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Acid Sphingomyelinase Is Required for Protection of Effector Memory T Cells against Glucocorticoid-Induced Cell Death

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The activity of acid sphingomyelinase (aSMase) was previously reported to be involved in glucocorticoid-induced cell death (GICD) of T lymphocytes. This mechanism in turn is believed to contribute to the therapeutic efficacy of glucocorticoids (GCs) in the treatment of inflammatory diseases. In this study, we reassessed the role of aSMase in GICD by using aSMase knockout mice. The absence of aSMase largely abolished the partial protection that effector memory CD4+ T cells in wild-type mice possess against GICD. Reduced IL-2 secretion by aSMase-deficient CD4+ T cells suggested that a lack of this important survival factor might be the cause of these cells’ enhanced susceptibility to GICD. Indeed, addition of IL-2 restored the protection against GICD, whereas neutralization of IL-2 abrogated the otherwise protective effect seen in wild-type effector memory CD4+ T cells. The therapeutic implications of the altered sensitivity of aSMase-deficient T cells to GICD were assessed in models of inflammatory disorders; namely, experimental autoimmune encephalomyelitis and acute graft-versus-host disease. Surprisingly, GC treatment was equally efficient in both models in terms of ameliorating the diseases, regardless of the genotype of the T cells. Thus, our data reveal a hitherto unrecognized contribution of aSMase to the sensitivity of effector memory CD4+ T cells to GICD and call into question the traditionally attributed importance of GICD of T cells to the treatment of inflammatory diseases by GCs. The Journal of Immunology, 2011, 187: 000–000.

Glucocorticoids (GCs) are the most widely prescribed drugs worldwide and are used to treat a variety of inflammatory disorders including multiple sclerosis (MS) and acute graft-versus-host disease (aGvHD). They mainly act through the glucocorticoid receptor (GR), a ligand-activated transcription factor that binds to specific DNA elements in the promoter and enhancer regions of many genes or alternatively interacts with other transcription factors such as NF-κB or AP-1 (1). Leukocytes are highly responsive to GC treatment, which results in the modulation of cytokines, chemokines, and adhesion molecules. In addition, GCs also induce apoptosis of T and B cells and thereby contribute to the regulation and resolution of inflammatory responses (1).

Apoptosis can be induced by ligation of death receptors via the extrinsic pathway or in response to certain cellular and molecular stimuli via the intrinsic mitochondrial pathway (2). It is currently accepted that glucocorticoid-induced cell death (GICD) proceeds via this intrinsic pathway and involves members of the Bcl-2 family (3). Immature thymocytes are particularly sensitive to GICD, whereas mature thymocytes are largely resistant due to the upregulation of Bcl-xl (4). Similarly, activated T cells with a phenotype characteristic for effector memory cells are less sensitive to GICD than naive T cells, possibly due to survival signals after TCR engagement. The pathway by which GCs induce lymphocyte apoptosis has not yet been fully resolved, but the molecules involved seem to differ between thymocytes and mature T cells, with only the former cell type strictly depending on caspase activation (5). Experiments in vivo revealed that overexpression of GR increases the sensitivity of thymocytes and mature T cells to GICD, whereas abrogating GR dimerization takes away the same (6–8). It is widely assumed that GICD contributes to the beneficial effects of GC in the treatment of inflammatory diseases such as MS and aGvHD (1). Consistently, GC administration to rodents suffering from experimental autoimmune encephalomyelitis (EAE) as well as to MS patients increases the number of apoptotic T cells, the latter being accompanied by an amelioration of the clinical symptoms (9, 10).

Acid sphingomyelinase (aSMase) is a lysosomal enzyme that catalyzes the hydrolysis of membrane-resident sphingomyelin into ceramide and phosphorylcholine. The importance of aSMase is highlighted by the discovery that Niemann–Pick disease (NPD),
a severe neurologic disorder, is caused by mutations in the aSMase gene (11). In addition, aSMase has been implicated in the functioning of lipid rafts, which form essential signaling platforms. Consequently, aSMase deficiency disturbs signaling by membrane receptors such as CD28 and exocytosis of vesicles. Furthermore, aSMase is involved in the induction of apoptosis through the generation of ceramide. In view of the importance of aSMase in these different processes, it is comprehensible that aSMase knockout mice show immunological defects and a disturbed apoptotic induction (11). Among others, they are characterized by a defective clearance of Listeria monocytogenes (12), altered CD28 signaling (13), disturbed IL-2 secretion (14), and impaired cellular cytotoxicity (13). Moreover, aSMase is involved in UV-induced apoptosis of HeLa cells (15) and Fas-induced T cell apoptosis (16). Several reports also implicated aSMase in GICD. Pharmacological approaches revealed that GCs cause aSMase activation in thymocytes, resulting in ceramide production and consequently apoptosis induction (17, 18). Other experiments identified ceramide production and aSMase activity as events occurring during GICD but prior to caspase activation (19, 20). Hence, aSMase activity seems to be essential for GICD.

The role of aSMase in inflammatory diseases has not been addressed to date, with the exception of a recent report claiming that induction of aGvHD was impaired in aSMase-deficient recipient mice (21). Whereas this points to a potentially important role of aSMase in non-hematopoietic host cells, clinical observations did not support this notion, as NPD patients succumb normally to aGvHD (22). Alternatively, aSMase could play a role in the T cells that cause aGvH, as it is required for the release of cytotoxic effector molecules such as perforin (13), and as the use of perforin-deficient T cells resulted in a delayed onset of aGvH (23, 24). In view of the fact that in the clinic, aGvHD is widely treated with GCs, it is also conceivable that an altered sensitivity of T cells to GICD affects the efficacy of this treatment regimen. Thus, a hitherto unrecognized role of aSMase in the alloreactive T cells that mediate aGvH cannot be excluded.

In this study, we reassessed the role of aSMase in GICD based on results from experiments using aSMase knockout mice. Our results argue against a general requirement of aSMase in GICD as wild-type controls. However, we identified a specific role of aSMase in protecting effector memory T cells against GICD and provide evidence that this is linked to a supportive role of aSMase in IL-2 secretion. Unexpectedly, the efficacy of GC therapy was not affected by the lack of aSMase in two models of T cell-driven inflammatory disorders, suggesting that GICD of CD4+ T cells with an activated effector memory phenotype is not needed for the treatment of MS and aGvHD.

Materials and Methods

Mice

C57BL/6 and BALB/c wild-type mice as well as B6.SJL-PippcrPeplb/BoyJ mice (CD45.1 congenic C57BL/6J, originally obtained from The Jackson Laboratory, Bar Harbor, ME) were bred under specific pathogen-free (SPF) conditions in our animal facility in Göttingen. aSMase knockout mice that had been back-crossed to the C57BL/6 background (11) were bred under SPF conditions in our animal facility in Cologne. All animal experiments were conducted according to ethical standards of humane animal care and approved by the authorities of Lower Saxony and North Rhine-Westphalia, respectively.

Flow cytometry

Lymphocytes were isolated from thymus, spleen, or lymph nodes by passing the freshly isolated organs through a 40-μm nylon mesh, washed in FACS buffer (PBS with 0.5% BSA and 0.05% NaN3), and counted. All Abs and reagents were obtained from BD Biosciences (Heidelberg, Germany) or BioLegend (Uithoorn, The Netherlands) and directed against the following Abs (clone name in parentheses): CD3 (145-2C11 or 17A4), CD4 (RM4-5), CD8a (53-6.7), CD11a (2D7), CD44 (IM7). CD45.1 (A20), CD45.2 (104), βTCR (H57-597), B220 (RA3-6B2), active caspase-3 (C92-605), Bcl-2, and annexin V. The Abs and annexin V were directly labeled with FITC, PE, PerCP, PE-Cy7, Cy5, allophyocyanin, or allophycocyanin-Cy7. Staining was performed as previously described (25) and analyzed using a FACS Canto II device with the capacity for detecting six fluorescent dyes (BD Biosciences). Intracellular flow cytometry was performed according to the manufacturer’s instructions.

Cell culture experiments

Thymocytes or peripheral T cells from spleen or lymph nodes were cultured in RPMI 1640 medium (Invitrogen, Karlsruhe, Germany) with Glutamax, 10% FCS, and 1% standard antibiotics for 20 h in the presence of different concentrations of water-soluble dexamethasone (Dex) (Sigma, Taufkirchen, Germany). In some cases, recombinant human IL-2 (kindly donated by Th. Hüning, Würzburg, Germany) was supplemented at a concentration of 300 U/ml over the whole incubation time. For neutralization purposes, an anti-mouse IL-2 mAb (clone JES5-12H4; BioLegend) was added at a concentration of 10 μg/ml to the lymphocyte cultures, and Dex treatment was started with a delay of 6 h.

Lymphocytic choriomeningitis virus infection and IL-2 secretion assay

Mice were infected i.v. with 105 IU of the lymphocytic choriomeningitis virus (LCMV), strain WE. On day 8 postinfection, CD4+ T cells were magnetically enriched from splenic single-cell suspensions using MACS technology (Miltenyi Biotec, Bergisch Gladbach, Germany), according to the manufacturer’s instructions, and used as responder cells at a density of 1 × 10^6 cells/ml. To stimulate LCMV epitope-specific secretion of IL-2 by the CD4+ responder cells, splenic single-cell suspensions of naive mice were loaded as APCs with the peptide gpEpol-40-48, resembling an MHC class II-restricted epitope of the glycoprotein of LCMV, at a concentration of 10^-6 M. Responder cells and peptide-loaded spleen cells were coincubated at a ratio of 1:1 for 24 h before harvesting the cell-free supernatants. IL-2 was quantified by ELISA according to the instructions of the manufacturer (R&D Systems, Wiesbaden, Germany).

Adoptive transfer experiments

To generate bone marrow chimeraemic mice, CD45.1 congenic C57BL/6 mice (B6-SJL-PippcrPeplb/BoyJ) were placed in a customized Perspex box and received a total body dose of 11.5 Gy at the age of 8–12 wk. Radiation treatment was delivered at a rate of 1 Gy/min by an RS 225 X-Ray Research System (Gulmay Medical Systems, Camberley, Surrey, U.K.) operated at 200 kV, 15 mA, and with 0.5-mm Cu filtration. On the following day, 2 × 10^6 bone marrow cells were isolated aseptically from tibia and femur of 6- to 12-wk-old CD45.2 congenic mice (wild type or aSMase deficient) and engrafted by i.v. injection into the tail vein of irradiated recipient mice. Six weeks after transplantation, reconstitution efficacy was analyzed by flow cytometry via monitoring of the peripheral blood chimerism using the CD45.1/CD45.2 isogenic system. Chimeric mice were used for EAE induction 6 wk after irradiation.

EAE induction and treatment protocol

Chimeric mice were immunized with 50 μg MOG35-55 peptide in CFA and treated twice with pertussis toxin as previously described (25). Animals were weighed and scored daily for clinical signs of the disease on a scale from 0 to 10 depending on its severity. Scores were as follows: 0, normal; 1, reduced tone of tail; 2, limp tail, impaired righting; 3, absent righting; 4, gait ataxia; 5, mild paraparesis of hindlimbs; 6, moderate paraparesis; 7, severe paraparesis or paraplegia; 8, tetraparesis; 9, moribund; 10, death. To analyze the effects of GC therapy, dexamethasone-21-dihydrogen-phosphate solution (Ratiopharm, Ulm, Germany) was injected i.p. at a concentration of 100 mg/kg on 3 consecutive days. Infiltrating cells from the spinal cord of EAE mice were isolated as described (25).

T cell purification and bone marrow depletion for graft-versus-host disease induction

Bone marrow cells for aGvHD experiments were isolated aseptically from tibia and femur of C57BL/6 wild-type mice and then T cell-depleted using anti-CD90 (Thy1.2) microbeads in combination with the autoMACS system (both from Miltenyi Biotec). Purity of the obtained bone marrow cells was...
confirmed by staining against CD3ε and TCRβ with subsequent FACS analysis.

T cells were isolated from submandibular, axillary, and mesenterial lymph nodes as well as spleen of aSMase knockout mice or wild-type littermates. Single-cell suspensions were prepared by passing the cells through a 40-μm nylon mesh. T cells were purified using the Pan T Cell Isolation Kit II and the autoMACS system (both from Miltenyi Biotech); their purity was assessed via FACS analysis for TCRβ, B220, CD4, and CD8α and routinely >95%.

**aGvHD induction and treatment protocol**

Male BALB/c recipient mice, aged 8–10 wk, were housed in IVC cages under SPF conditions, and food and water were provided ad libitum. The drinking water was supplemented with 25 μg/ml neomycin from 1 d prior to irradiation at 28 d after transplantation to prevent infections. The recipient mice received a single total body dose of 8.5 Gy (see earlier) and were injected via the tail vein the following day with 1 × 10^7 T cell-depleted bone marrow cells without (control) or with 2 × 10^6 purified T cells. Mice were monitored daily for survival, and their health status was assessed according to five clinical parameters (hunched posture, decrease in activity, fur ruffling, diarrhea, and weight loss), each of which received a score from 0 to 2, resulting in a total score between 0 and 10 (26). Mice were sacrificed for ethical reasons if they reached a score of 7 or greater.

**Statistical analysis**

Statistical analysis was generally performed by unpaired t tests and the data depicted as mean ± SEM (*p < 0.05, **p < 0.01, ***p < 0.001; n.s., p > 0.05). To determine differences with respect to the EAE or aGvHD disease courses, the whole curves were compared between experimental groups by Mann–Whitney U test, either over the whole observation period or starting at the day after the first treatment. Statistical analysis of survival curves was achieved by log-rank test. GraphPad Prism software was used in all cases.

**Results**

**Induction of T cell apoptosis by GC does not require aSMase**

Previous studies based on pharmacological approaches indicated that aSMase plays a critical role in GICD via production of ceramide (17, 19). To reassess those findings without the methodological drawbacks of inhibitor studies, we used mice genetically deficient in aSMase. To analyze thymocyte apoptosis induced by Dex in vitro, total thymocytes were isolated from aSMase knockout mice and wild-type littermates and incubated with graded concentrations of Dex for 20 h. Cell survival was determined by flow cytometry using annexin V staining in combination with mAbs directed against CD3ε, CD4, and CD8α. Identical sigmoidal survival curves for aSMase-deficient and wild-type CD4+CD8α double-positive (DP) thymocytes were observed from 10^{-7} M to 10^{-3} M Dex (Fig. 1A). Essentially the same results were obtained for CD4+ single-positive (SP) thymocytes (Fig. 1B) and CD8+ SP thymocytes (data not shown) of both genotypes. Similarly, analysis of splenic T cells did not reveal any significant difference in the sensitivity to GICD between peripheral aSMase knockout and wild-type CD4+ (Fig. 1C) or CD8α (data not shown) T lymphocytes.

**FIGURE 1.** Involvement of aSMase in GICD of thymocytes and peripheral T cells. A–C. Thymocytes or splenocytes from wild-type and aSMase knockout mice were incubated with graded concentrations of Dex for 20 h followed by FACS analysis of CD3ε, CD4, CD8α, and annexin V binding. Cell type-specific survival was normalized to untreated control cultures, which were set to 100% (n = 6). D–F. Wild-type and aSMase knockout mice were injected with 100 mg/kg dexamethasone-21-dihydrogenphosphate or PBS as a control as described earlier. Infiltrating cells from the liver of aGvHD mice were isolated as described (27).
To assess GICD in vivo, aSMase knockout mice and wild-type littermates were injected with 100 mg/kg Dex, and 20 h later, thymocyte and splenic T cell numbers were determined. As reported previously (4), numbers of DP thymocytes in wild-type mice were strongly decreased by Dex treatment (Fig. 1D), whereas CD4^+ SP T cells (Fig. 1E) and CD8^+ SP T cell numbers (data not shown) were unaffected. No significant differences in the thymocyte numbers belonging to each subpopulation were detectable between aSMase knockout and control mice before or after Dex injection (Fig. 1D, 1E). The numbers of splenic CD4^+ and CD8^+ T cells (Fig. 1F and data not shown) were diminished after Dex application but showed no differences between both genotypes. These data reveal that aSMase activity is not needed for GICD of thymocytes and peripheral T cells in vitro and in vivo.

**Protection of effector memory CD4^+ T cells against GICD is abrogated in aSMase knockout mice**

CD4^+ T cells with an activated phenotype characteristic for effector memory cells show more resistance to GICD than naive T cells, although protection against apoptosis is not complete. It had been hypothesized that this difference is due to TCR-mediated survival signals that counteract GR signaling during apoptosis induction (28). Costaining of several cell surface molecules revealed that effector memory CD4^+ T cells (29) defined as LFA-1^high (30), CD44^+ (31), or CD62L^- (32) represent largely identical lymphocyte subpopulations (Supplemental Fig. 1A). To determine whether aSMase is involved in conferring resistance of effector memory CD4^+ T cells to GC action, splenic T cells were incubated in vitro with graded concentrations of Dex and analyzed for the induction of apoptosis by flow cytometry. Cleavage of caspase-3 is a hallmark of apoptosis and occurs after GC exposure of T cells (25). Therefore, we determined the relative survival of LFA-1^high CD4^+ T cells after 12 and 20 h in culture by intracellular staining for active caspase-3. Dex hardly influenced the number of live effector memory CD4^+ T cells in wild-type cultures but significantly diminished them when aSMase was absent (Fig. 2A and Supplemental Fig. 1B). This suggests that the resistance of effector memory CD4^+ T cells toward GICD is reduced in aSMase knockout mice.

Externalization of phosphatidyl serine residues that are recognized by annexin V and activation of caspase-3 are largely concordant during GICD, for which reason both markers can be similarly used to investigate CD4^+ T cell apoptosis. In the following experiments, we therefore analyzed annexin V binding again. To assess consequences of the increased susceptibility of effector memory CD4^+ T cells from aSMase knockout mice to GICD, we determined changes in their relative number after incubation with Dex. The frequency of annexin V-negative CD4^+ T cells with an effector memory phenotype increased at higher Dex concentrations (Fig. 2B and Supplemental Fig. 1C). Whereas LFA-1^high cells constitute ∼10% of all annexin V-negative CD4^+ T cells in untreated spleens, their relative numbers in wild-type cell cultures reached almost 40% at 10^-7 M Dex, due to their resistance against GICD (Fig. 2A). In contrast, this protective effect was largely abolished in aSMase-deficient cell cultures with only ∼15% of LFA-1^high annexin V-negative CD4^+ T cells even at the highest concentration of Dex (Fig. 2B). Similar results were obtained for the relative numbers of CD44^- or CD62L^- annexin V-negative CD4^+ T cells (Supplemental Fig. 1C).

To assess the relevance of these findings in vivo, aSMase knockout and wild-type control mice were injected with 100 mg/kg Dex i.p., and 20 h later, the numbers of LFA-1^high CD4^+ and CD44^- CD4^+ T cells were determined in their spleens. Similar to the in vitro data, the absolute number of effector memory CD4^+ T cells in wild-type mice was only marginally affected by Dex (Fig. 2C and Supplemental Fig. 2). By contrast, we found a significantly lower number of effector memory CD4^+ T cells in aSMase knockout mice after Dex treatment. Concomitantly, the relative number of effector memory CD4^+ T cells among all CD4^+ T cells in wild-type mice significantly increased after Dex treatment, whereas it remained unaltered in aSMase knockout mice (Fig. 2C and Supplemental Fig. 2). Taken together, these data...
suggested that aSMase is involved in protecting effector memory CD4+ T cells against GICD both in vitro and in vivo.

**Diminished IL-2 secretion by aSMase knockout T cells abrogates the protection of effector memory CD4+ T cells against GICD**

We and others have reported that the secretion of selected cytokines is impaired in the absence of aSMase (13, 14). To assess the involvement of aSMase in Ag-specific secretion of IL-2 by CD4+ T cells as a crucial factor for their proliferation and survival, we resorted to the vigorous Ag-specific T cell response of mice acutely infected with LCMV. At day 8 postinfection with LCMV (i.e., at the peak of T cell activity in this model), CD4+ splenocytes were restimulated ex vivo with the MHC class II-restricted LCMV peptide gp61–88, and 24 h later, the IL-2 levels were determined in the supernatants. Extending previous reports (13, 14), aSMase-deficient CD4+ T cells secreted less than half the amount of IL-2 compared with wild-type cells (Fig. 3A).

To assess whether the diminished IL-2 secretion enhanced the susceptibility of aSMase-deficient effector memory CD4+ T cells to GICD, we treated peripheral T cells from mice of both genotypes with 10−7 M Dex, either in the absence or the presence of supplementing IL-2. Exogenously added recombinant IL-2 restored protection of effector memory CD4+ T cells against GICD in aSMase knockout cultures so that in the latter, relative survival of LFA-1high CD4+ T cells was no longer reduced after Dex treatment but even slightly higher than that in wild-type cultures (Fig. 3B and Supplemental Fig. 3A). Concomitantly, addition of IL-2 to aSMase cultures also restored the diminished relative number of LFA-1high and CD44+ cells amongst the annexin V-negative CD4+ T cells after Dex treatment to almost wild-type levels (Fig. 3B and Supplemental Fig. 3A). Conversely, neutralizing IL-2 with a mAb in wild-type cultures treated with Dex resulted in a significantly reduced survival of LFA-1high CD4+ T cells and diminished the relative numbers of LFA-1high and CD44+ cells amongst the annexin V-negative CD4+ T cell subpopulation (Fig. 3C and Supplemental Fig. 3B). These data reveal that IL-2 is an important protective factor for effector memory CD4+ T cells against GICD and that aSMase deficiency leads to an enhanced susceptibility of effector memory CD4+ T cells to GICD due to the reduced secretion of IL-2.

To explore further the mechanism by which aSMase affects the susceptibility of effector memory CD4+ T cells to GICD, we analyzed the level of antiapoptotic proteins by flow cytometry (Fig. 3D). In the absence of aSMase, the level of Bcl-XL in LFA-1high CD4+ T cells was considerably lower compared with that of controls (specific mean fluorescence intensity: 3592 ± 318 in wild-type versus 2661 ± 113 in knockout cells, n = 3). In contrast, Bcl-2 expression was similar in both genotypes (data not shown). This suggests that reduced IL-2 secretion results in diminished Bcl-XL expression thus decreasing the resistance of aSMase-deficient LFA-1high CD4+ T cells to GICD.

**GC therapy of EAE is unaffected in aSMase knockout mice**

EAE is mediated by T cells that recognize myelin Ags, and T cells expressing high levels of LFA-1 are particularly important for the pathogenesis of EAE because they constitute >80% of the infiltrating CD4+ T cells in the spinal cord of disease-affected mice (Supplemental Fig. 4A). EAE can be treated by administration of GCs in a manner similar to human MS (25, 33), and genetic manipulation of GR levels in T cells affects the clinical response of EAE to GC treatment (8, 25). This raised the question whether the effect of aSMase deficiency on GICD has any impact on the efficacy of GC therapy.

Because aSMase knockout mice start to develop neurologic symptoms of NPD at ∼3 mo of age (11), we were worried that this may compromise proper assessment of clinical EAE symptoms. Therefore, we generated bone marrow chimeras by using aSMase knockout mice and wild-type littermates as donors. We immunized them with MOG35–55 peptide according to our standard protocol to induce EAE (25). Chimeric mice reconstituted with bone marrow of either genotype showed the typical disease course known for C57BL/6 mice and did not differ in the kinetics or severity of the disease (Fig. 4A).

**FIGURE 3.** IL-2 is involved in the protection of effector memory T cells against GICD. A, Wild-type and aSMase knockout mice were i.v. infected with LMCV. After 8 d, CD4+ splenocytes were isolated, stimulated with gp61–88 peptide, and 24 h later, IL-2 was measured in the supernatants. Extending previous reports (13, 14), aSMase-deficient CD4+ T cells secreted less than half the amount of IL-2 compared with wild-type cells (Fig. 3A).

B, Peripheral T cells from lymph nodes and spleens of wild-type and aSMase knockout mice were incubated without (“con”) or with 10−7 M Dex for 20 h followed by FACS analysis. Parallel cultures of aSMase knockout cells were treated with 300 U/ml recombinant human IL-2. The relative survival of LFA-1high CD4+ T cells was determined based on the absence of annexin V binding and normalized to control cultures (n = 10, left panel); the relative number of LFA-1high cells amongst the annexin V-negative CD4+ T cells is depicted in the right panel. C, Peripheral T cells from lymph nodes and spleen of wild-type mice were incubated with or without 10 μg/ml anti-mouse IL-2 mAb for 6 h and treated without (“con”) or with 10−7 M Dex for another 14 h. The relative survival of LFA-1high CD4+ T cells was determined based on the absence of annexin V binding and normalized to control cultures (n = 4, left panel); the relative number of LFA-1high cells amongst the annexin V-negative CD4+ T cells is depicted in the right panel. D, LFA-1high CD4+ T cells were analyzed for the expression of Bcl-XL by intracellular flow cytometry. Isotype controls confirmed the specificity of the stainings (data not shown) and were used as a basis to calculate the specific mean fluorescence intensity mentioned in the text. One representative experiment of three is shown. *p < 0.05, **p < 0.01, ***p < 0.001, n.s., p > 0.05. ko, aSMase knockout; wt, wild-type.
sensitivity of aSMase knockout CD4+ T cells with an effector memory phenotype to GICD had any effect on the treatment of EAE with GCs in vivo. Three consecutive injections of 100 mg/kg dexamethasone-21-dihydrogenphosphate or PBS as a control for 3 d after reaching an average disease score of 3 (indicated by arrows).

Discussion

It was previously reported that aSMase activity is involved in the induction of apoptosis in T cells by different stimuli including GCs (17). In addition, GICD was hypothesized to contribute at least in part to the efficacy of GCs in the treatment of various inflammatory diseases including MS and aGvHD (1, 34). It is against this background that we decided to explore the role of disease under the conditions used in our laboratory, but regardless of this, there was no difference in mortality in the early phase of the disease between mice receiving aSMase-deficient T cells and those receiving wild-type T cells (Fig. 5A).

Because the deficiency of aSMase in the adoptively transferred allogeneic T cells had no effect on the course of aGvHD, we could assess the role of aSMase in GC therapy using the same experimental system. We treated recipients of wild-type or aSMase-deficient T cells with 100 mg/kg dexamethasone-21-dihydrogenphosphate from days 3 to 6. The reduction of morbidity in the early phase achieved by GC administration was comparable in both groups but did not impact overall survival in either group (Fig. 5A, 5B). Similar results were obtained when whole splenocyte preparations were used to induce aGvHD (5 × 10^6 T cell-depleted bone marrow cells and 4 × 10^6 splenocytes; data not shown). We conclude that increased GICD of CD4+ T cells with an effector memory phenotype due to the lack of aSMase does not significantly impact the efficacy of GCs in the treatment of aGvHD.

FIGURE 5. Disease course of aGvHD, overall survival and response to GC therapy. Wild-type BALB/c mice were lethally irradiated and received 1 × 10^7 T cell-depleted C57BL/6 wild-type bone marrow cells together with 2 × 10^6 T cells from either C57BL/6 (wild-type) or aSMase knockout mice to induce aGvHD. Some mice received only bone marrow cells but no T cells (“BM only”). One group of recipient mice each was treated from days 3 to 6 with 100 mg/kg dexamethasone-21-dihydrogenphosphate i.p. or PBS as a control (indicated by arrows). A, Kaplan–Meier survival analysis to determine mortality due to aGvHD induction (n = 7–10). B, Mean clinical scores of the same mice as depicted in A were obtained by daily monitoring during the first 9 d after disease induction; afterward, the health status was assessed twice a week. *p < 0.05, n.s., p > 0.05. ko, aSMase knockout; wt, wild-type.
aSMase in GICD and its impact on T cell-dependent inflammatory diseases.

Current models suggest a requirement for aSMase in GICD via production of ceramide (17–20), yet we did not find any general difference in cell survival after Dexam treatment, either in vitro or in vivo. This applies both to DP and SP thymocytes and mature CD4⁺ T cells in peripheral lymphoid organs and argues against the notion that aSMase is essential for GICD. However, we found that the sensitivity of effector memory CD4⁺ T cells to GC, either defined as LFA-1high, CD44⁺, or CD62Llow, was enhanced in the absence of aSMase. It is cells of this phenotype that are crucial for the pathogenesis of many inflammatory diseases (e.g., they encompass the majority of CD4⁺ T cells found in the spinal cord of EAE mice and in the organs of mice with aGvHD). This observation was unexpected because wild-type CD4⁺ T cells with an effector memory phenotype are partially protected against GICD and therefore become enriched after Dexam treatment. Why does lack of aSMase increase the sensitivity of these cells to apoptosis? One possible explanation could be that aSMase-deficient CD4⁺ T cells are impaired in their capacity to secrete cytokines due to the defective fusion of secretory vesicles with the plasma membrane (13, 14). Indeed, we could show that Ag-specific aSMase-deficient CD4⁺ T cells secrete diminished amounts of IL-2 and that supplementing IL-2 rescued them from GICD. Our finding that effector memory CD4⁺ T cells from aSMase knockout mice express lower levels of the antiapoptotic protein Bcl-Xl provides a mechanistic explanation for their increased susceptibility to GICD and is in line with reports showing that IL-2 promotes T cell survival by upregulating Bcl-X₁ (35–37). The fact that CD4⁺ T cells with an effector memory phenotype are partially spared from apoptosis induction by GCs could have important physiological implications. It is tempting to speculate that GICD curtails combat infection.

In view of the presumed role of GICD in suppressing inflammation and the reduced resistance of T cells with an effector memory phenotype against it, we expected GC therapy of T cell-driven immune responses to be altered in aSMase knockout mice. However, this was not the case in two different disease models. The course and severity of EAE were identical both in wild-type and aSMase knockout bone marrow chimeric mice, and, surprisingly, the therapeutic efficacy of GCs is not influenced by aSMase deficiency, or GICD is not essential for GC therapy of EAE at all.

Our results obtained in a murine model of aGvHD further support the notion that GICD of CD4⁺ T cells with an effector memory phenotype is not crucial for the efficacy of GC therapy of inflammatory disorders. In the case of aGvHD, the massive activation and expansion of alloreactive T cells may result in sufficient levels of IL-2 to protect the T cells from GICD, even when being aSMase deficient. Alternatively, the sensitivity of CD4⁺ T cells with an effector memory phenotype to GICD may be irrelevant for the treatment of aGvHD by GC because these drugs can also act by downregulating alternative T cell activities, by inhibiting T cell migration (40), or by modulating other cell types and tissues.

Taken together, our findings refute the previous notion that aSMase is essential for GICD of T lymphocytes but support the view that aSMase contributes to the protection of effector memory CD4⁺ T cells against GICD by facilitating IL-2 secretion. Additionally, our observation that the capacity of GC to ameliorate EAE and aGvHD does not depend on aSMase activity adds to the growing body of evidence that GICD of effector T cells is largely irrelevant for the efficacy of this widespread therapeutic regimen for inflammatory disorders (10, 40).

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Disclosures

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