The CD40-ligand stimulates T-lymphocytes via the neutral sphingomyelinase: A novel function of the CD40-ligand as signalling molecule

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Abstract Recent results suggest an activation of T-lymphocytes via the CD40L implying a dual function of this ligand involved in the activation of both B- and T-lymphocytes [1–4]. Here, we provide evidence that activation of T-lymphocytes via CD40L results in activation of a neutral but not an acidic sphingomyelinase correlating with a consumption of sphingomyelin and a release of ceramide. Activation of the neutral sphingomyelinase by the CD40L seems to involve a novel signalling cascade since it is independent of CD40L induced protein kinase activation or association of the neutral sphingomyelinase with the CD40L.

Key words: CD40 ligand; Neutral sphingomyelinase; T lymphocyte; Tyrosine kinase

1. Introduction

The interaction of the CD40 receptor on B cells with the CD40-ligand (CD40L, gp39 or TRAP) on T-lymphocytes has been shown to be crucial for proliferation, differentiation, isotype switching and survival of B-lymphocytes in germinal centers [5,6]. Disruption of the physiological interaction of the CD40L with its receptor, e.g. by mutations of the CD40L or in experimental knock-out models, results in the development of immunodeficiencies, in particular the X-linked hyper-IgM syndrome [1,7–14]. This syndrome is characterized by an immunodeficiency with normal or elevated IgM levels and inefficient synthesis of IgG or IgA due to impaired B-cell stimulation [7–11]. The CD40L has been shown to be expressed on T-lymphocytes, mast cell, basophils, NK cells, monocytes and even in B lymphocytes [15–19]. The CD40L is a 39 kDa type II membrane glycoprotein with a 22 amino-acid cytoplasmic amino terminal tail. The cytoplasmic domains of mouse and human CD40L are 82% identical [6] implying and important role of the cytoplasmic domain in CD40L function, however, the domain lacks any known enzymatic activity [6]. In fact, some data indicate that binding of the CD40L to the CD40-receptor may not only result in B cell activation but also in stimulation of T-lymphocytes [1–4].

In the present study we provide evidence for a novel signalling pathway coupled to the CD40L. Triggering T-lymphocytes via the CD40L results in a protein kinase independent activation of the neutral sphingomyelinase. Activation of the neutral sphingomyelinase correlates with a CD40L induced consumption of sphingomyelin and release of ceramide which are also not affected by protein kinase inhibitors.

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2. Methods

2.1. Cell culture and stimulation

All reagents were purchased from Sigma, if not otherwise cited. Murine CD40L positive or negative EL4 T-lymphocytes [20] were grown in RPMI-1640 medium supplemented with 10% fetal calf serum, 10 mM HEPES (pH 7.4), 2 mM L-glutamine, 100 μM non essential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin (all from Life Technologies, Eggenstein, Germany) and 50 μM β-mercaptoethanol.

Herbimycin A (Calbiochem, Bad-Soden, Germany), a specific inhibitor of src-like tyrosine kinases, or staurosporine, a broad kinase inhibitor, were added to the cells 12 h or 5 min, respectively, prior to any assay at a concentration of 10 μM for herbimycin A or 1 μM for staurosporin. For activation, cells were washed twice in sterile HEPES/saline (H/S, 132 mM NaCl, 20 mM HEPES, 5 mM KCl, 1 mM $CaCl_2$, 0.7 mM $MgCl_2$, 0.8 mM $MgSO_4$) and stimulated at 37°C with 2 μg/ml monoclonal anti-mouse CD40L-antibody (Pharmingen, San Diego, CA, USA).

2.2. Sphingomyelin consumption

Cells ($5 \cdot 10^6$/sample) were metabolically labelled by incubation with 1 μCi/ml [methyl-^{3}H]choline chloride (60–90 Ci/mmol; NEN-DuPont, Bad-Homburg, Germany) for 48 h in complete RPMI. Cells were washed in H/S and stimulated via the CD40L using 2 μg/ml monoclonal anti-mouse CD40L-antibody. Lipids were extracted by addition of 120 μl 0.22 M HCl, 2.7 ml $CHCl_3$:CH_3OH (2:1), 0.9 ml of $CHCl_3$ and 0.9 ml of 1 M KCl. The organic phase was collected, dried, resuspended in $CHCl_3$:CH_3OH (1:1) and lipids were separated on G60 silica gel thin layer chromatography plates (Machery-Nagel, Dueren, Germany) using $CHCl_3$:CH_3OH:H_2O:acetic acid (50:30:8:4) as solvent system. Sphingomyelin spots were identified in I_2-vapor by co-migration with standards, scrapped from the plate and radioactivity was determined by liquid scintillation counting.

2.3. Activity of acidic and neutral sphingomyelinase

For acidic sphingomyelinase activity measurements cells (10^7/sample) were stimulated with anti-CD40L, lysed in ice-cold 50 mM Tris (pH 7.4), 10 mM bacitracin, 1 mM benzamidine, 10 mM Na_3VO_4, 10 μg/ml each aprotinin and leupeptin, 0.1 mg/ml soybean trypsin inhibitor and 0.2% Triton X-100 and immediately sonicated for 10 s. Sonication was repeated three times and insoluble cell debris was pelleted by 5 min centrifugation at $600 \times g$. An equal amount of 50 mM Tris (pH 7.4), 3% NP40, 1% Triton X-100, 10 mM Na_3VO_4, 100 μg/ml each aprotinin and leupeptin ('ASM lysis buffer') was added to the samples and ASM was immunoprecipitated from the supernatants using a goat-anti-ASM-antiserum previously characterised [21]. Samples were incubated for 4 h at 4°C, immunocomplexes were immobilized by addition of 20 μl protein A/G coupled agarose (Santa Cruz Inc., Santa Cruz, CA) and further incubation for 60 min at 4°C. The immunoprecipitates were washed three times in lysis buffer followed by additional three washes in 50 mM sodium acetate (pH 5.0), 0.2% Triton X-100, 1 mM Na_3VO_4 and 10 μg/ml each aprotinin and leupeptin. Washed immunoprecipitates were incubated with [^{14}C] sphingomyelin (0.5 μCi/sample, 54.5 mCi/mmol; NEN-DuPont) in 250 mM sodium acetate (pH 5.0), 1.3 mM EDTA, 0.05% NP40 (assay buffer) for 30 min at 37°C. The substrate [^{14}C]sphingomyelin was dried and solubilized by 10 min bath sonication in assay buffer. Samples were then extracted with chloroform : methanol (2 : 1) and H_2O, the

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upper phase was collected and radioactivity reflecting the degradation of [^{14}C]sphingomyelin was determined by liquid scintillation counting.

The activity of the ASM in whole cell lysates was determined by lysis of the cells in a buffer consisting of 0.1% Triton X-100, 50 mM sodiumacetate, 1 mM Na_3VO_4 and 10 μg/ml each aprotinin and leupeptin. Extracts were sonicated centrifuged at 600×g for 5 min and the supernatants were added to the same volume of 0.5 μCi/sample [^{14}C]sphingomyelin in 250 mM sodiumacetate (pH 5.0, 1.3 mM EDTA and 0.05% NP-40. [^{14}C]sphingomyelin was prepared as described above. Samples were incubated, extracted and counted as described for the immunoprecipitates.

For determination of neutral sphingomyelinase activity, cells were lysed in a buffer consisting of 20 mM HEPES (pH 7.4), 5 mM Dithiothreitol, 2 mM EDTA, 10 mM $MgCl_2$, 0.1 mM Na_2MoO_4, 10 mM β-glycerophosphate, 7.5 μM ATP, 1 μM PMSF, 10 μM leupeptin and 0.2% Triton-X100. Extracts were processed as above with 0.5 μCi/sample [^{14}C]sphingomyelin as substrate. The substrate was solubilized by bath sonication for 10 min in a buffer consisting of 20 mM HEPES (pH 7.4), 1 mM $MgCl_2$ and 0.2% Triton X-100 and 75 μl of the substrate were added to 100 μl lysates.

2.4. Co-immunoprecipitation of neutral sphingomyelinase

Cell ($2{\cdot}10^7$/sample) stimulation was terminated by lysis in 50 mM Tris (pH 7.4), 10 mM Bacitracin, 1 mM benzamidine, 10 mM Na_3VO_4, 10 μg/ml each aprotinin and leupeptin, 0.1 mg/ml soybean trypsin inhibitor and 0.2% Triton X-100 (buffer A) or, alternatively, in 25 mM HEPES (pH 7.4), 2% NP40, 1% Triton X-100, 125 mM NaCl, 10 mM each NaF, Na_3VO_4 and sodium pyrophosphate and 10 μg/ml of each aprotinin and leupeptin (buffer B). After three rounds of sonication for 10 s each, DNA and cell debris were pelleted by centrifugation at 20000×g for 15 min and the supernatants were subjected to immunoprecipitation overnight at 4°C using 3 μg anti-DC40L antibodies/sample (Pharmingen, San Diego, CA). After addition of protein A/G-coupled agarose (Santa Cruz Inc.) incubation was continued for at least 60 min. Immunocomplexes were washed twice times in lysis buffer, three times in 20 mM HEPES (pH 7.4), 1 mM $MgCl_2$ and 0.2% Triton X-100 with 0.05 mCi/sample [^{14}C]-sphingomyelin and finally extracted as above. Degradation of [^{14}C]sphingomyelin reflecting the activity of any co-immunoprecipitated neutral sphingomyelinase was determined by liquid scintillation counting. Control immunoprecipitates were performed with an irrelevant monoclonal mouse Immunoglobulins.

2.5. Determination of ceramide

For determination of ceramide, cells were stimulated as above for the indicated times and the lipids were extracted with $CHCl_3$:CH_3OH:HCl (100:100:1). The organic phase was dried under vacuum, diacylglycerol was digested by mild alkaline hydrolysis in 0.1 M methanolic KCl, the samples were reextracted as above, dried and solubilized by 10 min bath sonication in 7.5% (w/v) *n*-octyl-β-glucopyranoside, 5 mM cardiolipin, 1 mM diethylenetriamine-pentaacetic acid (DETAPAC). After sonication 40 μg/ml of purified *E. coli* DAG-kinase (Amersham, Braunschweig, Germany) in 70 μl reaction buffer (100 mM imidazole-HCl (pH 6.6), 100 mM NaCl, 25 mM $MgCl_2$ and 2 mM EGTA) supplemented with 10 μCi/sample [^{32}P]γATP and 1 mM ATP were added and the samples were incubated at 22°C for 30 min. Kinase reaction was stopped by addition of 1 ml $CHCl_3$:CH_3OH:HCl (100:100:1), 170 μl of salt solution (10 mM HEPES (pH 7.2), 135 mM

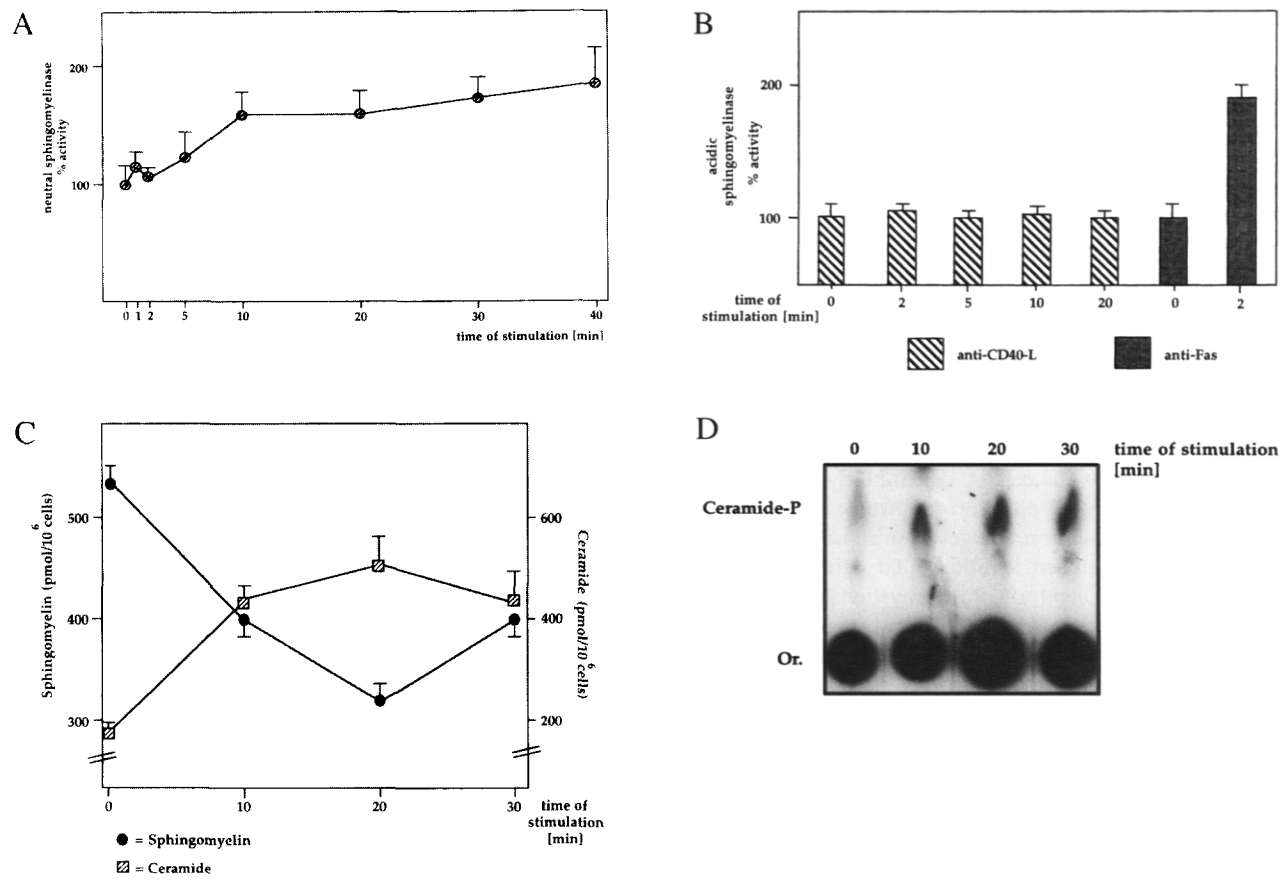

Fig. 1. Stimulation of T-lymphocytes via the CD40L results in activation of the neutral sphingomyelinase (A) but not the acidic sphingomyelinase (B). Activation of the neutral sphingomyelinase correlates with a consumption of sphingomyelin and a release of ceramide upon CD40L triggering (C). Cells were stimulated via the CD40L for the indicated time, lysed and activity of the neutral or acidic sphingomyelinase was determined in the lysates by degradation of the substrate [^{14}C]sphingomyelin. Consumption of sphingomyelin or release of ceramide was measured by organic extraction of stimulated or unstimulated cells and separation of lipids by TLC for sphingomyelin or incubation of the extracts with a ceramide sensitive kinase and [^{32}P]γATP followed by TLC-separation and autoradiography for ceramide. Panel C shows the change of sphingomyelin and ceramide in pmol/10^6 cells. Panel D displays a representative autoradiography from a TLC-plate obtained after incubation of the extracts with ceramide sensitive kinase. Experiments were repeated at least three times with similar results.

NaCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$ and 5.6 mM glucose) and 30 μl of 100 mM EDTA. The lower organic phase was dried, resuspended in $CHCl_3$:CH_3OH (1:1) and resolved by TLC using $CHCl_3$:CH_3OH: acetic acid (65:15:5, v/v/v) as solvent. Spots were visualized by autoradiography, scraped from the plate and analyzed by liquid scintillation counting.

The ceramide content of the samples was determined by comparison with a standard curve comprised of known quantities of DAG or ceramide.

3. Results

We have recently demonstrated the activation of two distinct signalling pathways upon cellular stimulation via the CD40-ligand. First, CD40L triggering results in an activation of a pathway from src-like tyrosine kinases, which seem to couple the CD40L to Rac 1 and JNK/p38-K (B. Brenner et al., submitted for publication). Second, CD40L triggering induces a herbimycin A sensitive activation of PLCγ, the release of IP_3 and Ca^{2+} and finally the activation of PKC. In order to identify novel signalling pathways involved in cellular activation via the CD40L, we tested whether stimulation of CD40L positive mouse EL-4 T-cells with a monoclonal anti-CD40L antibody results in an alteration of lipid metabolism. The results (Fig. 1A) show an approximately 1.8-fold activation of the neutral sphingomyelinase activity whereas no stimulation of the acidic sphingomyelinase could be detected (Fig. 1B). Stimulation of Jurkat cells via the CD95 receptor resulted in an approximately 2-fold stimulation of the acidic sphingomyelinase (Fig. 1B) showing that the acidic sphingomyelinase can be activated in these cells under the present assay conditions. Fig. 1B displays the results detecting the ASM activity in cell lysates, similar results were obtained by measuring the activity of immuoprecipitated ASM. The activity of the neutral sphingomyelinase was observed as early as 10 min and remained increased for more than 40 min. The activation of the neutral sphingomyelinase correlated with a consumption of cellular sphingomyelin and a release of ceramide (Fig. 1C and D).

The neutral sphingomyelinase has been only biochemically characterised and the gene is not cloned. Furthermore almost nothing is known about the mechanism of neutral sphingomyelinase activation in the cell. A recent study demonstrated that the FAN-protein [22] plays an important role in neutral sphingomyelinase regulation, however, no further or direct signalling events have been identified. To gain some insight into the mechanism of neutral sphingomyelinase activation by the CD40L, we tested the involvement of protein kinases. Cellular activation via CD40L has been previously shown to result in a rapid stimulation of p56lck (B. Brenner et al., submitted for publication). Thus, src-like tyrosine kinases are possible candidates for an upstream regulator of the neutral sphingomyelinase upon CD40L triggering. However, the activation of the neutral sphingomyelinase was not affected by preincubation of the cells with the broad kinase inhibitor staurosporine or the src-like tyrosine kinase specific inhibitor herbimycin A (Fig. 2A) indicating that the neutral sphongomyelinase is not regulated via a protein tyrosine kinase upon CD40L mediated cell stimulation. This notion is supported by our failure to show any neutral sphingomyelinase activity in immunoprecipitates performed with the monoclonal anti-phosphotyrosine antibody 4G10 (Fig. 2B). The lack of neutral sphingomyelinase inhibition after preincubation with staurosporine or herbimycin A correlated with a failure of the inhibitors to change the consumption of sphingomyelin or the release of ceramide after treatment with anti-CD40L antibodies (Fig. 2C).

To further elucidate the mechanism of neutral sphingomyelinase activation we tested the activity of the neutral sphingomyelinase in immunoprecipitates of the CD40L. Association of proteins is a very common motif involved in the activation or targeted localization of distinct proteins involved in cellular signalling. Furthermore, the FAN protein which seems to couple some receptors with the neutral sphingomyelinase has been shown to associate with the TNF-receptor. If the CD40L associate with the neutral sphingomyelinase prior or after cellular stimulation, CD40L immunoprecipitates should contain a sphingomyelinase activity. However, no associated sphingomyelinase could be detected in CD40L immunoprecipitates obtained from either unstimulated or stimulated EL-4 cells (Table 1). The same results were obtained using two different buffers resembling very mild (buffer A) or stronger (buffer B) lysis conditions. These data suggest that two proteins do not associate.

4. Discussion

Our data provide evidence for a novel function of the CD40L, i.e. the activation of the neutral sphingomyelinase in T-lymphocytes. In this concept, the CD40L behaves as a bifunctional molecule, first as the ligand for the CD40-receptor and simultaneously as receptor molecule activating T-lymphocytes. Previous data from our laboratory showed a rapid activation of the Src-like tyrosine kinase p56lck upon triggering the CD40L. These studies demonstrated that cellular activation via CD40L also result in a stimulation of PLCγ, a release of Ca^{2+} and IP_3, an activation of PKC and, on the other hand, a stimulation of Rac 1 as well as of JNK/p38-K. The activation of these two signalling pathways seems to depend on the function of the src-like tyrosine kinases p56lck since inhibition with herbimycin A prevented the stimulation

Table 1
The neutral sphingomyelinase does not associate with the CD40L prior or after cellular stimulation via the CD40L

Time of stimulation	Whole cell lysates	CD40L-Ipt. buffer A	CD40L-Ipt. buffer B
	(% activity)		
0 min	100 ± 9	1.9 ± 0.4	1.5 ± 0.3
30 min	180 ± 11	2.1 ± 0.7	1.7 ± 0.5

Cells were stimulated via the CD40L for 30 min, lysed in buffer A or B as described above and the CD40L was immunoprecipitated using an anti-CD40L antibody. Neither the immunoprecipitates prepared under very mild lysis conditions (buffer A) nor with stronger lysis conditions (buffer B) contained any neutral sphingomyelinase activity indicating that the two proteins do not associate. Control lysates (using buffer A) obtained from an aliquot of the same cells showed significant neutral sphingmyelinase activity. CD40L immunoprecipitates were also prepared 5, 10, 20 or 40 min after stimulation via the CD40L and did not show any associated neutral sphingomyelinase activity.

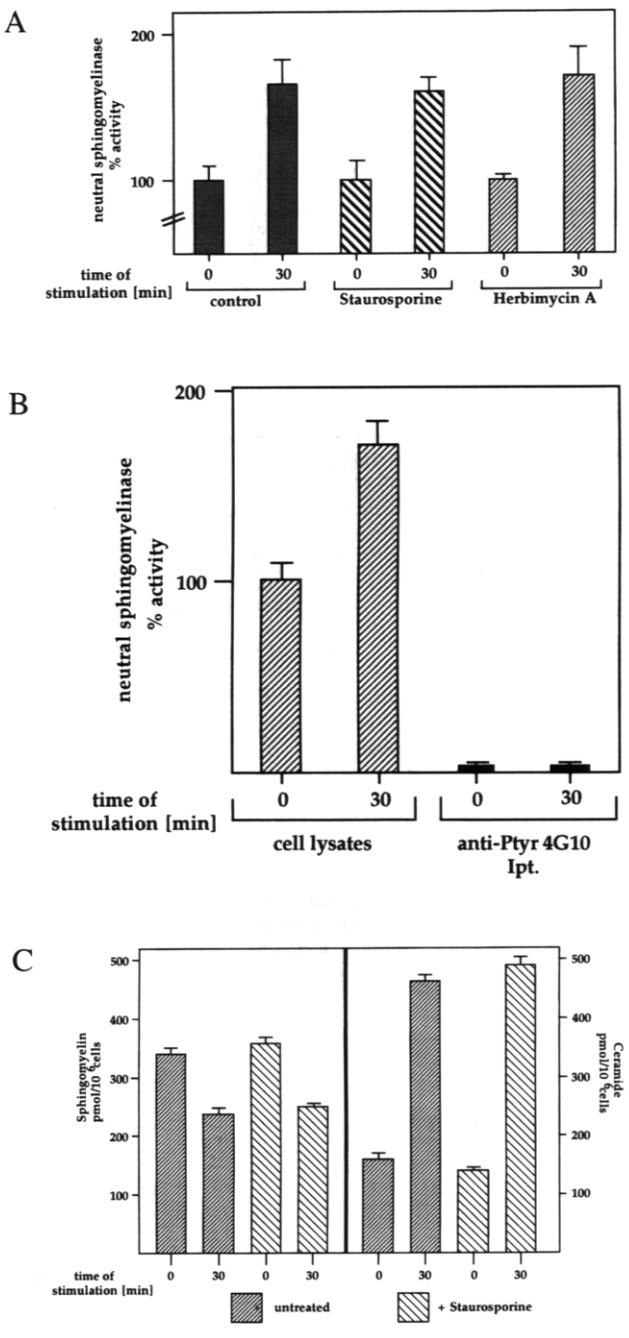

Fig. 2. Activation of the neutral sphingomyelinase (A and B), consumption of sphingomyelin (C) or release of ceramide (D). after CD40L triggering is not regulated by protein kinases. Preincubation of EL4 cells with 1 μM staurosporine or 10 μM herbimycin A does not alter the activation of neutral sphingomyelinase after stimulation via the CD40L (A). Likewise, immunoprecipitates performed with the monoclonal anti-phosphotyrosine antibody 4G10 did not contain any neutral sphingomyelinase is not directly phosphorylated (B). Finally, the consumption of cellular sphingomyelin or the release of ceramide are not altered after preincubation of the cells with staurosporine of herbimycin A (C).

of PLCγ/PKC as well as of Rac 1 and JNK/p38-K. However, the activation of the neutral sphingomyelinase seems to be completely independent of tyrosine or serine/threonine kinase activity since pretreatment with the broad kinase inhibitor staurosporine or the Src-like tyrosine kinase blocker herbimycin A did not affect the stimulation of the neutral sphingomyelinase by CD40L.

A recent study suggested a regulation of the neutral sphingomyelinase via the FAN-protein, which associates with the TNF-receptor [22]. However, co-immunoprecipitation experiments with anti-CD40L did not detect any neutral sphingomyelinase associated with the CD40L suggesting that, at least under the present conditions, the two proteins do not form a complex prior or after cellular stimulation via the CD40L. Since the antibody binds to the extracellular domain of the CD40L, it seems to be unlikely that the lack of neutral sphingomyelinase in the precipitates is due to a steric inhibition of the immunoprecipitation antibody with the associated neutral sphingomyelinase. However, since no antibodies to the neutral sphingomyelinase are available a positive control for the immunoprecipitates can not be included, thus, we can not completely exclude that our failure to show an association of the neutral sphingomyelinase with the CD40L is due to a trivial technical reason.

FAN associates with the TNF-receptor via a domain consisting of a specific motif (QKWEDSAHK) [22]. This domain is lacking in the CD40L (the CD40L contains a domain with weak homology (QxxxxSAxx), thus, it might be possible that the CD40L activates the neutral sphingomyelinase via a completely novel signalling mechanism.

Our results suggested a novel function of the CD40L, i.e. the ligand may also function as a receptor. The new concept is supported by previous findings. First, the best evidence for a stimulatory function of the CD40L for T-lymphocytes is provided by CD40-receptor knock-out mice [1]. These mice lack germinal centers, however, the formation of germinal centers upon injection of the antigen DNP-KLH can be initiated by co-injection of soluble CD40L-Fc molecules implying that cellular stimulation via the CD40L has a crucial function in the initiation of germinal center formation. These data suggest that the interaction of the CD40-receptor with the CD40L results in a stimulation of the T-lymphocytes and is required for normal T-cell function. Second, highly purified peripheral T-cells responded to anti-CD40L and anti-TCR/CD3 plus anti-CD28 triggering with a much higher IL-4 synthesis than observed after stimulation without involvement of the CD40L [2]. Likewise, co-incubation of CD40-receptor transfected P815 cells with human small, $CD4^+$, $CD40L^+$ T-cells enhanced anti-CD3 induced T-cell proliferation and the formation of CTL [3]. These data point to a stimulation of the T-cells via the CD40L, however, it might be also possible that transfected P815 cells are activated after CD40L binding and stimulate the T-cels via an unknown mechanism, e.g. by release of some cytokines.

Third, interaction of the CD40L with the CD40-receptor results in downregulation and internalization of the CD40L on the surface of T-cells, which may terminate the stimulation of the B-cells [4]. The internalization process might be mediated by a CD40L initiated active signalling mechanism.

Fourth, patients with X-linked Hyper-IgM syndrome due to CD40L mutations are highly susceptible to opportunistic infections, e.g. Pneumocystis carinii pneumonia and cryptosporidial diarrhoe [6]. These infections are T-cell dependent and indicate a defect of cellular T-cell function; however, this defect could be also explained by the lack of an interaction of the defective CD40L with the CD40-receptor on other cells,

e.g. monocytes or antigen presenting cells, which might be required for efficient stimulation of T-cells.

A signalling function of the CD40L is further supported by the finding that other ligands, in particular the CD30 ligand and the OX40-ligand of the TNF-ligand family exhibit similar dual functions [23,24].

However, application of activating CD40 receptor-antibodies to CD40L knock-out mice restores cellular and humoral immune responses to adenoviral vectors via a B7.2-CD28 dependent mechanism [25]. Thus, the CD40L may not be absolutely required for the stimulation of T-cells under these conditions and the CD40L may function predominantly as a co-stimulatory molecule which can be replaced by other strong co-stimuli or by upregulation of other stimulatory receptors replacing the function of the CD40L in the CD40-receptor knock-out mice.

In summary, we provide evidence for a novel function of the CD40L as a receptor molecule. One of the pathways activated by the CD40L couples the CD40L directly or indirectly to an activation of the neutral sphingomyelinase resulting in consumption of cellular sphingomyelin and release of ceramide.

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