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# Type II anti-CD20 mAb-induced lysosome mediated cell death is mediated through a ceramide-dependent pathway

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ABSTRACT

In the past decade, monoclonal antibodies (mAbs) have revolutionized the treatment of non-Hodgkin lymphomas (NHLs). Although Fc-dependent mechanisms of mAb-mediated tumor clearance have been extensively studied, the ability of mAbs to directly evoke programmed cell death (PCD) and the underlying mechanisms involved remain unclear. It is well established that type II anti-CD20 mAb (Tositumomab) potently evoked PCD through a caspases-independent, lysosome-mediated process, which is related to homotypic adhesion (HA) in NHL cell lines. Herein, we reveal that the induction of ceramide generation by anti-CD20 mAbs directly correlates with their ability to induce PCD. The inhibition of ceramide abrogated Tositumomab-induced PCD indicating that ceramide is required for the execution of cell death. Further experimental results revealed that ceramide was generated downstream of mAb-induced HA and upstream of lysosome leakage. These findings provide further insights into a previously unrecognized role for ceramide generation in mediating PCD evoked by type II anti-CD20 mAbs in Burkitt's lymphoma cells. This newly characterized cell death pathway may potentially be exploited to eliminate malignant cells.



## 1. Introduction

Monoclonal antibodies (mAb) directed to surface antigens on malignant B cells has proven the most clinically effective, with the anti-CD20 mAb, Rituximab, being the first to be approved by the US Food and Drug Administration (FDA) for the treatment of cancer [1]. Although clinical applications have proven unprecedented success of Rituximab in treating a variety range of non-Hodgkin lymphomas (NHLs), only 48% of patients respond to the treatment, with a complete remission (CR) rate of less than 10% [2]. Moreover, tumor relapse is inevitable for most patients [3,4]. Previous studies demonstrated that the exhaustion and anergy of effector cells and complements are involved in the limitations of Rituximab based immunotherapy [5,6]. In an attempt to further improve therapeutic outcomes and develop novel therapies for rituximab refractory patients, many next-generation mAbs directed against CD20 with improved ability in eliciting programmed cell death (PCD) have been developed by the pharmaceutical industry [1,7–9]. Central to this task is the need to identify the critical effector mechanisms involved in mAb therapy.

In addition to "classical" Fc-dependent effector mechanisms such as complement-dependent cytotoxicity (CDC) and antibody-dependent cellular cytotoxicity (ADCC), type II anti-CD20 mAbs can trigger intracellular signaling and directly induce PCD in targeting Non-Hodgkin lymphoma (NHL) cells [1,2,6,10]. *Andrei Ivanov's* study firstly reported that Tositumomab (a type II anti-CD20 mAb) can elicit strong homotypic adhesion (HA), followed by a swelling and/or collapse of lysosomal compartment in human lymphoma and leukemia cells [1]. The involvement of lysosomes in type II mAb induced PCD was also confirmed by *Alduaij W* et al.'s study, in which a novel type II mAb, GA101, can induce strong PCD in NHL cells through the lysosome mediated pathway [11]. However, the exact mechanism concerning the relationships between mAb-evoked-HA and the specific lysosome alteration remains unclear.

Previous studies have demonstrated that ceramide plays an important role in PCD induced by a variety of antineoplastic drugs and cytokines [12–15]. It should be mentioned that sphingolipids are important constituents of cytomembrane, which can either serve as substrates for or be produced by enzymes activated in response to cellular stress [12]. Ceramide can be generated during

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sphingomyelin catabolism by various sphingomyelinases during the responses to these stresses and acts as a second messenger in sphingomyelin signaling pathway, which has been identified as an important mediator in cell differentiation, survival, and apoptosis [12,16–18]. Besides, ceramide can be produced by *de novo* synthesis responding to similar agents identified for activation of sphingomyelinases [19,20].

Herein, for the first time we discovered that ceramide also participates in Tositumomab induced lysosome-mediated PCD. And more importantly, the relationships among Tositumomab-mediated HA, lysosome leakage and ceramide generation are deeply investigated. Further clarification of the mechanism of mAb-induced PCD may provide the potential for improved therapeutic efficacy through the development of optimized next-generation mAbs, mAb derivatives and novel, mechanism-based combination therapies in curing Burkitt's lymphoma.

## 2. Materials and methods

### 2.1. Cell lines, antibodies and reagents

Two human Burkitt's lymphoma cell lines, Daudi and Ramos, were obtained from the American Type Culture Collection (ATCC). Cells were maintained in RPMI 1640 media supplemented with 10% FCS (Gibco, Invitrogen) at 37 °C, 5% CO2. Rituximab (C2B8, trade name: Mabthera) and Tositumomab (B1, trade name: Bexxar) were purchased from Roche and GlaxoSmithKline (GSK, UK), respectively. All the other antibodies used in immunofluorescence experiments were obtained from Invitrogen Co. Ltd (California, USA). $C_2$-ceramide (N-acetylsphingosine), Fumonisin Bl (FB1), latrunculin B (LatB) and rabbit anti-human $(Fab')_2$ fragments (RAH) were purchased from Sigma–Aldrich (St Louis, USA).

### 2.2. Detection of programmed cell death by flow cytometer

Burkitt's lymphoma cells experienced PCD were assessed by flow cytometer (FCM) after Annexin V & Propidium Iodide (PI) staining. Briefly, cells were incubated with 10 μg/ml Rituximab, Tositumomab or different concentrations of $C_2$-Ceramide for 16 h. After washing, cells were stained with Alexa Fluor-488 Annexin-V&PI (Invitrogen, California, USA) and analyzed by a FCM (Beckman Coulter). For PCD inhibition assays, Z-VAD-FMK (Promega) or FB1 with different concentrations were added 1 h prior to the addition of therapeutic agents. And cells successively incubated with Rituximab (10 μg/ml) and RAH (20 μg/ml) were used as a positive control.

### 2.3. Determination of cellular ceramide by HPLC

The cellular ceramide level was determined by High Performance Liquid Chromatography (HPLC) following *Shinji Soeda's* methods [21]. Briefly, harvested cells were suspended in 200 μl of 0.25 M sucrose in PBS and disrupted by sonication. After centrifuge (800 g × 10 min), the pellet was washed, resuspended in 200 μl of PBS and mixed with 4.0 ml of chloroform/methanol (2:1) for 30 min. The resulting samples were added with 1 ml of Milli-Q water followed by centrifuge. The lower phase was collected and evaporated to dryness. The residue was dissolved in 100 μl chloroform and reacted at −20 °C for 3 h with 10 μl of 100 mM (+)-6-methoxy-α-methyl-2 naphthaleneacetic acid (NAP, Sigma–Aldrich, St Louis, USA), 100 mM N,N′-dicyclohexylcarbodiimide (DCC, Sigma–Aldrich, St Louis, USA) and 100 mM 4-dimethylaminopyridine (DMAP, 4-dimethylaminopyridine). After evaporation, the residue was suspended in 15 μl chloroform, and then mixed with 2 ml of hexane. After centrifuge, the upper phase was collected and filtered using a 0.45 μm membrane filter (Millipore, Massachusetts, USA). 20 μl of the filtrate was injected into an Econosphere CN 5U column (4.6 × 250 mm, Alltech, Chicago, USA). The derivatized ceramide was separated from by-products with 3% 2-propanol in *n*-hexane as the mobile phase with a flow rate of 2.0 ml/min. The effluent was monitored fluorometrically at a wavelength of 230/352 nm (excitation/emission).

### 2.4. Determination of mAb elicited homotypic adhesion in Burkitt's lymphoma cells

For the determination of mAb-elicited-HA, cells were incubated with 10 μg/ml anti-CD20 mAbs or different concentrations of ceramide for 4 h and cell morphology was observed by light microscopy. For HA inhibition assays, FB1 or LatB was added 1 h before the addition of Tositumomab.

### 2.5. Determination of lysosomal permeability

For the Determination of lysosomal permeability, cells were labeled with 75 nM Lyso-Tracker Red DND (Invitrogen, California, USA) at 37 °C for 10 min in the dark and assessed by FCM and Confocal Laser Scanning Microscopy (CLSM, Olympus FV1000). Unlabeled cells were used as a background control [1].

### 2.6. Determination of cathepsin B release in cytoplasm by CLSM

Released cathepsin B in cytoplasm was detected by CLSM following *Andrei Ivanov's* methods [1]. Briefly, harvested cells were placed onto poly-D-lysine (sigma-Aldrich, St Louis, USA) coated sterilized microscope slides, fixed in 4% paraformaldehyde, and permeabilized by 0.3% Triton X-100. Each sample was then incubated with cathepsin B mouse mAb at room temperature for 1 h and stained by Alexa Fluor® 488 Rabbit Anti-Mouse IgG (H + L) secondary antibodies in the dark. After washing, samples were observed using a CLSM.

### 2.7. Statistical analysis

Error bars represent the SEM of 3 independent experiments unless otherwise stated. To compare the difference between the experimental groups, an unpaired, 2-tailed *t* test was calculated. The number of asterisks displayed on the figures represents the degree of statistical significance as determined by *p* values as follows: $^{*}p < 0.05$, $^{**}p < 0.01$. All data were processed with SPSS 10.0 software.

## 3. Results

### 3.1. Tositumomab efficiently induces PCD in Burkitt's lymphoma cells in a caspase-independent manner

Induction of PCD was evaluated by FCM following Annexin V & PI staining. As indicated in Fig. 1A, Tositumomab (type II mAb) induced a substantially higher level of PCD than that induced by Rituximab (type I mAb) and No Treatment (NT) in both Daudi and Ramos cells. Results of PCD inhibition assays indicated that a cell-permeable pan-caspases inhibitor (Z-VAD-FMK), which can efficiently inhibit RAH-crosslinked-Rituximab evoked apoptosis in a does dependent manner, was unable to prevent Tositumomab-induced PCD over a range of concentrations from 10 to 30 μM (Fig. 1B). These results clearly demonstrated that type II but not type I anti-CD20 mAb can evoke significant PCD in Burkitt's lymphoma cells in a caspase-independent manner.

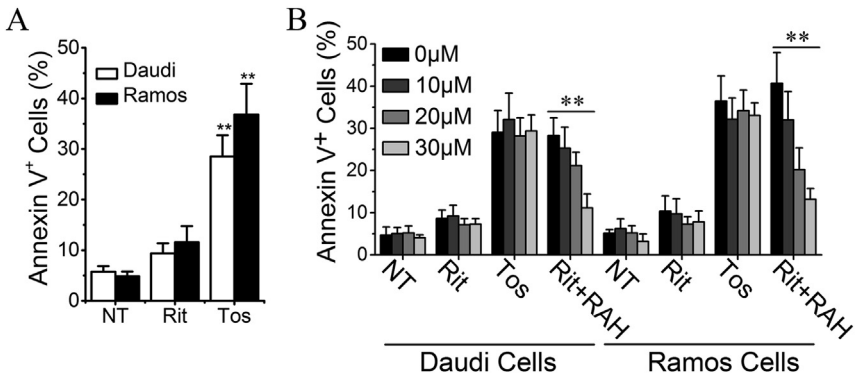


**Fig. 1.** Tositumomab can induce caspase-independent cell death in Burkitt's lymphoma cells. (A) Daudi and Ramos cells were incubated with 10 μg/ml Rituximab or Tositumomab and PCD was analyzed by FCM following Annexin V & PI staining. (B) Z-VAD-FMK (a pan-caspase inhibitor) over a range of 0–30 μM was incubated with Daudi and Ramos cells 1 h prior the addition of therapeutic antibodies. PCD was analyzed using the Annexin V & PI assay. Data are mean ± SEM of atleast 3 separate experiments, $^{**}p < 0.01$.

### 3.2. Tositumomab induced PCD correlates with the generation of ceramide

Based on earlier observation that ceramide plays an important role in PCD induced by a variety of chemotherapeutic drugs and cytokines [12–17], we subsequently investigated the correlation between Tositumomab-induced cell death and ceramide generation. Firstly, we investigated the ability of Tositumomab to stimulate the production of intracellular ceramide in Burkitt's lymphoma cells. Fig. 2A demonstrates a robust increase of ceramide levels in cells treated with Tositumomab but not Rituximab, in keeping with their ability to trigger programmed cell death. To link the induction of ceramide to cell death, cells were treated with different concentrations of ceramide and stained with Annexin V & PI to identify dead cells. Before Annexin V & PI staining, the relative and absolute intracellular ceramide levels after the treatment of different concentrations of ceramide in culture media have been investigated in our preliminary studies (Supplementary Fig. 1) and the ceramide concentrations used in subsequent experiments are based on these results. Fig. 2B indicates that ceramide over a range of concentrations from 5 to 30 μM can evoke significant cell death in Daudi and Ramos cells in a dose-dependent manner.

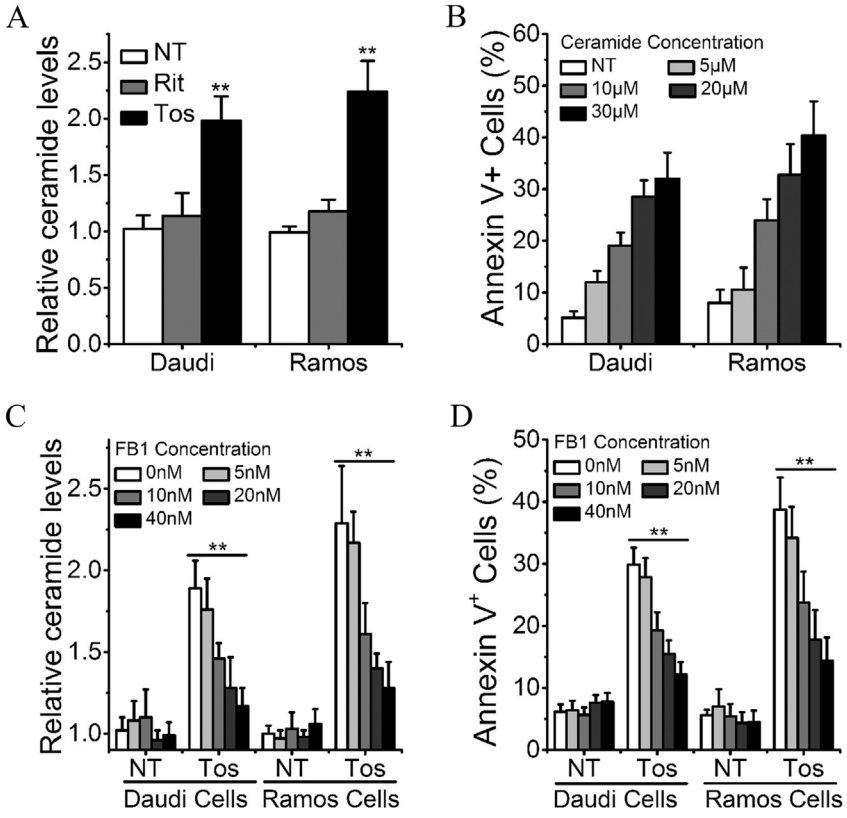


**Fig. 2.** mAb-induced ceramide triggers cell death in Burkitt's lymphoma cells. (A) Daudi and Ramos cells were treated with Tositumomab or Rituximab, and intracellular ceramide levels were determined by HPLC. (B) Burkitt's lymphoma cells were incubated with different concentrations of ceramide and cell death was analyzed using the Annexin V&PI assay. (C–D) Fumonisin BI (FB1) over a range of 0–40 nM was incubated with lymphoma cells for 1 h prior the addition of Tositumomab. After 16 h, ceramide levels were determined by HPLC (C) and cell death was analyzed using the Annexin V & PI assay (D). Data are mean ± SEM (n = 3) of atleast 3 experiments, $^{**}p < 0.01$.

Based on the above results, we hypothesized that Tositumomab induced PCD and ceramide generation might be closely related. To verify this hypothesis, we determined intracellular ceramide content and Tositumomab induced cell death in the absence or presence of FB1, which acts as an inhibitor of dihydroceramide synthase (a key enzyme regulating the conversion of sphinganine to dihydroceramides in the synthesis of cellular ceramide) [22]. Fig. 2C indicates that the increased ceramide levels evoked by Tositumomab can be significantly reduced by FB1 in a dose-dependent manner from 5 to 40 nM. More importantly, the Tositumomab-mediated PCD can also be significantly prevented by FB1 in both Daudi and Ramos cell lines (Fig. 2D). These experimental results revealed that Tositumomab evoked ceramide production coincides with programmed cell death, which confirms that these 2 processes are directly related.

### 3.3. Ceramide generation lies downstream of homotypic adhesion

The above mentioned experimental results, along with previous studies, have indicated a direct correlation among Tositumomab induced ceramide generation, homotypic adhesion and lysosome leakage in Burkitt's lymphoma cells [1,7]. In order to verify the causal relationship among them, we investigated whether the dihydroceramide synthase inhibitor (FB1) can negatively influence the mAb-evoked HA and lysosome alteration. For determining the casual relationship between HA and ceramide generation, 40 nM FB1 was incubated with Burkitt's lymphoma cells for 1 h before Tositumomab treatment. Experimental results indicated that both Daudi and Ramos cells were undergoing more pronounced HA following treatment with Tositumomab than Rituximab, pretreatment with FB1 has no significant influence on mAb-induced HA

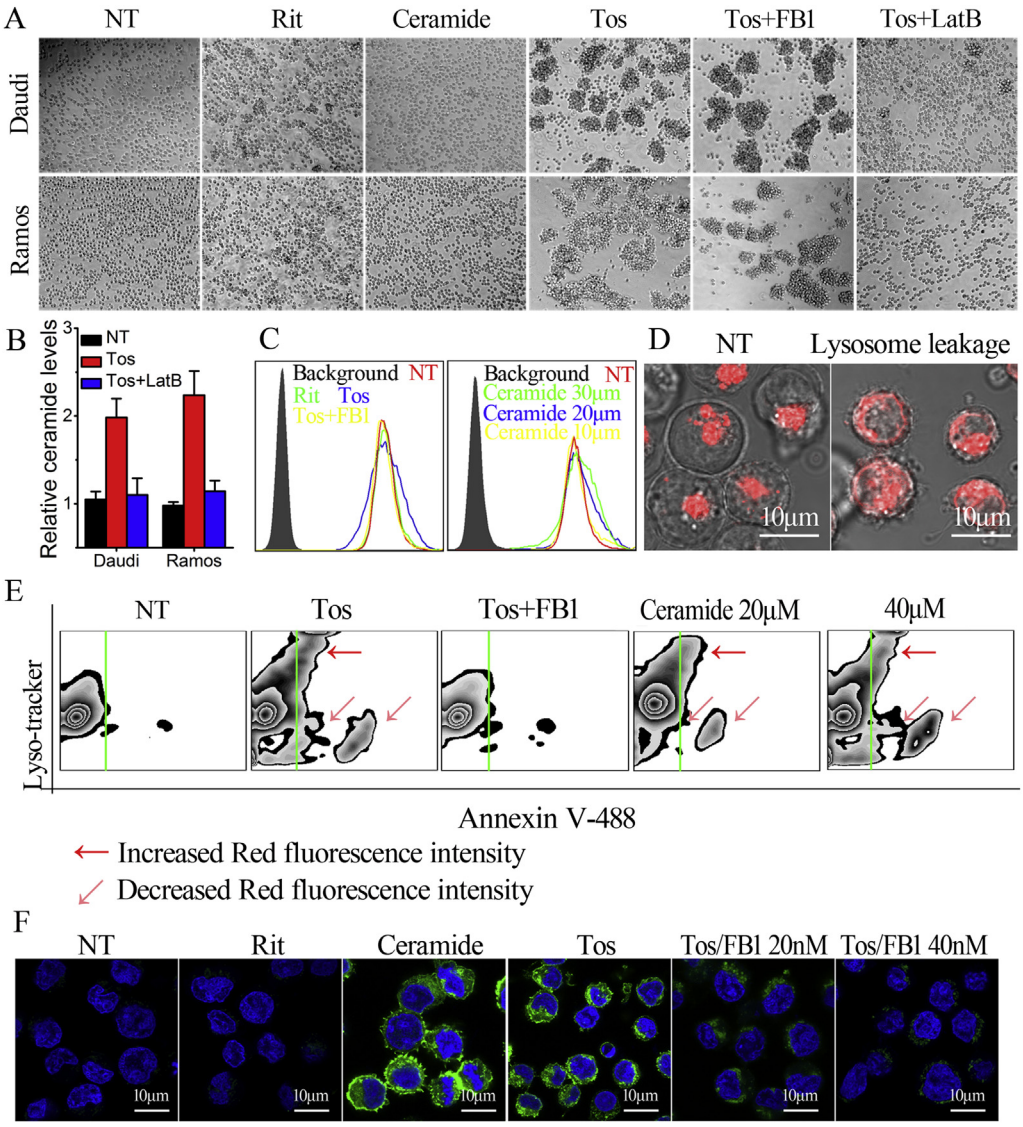


**Fig. 3.** The correlation among HA formation, ceramide generation and lysosome leakage induced by Tositumomab. (A) Cells were incubated with Rituximab, Tositumomab or $C_2$-ceramide for 4 h and cell morphology was observed by light microscopy. For HA inhibition assays, FB1 (40 nM) and LatB (10 μM) were incubated with Burkitt's lymphoma cells for 1 h prior the addition of Tositumomab. Magnification times: 20 × 20. (B) Daudi and Ramos cells were pretreated with/without 10 μM LatB prior to Tositumomab treatment, and cellular HPLC were determined by HPLC. (C–D) Ramos cells were incubated with Tositumomab with the absence/presence of FB1 or treated with ceramide at different concentrations for 16 h. After labeling with lyso-tracker, total lysosomal volume in cells was detected using FCM (C) and conforcal microscopy (D). (E) After the treatment of Tositumomab or $C_2$-Ceramide, Ramos cells were labeled with LysoTracker-Red and annexin V-488. Zebra plot describes the FCM results of dual labeling. (F) Confocal microscopy of cathepsin B staining (green). DNA was counterstained with DAPI (blue). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Fig. 3A). What's more, comparing with no treated Burkitt's lymphoma cells, no obvious HA was observed after the treatment of 30 μM ceramide (Fig. 3A), at the concentration of which considerable PCD can be significantly evoked (Fig. 2B). Taken together, these data suggest that FB1 can significantly inhibit the generation of ceramide but not the formation of HA. Thus, we hypothesized that the increased intracellular ceramide levels lies downstream of Tositumomab induced HA. In order for confirming this hypothesis, we performed experiments to investigate the relationship between changes in HA and ceramide biosynthesis. It has been reported that Tositumomab evoked HA would require ultrastructural reorganization of the actin cytoskeleton [1], thus we investigated the effects of blocking this activity on HA and cell death. Experimental results revealed that the actin polymerization inhibitor, latrunculin B (LatB, 10 μg/ml), which can efficiently inhibit HA formation (Fig. 3A), can significant prevent the intracellular ceramide generation induced by Tositumomab (Fig. 3B). Based on these results, we can draw the conclusion that type II anti-CD20 mAb evoked ceramide generation lies downstream of HA formation.

### 3.4. Ceramide generation lies upstream lysosome leakage

Given the functional significance of ceramide production and lysosome leakage in mAb induced cell death, we next aimed to delineate where the production of ceramide lies up- or downstream in Tositumomab induced lysosome alteration [1,7]. As shown in Fig. 3C, the distribution of lyso-tracker fluorescence (FL-2, Red) in Ramos cells experienced a visible increase and decrease after Tositumomab treatment (blue histograms in the left panel) compared to normal (red histograms) and Rituximab treated cells (green histograms). CLSM results clearly explained the specific alteration in fluorescence distribution, which revealed that in normal cells, cellular lysosomes were labeled as a relatively small and confined organelle, while after Tositumomab treatment, an enlargement of red fluorescence labeled compartments and diffusion of red fluorescence in the cytoplasm was detected (Fig. 3D). To directly correlate the lysosomal changes with cell death, we performed dual labeling of cells with LysoTracker and annexin V-488 in FCM assessment (Fig. 3E). As expected, the decrease (pink arrows) and increase (red arrows) of lysosomal labeling with LysoTracker was coincident with annexin V staining, indicating that cell death is directly related to the lysosome alteration. We and others all revealed that Burkitt's lymphoma cells were successively undergoing a swelling of lysosomes (enlargement of red fluorescence labeled compartments) and the collapse of lysosomal compartment (diffusion of red fluorescence in the cytoplasm) after Tositumomab treatment. Encouragingly, this distinct alteration of lysosome can be effectively prevented by FB1. What's more, $C_2$-ceramide can evoke similar alteration in lysosommes in a dose dependent manner (Fig. 3C and E).

For further confirmation, we performed IF staining for cathepsin B, a well-known lysosomal component (Fig. 3F). CLSM results revealed that a substantial increase in cathepsin B (green fluorescence) was found throughout the cytoplasm of Tositumomab and ceramide treated cells, which can be successfully prevented by FB1. From the above results, we can conclude that the mAb-induced ceramide generation occurs up-stream of lysosome leakage.

## 4. Discussion

Increasing evidence suggests that ceramide may play a central role in mediating various forms of PCD through the activation of pro-death signal transduction programs [12–20]. In this study, we investigated the involvement of ceramide in the direct lysosome mediated PCD evoked by type II anti-CD20 mAb (Tositumomab) and identified several key findings. Firstly, the ability of anti-CD20 mAbs to elicit PCD directly correlated with their ability to generate ceramide and $C_2$-ceramide can induce PCD in Burkitt's lymphoma cells in a dose dependent manner similar to Tositumomab. Secondly, since FB1, which can negatively influence the endogenous production of cellular ceramide, can significantly prevent Tositumomab induced PCD in a dose dependent manner, we can conclude that ceramide production was required for Tositumomab induced PCD.

It has been reported that HA and the following lysosome leakage play important roles in type II anti-CD20 mAb mediated PCD [1,7,9]. Based on this lysosome dependent cell death, a novel type II anti-CD20 mAb, GA101, was designed and exhibited exceptional potent anti-lymphoma abilities in both pre-clinical studies and clinical trials [7,11,23]. In order to clarifying the causal relationships among intracellular ceramide levels and Tositumomab induced HA as well as lysosome leakage, we performed further experiments to determine the effects of FB1 and ceramide on HA formation and the lysosome leakage. Our experimental results indicated that ceramide can evoke typical lysosome leakage but not obvious HA in Burkitt's lymphoma cells, while FB1, which can successfully block lysosome leakage, seems incapable in preventing HA formation mediated by Tositumomab. In addition, the blockade of HA formation by LatB, can significant prevent Tositumomab eviked intracellular ceramide generation. Based on these results, we can sketch a detailed signaling transduction pathway of Tositumomab evoked cell death as shown in Fig. 4. Here, ceramide production occurs downstream of mAb mediated HA and upstream of lysosome alteration in targeting cells. It should be mentioned that, the inhibition of ceramide generation by FB1 also has a direct effect on lysosome leakage and cell death, which indicated that ceramide production must be the only and necessary intermediate pathway between HA and lysosome alteration.

The clarification of the mechanism of mAb-induced PCD may provide the potential for improved therapeutic efficacy through the development of optimized next-generation mAbs, mAb derivatives and novel, mechanism-based combination therapies in treating Burkitt's lymphomas. Based on our findings, ceramide might be a possible and effective bioactive reagent in suppressing malignant B cells. *Stephen A. Beers's* study demonstrated that Rituximab-mediated internalization of CD20 by malignant B cells led to reduced macrophage recruitment and the degradation of CD20/

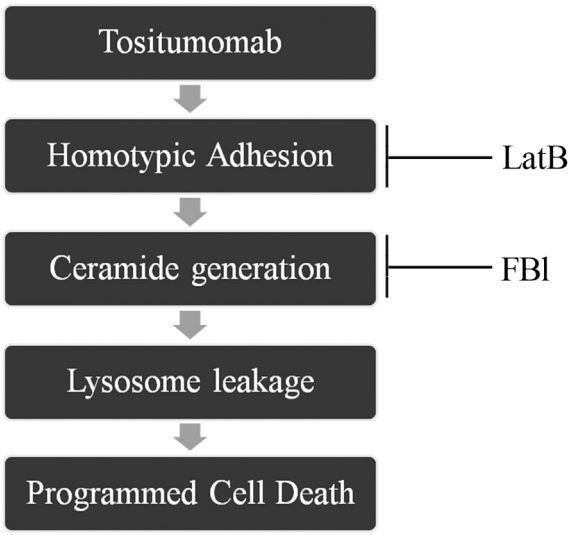


**Fig. 4.** Signal transduction pathway of Tositumomab induced programmed cell death.

mAb complexes, which limits the efficacy of CD20 based immunotherapy [24]. Currently, a variety of studies are focusing on constructing new anti-CD20 mAb or mAb derivatives in combating Rituximab resistance. However, the low expression of CD20 as a result of antigen modulation may strongly limit the therapeutic efficacy of such products. Since the involvement of ceramide in anti-CD20 mAb mediated PCD in lymphoma cells, maybe we can directly make use of this bioactive molecule in treating Burkitt's lymphomas regardless of surface CD20 expression. Also, the regulation of intracellular ceramide synthesis might be a promising method for improved therapeutic efficacy against Burkitt's lymphomas.

## Conflict of interest

The authors have no conflicts of interest to disclose.

## Transparency document

Transparency document related to this article can be found online at http://dx.doi.org/10.1016/j.bbrc.2015.01.026.

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.bbrc.2015.01.026.

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