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Delayed Contraction of the CD8⁺ T Cell Response toward Lymphocytic Choriomeningitis Virus Infection in Mice Lacking Serglycin

Mirjana Grujic,* Jan P. Christensen,* Maria R. Sørensen,* Magnus Abrink,† Gunnar Pejler,‡ and Allan R. Thomsen²*°

We previously reported that the lack of serglycin proteoglycan affects secretory granule morphology and granzyme B (GrB) storage in in vitro generated CTLs. In this study, the role of serglycin during viral infection was studied by infecting wild-type (wt) mice and serglycin-deficient (SG−/−) mice with lymphocytic choriomeningitis virus (LCMV). Wt and SG−/− mice cleared 10⁷ PFU of highly invasive LCMV with the same kinetics, and the CD8⁺ T lymphocytes from wt and SG−/− animals did not differ in GrB, perforin, IFN-γ, or TNF-α content. However, when a less invasive LCMV strain was used, SG−/− GrB⁺ CD8⁺ T cells contained ~30% less GrB than wt GrB⁺ CD8⁺ T cells. Interestingly, the contraction of the antiviral CD8⁺ T cell response to highly invasive LCMV was markedly delayed in SG−/− mice, and a delayed contraction of the virus-specific CD8⁺ T cell response was also seen after infection with vesicular stomatitis virus. BrdU labeling of cells in vivo revealed that the delayed contraction was associated with sustained proliferation of Ag-specific CD8⁺ T cells in SG−/− mice. Moreover, wt LCMV-specific CD8⁺ T cells from TCR318 transgenic mice expanded much more extensively in virus-infected SG−/− mice than in matched wt mice, indicating that the delayed contraction represents a T cell extrinsic phenomenon. In summary, the present report points to a novel, previously unrecognized role for serglycin proteoglycan in regulating the kinetics of antiviral CD8⁺ T cell responses. The Journal of Immunology, 2008, 181: 1043–1051.

Natural killer cells and CD8⁺ CTLs are major effectors in the battle against virus-infected and transformed cells. Through the TCR, CTL precursors recognize and bind a complex formed of antigenic peptide and MHC class I molecules on, e.g., virus-infected cells. Upon several such interactions, the CTLs become activated, resulting in redistribution of signaling and adhesion molecules at the contact area, followed by formation of additional contacts (1–3). As a consequence the primed cells will undergo substantial clonal expansion and, given the right additional signals (4), eventually differentiate into effector cells. Following the generation of effector CD8⁺ T cells, renewed triggering through the TCR complex induces the mobilization and exocytosis of preformed cytotoxic granules, and as a result, the APC, e.g., a virus-infected target cell, undergoes apoptotic cell death (reviewed in Refs. 5, 6). The major components of the cytotoxic granules are lytic molecules such as granzymes and perforin. Among various granzymes, granzyme B (GrB)¹ and granzyme A (GrA) are the most abundant (5, 7, 8). Additional components of the cytotoxic granules include chemokines (9), various lysosomal proteases and lysosomal membrane proteins (10, 11), and finally, proteoglycans (12, 13), i.e., high molecular weight molecules composed of a protein core in which one or more sulfated and thereby negatively charged glycosaminoglycan chains are attached (reviewed in Refs. 14, 15). Out of the various proteoglycan species, serglycin proteoglycan appears to be the dominant proteoglycan found within cytoplasmic granules of hematopoietic cells (16–19), and several studies have suggested that serglycin with chondroitin sulfate side chains is the main proteoglycan of the cytotoxic granules in CTLs and NK cells. There, the negatively charged serglycin proteoglycan can have the potential to form macromolecular complexes with positively charged molecules, such as GrB, GrA, perforin (20–23), and chemokines (9). Indeed, by analyzing Con A-induced CTLs derived from splenocytes of wild-type (wt) mice and serglycin-deficient (SG−/−) mice, we previously showed that serglycin proteoglycan is the dominating proteoglycan of CTLs, and that the lack of serglycin affected the ultrastructure of the secretory granule in the Con A-induced CTLs. Furthermore, we showed that the storage of GrB was severely affected in SG−/− Con A-induced CTLs, whereas, in contrast, the storage of GrA, perforin, and Fas ligand was not dependent on serglycin (24).

In light of the demonstrated role for serglycin in regulating secretory granule homeostasis in CTLs, we investigated in this study the possibility that serglycin might play a role in the control of viral infections in vivo. As our primary model, we used infection with lymphocytic choriomeningitis virus (LCMV), a noncytolytic arenavirus, which is a natural mouse pathogen that spreads to high titers in most organs of mice. Importantly, studies have shown that

¹University of Copenhagen, Institute of International Health, Immunology and Microbiology, Copenhagen, Denmark; ²Department of Medical Biochemistry and Microbiology, Uppsala University, and Department of Anatomy, Physiology and Biochemistry, Swedish University of Agricultural Sciences, Uppsala, Sweden

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2 Address correspondence and reprint requests to Dr. Allan R. Thomsen, Institute of International Health, Immunology and Microbiology, The Panum Institute Building 22.5, University of Copenhagen, 3C Blegdamsvej, DK-2200 Copenhagen N, Denmark. E-mail address: A.R.Thomsen@immi.ku.dk

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LCMV infection induces a potent CD8+ T cell response, which is solely responsible for the initial control of this virus infection (25, 26). We show that SG-/- mice develop a normal CD8+ T cell response, both with regard to the number of virus-specific CD8+ T cells and to their content of relevant effector molecules. Moreover, SG-/- mice eliminated the virus-infected cells as efficiently as matched wt mice. However, the subsequent contraction of the virus-specific CD8+ T cell population was markedly delayed in SG-/- mice, and adoptive transfer experiments using TCR transgenic cells point to a critical serglycin-dependent modification of the external environment regulating the T cell response.

Materials and Methods

Mice

C57BL/6 (B6) wt mice were obtained from Taconic M&B Farms. SG-/- mice, backcrossed to the B6 background, were bred locally from breeder pairs originally produced at the Uppsala University Transgenic Facility (Uppsala, Sweden) (27). Transgenic mice (Tcrl381) expressing a TCR for LCMV gp33-41 on ~60% of their CD8+ T cells were originally provided by H. Pircher and R. M. Zinkernagel (University of Zurich, Zurich, Switzerland), and the transgene has recently been transferred onto a B6.SJL (B6.SJL-Pipp/c®/Boa/Atac, CD45.1+; Taconic Farms) background. Seven- to 10-wk-old mice were used in all experiments, and animals from outside sources were always allowed to acclimatize to the local environment for at least 1 wk before use. All animals were housed under specific pathogen-free conditions as validated by screening of sentinel. All animal experiments were conducted according to national guidelines.

Virus

LCMV of the Armstrong strain clone 13 was used in most experiments. Unless otherwise stated, mice to be infected received a dose of 105 PFU of clone 13 in an i.v. injection of 0.5 ml. A few experiments were performed with Armstrong strain clone 53b, in which case the mice received an i.p. dose of 2 x 105 PFU/0.4 ml. Seeding stocks of both LCMV strains were provided by M. B. A. Oldstone (Scripps Clinic and Research Foundation, La Jolla, CA) (28). For infection with vesicular stomatitis virus (VSV) of the Indiana strain (29), mice were inoculated i.v. with 106 PFU in a volume of 0.3 ml.

In vivo BrdU labeling

Mice were given BrdU (Sigma-Aldrich) at 0.8 mg/ml in their drinking water the last 3 days before analysis (30, 31). BrdU-containing water was protected from light and changed daily.

Virus titration

Organ virus titers were assayed by an immune focus assay as previously described (32).

Quantitative PCRs for non-serglycin proteoglycan species

By use of the RNeasy midi kit (Qiagen), total RNA was extracted from splenic CD8+ T cells enriched by negative selection of MHC class II-, Ig-, or Fc receptor-positive cells by magnetic beads. mRNA was reverse transcribed to cDNA using RevertAid First strand cDNA synthesis kit (MBI Fermentas). Brilliant SYBR Green quantitative PCR Mastermix (Stratagene) was used as a control for nonspecific staining.

Cell preparations and adoptive transfer experiments

For flow cytometric analysis, spleens were aseptically removed and transferred to HBSS. Single cell suspensions were obtained by pressing the organs through a fine sterile steel mesh. The cells were washed twice with HBSS, and cell concentrations were adjusted in RPMI 1640 containing 10% FCS, supplemented with 2-ME, t-glutamine, and penicillin-streptomycin. In the case of adoptive transfer, single cell suspensions of the splenocytes from TCR318-transgenic (CD45.1+) mice were washed twice with HBSS and filtered through a 70-µm nylon cell strainer from BD Biosciences. The cell concentration was adjusted in HBSS and 1 x 10^6 cells in a volume of 0.3 ml were adoptively transferred into CD45.2+ recipients.

Monoclonal Ab for flow cytometry

The following mAbs were all purchased from BD Pharmingen as rat anti-mouse Abs: FITC-, PerCP Cy5.5-, or CyChrome-conjugated anti-CD8a; CyChrome-conjugated anti-CD4; CyChrome-conjugated anti-B220; PE-conjugated anti-GrB; PE-conjugated anti-TNF-α; PE-conjugated anti-IL-2; PE-conjugated anti-Va2; PE- or FITC-conjugated anti-perforin; PE- allophycocyanin-, or FITC-conjugated anti-IFN-γ; PE- or FITC-conjugated anti-CD44; PE-conjugated anti-CD62L; PE-conjugated anti-CD43 (clone 1B11); FITC-conjugated anti-NK1.1; FITC-conjugated anti-CD45.1; FITC-conjugated anti-BrDU; and matched isotype controls. The Bcl-2 staining kit and the annexin V apoptosis detection kit were also from BD Pharmingen. PE-conjugated anti-CD127 and FITC-conjugated anti-CD27 were from eBioscience.

Flow cytometric analysis

For visualization of adoptively transferred, virus-specific donor (CD45.1+, CD8+, Vα2+) cells, 1 x 10^6 splenocytes were stained with mAbs in FACS medium (PBS containing 10% rat serum, 1% BSA, and 0.1% NaN3) for 20 min in the dark and at 4°C. The cells were washed twice, and fixed with 1% paraformaldehyde in PBS.

GrB- and perforin-positive CTLs or NK cells were determined after direct intracellular staining of splenocytes, without prior peptide stimulation. A total of 2 x 10^6 splenocytes were surface stained, washed, fixed, permeabilized, and subsequently stained with GrB or perforin-specific mAbs as previously described (30). Splenocytes from noninfected mice served to provide a cut-off for GrB- and perforin-positive CTLs or NK cells.

For visualization of virus-specific (IFN-γ- and TNF-α-producing) CD8+ T cells, 2 x 10^6 splenocytes were resuspended in 0.2 ml of complete RPMI 1640 medium supplemented with 10 U of murine recombinant IL-2 (R&D Systems), 3 µM monensin (Sigma-Aldrich), and 1 µg/ml relevant peptide and incubated for 5 h at 37°C. The peptides used for LCMV-specific CD8+ T cells were gp33-41, gp276-286, and NP396-404 and for VSV-specific CD8+ T cells NP52-59. After incubation, cells were surface stained, washed, fixed, permeabilized, and stained with IFN-γ or (IFN-γ and TNF-α) specific mAbs as previously described (30). Isotype-matched Ab served as control for nonspecific staining.

When studying virus-specific BrdU+ and BrdU− (IFN-γ-producing) CD8+ T cells, splenocytes were subjected to peptide stimulation and surface staining as described. Afterward, the cells were washed and processed using a BrdU Flow kit (BD Biosciences) according to the manufacturer’s instructions.

Cells were analyzed using a FACScalibur (BD Biosciences), and at least 10^4 cells were gated using a combination of low angle and side scatter to exclude dead cells and debris. Data analysis was conducted using CellQuest software.

In vivo cytotoxicity assay

Splenocytes from naive B6 and B6.SJL mice were incubated with LCMV-specific peptides (gp33-41 and NP396-404) or irrelevant peptide for control (1 µg/10^6 cells/ml). Following incubation for 30 min at 37°C, the cells were washed and labeled with CFSE at 2 or 0.2 µM. Following another washing step, the labeled cells were mixed, and an equal number of each population (10^5 cells) was i.v. injected; 2 h later lymphocytes from the recipient spleens were isolated. Target cells were distinguished by expression of CD45.1 or the intensity of CFSE staining. The percentage of killing was calculated with the following equation: 100 – (percentage of LCMV peptide-labeled cells in infected/percentage of cells labeled with irrelevant peptide in infected)/(percentage of LCMV peptide-labeled cells in uninfected/percentage of cells labeled with irrelevant peptide in uninfected) x 100).

Clinical disease

Weight loss and mortality were used to evaluate the clinical severity of the LCMV infection. Mice were monitored daily for a period of 12 days after i.v. inoculation.

Statistical evaluation

Results were compared using the Mann-Whitney U test. A value of p < 0.05 was considered as evidence of statistical significance.
Results
SG<sup>−/−</sup> mice control acute LCMV infection as efficiently as wt mice
Initially we examined whether SG<sup>−/−</sup> mice can control an acute type of infection caused by a moderate dose (10<sup>6</sup> PFU administered i.v.) of highly invasive LCMV Armstrong strain clone 13. We determined the virus load in lymphoid (spleen) and nonlymphoid (lungs) organs on days 5, 8, and 10 postinfection (p.i.) (Fig. 1). Before the onset of an efficient T cell response (i.e., on day 5 p.i.) (25), similar virus loads were found in both lymphoid and nonlymphoid organs of SG<sup>−/−</sup> and wt mice. With the appearance of virus-specific CTLs (see below) the level of infection decreases and, notably, the rate of virus elimination was similar in wt and SG<sup>−/−</sup> mice (Fig. 1).

Similar capacity for in vivo cytotoxicity in SG<sup>−/−</sup> and wt mice
Because CTL-mediated killing of virus-infected cells is known to play a key role in the control of acute LCMV infection, we next evaluated the in vivo killing of fluorochrome-labeled target cells pulsed with peptides representing known LCMV-derived immunodominant epitopes (gp33–41 and NP396–404) (Fig. 2). When the target cells were transferred 8 days after infection, all relevantly labeled target cells were eliminated from the spleen in under 2 h, and this rapid kinetics of killing was seen irrespective of the mouse strain. At 30 days p.i., the capacity for in vivo killing was slightly reduced in both mouse strains as compared with 8 days p.i., but still no difference was found between SG<sup>−/−</sup> mice and similarly infected wt.

Characterization of virus-induced CTLs with regard to expression of lytic and nonlytic effector molecules
The result just described indicates that SG<sup>−/−</sup> mice develop a normal CD8<sup>+</sup> T cell response, and that the virus-specific CD8<sup>+</sup> T

![FIGURE 1.](image1) Spleen and lungs virus titers of LCMV clone 13 infected mice. Wt and SG<sup>−/−</sup> mice were infected with 10<sup>6</sup> PFU of LCMV Armstrong clone 13 i.v. On the indicated days p.i., mice were sacrificed, and spleens and lungs were recovered for titration of organ virus load. Data represent titers of individual mice, either wt (△) or SG<sup>−/−</sup> (□) mice.

![FIGURE 2.](image2) Similar capacity for in vivo killing in LCMV clone 13 infected SG<sup>−/−</sup> and wt mice. Peptide pulsed (gp33–41, NP396–404, or irrelevant) target cells were transferred into wt and SG<sup>−/−</sup> mice infected with 10<sup>6</sup> PFU of LCMV Armstrong clone 13 i.v. 8 days or 1 mo earlier. Spleens were harvested 2 h later, and target cell elimination was calculated; cells pulsed with irrelevant peptide served as reference for background killing. Results are presented as mean ± SE (n = 3–4 mice/group).

![FIGURE 3.](image3) GrB and perforin content of CD8<sup>+</sup> T cells upon infection with different clones of LCMV Armstrong strain. Wt and SG<sup>−/−</sup> mice were infected with either 10<sup>6</sup> PFU of LCMV clone 13 13 i.v. or 2 × 10<sup>6</sup> PFU of LCMV clone 53b i.p. On the indicated days p.i., mice were sacrificed, and splenocytes were stained for the cell surface markers CD8 and CD44, permeabilized and stained for intracellular GrB or perforin, followed by flow cytometry analysis. a, Representative dot plots of splenic GrB<sup>+</sup> CD8<sup>+</sup> T cells, obtained from LCMV clone 13 infected wt or SG<sup>−/−</sup> mice on day 8 p.i. b, The number of GrB<sup>+</sup> CD8<sup>+</sup> T cells, presented as mean ± SE (n = 5 mice/group), from wt and SG<sup>−/−</sup> mice. c, MFI of staining for GrB, as measured for GrB<sup>+</sup> CD8<sup>+</sup> T cells from either LCMV clone 13 or LCMV clone 53b infected wt and SG<sup>−/−</sup> mice, determined on day 8 p.i., p < 0.01 relative to wt mice determined by Mann-Whitney rank-sum test. d, Representative dot plots of splenic perforin-positive CD8<sup>+</sup> T cells, obtained from LCMV clone 13 infected wt or SG<sup>−/−</sup> mice on day 6 p.i. Numbers shown in the upper right corners of the panels in a and d represent the percentages of granzyme B<sup>+</sup> (a) and perforin<sup>+</sup> (d) CD8<sup>+</sup> T cells, respectively.
FIGURE 4. Lower rate of LCMV-specific CD8\(^+\) T cell contraction in SG\(^{-/-}\) than in wt mice. Wt and SG\(^{-/-}\) mice were infected i.v. with 10\(^7\) PFU of LCMV clone 13. Splenocytes, isolated at the indicated time points p.i., were stimulated for 5 h with peptides representing known LCMV-derived epitopes (gp33–41: gP276–286, and NP396–404), surface stained for CD8, and after permeabilization stained for intracellular IFN-\(\gamma\) and TNF-\(\alpha\). a. Representative dot plots of splenic gp33–41-specific IFN-\(\gamma\)- or TNF-\(\alpha\)-activated CD8\(^+\) T cells, obtained from LCMV clone 13 infected wt or SG\(^{-/-}\) mice on day 10 p.i. Numbers in the upper right corners of the panels represent the percentages of cytokine CD8\(^+\) T cells. b. The number of epitope-specific IFN-\(\gamma\)- or TNF-\(\alpha\)-activated CD8\(^+\) T cells. Data are mean \(\pm\) SE (\(n = 5\) mice/group) from wt or SG\(^{-/-}\) mice. *\(, p < 0.05\) relative to wt mice determined by Mann-Whitney rank-sum test.

cells in SG\(^{-/-}\) mice might not differ in antiviral activity from their wt counterparts. CD8\(^+\) T cells express many antiviral effector molecules, which can generally be sorted into two major groups: cytotoxic (granzymes and perforin) and noncytotoxic (cytokines and chemokines) molecules. Therefore, to further evaluate the LCMV-specific CD8\(^+\) T cell response, splenocytes isolated on different days after LCMV clone 13 infection were stained for cell surface markers CD8 and CD44, permeabilized, stained for intracellular GrB or perforin, and subsequently analyzed by flow cytometry.

The LCMV clone 13 infection induced a marked expansion of the CD8\(^+\) T cell population (>16-fold increase) in both wt and SG\(^{-/-}\) mice, peaking at day 10 p.i. Furthermore, most of the CD8\(^+\) T cells had an activated phenotype, i.e., expressing high levels of CD44. The frequency of GrB\(^+\) CD8\(^+\) T cells peaked already on day 8 p.i. (Fig. 3b), with \(~80\%\) of the splenic CD8\(^+\) T cells being positive for GrB at this time. At later time points, fewer of the activated CD8\(^+\) T cells expressed GrB, thus on day 10 p.i. only \(~40\%\) of splenic CD8\(^+\) T cells retained the expression of GrB (data not shown). However, the total number of GrB\(^+\) CD8\(^+\) T cells remained constant due to a further increase in the total number of splenic CD8\(^+\) T cells. Notably, SG\(^{-/-}\) mice did not differ from wt mice either in the number of GrB\(^+\) CD8\(^+\) T cells (Fig. 3b), nor in the content of GrB on a per cell basis (as evaluated by mean fluorescence intensity (MFI) of GrB\(^+\) CD8\(^+\) T cells) (Fig. 3, a and c). However, when we used another clone of LCMV Armstrong, the noninvasive clone 53b, SG\(^{-/-}\) GrB\(^+\) CD8\(^+\) T cells contained \(~30\%\) less GrB than matching wt GrB\(^+\) CD8\(^+\) T cells (Fig. 3c).

We also infected wt and SG\(^{-/-}\) mice with VSV, a non-natural mouse pathogen that replicates relatively inefficiently in mice and therefore induces a weaker CD8\(^+\) T cell response (33). When CD8\(^+\) T cells were analyzed at the peak of VSV infection (day 6 p.i.) (33), it was found that VSV-activated SG\(^{-/-}\) CD8\(^+\) T cells were not compromised in the storage of GrB (data not shown). Together, these results suggest that the storage of GrB may be partially dependent on serglycin, and that the extent of serglycin dependency might vary with the type of viral infection. Further characterization of the activated CD8\(^+\) T cells derived from virus-infected mice revealed that the lack of serglycin had no effect on the intracellular content of perforin (Fig. 3d).

To see whether a compensatory increase in other proteoglycan species could explain the normal or near-normal expression of GrB
in \(SG^{-/-}\) CD8\(^{+}\) T cells, splenic CD8\(^{+}\) T cells from both infected and uninfected mice of both mouse strains were enriched by negative selection of MHC class II-, Ig-, or Fc receptor-positive cells, and mRNA expression of glypican-1, syndecan-1, and glypican-4 was evaluated using real-time PCR. Compared with the expression of RANTES, which is a molecule that is clearly up-regulated in activated T cells, we detected hardly any change in the expression of proteoglycan species as a result of T cell activation, and most importantly, no marked difference between cells from SG \(^-/-\) and wt mice was revealed (data not shown).

Next we examined the generation of cytokine IFN-\(\gamma\)-producing and TNF-\(\alpha\)-producing CD8\(^{+}\) T cells in SG \(^-/-\) mice. A previous report shows that IFN-\(\gamma\) is important for the control of LCMV infection, in particular when using highly invasive virus strains (25). Splenocytes, isolated at different days after infection with LCMV clone 13, were stimulated ex vivo for 5 h with peptides representing known LCMV-derived epitopes (immunodominant gp33–41 and NP\(_{396-404}\) and subdominant gp276–286). Following incubation with peptide, the cells were stained for cell surface CD8, permeabilized, and stained for intracellular IFN-\(\gamma\) and TNF-\(\alpha\). As shown in Fig. 4a, flow cytometric analysis revealed that LCMV-specific CD8\(^{+}\) T cells from wt and SG \(^-/-\) mice do not differ in the content of either of these cytokines. The depicted results are representative of gp33–41-specific cells, but similar results were obtained with regard to the other two epitopes (data not shown).

**Prolonged proliferation and delayed contraction of the antiviral CD8 T cell population**

The results mentioned show that serglycin influences neither the rate of virus clearance, nor the cellular content of a number of CD8\(^{+}\) T cell effector molecules. We next investigated the possibility that serglycin might influence the kinetics of the CD8\(^{+}\) T cell response toward viral infection. To this end, splenocytes were isolated at various time points p.i., stimulated with gp33–41 and NP\(_{396-404}\) followed by quantification of the number of gp33–41\(^{+}\) and NP\(_{396-404}\)-specific CD8\(^{+}\) T cells positive for IFN-\(\gamma\) or TNF-\(\alpha\). Strikingly, these experiments revealed a marked delay in the contraction of the virus-specific CD8\(^{+}\) T cell response in mice lacking serglycin (Fig. 4b). Specifically, the number of IFN-\(\gamma\)- and TNF-\(\alpha\)-producing CD8\(^{+}\) T cells were similar in wt and SG \(^-/-\) mice at early time points p.i. (up to days 8–10), but at later time points (from day 10–12), the number of IFN-\(\gamma\)- and TNF-\(\alpha\)-producing CD8\(^{+}\) T cells was considerably higher (up to ~3-fold) in SG \(^-/-\) than in wt animals (Fig. 4b).

Up to 95% of effector CD8\(^{+}\) T cells undergo apoptosis during the contraction phase and only a small fraction of cells survive and subsequently differentiate into memory CD8\(^{+}\) T cells (34). Thus, the delayed contraction of the LCMV-specific CD8\(^{+}\) T cell pool in SG \(^-/-\) mice might be explained by reduced apoptosis, sustained proliferation of the cells, or both processes. We addressed these possibilities through in vivo labeling of dividing CD8\(^{+}\) T cells with BrdU. BrdU was given in the drinking water for 3 days before analysis of splenocytes. On day 12 p.i., the splenocytes were stimulated for 5 h with the appropriate peptides representing viral epitopes (NP\(_{396-404}\) for LCMV epitope; NP\(_{52-59}\) for VSV epitope), surface stained for CD8, permeabilized, and stained for intracellular IFN-\(\gamma\) and incorporated BrdU. On day 12 p.i. (LCMV infection) or on day 10 p.i. (VSV infection) the mice were killed, and the splenocytes were stimulated for 5 h with the appropriate peptides representing viral epitopes (NP\(_{396-404}\) for LCMV epitope; NP\(_{52-59}\) for VSV epitope), surface stained for CD8, permeabilized, and stained for intracellular IFN-\(\gamma\) and incorporated BrdU. As shown in Fig. 4a, flow cytometric analysis revealed that LCMV-specific CD8\(^{+}\) T cells from wt and SG \(^-/-\) mice do not differ in the content of either of these cytokines. The depicted results are representative of gp33–41-specific cells, but similar results were obtained with regard to the other two epitopes (data not shown).

**FIGURE 5.** Sustained proliferation of virus-specific CD8\(^{+}\) T cells in SG \(^-/-\) mice. Wt and SG \(^-/-\) mice were infected i.v. with either 10\(^3\) PFU of LCMV clone 13 or with 10\(^6\) PFU VSV. Three days after the analysis of splenocytes, mice were given water enriched with BrdU. On day 12 p.i. (LCMV infection) or on day 10 p.i. (VSV infection) the mice were killed, and the splenocytes were stimulated for 5 h with the appropriate peptides representing viral epitopes (NP\(_{396-404}\) for LCMV epitope; NP\(_{52-59}\) for VSV epitope), surface stained for CD8, permeabilized, and stained for intracellular IFN-\(\gamma\) and incorporated BrdU. a, Representative dot plots of NP\(_{396-404}\)-specific IFN-\(\gamma\)-producing CD8\(^{+}\) T cells, obtained from LCMV clone 13 infected wt or SG \(^-/-\) mice. Numbers shown in the top quadrants of the panels represent the percentages of Ag-specific (IFN-\(\gamma\)) CD8\(^{+}\) T cells positive (right side) for negative (left side) for BrdU incorporation, respectively. b, The number of NP\(_{396-404}\)- and NP\(_{52-59}\)-specific IFN-\(\gamma\)-producing CD8\(^{+}\) T cells, either nonproliferating (BrdU\(^{-}\)) or proliferating (BrdU\(^{+}\)), are presented. Data are mean ± SE (n = 4–5 mice/group) from wt or the SG \(^-/-\) mice. * p < 0.05 statistical significance relative to wt mice determined by Mann-Whitney rank-sum test.
apoptosis of the generated CD8+ T cells might also contribute to the delayed contraction (Fig. 5b, top).

To investigate the latter possibility further, we studied the expression of a number of factors known to participate in the regulation of apoptosis during the contraction phase (Fig. 6). However, the frequency of virus-specific CD127+ CD8+ T cells was similar in SG-/- and wt mice on day 10 p.i., as was the level of Bcl-2 expression in CD44highCD8+ T cells. To directly evaluate apoptosis, we also stained CD8+ T cells from day 10-infected mice with annexin V, but again we found no difference in staining intensity between the cells from the two mouse strains.

To see whether the delayed contraction in SG-/- mice had any influence on the quality of the surviving memory CD8+ T cells, splenocytes from both mouse strains were harvested around 4 mo p.i. and subjected to extended surface phenotyping (expression of CD127, CD27, CD43, and CD62L). This analysis did not reveal any differences between the virus-specific CD8+ T cells remaining in SG-/- mice and in matched wt (data not shown), indicating that the memory cells were qualitatively similar.

Finally, we investigated whether delayed contraction of the Ag-specific CD8+ T cell population would also be seen in another virus infection model. For this purpose, mice were infected with VSV, and on day 10 p.i., i.e., 3–4 days after the peak of the VSV-specific CD8+ T cell response, the number of the virus-specific BrdU+ and BrdU- IFN-γ+ CD8+ T cells was determined. Also in this model we detected more virus-specific CD8+ T cells, both proliferating (BrdU+) and nonproliferating (BrdU-), in SG-/- mice than in matched wt mice (Fig. 5b, bottom).

Sustained expansion of virus-specific CD8+ T cells is caused by a T cell external factor

Considering that one of the known functions of serglycin is to retain compounds within the secretory granule of hematopoietic cells (24, 27, 35), one potential explanation for the sustained proliferation of CD8+ T cells in mice lacking serglycin could be leakage of a T cell-stimulating factor from SG-/- hematopoietic cells. To address this possibility, 1 × 10^5 splenocytes from TCR318 transgenic mice in the SG-/- and wt mice on day 10 p.i., as was the level of Bcl-2 with annexin V, but again we found no difference in staining intensity between the cells from the two mouse strains.

To see whether the delayed contraction in SG-/- mice had any influence on the quality of the surviving memory CD8+ T cells, splenocytes from both mouse strains were harvested around 4 mo p.i. and subjected to extended surface phenotyping (expression of CD127, CD27, CD43, and CD62L). This analysis did not reveal any differences between the virus-specific CD8+ T cells remaining in SG-/- mice and in matched wt (data not shown), indicating that the memory cells were qualitatively similar.

Finally, we investigated whether delayed contraction of the Ag-specific CD8+ T cell population would also be seen in another virus infection model. For this purpose, mice were infected with VSV, and on day 10 p.i., i.e., 3–4 days after the peak of the VSV-specific CD8+ T cell response, the number of the virus-specific BrdU+ and BrdU- IFN-γ+ CD8+ T cells was determined. Also in this model we detected more virus-specific CD8+ T cells, both proliferating (BrdU+) and nonproliferating (BrdU-), in SG-/- mice than in matched wt mice (Fig. 5b, bottom).

Sustained expansion of virus-specific CD8+ T cells is caused by a T cell external factor

Considering that one of the known functions of serglycin is to retain compounds within the secretory granule of hematopoietic cells (24, 27, 35), one potential explanation for the sustained proliferation of CD8+ T cells in mice lacking serglycin could be leakage of a T cell-stimulating factor from SG-/- hematopoietic cells. To address this possibility, 1 × 10^5 splenocytes from TCR318 mice on a B6.SJL (CD45.1+) background expressing a gp33-41-specific TCR on approximately two-thirds of their CD8+ T cells was similar (Fig. 7a, top). One representative histogram from each mouse group is presented. Background (thin line histogram) represents staining with isotype control. Binding of annexin V to CD8+ T cells from naive mice. Expression of CD127 on gp33-41- and NP396-404-specific IFN-γ+ CD8+ T cells (top). The day after cell transfer, mice were infected i.v. with 10^6 PFU of LCMV clone 13, and on day 12 p.i., splenocytes were analyzed by flow cytometry either after surface staining for CD8, CD45.1, and Vα2 (a) or after gp33-41 peptide activation and subsequent staining for CD8, CD45.1, and intracellular IFN-γ (b). a. The number of donor cells (CD45.1+ Vα2+ leukocytes) in infected wt or SG-/- mice. Data are mean ± SE (n = 4 mice/group). b. The number of gp33-41-specific IFN-γ+ CD45.1+ donor or CD45.1+ endogenous CD8+ T cells in infected wt or in SG-/- mice. Data are presented as mean ± SE (n = 4 mice/group). In uninfected (wt and SG-/-) recipients, donor-derived CD8+ T cells were below the detection limit. *, p < 0.05 statistical significance relative to wt mice determined by Mann-Whitney rank-sum test.

FIGURE 7. Higher rate of proliferation of splenocytes from TCR318 transgenic mice in the SG-/- than in wt mice. A total of 10^5 splenocytes from TCR318 (on SJL background, CD45.1+) mice, expressing a gp33-41-specific TCR, were adoptively transferred into wt or SG-/- mice. The day after cell transfer, mice were infected i.v. with 10^6 PFU of LCMV clone 13, and on day 12 p.i., splenocytes were analyzed by flow cytometry either after surface staining for CD8, CD45.1, and Vα2 (a) or after gp33-41 peptide activation and subsequent staining for CD8, CD45.1, and intracellular IFN-γ (b). a. The number of donor cells (CD45.1+ Vα2+ leukocytes) in infected wt or SG-/- mice. Data are mean ± SE (n = 4 mice/group). b. The number of gp33-41-specific IFN-γ+ CD45.1+ donor or CD45.1+ endogenous CD8+ T cells in infected wt or in SG-/- mice. Data are presented as mean ± SE (n = 4 mice/group). In uninfected (wt and SG-/-) recipients, donor-derived CD8+ T cells were below the detection limit. *, p < 0.05 statistical significance relative to wt mice determined by Mann-Whitney rank-sum test.
These results indicate that the prolonged proliferation of virus-specific CD8+ T cells in SG−/− mice are caused by external factors readily available in the environment of virus-infected SG−/− mice.

SG−/− mice survive chronic infection, induced with a high viral dose

A common feature of many chronic viral infections is dysfunctional or deleted CD8+ effector T cells (36–39). Recent data indicate that in mice for which the T cell response is not inhibited, due to the absence of a functional PD-1 inhibitory pathway, death occurs upon infection with a high dose of highly invasive LCMV clone 13 (40). Considering that the SG−/− mice exhibit a prolonged T cell response, it was of interest to investigate whether they showed an altered response toward chronic virus infection. We therefore i.v. infected the mice with 106 PFU LCMV clone 13, and measured the body weight daily until day 12 p.i., when the CD8+ T cells normally have become exhausted. Like wt mice, all SG−/− animals survived the infection, and the mice did not differ substantially in loss of body weight (data not shown). Moreover, analysis of spleen virus titers revealed high virus levels of equal magnitude in the two groups. Together, these observations indicate that in mice for which the T cell response is not inhibited, SG−/− mice exhibit a prolonged proliferation of virus-specific CD8+ T cells, similar to mast cells, in which serglycin was shown to be essential for the development of mature secretory granules (27). We analyzed splenic NK cells with regard to the content of GrB at several time points during the innate immune response toward LCMV infection. The splenocytes were stained for cell surface CD8, CD4, CD8, and NK1.1, permeabilized, and stained for intracellular GrB. Subsequent flow cytometric analysis showed that SG−/− mice did not differ from wt animals in total numbers of NK cells (Fig. 8a). However, at the peak of the NK cell response (day 4 p.i.), we detected ~40% less GrB+ NK1.1+ cells in SG−/− mice compared with matched wt (Fig. 8b). Furthermore, according to MFI for GrB staining, GrB+ NK cells from SG−/− mice contain significantly less GrB than their wt counterparts (data not shown). Taken together these results point to a role for serglycin in the storage of GrB in virus-activated NK cells.

Discussion

Due to the development of a mouse strain with a targeted inactivation of the serglycin gene (27), a great body of information regarding the function of serglycin proteoglycans in various hematopoietic cells has been accumulated during the past few years. Thus, serglycin has been shown to be crucial for storage of various molecules in different hematopoietic cells: 1) various proteases in mast cells (27, 35), 2) elastase in neutrophils (41), 3) platelet factor 4 in platelets (42), and 4) GrB in Con A-induced CTLs (24). Considering the importance of serglycin in regulating secretory granule content and morphology in vitro-activated CTLs (24), we investigated in this study the possibility that serglycin may be important for antiviral responses in vivo. This role was addressed by infection of wt and serglycin null animals with LCMV, a viral model that is widely used in immunological studies, particularly in situations in which the in vivo significance of an effector molecule of the CTL population is addressed (e.g., in Refs. 25, 43).

We found that SG−/− mice eliminated LCMV from both lymphoid (spleen) and nonlymphoid (lungs) organs with similar kinetics as wt controls, which was somewhat unexpected considering 1) that serglycin deficiency results in reduced GrB storage in Con A-induced CTLs (24) and 2) that clearance of LCMV infection is impaired in mice lacking GrB (44). This finding indicates that SG−/− mice develop a functionally normal CD8+ T cell response. In support of such a notion, we found a similar capacity for in vivo killing of Ag-expressing target cells in the two mouse strains, and flow cytometric analysis of splenic CD8+ T cells revealed an equal number of perforin-positive and GrB+ CD8+ T cells in wt and SG−/− mice. Moreover, on a per cell basis (as estimated by MFI) the content of these cytolytic molecules was similar in the two mouse strains. In addition, flow cytometric analysis of splenic LCMV-specific CD8+ T cells (directed toward immunodominant peptides gp33–41 or NP396–404, or subdominant peptide gp276–286) revealed that SG−/− mice generated numbers of IFN-γ and TNF-α virus-specific CD8+ T cells similar to wt mice. Furthermore, the content of IFN-γ or TNF-α on a per cell basis was similar in SG−/− and wt CD8+ T cells.

An important implication of these results is that, although the storage of GrB is affected to a major extent by the lack of serglycin in Con A-induced CTLs in vitro (24), the level of stored GrB in vivo-activated CTLs following LCMV clone 13 infection is not substantially affected by the absence of serglycin. However, when infection with the less invasive LCMV clone 53b was studied, GrB+ CD8+ T cells from SG−/− mice were found to contain ~30% less GrB than similar cells from wt mice, indicating that under some conditions serglycin may also influence the retention of GrB within in vivo-generated effector CD8+ T cells.
The reasons for this variation in the role of serglycin could be several. First, other, non-serglycin proteoglycans, may compensate for the lack of serglycin. In line with such a possibility, CTLs were recently shown to express a number of proteoglycan species in addition to serglycin (24). However, when we analyzed the expression of a number of other proteoglycan species by quantitative PCR, we did not observe any compensatory increase, at least in LCMV clone 13-infected mice. Nevertheless, it is still possible that the proteoglycan expression patterns may differ between Con A-induced CTLs and in vivo-activated CTLs, and that the proteoglycan expression pattern may also differ depending on the nature of the virus infection. A second reason could be that serglycin to a certain extent is involved in the transport of GrB to the cytotoxic granules. It has been known for quite some time that GrB is targeted to the granules partly by the mannose-6P receptor pathway, whereas undefined pathways are responsible for the remaining transport of GrB into cytotoxic granules (45). Conceivably, the alternative mode of transportation of GrB to the cytotoxic granules could be serglycin-dependent (41, 46), and possibly, the relative dependence on the mannose-6P vs serglycin pathway may vary. For example, in a case of extensive synthesis of serglycin the transport of GrB into the cytotoxic granule might depend more on serglycin, whereas the production of serglycin may be lower under other circumstances and thus influence the storage of GrB within the CTLs to a lesser degree. Additionally, differential rates of GrB synthesis relative to the rate of serglycin synthesis may influence the extent to which serglycin regulates GrB storage in CTLs.

Interestingly, despite unimpaired virus clearance, we noted a markedly delayed contraction phase of the virus-specific CD8\(^+\) T cell pool in SG\(^/-\)/ mice. Compared with wt mice, almost twice as many LCMV-specific CD8\(^+\) T cells were present in SG\(^/-\)/ mice on day 10 p.i., and the pool of virus-specific CD8\(^+\) T cells continued to expand in the knockout animals between day 10 and 12 p.i., which is for \(\sim\)2 days more than in similarly infected wt mice. SG\(^/-\)/ mice also had more virus-specific CD8\(^+\) T cells on day 28 p.i., whereas little difference was observed after 2 mo, and importantly, the generated memory cells were phenotypically similar. The marker of virus-specific CD8\(^+\) T cells was intracellular cytokine production, as the primed T cells drastically reduce the expression of perforin and GrB after day 10 p.i., at which time virus was eradicated from the organs. Similarly delayed contraction followed infection with VSV, indicating that this phenomenon may be a general quality of SG\(^/-\)/ mice. The delayed contraction phase could potentially reflect reduced apoptosis in SG\(^/-\)/ mice, but we could not find any evidence supporting this possibility. In contrast, through BrdU labeling of dividing cells, we could show that the delayed contraction is associated with prolonged proliferation of the Ag-specific CD8\(^+\) T cells in infected SG\(^/-\)/ mice.

The reason for the prolonged proliferation of CD8\(^+\) T cells in mice lacking serglycin is intriguing. Serglycin is considered a multifunctional proteoglycan, being involved in the activation of enzymes (47), protecting them from inhibitors (48), transport of molecules into specific intracellular granules (41), retaining the molecules within these organelles (27, 35), and even playing a role in the delivery of certain molecules to their receptors outside the cell has been discussed (24). Importantly, all known functions of serglycin are most likely based on electrostatic interactions between the highly negatively charged serglycin proteoglycan and various positively charged molecules. Therefore, we favor the notion that the normal contraction of the CD8\(^+\) T cell response is dependent on an electrostatic interaction between serglycin proteoglycan and one or more basic compounds, and that the lack of serglycin reduces the impact of this control mechanism and, hence, leads to an increased rate of proliferation or decreased rate of apoptosis. The nature of the hypothetical basic compounds that regulate the CD8\(^+\) T cell response is not clear at present, but it is well known that following initial Ag-dependent triggering, subsequent clonal expansion is Ag-independent (49–51) with the amplitude of the primary response being strongly influenced by the availability of inflammation-associated cytokines (52, 53). Notably, a large number of growth factors, both T cell growth factors and growth factors for other cell types, are basic and are known to interact with proteoglycans (54–56), thus conforming well to the outlined scenario. Although we cannot with certainty explain how an interaction between serglycin and a tentative T cell growth factor would result in contraction of the T cell response, a likely possibility would be that the binding to serglycin may result in masking of receptor binding sites (57). An alternative, although related, mechanism could be that relevant T cell growth factors may be regulated proteolytically by proteases that are dependent on serglycin. For example, several mast cell proteases as well as neutrophil elastase are critically dependent on serglycin for their storage (27, 35, 41). Possibly, the lack of serglycin could thus result in reduced degradation of T cell growth factors as a result of decreased amounts of serglycin-dependent protease activity, eventually leading to an increased CD8\(^+\) T cell proliferation.

An important question is whether the dysregulation of CD8\(^+\) T cell contraction in SG\(^/-\)/ mice is intrinsic to the T cells or whether it reflects a change in the surrounding environment. We addressed this function by adoptive transfer of LCMV-specific CD8\(^+\) T cells from TCR318 transgenic mice into wt or SG\(^/-\)/ mice before LCMV infection. We found that wt donor cells expanded substantially more in SG\(^/-\)/ mice than in matched wt mice, indicating that the prolonged expansion of virus-specific CD8\(^+\) T cells in SG\(^/-\)/ mice to a large degree reflects factors in the external environment of these cells. In contrast, although we cannot formally exclude that a T cell internal component may contribute as well, the virtually identical expansion of endogenous SG\(^/-\)/ and exogenous wt CD8\(^+\) T cells in SG\(^/-\)/ recipients argues strongly against this possibility. Thus, we suggest that a serglycin binding factor, produced by hematopoietic cells, plays an important role in regulating the CD8\(^+\) T cell response during viral infection, and that the absence of serglycin leads to increased levels of this factor and, hence, an extended proliferative response. However, further work will be required to identify the factor involved and to precisely determine the mechanism by which serglycin regulates T cell responses during viral infection. Nevertheless, this study identifies a completely novel role for serglycin proteoglycan in the regulation of an adaptive immune response.

Disclosures
The authors have no financial conflict of interest.

References