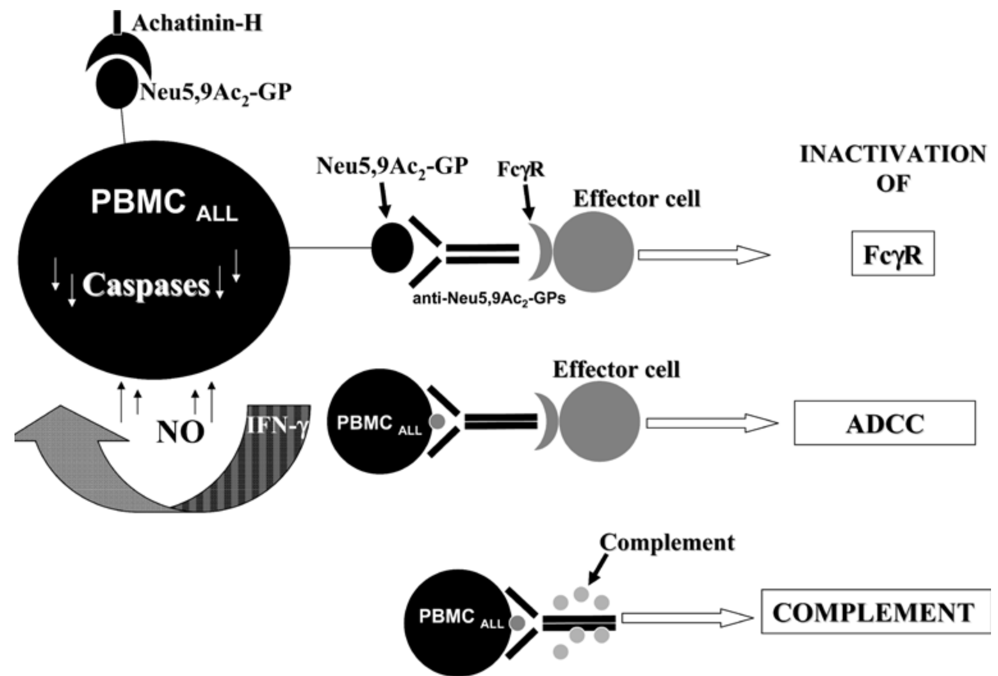

Fig. 6 A scheme of probable role of Neu5,9Ac_2-GPs and anti-Neu5,9Ac_2-GPs in enabling cells escape from immune surveillance

the 9-*O*-AcSA$^-$ cells [29]. However, 32-kDa pro-caspase was present in the cytosol of 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells in both the presence and absence of IFN-γ. A ten-fold more Annexin V positive 9-*O*-AcSA$^-$ cells were observed after exposure with LNAME (10 mM) in the presence of IFN-γ as compared to 9-*O*-AcSA$^+$ cells (Table 4), suggesting a protective role of the glycotope. The decreased Annexin V binding with 9-*O*-AcSA$^+$ cells appeared to be due to increased production of NO_2^- in these cells.

Neu5,9Ac_2-GPs are highly immunogenic

Neu5,9Ac_2-GPs were also found to be immunogenic in nature as reflected by high titers of anti-Neu5,9Ac_2-GPs in the sera of these patients as detected by ELISA using bovine submaxillary mucin as the coating antigen [13, 14, 20, 30]. The specificity of these anti-Neu5,9Ac_2-GPs toward the disease-specific glycotope (Neu5,9Ac_2α2-6GalNAc) was established by Western blot, flow cytometry and ELISA (Fig. 5). Hemagglutination assay and confocal microscopic studies further reconfirm the affinity of this antibody towards Neu5,9Ac_2-GPs [13, 14, 30].

Unfortunately, enhanced amount of disease-associated Neu5,9Ac_2 specific IgG antibodies was unable to trigger activation of FcγR, the complement cascade and cell mediated cytotoxicity (ADCC) in ALL [Fig. 6, 30]. These findings suggest defective triggering of a few Fc-glycosylation-sensitive effector functions in anti-Neu5,9Ac_2-GPs while their glycotope-binding ability remain unaffected. This disbalanced homeostasis is thereby helping the cells to evade the host defense.

Conclusion

We have thus conclusively established the induction of linkage-specific Neu5,9Ac_2-GPs (90 and 120 kDa) containing 9-*O*-AcSA(α2-6)GalNAc glycotope on PBMC of ALL patients [11, 12, 15–19]. Absence of Neu5,9Ac_2-GPs both on PBMC [11–19] and erythrocytes [25] of patients with other cross-reactive haematological disorders confirmed its disease specificity. The major significance of our finding has been (a) establishment of the disease-specific expression of Neu5,9Ac_2α2-6GalNAc in Neu5,9Ac_2-GPs_{ALL}, (b) their occurrence on all hematopoetic cells in $PBMC_{ALL}$ (c) the role of these 9-*O*-acetylated moeties as signaling molecules, and (d) the functioning of 9-*O*-acetylated sialic acid moieties in regulating survival of $PBMC_{ALL}$.

The increase in proliferation of $PBMC_{ALL}$ when incubated with Achatinin-H suggested disease-specific stimulation via interaction with Neu5,9Ac_2 (Fig. 2), which led to downstream production and release of IFN-γ (Fig. 3) suggesting a possible role of over-expressed disease-specific Neu5,9Ac_2 in regulating signaling for proliferation.

Since the serum of ALL patients was rich in a high level of IFN-γ as compared to other cytokines, the protective role of the glycotopes was established by exposing the cells (both 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$) to this cytokine. IFN-γ-induced increased expression of NO_2^- (Fig. 4a) and iNOS

(Fig. 4b) in 9-*O*-AcSA$^+$ cells as compared to 9-*O*-AcSA$^-$ cells demonstrated its differential regulatory effect on these two cell types. A 5-fold increase in activation of caspase-3 in 9-*O*-AcSA$^-$ cells was observed when availability of NO in the environment was further reduced which correlated well with increased apoptosis (Table 3). Using these 9-*O*-AcSA$^+$ and 9-*O*-AcSA$^-$ cells, we have demonstrated for the first time that increased caspase-3 activation (Table 3) and apoptotic parameters (Table 4) in the 9-*O*-AcSA$^-$ cells through decreased production of NO_2^- (Fig. 4a) and iNOS (Fig. 4b) suggesting a prominent role of this glycotope in the enhanced survival of $PBMC_{ALL}$.

The idea that Neu5,9Ac$_2$ is potentially immunogenic in nature led us to hypothesize similar downstream signaling events *in vivo*. The increased level of IFN-γ in the sera of these affected children may be due to *in vivo* interaction of anti-Neu5,9Ac$_2$-GPs with Neu5,9Ac$_2$-GPs in turn contributing to the survival of $PBMC_{ALL}$. Thus, a link possibly exists between the inductions of cytokine-mediated NO_2^- production by 9-*O*-AcSA$^+$ cells and protection of $PBMC_{ALL}$ from programmed cell death (Fig. 6).

Our study is in concurrence with other observations in B–CLL where cytokines, including IFN-γ, lead to the promotion of their survival possibly by regulating iNOS [31, 32]. Thus it appears that 9-*O*-acetylation of sialic acids is a unique manifestation to the $PBMC_{ALL}$ which possibly mediate the survival of lymphoblasts and may be considered to be one of the pathways for evading programmed cell death in childhood cancer.

Perspective

In consequence to our observation that a possible role of over-expressed disease-specific Neu5,9Ac$_2$-GPs exist in regulating signaling for proliferation, we hypothesize that controlled expression of these molecules may be exploited for therapeutic applications promising beneficial effects to the children with ALL. As little is known about the factors mediating the evasion of the leukemia cells from immune surveillance and its aggressive nature, studies on factors controlling their aberrant behavior may contribute to an improved prognosis for ALL patients.

Considering the phenomena of subclass-switching from IgG1 to IgG2, their heterogeneous glycosylation, preferential sialylation along with the impairment of function (activation of FcγR and complement) in ALL patients [30], the generation of customized antibody constructs bearing functional Fc domain of anti-Neu5,9Ac$_2$-GPs, and having a homogenous glycoform and a predetermined profile of functional potential might also lead to their effective functioning. Such customized antibodies might be used in conjugation with cytokine therapy to activate *in vivo* anti-cancer pathways for proper immune-surveillance in pediatric ALL.

It is our aspiration that these observations will have a far-reaching clinical relevance. Ideally, either the functioning of these Neu5,9Ac$_2$-GPs or the group of enzymes like *O*-acetyl transferase, *O*-acetyl esterase, sialyltransferase and sialidase responsible for regulating their expression could be exploited for pharmacological manipulation of the apoptotic pathways leading to clinical application.

However the presence of glycolipids and their *O*-acetylated relatives and their role in the disease remain to be investigated.

Acknowledgment This work was supported by the Department of Science and Technology and Indian Council of Medical Research, Council of Scientific and Industrial Research, Goverment of India. Our sincere thanks to Dr. R. Vlasak, Applied BioTechnology, University of Salzburg, Austria for providing *O*-acetyl esterase and Prof. Dr. R. Schauer Biochemisches Institut, Christian-Albrechts-Universität zu Kiel, Germany, for fluorimetric HPLC, and Prof. R. Brossmer, Biochemistry Center, Universität Heidelberg, Germany, for providing synthetic sialic acid analogues.

References

1. Schauer, R.: Achievements and challenges of sialic acid research. Glycoconj. J. **17**, 485–499 (2000)
2. Schauer, R.: Sialic acids: fascinating sugars in higher animals and man. Zoology **107**, 49–64 (2004)
3. Corfield, A.P., Myerscoug, N., Warren, B.F., Durdey, P., Paraskeva, C., Schauer, R.: Reduction of sialic acid O-acetylation in human colonic mucins in the adenoma–carcinoma sequence. Glycoconj. J. **16**, 307–317 (1999)
4. Kelm, S., Schauer, R.: Sialic acids in molecular and cellular interactions. Int. Rev. Cyt. **175**, 137–240 (1997)
5. Kohla, G., Stockfleth, E., Schauer, R.: Gangliosides with *O*-acetylated sialic acids in tumors of neuroectodermal origin. Neurochem. Res. **7**, 583–592 (2002)
6. Shi, W.X., Chammas, R., Varki, A.: Regulation of sialic acid 9-*O*-acetylation during the growth and differentiation of murine erythroleukemia cells. J. Biol. Chem. **271**, 31517–31525 (1996)
7. Cheresh, D.A., Reisfeld, R.A., Varki, A.P.: *O*-Acetylation of disialoganglioside GD3 by human melanoma cells creates a unique antigenic determinant. Science **225**, 844–846 (1984)
8. 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. Lett. **59**, 151–157 (1997)
9. Kamerling, J.P., Makovitzky, J., Schauer, R., Vliegenthart, J.F.G., Wember, M.: The nature of sialic acids in human lymphocytes. Biochim. Biophys. Acta **714**, 351–355 (1982)
10. Hoelzer, D., Gokbuget, N., Ottmann, O., Pui, C.H., Relling, M.V., Appelbaum, F.R., van Dongen, J.J., Szczepanski, T.: Acute lymphoblastic leukaemia. Hematol. (Am Soc Hematol Educ Program) **2002**, 162–192 (2002)
11. Mandal, C., Chatterjee, M., Sinha, D.: Investigation of 9-*O*-acetylated sialoglycoconjugates in childhood acute lymphoblastic leukemia. Br. J. Haematol. **110**, 801–812 (2000)
12. Sinha, D., Mandal, C., Bhattacharya, D.K.: Identification of 9-*O*-acetyl sialoglycoconjugates (9-*O*AcSGs) as biomarkers in child-

hood acute lymphoblastic leukemia using a lectin, AchatininH, as a probe. Leukemia **13**, 119–125 (1999)
13. Pal, S., Chatterjee, M., Bhattacharya, D.K., Bandhyopadhyay, S., Mandal, C., Mandal, C.: *O*-acetyl sialic acid specific IgM in childhood acute lymphoblastic leukaemia. Glycoconj. J. **18**, 529–537 (2001)
14. Pal, S., Chatterjee, M., Bhattacharya, D.K., Bandyopadhyay, S., Mandal, C.: Identification and purification of cytolyic antibodies directed against *O*-acetylated sialic acid in childhood acute lymphoblastic leukemia. Glycobiology **10**, 539–549 (2000)
15. Sinha, D., Bhattacharya, D.K., Mandal, C.: A colorimetric assay to evaluate the chemotherapeutic response of children with acute lymphoblastic leukemia (ALL) employing Achatinin H: a 9-*O*-acetyl sialic acid binding lectin. Leuk. Res. **9**, 803–809 (1999)
16. Sinha, D., Mandal, C., Bhattacharya, D.K.: Development of a simple, blood based lymphoproliferation assay to assess the clinical status of patients with acute lymphoblastic leukemia. Leuk. Res. **23**, 433–439 (1999)
17. Sinha, D., Mandal, C., Bhattacharya, D.K.: A novel method for evaluation of childhood acute lymphoblastic leukemia. Leukemia **13**, 309–312 (1999)
18. Pal, S., Ghosh, S., Bandyopadhyay, S., Mandal, C.N., Bandhyopadhyay, S., Bhattacharya, D.K., Mandal, C.: Differential expression of 9-*O*-acetylated sialoglycoconjugates on leukemic blasts: a potential tool for long-term monitoring of children with acute lymphoblastic leukemia. Int. J. Cancer **111**, 270–277 (2004)
19. Pal, S., Ghosh, S., Mandal, C., Kohla, G., Brossmer, R., Isecke, R., Merling, A., Schauer, R., Schwartz-Albiez, R., Bhattacharya, D.K., Mandal, C.: Purification and characterization of 9-*O*-acetylated sialoglycoproteins from leukemic cells and their potential as immunological tool for monitoring childhood acute lymphoblastic leukemia. Glycobiology **14**, 859–870 (2004)
20. Pal, S., Bandyopadhyay, S., Chatterjee, M., Bhattacharya, D.K., Minto, L., Hall, A.G., Mandal, C.: Antibodies against 9-*O*-acetylated sialoglycans: a potent marker to monitor clinical status in childhood acute lymphoblastic leukemia. Clin. Biochem. **37**, 395–403 (2004)
21. Chen, H.Y., Varki, A.: *O*-acetylation of GD3: an enigmatic modification regulating apoptosis? J. Exp. Med. **196**, 1529–1533 (2002)
22. Malisan, F., Franchi, L., Tomassini, B., Ventura, N., Condo, 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)
23. Mandal, C., Basu, S.: A unique specificity of a sialic acid binding lectin Achatinin-H from the hemolymph of *Achatina fulica* snails. Biochem. Biophys. Res. Commun. **148**, 795–801 (1987)
24. Sen, G., Mandal, C.: The specificity of the binding site of AchatininH, a sialic acid-binding lectin from *Achatina fulica*. Carbohydr. Res. **268**, 115–125 (1995)
25. Ghosh, S., Bandyopadhyay, S., Bhattacharya, D.K., Mandal, C.: Altered erythrocyte membrane characteristics during anemia in childhood acute lymphoblastic leukemia. Ann. Hematol. **84**, 76–84 (2005)
26. Vlasak, R., Krystal, M., Nacht, M., Palese, P.: The influenza C virus glycoprotein (HE) exhibits receptor binding (hemagglutinin) and receptor destroying (esterase) activities. Virology **160**, 419–425 (1987)
27. Young, H.A.: Interferon expression by B cells. Methods **11**, 112–115 (1997)
28. Ghosh, S., Bandyopadhyay, S., Pal, S., Das, B., Bhattacharya, D. K., Mandal, C.: Increased interferon gamma production by peripheral blood mononuclear cells in response to stimulation of overexpressed disease-specific 9-*O*-acetylated sialoglycoconjugates in children suffering from acute lymphoblastic leukaemia. Br. J. Haematol. **128**, 35–41 (2005)
29. Ghosh, S., Bandyopadhyay, S., Mallick, A., Pal, S., Vlasak, R., Bhattacharya, D.K., Mandal, C.: Interferon gamma promotes survival of lymphoblasts overexpressing 9-*O*-acetylated sialoglycoconjugates in childhood acute lymphoblastic leukaemia (ALL). J. Cell. Biochem. **95**, 206–216, (2005)
30. Bandyopadhyay, S., Bhattacharyya, A., Mallick, A., Sen, A.K., Tripathi, G., Das, T., Sa, G., Bhattacharya, D.K., Mandal, C.: Over-expressed IgG2 antibodies against *O*-acetylated sialoglycoconjugates incapable of proper effector functioning in childhood acute lymphoblastic leukemia. Int. Immunol. **17**, 177–191 (2005)
31. Buschle, M., Campana, D., Carding, S.R., Richard, C.C., Hoffbrand, A.V., Brenner, M.K.: Interferon gamma inhibits apoptotic cell death in B cell chronic lymphocytic leukaemia. J. Exp. Med. **177**, 213–218 (1993)
32. Levesque, M.C., Misukonis, M.A., O'Loughlin, C.W., Chen, Y., Beasley, B.E., Wilson, D.L., Adams, D.J., Silber, R., Weinberg, J. B.: IL-4 and interferon gamma regulate expression of inducible nitric oxide synthase in chronic lymphocytic leukemia cells. Leukemia **17**, 442–450 (2003)