Ceramide-independent CD28 and TCR signaling but reduced IL-2 secretion in T cells of acid sphingomyelinase-deficient mice

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Ceramide generated by lysosomal acid sphingomyelinase (aSMase) has been proposed to contribute to CD28 co-stimulatory signaling pathways. We used an aSMase-deficient mouse line (*asmase*$^{-/-}$) to elucidate the role of the aSMase in splenocytes stimulated with either a combination of anti-CD3 and anti-CD28 antibodies, the lectin concanavalin A (Con A) or the superantigen staphylococcal enterotoxin B. All stimuli were shown to induce IL-2 expression, Con A additionally triggered the expression of high-affinity IL-2 receptor. However, in *asmase*$^{-/-}$ mice secretion of IL-2 was significantly reduced, whereas the intracellular IL-2 levels were elevated. Proliferation of anti-CD3/anti-CD28 or Con A-stimulated aSMase-deficient splenocytes was reduced up to 50 % after 72 h in comparison to wild-type cells. We conclude that ceramide generated by aSMase is not involved in CD28 signal transduction, but rather a perturbation of the secretory system is responsible for the impaired proliferation of aSMase-deficient splenocytes.

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1 Introduction

T cell activation is known to require an orchestrated interaction of different cell surface receptors. Apart from the binding of the TCR-CD3 complex to CD4 or CD8, co-stimulatory signals are required to generate maximal T cell response. The molecular mechanism concerning TCR-initiated signaling (reviewed in [1]) or co-stimulatory signaling [2] is discussed controversially and awaits clarification.

The first known step after TCR binding to a specific MHC-peptide complex is tyrosine phosphorylation of CD3 chains [3, 4]. Among other downstream events, hydrolysis of phosphoinositides with a sustained elevation of intracellular free Ca^{2+} is initiated (reviewed in [5]). The rate of intracellular Ca^{2+} elevation either by a release from intracellular stores or influx determines the extent of T cell clonal expansion. Apart from phosphoinositides, a sphingomyelin breakdown product, sphingosine, has been discussed either to enhance [6] or inhibit [7] the Ca^{2+} release and/or influx. Thus, alterations in sphingosine levels may influence T cell proliferation rates, for instance by down-regulation of calcium-dependent IL-2 gene expression [8] or impairment of Ca^{2+}-mediated exocytotic vesicle fusion [5].

Certain molecules on APC have been identified which enhance T cell responses [9–13]. Considerable attention has been drawn to the glycoproteins B7.1 (CD80) and B7.2 (B70, CD86) which differentially regulate T cell clonal expansion [13, 14]. Their natural receptors are CD28 and the structurally related cytotoxic T lymphocyte-associated molecule-4 (CTLA-4). Binding of B7 to CD28 lowers the threshold needed for T cell activation and a prolongation of the response [1]. Transcription and stability of lymphokine mRNA, in particular those encoding IL-2, IL-4, IFN-γ and TNF-α, are increased [15, 16]. Expression of CTLA-4, high-affinity IL-2 receptor, CD40 ligand and anti-apoptotic molecule bcl-x_L is up-regulated [2]. Blocking of either B7 ligands or CD28 receptor synergistically with TCR stimulation abrogates T cell activation and leads to anergy in most T cell subsets [17, 18]. Binding of B7 to CTLA-4 is thought to antagonize B7-CD28 signaling [1].

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Abbreviations: aSMase: Acid sphingomyelinase **WT:** Wild-type **SEB:** Staphylococcal enterotoxin B **BrdU:** 5-bromo-2'-deoxyuridine

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Ligand binding-induced tyrosine phosphorylation is thought to initiate the CD28 signal transduction cascade via recruitment of phosphatidylinositol 3'-kinase (PI3-kinase) [19]. This kinase has been connected to mitogenesis and apoptosis [20]. The cascade is thought to split into a calcium-dependent TCR-analogous pathway and a calcium-independent CD28-specific pathway [21]. The former is sensitive to cyclosporin A (CsA) and leads to NF-AT activation. The latter initiates the mitogen-activated protein kinase (MAPK)-cascade via $p21^{ras}$ (eventually resulting in AP-1 and NF-κB formation) and protein kinase C (PKC). MAPK activation was reported to require both PI3-kinase and acid sphingomyelinase (aSMase) activity [22–24]. Both the lysosomal aSMase and the neutral sphingomyelinase, located in the plasmamembrane, are sphingomyelin-specific phospholipases C that generate ceramide and phosphocholine. Ceramide released by either of these enzymes has been thought to participate in different signal transduction pathways coupled to TNF receptor, Fas, CD28, nerve growth factor receptor and others [25].

Here we used an aSMase-deficient (*asmase*$^{-/-}$) mouse [26] to elucidate the role of aSMase in CD28 and TCR signaling after anti-CD3/anti-CD28, lectin and superantigen stimulation. We present experimental evidence that ceramide released in the aSMase-activated "sphingomyelin cycle" is not involved in CD28-activated signal transduction. However, IL-2 secretion is strongly impaired in activated T cells of *asmase*$^{-/-}$ mice, while the IL-2 synthesis and IL-2 receptor expression remain unaltered and comparable to wild-type (WT) control levels.

2 Results

2.1 Proliferative response of *asmase*$^{-/-}$ splenocytes to mitogenic challenges

Spleen cells of WT and aSMase-deficient (*asmase*$^{-/-}$) mice were stimulated for up to 72 h with the phorbol ester PMA (15 ng/ml) and calcium ionophore (250 ng/ml), with the lectin Con A at different concentrations (2.5 and 5 μg/ml), and by anti-CD3/anti-CD28 treatment (1 μg/ml each). Cell proliferation was measured initially by 5-bromo-2'-deoxyuridine (BrdU) and [^{3}H]thymidine uptake. The two methods gave identical results. Therefore, the BrdU proliferation ELISA was used in all further experiments. Fig. 1 demonstrates that T cells derived from *asmase*$^{-/-}$ mice stimulated with anti-CD3/anti-CD28 and Con A showed a reduced proliferative response to the mitogens compared to WT T cells. After 72 h, WT cells exceeded the aSMase-deficient T cells by approximately 50 %. No significant differences in the proliferation potential were observed in WT and *asmase*$^{-/-}$ T cells in response to a combination of PMA and ionomycin.

2.2 IL-2 production and secretion of *in vitro* stimulated *asmase*$^{-/-}$ T cells

The production of IL-2 was determined by intracellular staining of IL-2-positive splenocytes after stimulation with either a combination of anti-CD3 and anti-CD28 antibody (Fig. 2A), Con A (Fig. 3A) or staphylococcal

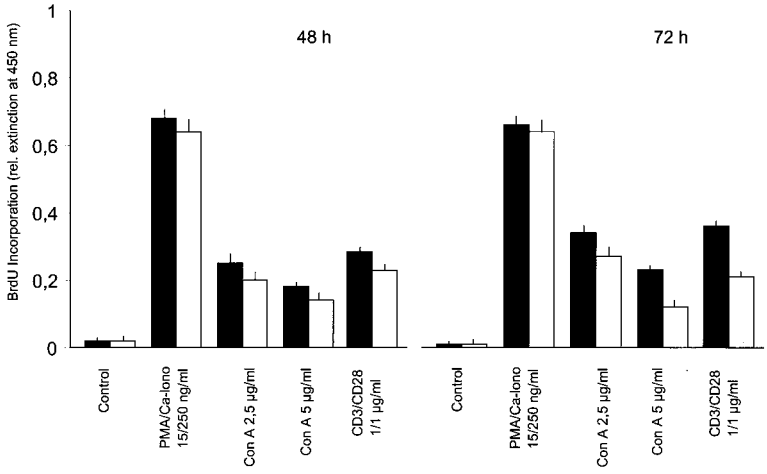

Figure 1. Splenocyte proliferation measured by BrdU incorporation ELISA. Splenocytes (1×10^5 cells/well) of pooled WT ($n = 3$, filled columns) and aSMase-deficient mice ($n = 3$, open columns) were cultured in 96-well flat-bottom plates and stimulated with PMA/Ca^{2+} ionophore (15 ng/250 ng/ml), Con A (2.5 or 5 μg/ml) or anti-CD3/anti-CD28 antibodies (1 μg/ml each) for 48 and 72 h. Each data point represents the mean value ± SD of six repeats per stimulant.

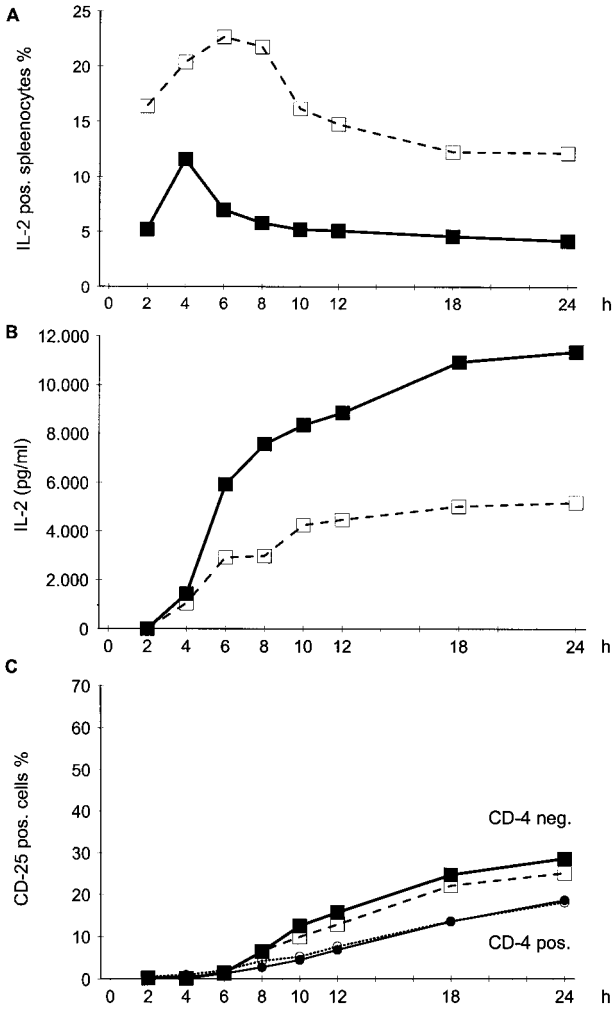

Figure 2. Spleen cells (2×10^6 cells/well) were cultured in 24-well flat-bottom plates and stimulated with 10 μg/ml anti-CD3 and 1 μg/ml anti-CD28 for the times (h) indicated on the abscissa. (A) Relative number of IL-2-positive WT (filled squares) or aSMase-deficient (open squares) splenocytes. Cells ($0.5–1 \times 10^6$) were pelleted by centrifugation. Cell pellets were resuspended in PBS containing 0.5 % BSA, 0.02 % NaN_3 and 0.5 % saponin and incubated with 5 μg/ml rat anti-mouse IL-2 Ab for 15 min at 4 °C in the dark; 10 000 fixed splenocytes in the gate were analyzed by flow cytometry. (B) IL-2 secretion of WT (filled squares) or *asmase*$^{-/-}$ (open squares) splenocytes. Cell culture supernatants were analyzed with IL-2 ELISA. (C) Expression of high-affinity IL-2 receptor on WT (filled squares) or aSMase-deficient (open squares) $CD4^+$ and $CD4^-$ splenocytes, respectively. Cells were simultaneously incubated with 1 μg/ml FITC-conjugated anti-CD25 and 2 μg/ml PE-conjugated anti-CD3 Ab at room temperature. After washing with 1 ml PBS, cells were resuspended in PBS/0.5 % BSA/0.02 % NaN_3 and analyzed by flow cytometry as described above.

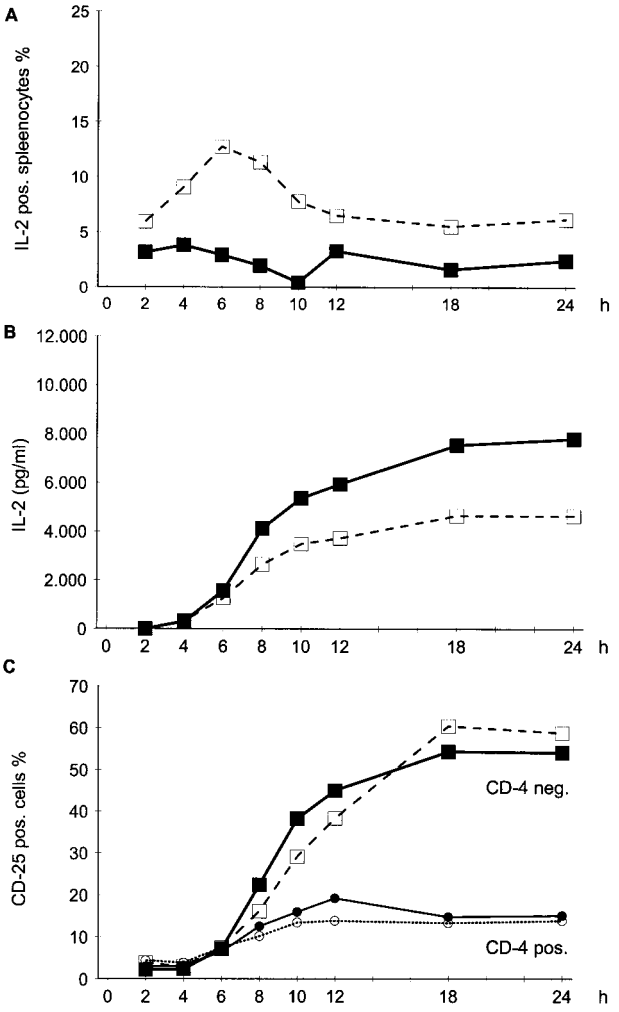

Figure 3. Splenocytes (2×10^6 cells/well) were cultured in 24-well flat-bottom plates and stimulated with 5 μg/ml Con A. Kinetic analyses of the relative numbers of IL-2-positive splenocytes (A), IL-2 content of cell culture supernatants (B) and high-affinity IL-2 receptor expression on $CD4^+$ and $CD4^-$ splenocytes, respectively (C), were performed as described in the legend of Fig. 2. The abscissa indicates periods of stimulation (h). WT splenocytes: filled squares, aSMase-deficient splenocytes: open squares.

enterotoxin B (SEB) (Fig. 4A). All samples of aSMase-deficient cells generally revealed a higher number of IL-2-positive cells compared to WT cells. Upon CD3 and CD28 stimulation, IL-2-positive WT splenocytes doubled from a 5 % base level to 10 % after 4 h. After 8–10 h, intracellular IL-2 was down-regulated to normal amounts. Numbers of IL-2-positive *asmase*$^{-/-}$ cells rose from about 12 % base level to 23 % after 4 h and normalized in a time course similar to WT splenocytes. While IL-2 levels in WT splenocytes remained constant within a 24-h period (2–4 % positive cells), the number of IL-2

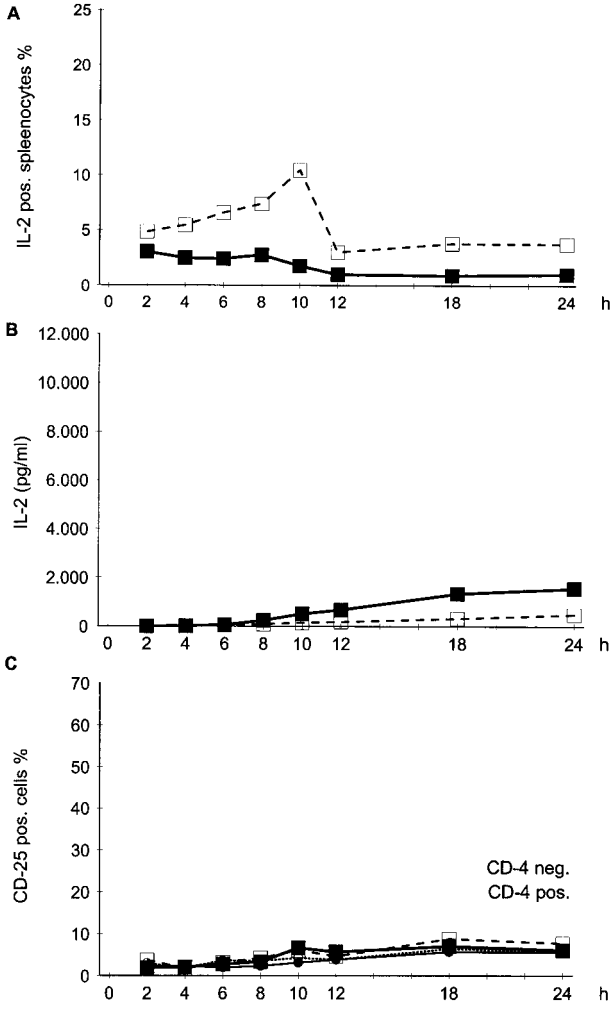

Figure 4. Splenocytes (2×10^6 cells/well) were cultured in 24-well flat-bottom plates and stimulated with 5 μg/ml SEB. Kinetic analyses of the relative numbers of IL-2-positive splenocytes (A), IL-2 content of cell culture supernatants (B) and high-affinity IL-2 receptor expression on $CD4^+$ and $CD4^-$ splenocytes, respectively (C), were performed as described in the legend of Fig. 2. The abscissa indicates periods of stimulation (h). WT splenocytes: filled squares, aSMase-deficient splenocytes: open squares.

positive *asmase*$^{-/-}$ cells doubled after 6 h of Con A challenge and after 10 h of SEB challenge, respectively. Later on, cell numbers decreased to base levels within 2–4 h. In order to correlate production and secretion of IL-2, we determined the IL-2 content of cell culture supernatant after anti-CD3/anti-CD28, lectin and superantigen stimulation (Figs. 2B, 3B and 4B). After a challenge with anti-CD3/anti-CD28, WT and *asmase*$^{-/-}$ cells secreted high amounts of IL-2 between 4 h and 18 h. Secretion reached and remained on a maximum level between 18 h and 48 h at about 11 ng/ml for WT cells and about 5 ng/ml for *asmase*$^{-/-}$ cells (data not shown). After Con A stimulation, secretion started analogously between 4 h and 18 h; after SEB challenge IL-2 secretion started after 6 h with a delay of 2 h compared to lectin stimulation. The final IL-2 secretion level between 18 h and 48 h was twice (Con A) or even three times as high (SEB) in WT than in *asmase*$^{-/-}$ splenocytes. For SEB, however, within the time of the experiment (48 h), no plateau was reached (data not shown).

2.3 IL-2 receptor α chain expression of *in vitro* stimulated *asmase*$^{-/-}$ T cells

After splenocyte stimulation with Con A, the IL-2 receptor α chain (CD25) expression rose about 10-fold within 12 h on $CD4^+$ cells and about 60-fold within 18 h on $CD4^-$ lymphocytes (Fig. 3C). CD25 expression on aSMase-deficient T cells was slightly delayed and never reached WT level. The same observation was made for $CD4^-$ cells, except that after 18 h even more *asmase*$^{-/-}$ cells were found CD25 positive than WT lymphocytes. Upon anti-CD3/anti-CD28 treatment of splenocytes, CD25 expression rose about 30-fold in the time of observation in WT $CD4^-$ cells (Fig. 2C). Expression in *asmase*$^{-/-}$ T cells lagged closely behind WT levels. Expression in $CD4^+$ cells was comparable to that triggered by Con A. SEB stimulation did not lead to a significant increase in CD25 expression in neither genotype (Fig. 4C).

3 Discussion

We addressed the question whether aSMase or its reaction product ceramide is important for TCR- or CD28-mediated signal transduction. *asmase*$^{-/-}$ splenocytes stimulated with Con A proliferated significantly slower than WT cells, while only marginal effects were observed after PMA/Ca^{2+}-ionophore treatment of the same cells. This is due to a strongly reduced IL-2 secretion after Con A challenge, which is only half as high in aSMase-deficient splenocytes as in splenocytes of WT controls. After a combined treatment of the cells with anti-CD3 and anti-CD28 antibody, IL-2 secretion and proliferation was impaired in a similar manner. Surprisingly, in both cases *asmase*$^{-/-}$ cells accumulate IL-2 intracellularly. We therefore conclude that secretion is impaired rather than IL-2 gene transcription, mRNA stabilization or translation. Additionally, the expression of high-affinity IL-2 receptor was only slightly different in both genotypes. This suggests an intact CD28 signal transduction pathway.

Generally the same results were obtained after TCR stimulation with the superantigen SEB. With a small time

shift of about 2 h, intracellular accumulation of IL-2 and diminished IL-2 secretion was observed correspondingly to lectin stimulation. CD25 expression was neither enhanced in aSMase-deficient nor in WT splenocytes.

Ceramide has been considered to contribute to many different signaling events. Among others [25], it was reported to be released after CD28-B7 binding [22–24]. We could not confirm these results. On the contrary, we observed higher intracellular IL-2 levels in *asmase*$^{-/-}$ cells which correlate with a higher number of mRNA transcripts [27]. Splenocytes from both the aSMase-deficient and the WT mice showed an almost equal proliferation in response to PMA/ionomycin stimulation which mimics TCR-CD3- and CD28-mediated signal transduction [28]. However, proliferation of *asmase*$^{-/-}$ splenocytes was reduced after Con A or anti-CD3/anti-CD28 challenge caused by impaired IL-2 secretion.

Activation of a phosphatidylinositol-specific phospholipase C (PI-PLC) and PI3-kinase are early events in TCR and CD28 signal transduction [5, 19]. Sphingomyelin that is highly accumulated in membranes of aSMase-deficient cells has been shown to be a potent inhibitor of PI-PLC [29], suggesting that the enzyme is less active in *asmase*$^{-/-}$ splenocytes. Since the Ca^{2+} ionophore directly elevates intracellular Ca^{2+} concentrations and therefore bypasses the inositol-1,4,5-trisphosphate-induced Ca^{2+} release, we conclude that reduced Ca^{2+} concentrations in *asmase*$^{-/-}$ cells might be responsible for impaired vesicle fusion. Ca^{2+} concentrations appear to be sufficiently high to induce IL-2 and IL-2R expression.

Sphingomyelin and cholesterol are generally associated [30]. Sphingomyelin accumulation in the plasmamembrane of *asmase*$^{-/-}$ splenocytes may change its biophysical properties (*e.g.* membrane fluidity) and thereby alter the function of PI-PLC, PKC and/or PI3-kinase. Vesicular transport and fusion may also be impeded. Perturbed sphingolipid synthesis has been shown to affect protein and lipid trafficking through the secretory system, suggesting that vesicular transport along the secretory pathway is coupled to sphingolipid metabolism [31]. The fungal metabolite brefeldin A (BFA) has been reported not only to inhibit protein transport from cis- to trans-Golgi, but also to induce sphingomyelin hydrolysis [32]. Whether the reaction product ceramide regulates membrane fluidity and facilitates vesicular budding remains to be proven.

The role of ceramide released by aSMase in signal transduction has been questioned recently. We have shown that NF-$\varkappa$B is activated upon TNF-α stimulation indiscriminately in *asmase*$^{-/-}$ and WT embryonic fibroblasts [33]. Others have provided evidence against a role of ceramide in Fas-mediated signal transduction [34]. The results reported here suggest that ceramide-dependent signal transduction processes in T cell proliferation triggered by CD28 and TCR stimulation are unimpaired; however, alterations of the physical properties of the membrane by sphingomyelin accumulation in aSMase-deficient splenocytes might be responsible for an impaired secretion of IL-2.

4 Materials and methods

4.1 Animals

WT mice (C57BL/6 × 129/01a) and aSMase-deficient mice (C57BL/6 × 129/01a *asmase*$^{-/-}$) were generated as described [26]. Mice were used at 8–10 weeks of age.

4.2 Cell culture conditions and reagents

Splenocytes of WT ($n = 3$) and aSMase-deficient mice ($n = 3$) were harvested, pooled and plated in 24-well flat-bottom plates at 2×10^6 cells/ml cell culture medium (RPMI 1640 containing 10 % FCS and 100 U/ml penicillin/streptomycin; PAA Laboratories GmbH, Coelbe, Germany) using standard methods. Cells were stimulated with a combination of 10 μg/ml anti-CD3-ε (hamster IgG, clone 145-2C11, PharMingen) and 1 μg/ml anti-CD28 (hamster IgG, clone 37.51, PharMingen), 5 μg/ml SEB (Fluka, Sigma-Aldrich Chemie GmbH) or 5 μg/ml mitogen Con A (Boehringer Mannheim). Splenocytes and cell culture supernatants were collected at the times indicated in the figures. Supernatants were stored at −80 °C until analyzed. Cells were washed once with PBS, resuspended in 500 μl PBS and fixed in 4 % formaldehyde in PBS (Merck, Darmstadt, Germany). After fixation for 20 min at room temperature, cells were washed with PBS, resuspended in PBS/0.5 % BSA/0.02 % NaN_3 and stored at 4 °C until stained.

4.3 Intracellular immunofluorescence staining of IL-2

For staining, 0.5×10^6–1×10^6 cells were centrifuged for 10 min at 300 × g. Cell pellets were resuspended and incubated with 5 μg/ml rat anti-mouse IL-2 Ab (RIgG2a, clone S4B6, PharMingen, Hamburg, Germany) in PBS/0.5 % BSA/0.02 % NaN_3 containing 0.5 % saponin (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany) for 15 min at 4 °C in the dark. After washing with 1 ml PBS/0.5 % BSA/0.02 % NaN_3/0.5 % saponin, cells were resuspended in 500 μl PBS/0.5 % BSA/0.02 % NaN_3 and stored at 4 °C until analyzed by flow cytometry. 10 000 fixed splenocytes in the gate were used for analysis of intracellular staining (FACScan and CELL Quest research software, Becton Dickinson GmbH, Heidelberg, Germany) [35, 36].

4.4 Immunofluorescence staining of IL-2 receptor α chain (CD25)

Cells were incubated for 10 min at room temperature with 1 μg/ml FITC-conjugated anti-CD25 (RIgM, clone 7D4) and 2 μg/ml PE-conjugated anti-CD3e (AHIgG, 145-2C11; both Ab from PharMingen, Hamburg, Germany). After washing with 1 ml PBS, cells were resuspended in PBS containing 0.5 % BSA and 0.02 % NaN_3, stored at 4 °C and analyzed as described above.

4.5 IL-2 ELISA

Supernatants were collected in 1.5 ml Eppendorf cups, centrifuged for 10 min at 300 × g and aliquots stored at −80 °C. IL-2 was determined by ELISA (Biotrak™, Amersham Life Science, Amersham, GB) according to the manufacturer's manual.

4.6 Proliferation assays

Splenocytes (1×10^5) from WT ($n = 3$) and aSMase-deficient mice ($n = 3$) were incubated in 96-well flat-bottom plates (Falcon 3072, Becton Dickinson GmbH, Heidelberg, Germany) in 200 μl RPMI 1640 medium supplemented with 10 % FCS and 100 U/ml penicillin/streptomycin. Cells were stimulated by the addition of PMA/ionomycin (15 ng/250 ng per ml; Sigma-Aldrich Chemie GmbH), Con A (2.5 and 5 μg per ml) or a combination of anti-CD3 and anti-CD28 antibody (1 μg/ml each) for 48 h and 72 h. Cell proliferation was measured by either using the BrdU-Cell Proliferation ELISA (Boehringer Mannheim, Germany), following the manufacturer's instructions, or by a modified [^{3}H]thymidine incorporation method [37]. In brief, cells were soaked onto a nitrocellulose membrane (Schleicher & Schüll BA85, Dassel, Germany) followed by three washes with 10 % trichloroacetic acid (TCA), 5 % TCA and 95 % ethanol, respectively. Air-dried filters were analyzed in a liquid scintillation counter (Wallac 1409, Turku, Finnland) with polar Bray solution.

5 References

1 **Shaw, A. S. and Dustin, M. L.,** Making the T cell receptor go the distance: A topological view of T cell activation. *Immunity* 1997. **6:** 361–369.

2 **Rudd, C. E.,** Upstream-downstream: CD28 cosignaling pathways and T cell function. *Immunity* 1996. **4:** 527–534.

3 **DeFranco, A. L.,** Signaling pathways activated by protein tyrosine phosphorylation in lymphocytes. *Curr. Opin. Immunol.* 1994. **6:** 364–371.

4 **Senfton, B. M. and Taddie, J. A.,** Role of tyrosine kinases in lymphocyte activation. *Curr. Opin. Immunol.* 1994. **6:** 372–379.

5 **Premack, B. A. and Gardner, P.,** Signal transduction by T cell receptors: mobilization of Ca and regulation of Ca-dependent effector molecules. *Am. J. Physiol.* 1992. **263:** C1119–C1140.

6 **Sakano, S., Takemura, H., Yamada, K., Imoto, K., Kaneko, M. and Ohshika, H.,** Ca^{2+} mobilizing action of sphingosine in Jurkat human leukemia cells. Evidence that sphingosine releases Ca^{2+} from inositol trisphosphate- and phosphatidic acid-sensitive intracellular stores through a mechanism independent of inositol trisphosphate. *J. Biol. Chem.* 1996. **271:** 11148–11155.

7 **Breittmayer, J. P., Bernard, A. and Aussel, C.,** Regulation by sphingomyelinase and sphingosine of Ca^{2+} signals elicited by CD3 monoclonal antibody, thapsigargin, or ionomycin in the Jurkat T cell line. *J. Biol. Chem.* 1994. **269:** 5054–5058.

8 **Negulescu, P. A., Shastri, N. and Cahalan, M. D.,** Intracellular calcium dependence of gene expression in single T lymphocytes. *Proc. Natl. Acad. Sci. USA* 1994. **91:** 2873–2877.

9 **Lafferty, K. J., Prowse, S. J., Simeonovic, C. J. and Warren, H. S.,** Immunobiology of tissue transplantation: a return to the passenger leukocyte concept. *Annu. Rev. Immunol.* 1983. **1:** 143–173.

10 **Janeway, C. A. Jr., Dianzani, U., Portoles, P., Rath, S., Reich, E. P., Rojo, J., Yagi, J. and Murphy, D. B.,** Cross-linking and conformational change in T-cell receptors: role in activation and in repertoire selection. *Cold Spring Harb. Symp. Quant. Biol.* 1989. **54:** 657–666.

11 **Schwartz, R. H.,** Acquisition of immunologic self-tolerance. *Cell* 1989. **57:** 1073–1081.

12 **Liu, Y. and Linsley, P. S.,** Costimulation of T-cell growth. *Curr. Opin. Immunol.* 1992. **4:** 265–270.

13 **Kuchroo, V. K., Das, M. P., Brown, J. A., Ranger, A. M., Zamvil, S. S., Sobel, R. A., Weiner, H. L., Nabavi, N. and Glimcher, L. H.,** B7-1 and B7-2 costimulatory molecules activate differentially the Th1/Th2 developmental pathways: application to autoimmune disease therapy. *Cell* 1995. **80:** 707–718.

14 **Lentschow, D. J., Herold, K. C., Rhee, L., Patel, B., Koons, A., Gin, H., Fuchs, E., Singh, B., Thompson, C. B. and Bluestone, J. A.,** CD28/B7 regulation of Th1 and Th2 subsets in the development of autoimmune diabetes. *Immunity* 1996. **5:** 285–293.

15 **Linsley, P. S., Brady, W., Grosmaire, L., Aruffo, A., Damle, N. K. and Ledbetter, J. A.,** Binding of the B cell activation antigen B7 to CD28 costimulates T cell proliferation and interleukin-2 mRNA accumulation. *J. Exp. Med.* 1991. **173:** 721–730.

16 **Lindsten, T., June, C. H., Ledbetter, J. A., Stella, G. and Thompson, C. B.,** Regulation of lymphokine messenger RNA stability by a surface-mediated T cell activation pathway. *Science* 1989. **244:** 339–343.

17 **Eynon, E. E. and Parker, D. C.,** Small B cells as antigen-presenting cells in the induction of tolerance to soluble protein antigens. *J. Exp. Med.* 1992. **175:** 131–138.

18 **Harding, F. A., McArthur, J. G., Gross, J. A., Raulet, D. H. and Allison, J. P.,** CD28-mediated signaling costimulates murine T-cells and prevents induction of anergy in T-cell clones. *Nature* 1992. **356:** 607–609.

19 **Truitt, K. E., Hicks, C. M. and Imbodden, J. B.,** Stimulation of CD28 triggers an association between CD28 and phosphatidylinositol 3-kinase in Jurkat T-cells. *J. Exp. Med.* 1994. **179:** 1071–1076.

20 **Kapeller, R. and Cantley, L. C.,** Phosphatidylinositol 3-kinase. *Bioessays* 1994. **15:** 565–576.

21 **June, C. H., Bluestone, J. A., Nadler, L. M. and Thompson, C. B.,** The B7 and CD28 receptor families. *Immunol. Today* 1994. **15:** 321–331.

22 **Edmead, C. E., Patel, Y. I., Wilson, A., Boulougouris, G., Hall, N. D., Ward, S. G. and Sansom, D. M.,** Induction of activator protein (AP)-1 and nuclear factor-kB by CD28 stimulation involves both phosphatidylinositol 3-kinase and acidic sphingomyelinase signals. *J. Immunol.* 1996. **157:** 3290–3297.

23 **Chan, G. and Ochi, A.,** Sphingomyelin-ceramide turnover in CD28 costimulatory signaling. *Eur. J. Immunol.* 1995. **25:** 1999–2004.

24 **Boucher, L. M., Wiegmann, K., Fütterer, A., Pfeffer, K., Machleidt, T., Schütze, S., Mak, T. W. and Krönke, M.,** CD28 signals through acidic sphingomyelinase. *J. Exp. Med.* 1995. **181:** 2059–2068.

25 **Hannun, Y. A.,** Functions of ceramide in coordinating cellular responses to stress. *Science* 1996. **274:** 1855–1859.

26 **Otterbach, B. and Stoffel, W.,** Acid sphingomyelinase-deficient mice mimic the neurovisceral form of human lysosomal storage disease (Niemann-Pick disease). *Cell* 1995. **81:** 1053–1061.

27 **Lewis, D. B., Prickett, K. S., Larsen, A., Grabstein, K., Weaver, M. and Wilson, C. B.,** Restricted production of interleukin 4 by activated human T cells. *Proc. Natl. Acad. Sci. USA* 1988. **85:** 9743–9747.

28 **Su, B., Jacinto, E., Hibi, M., Kallunki, T., Karin, M. and Ben-Neriah, Y.,** JNK is involved in signal integration during costimulation of T lymphocytes. *Cell* 1994. **77:** 727–736.

29 **Momchilova-Pankova, A. B., Markovska, T. T., Yanev, E. I. and Koumanov, K. S.,** Phospholipase C activities in rat liver plasma membranes depend on the phospholipid composition. *J. Lipid Mediat Cell Signal* 1994. **9:** 235–246.

30 **Shiao, Y. J. and Vance, J. E.,** Sphingomyelin transport to the cell surface occurs independently of protein secretion in rat hepatocytes. *J. Biol. Chem.* 1993. **268:** 26085–26092.

31 **Rosenwald, A. G., Machamer, C. E. and Pagano, R. E.,** Effects of sphingolipid synthesis inhibitor on membrane transport through the secretory pathway. *Biochemistry* 1992. **31:** 3581–3590.

32 **Linardic, C. M., Jayadev, S. and Hannun, Y. A.,** Brefeldin A promotes hydrolysis of sphingomyelin. *J. Biol. Chem.* 1992. **267:** 14909–14911.

33 **Zumbansen, M. and Stoffel, W.,** Tumor necrosis factor α activates NF-$\varkappa$B in acid sphingomyelinase-deficient mouse embryonic fibroblasts. *J. Biol. Chem.* 1997. **272:** 10904–10909.

34 **Sillence, D. J. and Allan, D.,** Evidence against an early signaling role for ceramide in Fas-mediated apoptosis. *Biochem. J.* 1997. **324:** 29–32.

35 **Vikinson, A., Pederson, K. and Muller, D.,** Enumeration of IFN-gamma producing lymphocytes by flow cytometry and correlation with quantitative measurement of IFN-gamma. *J. Immunol. Methods* 1994. **173:** 219–228.

36 **Assenmacher, M., Schmitz, J. and Radbruch, A.,** Flow cytometric determination of cytokines in activated murine T helper lymphocytes: expression of interleukin-10 in interferon-gamma and in interleukin-4-expressing cells. *Eur. J. Immunol.* 1994. **24:** 1097–1101.

37 **Nash, P. V. and Mastro, A. M.,** Activation of primary lymphocytes requires prolonged lectin stimulation. *J. Leukoc. Biol.* 1993. **53:** 73–78.

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