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Stimulation of CD95-induced apoptosis in T-cells by a subtype specific neutral sphingomyelinase inhibitor

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Abstract

Neutral sphingomyelinase (nSMase) has been supposed to be involved in the activation of anti-apoptotic genes and, thus, could well sustain autoimmune reactions by preventing activation induced death of autoreactive T-cells. When screening cellular extracts for SMase activity in the range between pH 6.5 and 8.5 various murine tissue samples as well as cell lines of murine and human origin displayed peaks of activity, both, at pH 7.0 and 8.0. In contrast, T-cells (human T-cell lymphoma and PHA stimulated murine lymph node cells) and monocytic leukemia cells were lacking SMase activity at pH 8.0. Only one peak of activity was found at pH 7.0. Recently we described an inhibitory compound, C11AG which selectively suppresses nSMase activity. In dose–response assays using cellular extracts the pH 7.0 nSMase turned out to be almost 100-fold more sensitive to the inhibitor than the pH 8.0 nSMase. In Jurkat T-cell lymphoma cells lacking the pH 8.0 nSMase, treatment with C11AG enhanced sensitivity to apoptosis: the concentration of CD95-specific antibody anti-APO1 could be lowered by six-fold in order to induce cell death. Concomitantly the expression of the anti-apoptotic protein A1 was found to be down-regulated. In the joints of arthritic mice, apoptosis of T-cells was stimulated after application of C11AG. Accordingly, C11AG displayed curative effects on experimental arthritis: swelling and inflammation were found to be significantly alleviated.

Keywords: Neutral sphingomyelinase; Sphingomyelinase inhibitors; Apoptosis; Autoimmune diseases; Arthritis; Undecylideneaminoguanidine

1. Introduction

There exist apparently several distinctive nSMase activities: more than one gene coding for proteins with nSMase activity have been cloned [1–3]. Their products were described to differ in tissue specificity and/or cellular localization. Overexpression of nSMase1 in Jurkat cells resulted in dramatically elevated nSMase activity. Yet, no influence on CD95-induced ceramide production and apoptosis could be observed [4,5]. In contrast, in a T-cell hybridoma a correlation between the induction of apoptosis by T-cell receptor crosslinking and nSMase1 activity was found [6]. Contradictory results were obtained when synthetic, cell permeable ceramide was added to cells, in that either induction or in some cases, protection from apoptosis was found [7,8].

Recently, we described C11AG as a selective inhibitor of plasma membrane associated nSMase from macrophages [9]. In order to characterize the specificity of this inhibitor in more detail, we analyzed the effects of C11AG on SMase activity from various cell lines and tissue samples. In membrane extracts from mouse macrophages nSMase activity was inhibited already at a concentration of 1 μg/ml, while the concentration of the inhibitor had to be elevated by 100-fold in order to accomplish the same effect in extracts from mouse brain (data not shown). It became obvious that nSMase activity could be differentiated by its sensitivity to C11AG. Here we describe two different Mg dependent nSMase activities with an optimum pH of 7.0 or 8.0. Interestingly, monocytes and T-cells express only the pH 7.0 nSMase, which is preferentially inhibited by C11AG. The tissue specificity of pH 7.0 nSMase and the selectivity of C11AG allowed us to evaluate the functional activity of the pH 7.0 enzyme in apoptosis. Our results indicate that this specific nSMase is involved in the regulation of anti-apoptotic gene expression. Consequently activation-induced cell death can be readily stimulated by C11AG.

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2. Material and methods

2.1. Cell lines

Cell lines were obtained from the American Type Culture Collection. TREV (B-cell line from a Niemann-Pick patient) and DAV (B-cell line from a healthy donor) were a kind gift of Thierry Levade (Laboratoire de Biochimie, Toulouse, France). Cells were cultured in RPMI, 10% fetal calf serum. Cytotoxicity assays: after incubation at 37 °C, cells were diluted with Trypan blue and the number of surviving cells was determined in a Neubauer hematocytometer.

Enrichment of peripheral blood derived T-cells: peripheral blood cells were purified from buffy coat, through Ficoll-Hypaque gradient sedimentation, and washed twice with phosphate-buffered saline (PBS) and were passed over nylon wool. Cells (1×10^6 cells/ml) were suspended in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% fetal calf serum and 1 μg/ml phytohemagglutinin (PHA; Sigma, München) and were incubated at 37 °C. Two days later cells were analyzed for the expression of the T-cell marker CD3 by FACS analysis using the monoclonal antibody OKT-3. More than 95% of the population were found to be positive.

2.2. Animals and treatment

DBA I mice were obtained from Charles River at the age of 6–8 weeks. Mice were kept under specific pathogen free conditions and were fed with sterilized TAP food and water ad libitum.

The collagen induced arthritis model was described in detail [10]. Mice were injected s.c. with 100 μg collagen type II (Sigma, Munich) in 0.1 ml with complete Freud's adjuvant. The 28 days later animals were treated i.p. with 50 μg collagen, dissolved in 0.2 ml PBS. Stock solutions (5 mg/ml) were prepared by dissolving collagen in 0.1% acetic acid.

Symptoms were graded for all four feet as described [10]. Swelling of foot pads was assessed by determination of the thickness of foot pads using calipers. For apoptosis assays cells infiltrating the joints were collected. Animals were killed by cervical dislocation and the foot pad and ankle joints were washed with 2× 1 ml PBS. Cells were collected by centrifugation at $1000 \times g$. For TUNEL assays 5000 cells were pelleted on glass slides in a Cyto Spin centrifuge.

Where indicated, animals were treated with C11AG, a specific inhibitor of nSMase from macrophages which has been described recently [9]. C11AG was received from BioSphings AG, Frankfurt. Animals were treated subcutaneously with a volume of 0.2 ml/20 g body weight. For in vivo application the chloride of C11AG was dissolved between 0.5 and 30 mg/ml in 0.9% NaCl. For tissue culture and enzyme inhibition experiments C11AG was dissolved in ethanol at a concentration of 1–10 mg/ml.

2.3. nSMase assays

Membrane extracts of cultured cell lines were prepared as previously described [9]. Mouse organs were frozen in liquid nitrogen immediately after isolation from animals that had been killed by cervical dislocation. Pieces of approximately 300 mg frozen tissue were powdered in a Mikrodismembrator (Braun, Melsungen). The powder was suspended in 0.5% Triton X 100 to a concentration of 10 mg/ml. After centrifugation at $10{,}000 \times g$ the supernatant was used as an enzyme source. For a reaction volume of 100 μl, 5 μl of organ extract was used. All SMase reactions were carried out in the presence of 0.5% Triton X 100 and 10 mM $MgCl_2$. ^{14}C labeled sphingomyelin from bovine brain (Amersham) served as a substrate. After incubation at 25 °C for 30 min samples were extracted with chloroform/methanol (4/1) and 10 μl aliquots of the supernatant were applied to silica gel 60 TLC plates (Merck, Darmstadt) and were run as described [9]. ^3H choline, ^{14}C phosphorylcholine and ^{14}C sphingomyelin served as markers. Radioactivity in phosphorylcholine spots was quantified in a Digital Autoradiograph (Berthold).

2.4. Western blot

Details of the Western blot method were described previously [9]. Shortly, cells were collected by centrifugation, washed with PBS and lysed in 100 μl LSB/10^6 cells. Proteins were separated on 12.5% (for A1) or 10% polyacrylamide gels and transferred to nylon membranes by the semi-dry method. Antibodies (Santa Cruz, Munich) were diluted in 5% fat-free milk powder. A peroxidase coupled rabbit antibody served as secondary antibody and ECL (Amersham) was used as a substrate.

2.5. Proliferation assays and FACS analysis

Proliferation assay: Proliferation was determined by ^3H-thymidine incorporation for 8 h after cells had been incubated for 72 h at 37 °C in 96-well plates. Cells were harvested on glass fiber mats using a TOMTEC harvester and radioactivity was determined in a Beta Plate counter.

FACS analysis: Cell samples were incubated with 10 μg/ml of the following monoclonal antibody: unconjugated anti-mouse CD4 (clone YTA 3.2.1) or CD8 (clone YTS 169.4.2.1). Cells were subsequently incubated with the appropriate anti-mouse secondary fluorescein or Texas red labeled antibody. Negative controls were incubated with a non-binding primary antibody and the same secondary reagents as described. Flow cytometry followed routine procedures. A fluorescence-activated cell sorter (Becton Dickinson, Sunnyvale, CA) was used to analyze

lymphocyte cell populations. Events were analyzed using appropriate forward and side scatter settings and gates discerning the relevant cell populations.

2.6. Apoptosis assay

For TUNEL assay, a commercial kit (Roche Diagnostics) using a FITC labeled nucleotide was used according to instructions of the supplier.

3. Results

3.1. Tissue distribution of nSMases which differ in their pH optimum

It has been described that nSMases vary in enzyme activity in dependence of the pH. To discern the tissue distribution of different nSMases we determined the enzyme activity in various cell lines and tissues under different pH conditions. ^{14}C sphingomyelin was incubated with membrane extracts and the cleavage product phosphorylcholine was quantified after separation of the reaction products on TLC plates in a Digital Autoradiograph. Analysis by TLC allows simultaneous monitoring of further phospholipases, such as PLA2 or PLD that might be present in the extracts. However, under our experimental conditions, we observed exclusively sphingomyelinase activity. In Jurkat cells SMase activity could be observed between pH 6.0 and 7.5. The peak being at pH 7.0. No phosphorylcholine was detected at pH 8.0 and 8.5. Instead, two pH optima could be seen with B-cell lines Raji and KE37, one between pH 6.5 and 7.5, and an additional one between pH 7.5 and 8.5. The relative amount of the two activities was variable in different cell lines. In Raji cells similar activity was detected at pH 8.0 and at pH 7.0. In KE37 cells about seven-fold more phosphorylcholine was produced at pH 8.0 (Fig. 1). The pH 7.0 activity fails to cleave Sphingomyelin at pH 8.5, while the pH 8.0 activity is neglectible at pH 6.5. Thus, at least two different nSMases could be defined by their pH-dependent enzyme activity. Both enzyme activities were found to be Mg dependent (data not shown). A subsequent screening of human cell lines and mouse tissues is listed in Table 1. Both nSMases were found in liver, brain, muscle, spleen and kidney. Brain tissue displayed the highest activity of both activities. In human cell lines both nSMases were detected in Hela (cervix carcinoma), KB (squamous cell carcinoma), HTB72 (melanoma), DANG (pancreatic cancer cell line) as well as in B-cell lymphomas. Yet in T-cell lymphomas, PHA stimulated lymph node cells from mice and the monocytic leukemia line HL60 nSMase activity was observed only at pH 6.5.

3.2. Selective inhibition of pH 7.0 nSMase by C11AG

We recently described the drug C11AG [9], which had been shown to block efficiently nSMase activity in macrophages. As macrophages express only nSMase activity with a pH optimum at pH 7.0 it was of interest, whether C11AG would block also the nSMase activity with an optimum at pH 8.0. In order to exclude that the inhibitor

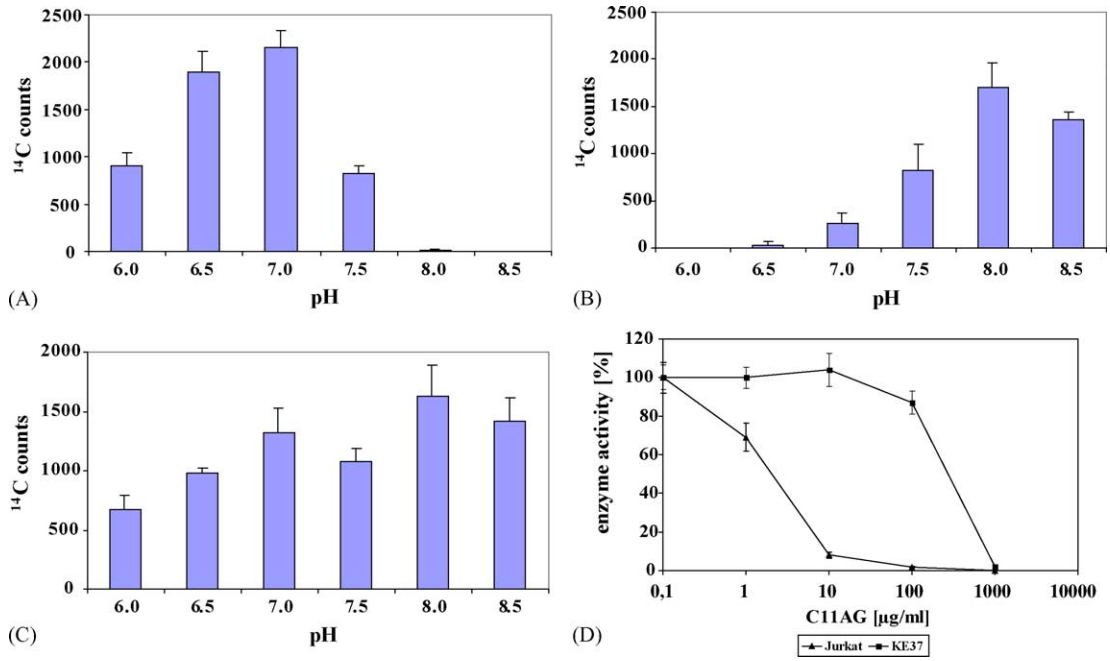

Fig. 1. nSMase activity in B- and T-cell lymphomas. SMase activity was determined at different pH conditions in membrane extracts from Jurkat (A), KE37 (B) and Raji (C) cells. (D) Inhibitory effect of C11AG on nSMase activity in membrane extracts from Jurkat (triangles) and KE37 (squares) cells. All activities were determined at pH 7.5. The 100% enzyme activity corresponds with the uninhibited control reaction. All assays were run in triplicate. Mean values ± standard deviation are indicated.

Table 1
nSMase activity in mouse tissues and human tumor cell lines

Tissue/cell line	pH 6.5 (^{14}C PCH counts)	pH 8.5 (^{14}C PCH counts)
Mouse		
Liver	2.3×10^3	11.5×10^3
Brain	39.7×10^3	50.8×10^3
Muscle	1.29×10^3	0.12×10^3
Spleen	1.32×10^3	0.46×10^3
Kidney	1.14×10^3	0.26×10^3
PHA stimulated lymph node cells	0.2×10^3	0.0×10^3
Human		
PHA stimulated peripheral blood cells (>95% T-cells)	0.3×10^3	0.0×10^3
Raji/B-cell lymphoma	2.3×10^3	1.8×10^3
DG75/B-cell lymphoma	1.67×10^3	3.4×10^3
DAV/B-cell lymphoma	3.6×10^3	1.7×10^3
TREV/B-cell lymphoma	4.7×10^3	3.1×10^3
H9/T-cell lymphoma	6.3×10^3	0.0×10^3
HSB/T-cell lymphoma	8.3×10^3	0.0×10^3
Jurkat/T-cell lymphoma	3.1×10^3	0.0×10^3
HL60/acute myeloid leukemia	0.52×10^3	0.0×10^3
Hela/cervix carcinoma	2.3×10^3	0.0×10^3
KB/squamous cell carcinoma	0.44×10^3	0.0×10^3
HTB72/melanoma	1.9×10^3	0.0×10^3
DANG/pancreas carcinoma	1.6×10^3	0.2×10^3

nSMase activity was determined in membrane extracts from cell lines or tissues as described in Section 2.

might be affected per se by the reaction conditions (i.e. the pH), both enzyme activities were assayed under the identical pH. Both enzymes are active at pH 7.5, albeit suboptimally. When Jurkat cells, which express only the nSMase with a pH optimum of 7.0, were used as a source for nSMase a concentration of 2.8 μg/ml C11AG resulted in a 50% inhibition of nSMase activity (Fig. 1D). KE37 cells overexpress the nSMase with an optimum at pH 8.0 by seven-fold. In order to obtain 50% inhibition as much as 290 μg/ml C11AG were required (Fig. 1D). Hence C11AG is rather selectively inhibiting the nSMase with a pH optimum of 7.0 and is 100-fold less active on the enzyme with a pH 8.0 optimum. Similar results were obtained, when the inhibitory activity of C11AG was assayed at the pH optimum of the two nSMase activities (data not shown).

3.3. Expression of anti-apoptotic gene A1 is prevented by C11AG

Activation of MAP kinases and nSMase is triggered by TNF. It was suggested that nSMase might be involved in the regulation of protection from TNF-induced apoptosis [11]. Therefore, we studied the effect of C11AG on the induction of apoptosis. As a model system we used Jurkat cells, which express only the pH 7.0 nSMase. Apoptosis was induced either by the CD95-specific monoclonal antibody APO-1 or, by the protein kinase inhibitor H7. C11AG was used at a concentration of 1 μg/ml which had no significant influence (data not shown) on the growth rate of Jurkat cells. APO-1 as well as H7 effectively induced apoptosis. However, in the presence of C11AG cells were found to be six-fold more sensitive to APO-1 (IC50: 7 ng/ml APO-1). In the absence of C11AG 43 ng/ml APO-1 were required. The IC50 for H7 remained unaltered, i.e. it was 62 and 57 nM in the presence or absence of C11AG, respectively (Fig. 2A).

At which step leading to apoptosis does C11AG interact? Cleavage of FLICE is an early event in apoptosis [12]. After recruitment to the death inducing complex auto-cleavage of FLICE is initiated and apoptosis is executed [13]. We monitored CD95-induced auto-cleavage of FLICE in Western blots. Neither C11AG nor APO-1 had an impact on the cleavage of FLICE at non-toxic concentrations. Instead, in cells treated with a combination of both a significant cleavage was observed (Fig. 2B). As it has been speculated that nSMases are involved in gene expression we decided to analyze the effect of C11AG on the level of expression of proteins that affect the auto cleavage of FLICE. Proteins of the Bcl2 family interfere with activation of FLICE [14,15]. We found that C11AG inhibited the expression of the anti-apoptotic gene A1. Bcl2 and Bcl_{XL}/Bcl_{XS} remained unaf-

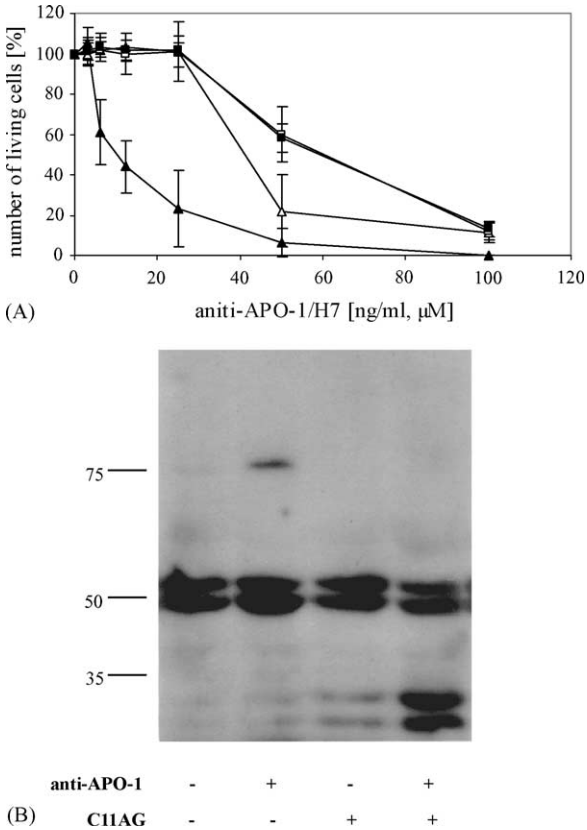

Fig. 2. Stimulation of CD95-induced apoptosis by C11AG. (A) Jurkat cells were treated with various concentrations of anti-APO-1 or protein kinase inhibitor H7 in the presence or absence of 1 μg/ml C11AG. After 24 h the number of living cells was determined. Open squares: H7; filled squares: H7 + 1 μg/ml C11AG; open triangles: anti-APO-1; filled triangles: anti-APO-1 + 1 μg/ml C11AG. Mean values of four samples each ± standard deviation. (B) Jurkat cells were treated with 20 ng/ml anti-APO-1, 1 μg/ml C11AG or with both agents. After 2 h of incubation at 37 °C cells were collected and FLICE was revealed in a Western blot.

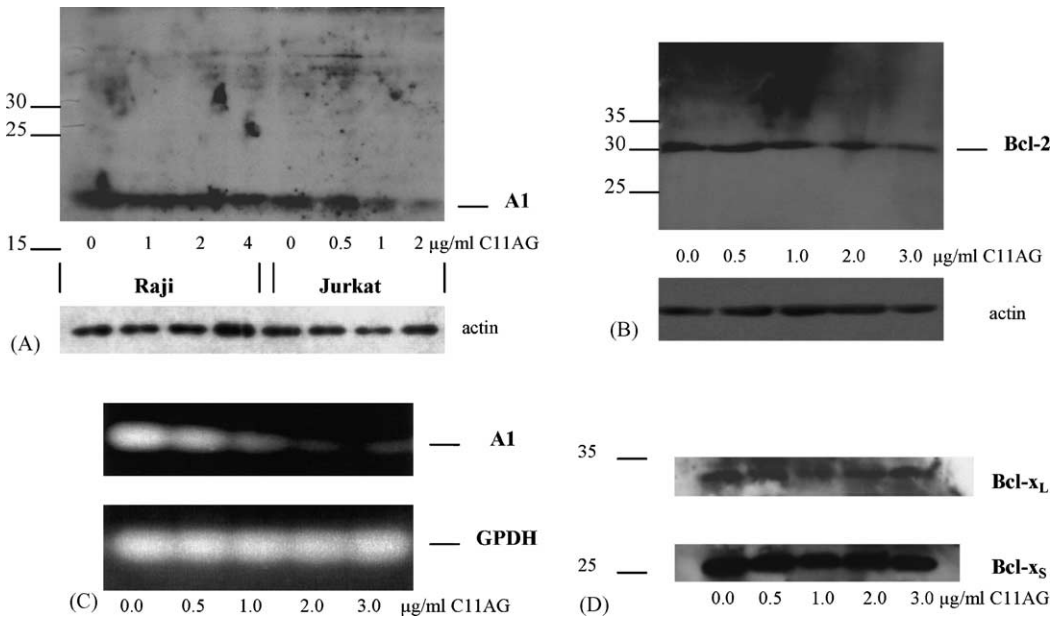

Fig. 3. Inhibition of A1 expression by C11AG in Jurkat cells. Cells were treated for 24 h with various concentrations of C11AG. (A), (B) and (D) Western blot analysis. (A) Raji and Jurkat cells, anti-A1 specific antibody. Lower panel: the filter was reprobed with an actin specific monoclonal antibody. (B) Jurkat cells, anti-Bcl2 specific antibody. Lower panel: the filter was reprobed with an actin specific monoclonal antibody. (D) The filter from (B) was reacted with an antibody specific for Bcl_{XL} and Bcl_{XS} after stripping. (C) RT-PCR, Jurkat cells.

fected (Fig. 3B–D). Inhibition of A1 could be demonstrated by Western blotting and semi-quantitative RT-PCR. It is noteworthy that C11AG had no influence on the A1 expression in Raji cells, which express both, pH 7.0 and pH 8.0 nSMases. Thus, C11AG seems to prevent the up-regulation of at least one anti-apoptotic gene via an nSMase with a pH optimum of 7.0.

3.4. Therapeutic efficacy of C11AG in collagen induced arthritis

Auto-aggressive T-cells are supposed to be resistant toward CD95-induced apoptosis [16–18]. Stimulation of apoptosis initiated by interaction of CD95 with CD95L could be a way to eliminate autoreactive T-cells. As shown above, C11AG sensitizes T-cells to this type of apoptosis. The question arises whether the inhibitor might also act in vivo. As a model we chose collagen induced arthritis in mice. Three groups of 10 animals each were injected with 100 μg collagen in complete Freud's adjuvant. At day 21 the animals of one group were treated subcutaneously with 200 mg/kg C11AG. All animals were challenged with 50 μg collagen i.p. at day 28. At day 35 the animals of one of the two groups that had received exclusively collagen were treated with 200 mg/kg C11AG. All animals treated with collagen alone developed strong symptoms of arthritis within 7 days after the boost (Fig. 4A). Thickness of foot pads was almost doubled in collagen treated animals. Animals which had been treated with C11AG at day 21 were only slightly affected. Animals that were treated with C11AG 7 days after the boost strongly reacted within 24 h: foot pad thickness had returned to almost normal values (Fig. 4B). Inflammatory symptoms such as erythema and edema were also found to be alleviated after C11AG treatment.

Three animals of each group were sacrificed 24 h after C11AG treatment and draining lymph nodes were collected. Proliferation assays revealed an increased ^{3}H thymidine incorporation in lymph node cells from arthritic mice (Fig. 4C), which could be further stimulated by collagen. A significantly reduced proliferation rate was found in lymph node cells from animals which had been treated with C11AG 24 h before isolation of cells. No profound increase in the proliferation as compared to that of non-arthritic controls was observed in cells isolated from animals which had been injected with C11AG at day 21. In order to analyze the effect of C11AG on resting lymph node cells, non-immunized animals were treated with 300 mg/kg C11AG. Lymph node cells were isolated 24 h later and stimulated with 7.5 μg/ml Con A and the rate of proliferation was determined by ^{3}H thymidine incorporation assays (Fig. 4E). A strong stimulation of cell proliferation was obtained in both, cells from treated and untreated animals. In addition we analyzed the ratio of CD4 and CD8 positive T-cells in lymph nodes of arthritic mice (Fig. 4D). In healthy mice 17% CD4 and 13% CD8 positive cells were found. In arthritic mice the amount had increased to 31 and 30%. In C11AG treated arthritic mice the number of CD8 positive T-cells was reduced to 14.5%, while CD4 positive cells were only slightly reduced to 25%.

In order to strengthen the hypothesis that apoptosis is involved in the anti-inflammatory effect of C11AG on collagen induced arthritis in mice, cells infiltrating the

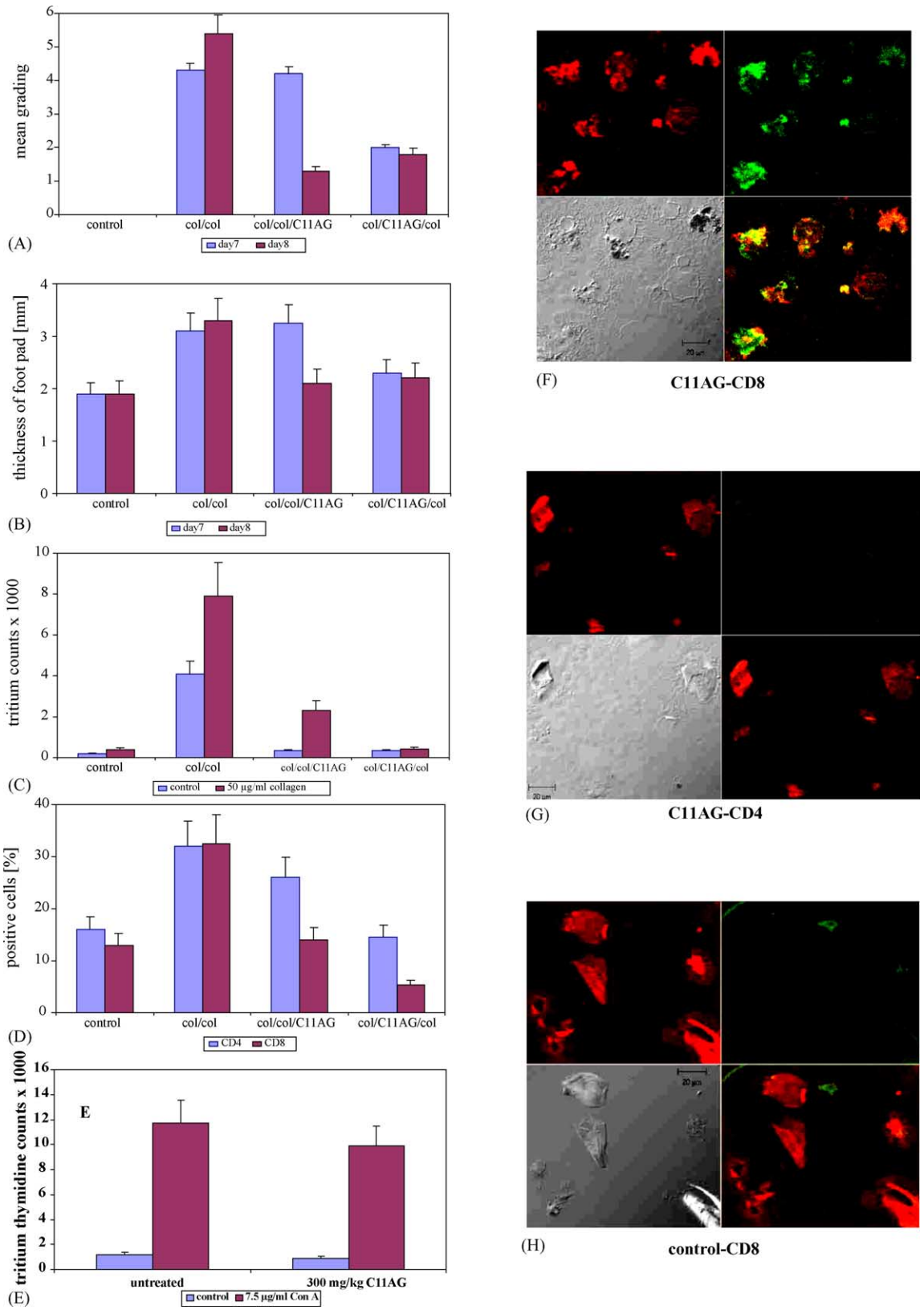

Fig. 4. Curative effects of C11AG on collagen induced arthritis. (A)–(D) Three groups of 10 animals were injected s.c. with collagen or with PBS (control). At day 21 the animals of one group were treated with 200 mg/kg C11AG s.c. (col/C11AG/col). At day 28 all collagen immunized animals were boosted with collagen i.p. At day 35 the mice of one group (col/col/C11AG) were treated with 200 mg/kg C11AG s.c., a placebo group was treated wit 0.9% NaCl (col/col). (A) Symptoms were scored at days 35 and 36 (days 7 and 8 after boost). (B) Thickness of foot pads was determined at days 35 and 36 (days 7 and 8 after boost). (C) Three animals of each group were sacrificed at day 36 and draining lymph nodes were removed. In lymphocyte cultures ^{3}H thymidine incorporation was determined in the presence and absence of collagen. (D) The number of CD4 and CD8 positive lymph node cells was determined by flow cytometry at day 36

inflamed joints were collected 12 h after C11AG or placebo treatment (day 7 after collagen boost, three animals each). Flow cytometry revealed a significant reduction in the percentage of CD8 + T-cells and of macrophages (data not shown). TUNEL staining revealed only a few cells with fragmented DNA in the joints of control animals. Dead cells were readily detected in the joints of C11AG treated animals. Most of the TUNEL positive cells reacted with a CD8 specific antibody, while a CD4 specific antibody failed to stain cells that were positive in the TUNEL assay (Fig. 4F).

4. Discussion

Two nSMase activities with optima at pH 7.0 or 8.0, respectively, can be distinguished in human tissue culture cells and in tissues from mice. Currently we cannot define whether these two activities are resulting from two different iso-enzymes. It is also conceivable that post-translational modifications of the same protein are responsible for the different pH optima. Yet, we found that the ratio of the two activities is stable for each cell type and independent from cell growth or from the metabolic state of the cell. Notably, T-cells express exclusively the pH 7.0 nSMase. We favour the existence of at least two different iso-forms of membrane associated nSMase. We can also rule out that one of the activities is correlated with acidic SMase. In a B-cell line from a Niemann-Pick patient (TREV), which is completely devoid of acidic SMase both nSMase activities were detected. For none of the cloned nSMase a protective function in CD95-induced apoptosis has been found. Therefore, it is likely that the target of C11AG is a still undescribed nSMase.

Surprisingly, we found that the nSMase inhibitor C11AG is 100-fold more active on the pH 7.0 nSMase activity. There is evidence that in some cases the pH 8.0 enzyme can substitute the inhibited pH 7.0 enzyme. In Raji cells expression of the anti-apoptotic protein A1 remained unaffected by treatment with C11AG. Whether this is a common feature of the two nSMases or whether both enzymes have also separate functions remains to be evaluated.

In the presence of the nSMase inhibitor C11AG the expression of the anti-apoptotic A1 gene was found to be down-regulated. However, Bcl2 and Bcl_{XL}/Bcl_{XS} expression remained unaffected. This observation may be explained by the fact that several transcription factors can be involved in the regulation of one specific gene. As a consequence, inhibition of only one transcription factor may not necessarily result in inhibition of transcription of a given gene, while other genes may not be transcribed any longer. Nevertheless, the reduction of the level of A1 protein could well account for the increased sensitivity of Jurkat cells to CD95 in the presence of C11AG. This assumption is in agreement with the observation that TNF-induced apoptosis is blocked by A1 [19]. Furthermore, it was shown that an increased expression of Bcl_{XL} and A1 promotes the survival of $CD8^+$ T lymphocytes [20]. The expression of the A1 gene is regulated by NFκB [19]. We have shown recently that C11AG prevents LPS induced NFκB activation, both, in vitro and in vivo [21]. In addition, we found that C11AG prevents liberation of NFκB from its inhibitor in Jurkat cells after crosslinking of CD95 [data not shown].

Collagen induced arthritis could either be prevented and/or alleviated by C11AG. We could demonstrate in vivo that C11AG preferentially affects activated T-cells and rather selectively drives activated CD8 + T-cells into death. Already 1 day after C11AG treatment a significant loss of collagen specific T-cells was observed in proliferation assays. Therefore, it is likely that the anti-arthritic effect of C11AG is based on the loss of collagen specific T-cells. Although the TUNEL assay is not a good prove for apoptosis it shows preferential death of $CD8^+$ T-cells. Our in vitro experiments with Jurkat cells show that C11AG can sensitize cells to CD95-induced apoptosis. Whether the rapid loss of $CD8^+$ T-cells in the collagen induced arthritis model is due to the same effect remains to be further elucidated.

C11AG exerts its activity already within a few hours after treatment: cleavage of FLICE after crosslinking of CD95 was found to be stimulated by C11AG treatment after 2 h.

Immunization with collagen followed by even 3 weeks of continuous C11AG treatment led to similar titers of collagen specific antibodies (data not shown). This result confirms that C11AG leaves the function of B-cells, which express high levels of pH 8.0 nSMase, unaffected.

The therapy of severe autoimmune diseases has long been based on a persisting application of anti-inflammatory agents or even cytostatica, both treatment methods being burdened by severe side effects. We demonstrate here that a single application of an inhibitor specific for one subtype of nSMase efficiently cures an autoimmune disease in the absence of overt side effects.

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(three animals each). (E) Mice were treated with 300 mg/kg C11AG s.c. or with 0.9% NaCl. The 24 h later lymph node cells were isolated and ^3H thymidine incorporation was determined in the presence and absence of 7.5 μg/ml Con A. Three animals each. (F)–(H) Arthritis was induced in mice with collagen. At day 10 after boosting 200 mg/kg C11AG was injected s.c. After killing of the animals infiltrating cells were collected from foot pads with PBS. After staining with an antibody specific for CD4 or CD8 and a Texas red coupled secondary antibody, cells were fixed with formaldehyde and collected on slides in a Cyto Spin centrifuge. Apoptotic cells were stained with a TUNEL assay kit using FITC labeled BUdR. Confocal laser scanning microscope photographs of the same area. (F) C11AG treated animal, antibody specific for CD8. (G) C11AG treated animal, antibody specific for CD4. (H) PBS treated animal, antibody specific for CD8.

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