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Fas/FasL signaling is critical for the survival of exhausted antigen-specific CD8$^+$ T cells during tumor immune response

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ABSTRACT

Antigen (Ag)-specific activated CD8$^+$ T cells are critical for tumor elimination but become exhausted, and thus, dysfunctional during immune response against the tumor due to chronic antigen stimulation. The signaling of immune checkpoint receptors is known to be a critical component in this exhaustion; however, the fate of these exhausted CD8$^+$ T cells remains unclear. Therefore, to elucidate this, we followed the fate of Ag-specific CD8$^+$ T cells by directly visualizing them using MHC class I tetramers coupled with ovoalubumin$_{257–264}$ in C57BL/6 mice inoculated with EG.7. We found that the number of generated Ag-specific activated CD8$^+$ T cells decreased via apoptosis during a prolonged tumor immune response. However, the number of Ag-specific CD8$^+$ T cells was significantly higher in Fas ligand (FasL)-dysfunctional gld mice than in control mice, resulting in suppressed tumor growth. In contrast, the enforced expression of Bcl-2 failed to rescue apoptosis of the exhausted CD8$^+$ T cells following EG.7 inoculation. These results suggest that Fas/FasL signaling is critical for the survival of exhausted CD8$^+$ T cells during the tumor immune response.

1. Introduction

Upon encountering a pathogenic microbe, naive antigen (Ag)-specific CD8$^+$ T cells proliferate and differentiate into effector CD8$^+$ T cells during the expansion phase. Most activated T cells subsequently die by apoptosis during the contraction phase, but a few survive to become memory cells, which persist for a long period of time (Wong and Pamer, 2003; Ahmed and Gray, 1996; Schluns and Lefrancois, 2003). However, in chronic antigen stimulation, such as that occurs with chronic infections or tumors, CD8$^+$ T cells become exhausted, and thus, dysfunctional, resulting in failure to eliminate the bacteria or cancer (Barber et al., 2006; Wherry et al., 2007; Wherry and Kurachi, 2015; Pauken and Wherry, 2015). This exhaustion is characterized by a decreased proliferative capacity, loss of cytokine secretion, reduced cytotoxic killing abilities, and phenotypic changes, as well as an increase in inhibitory receptors, including programmed cell death protein 1 (PD-1), all of which can be restored by blockading the inhibitory receptors of the exhausted CD8$^+$ T cells (Kim and Ahmed, 2010; Sakuishi et al., 2010; Ostrand-Rosenberg et al., 2014). Although the exhausted T cells may be physically deleted through apoptosis, the fate of Ag-specific activated CD8$^+$ T cells in tumor immune response remains unclear. Because the number of Ag-specific effector CD8$^+$ T cells is dependent on the number of T cells that survive apoptosis, identification of the molecular mechanisms responsible for activated T cell apoptosis following continuous T cell antigen receptor (TCR)-mediated activation is important for understanding how effector cells survive during the tumor immune response.

Primary TCR-mediated activation can result in at least two types of cell death in activated T cells, activation-induced cell death (AICD) (Schluns and Lefrancois, 2003), also known as Ag-driven apoptosis, and activated T cell autonomous cell death (ACAD), also known as growth factor withdrawal-induced apoptosis (Marrack and Kappler, 2004; Hildeman et al., 2002a; Marsden and Strasser, 2003). Previous studies have shown that ACAD is responsible for the death of the majority of

Abbreviations: ACAD, activated T cell autonomous cell death; Ag, antigen; AICD, activation-induced cell death; FasL, Fas ligand; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; LAG-3, lymphocyte-activation gene 3; LCMV, chronic lymphocytic choriomeningitis virus; LN, lymph node; OVA, ovoalubumin; PBS, phosphate-buffered saline; PD-1, programmed cell death protein 1; PE, phycoerythrin; RPMI, Roswell Park Memorial Institute; s.c., subcutaneously; TCR, T cell antigen receptor; TNF, tumor necrosis factor; TNFR1, tumor necrosis factor 1; TILs, tumor-infiltrating lymphocytes

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activated T cells responding to a foreign Ag (Ostrand-Rosenberg et al., 2014; Schluns and Lefrancois, 2003; Marsden and Strasser, 2003) but that this can be prevented by the enforced expression of Bcl-2, indicating that the upregulation of Bcl-2 in effector T cells plays a critical role in preventing activated T cell death by ACAD during the contraction phase (Hildeman et al., 2002a; Pellegrini et al., 2003; Van Parijs et al., 1998) Common γ cytokines such as IL-15 and IL-7 have been shown to play a critical role in the upregulation of Bcl-2 and the survival of effector $CD8^+$ T cells during the contraction phase of acute bacterial infection (Nakajima et al., 1997; Vella et al., 1998; Gett et al., 2003; Yajima et al., 2006). However, the expression of memory markers, such as the IL-7 receptor α chain (CD127), is decreased in exhausted $CD8^+$ T cells upon chronic Ag stimulation against a tumor (Kim and Ahmed, 2010; Sakuishi et al., 2010; Ostrand-Rosenberg et al., 2014). Therefore, commonγ cytokines such as IL-7 may not prevent apoptosis in exhausted $CD8^+$ T cells during the tumor immune response.

The other kind of cell death, AICD, is mainly triggered through cell surface proteins of the tumor necrosis factor (TNF) receptor family, including Fas (Ju et al., 1995; Sytwu et al., 1996; Nagata and Suda, 1995). Cross-linking of Fas via ligation by the Fas ligand (FasL) activates the Fas-associated death domain, which triggers the activation of intracellular caspases, leading to apoptosis (Ashkenazi and Dixit, 1998). Previous studies using Fas/FasL mutants or Fas/tumor necrosis factor 1 (TNFR1)-deficient mice have demonstrated that either Fas/FasL or TNFR1 is required for T cell death during chronic infections (Zhou et al., 2002). Therefore, it is possible that apoptosis of exhausted $CD8^+$ T cells is induced by AICD following the continuous stimulation by a tumor antigen during the tumor immune response.

In this study, we followed the fate of Ag-specific $CD8^+$ T cells by directly visualizing them using MHC class I tetramers coupled with ovoalubumin $(OVA)_{257–264}$ following EG.7 inoculation. We also examined the mechanism of apoptosis in exhausted Ag-specific $CD8^+$ T cells during tumor immune response using FasL mutant mice or Bcl-2 transgenic mice. Our results demonstrate that the survival of exhausted $CD8^+$ T cells is critically dependent on Fas/FaL signaling, but not on the overexpression of Bcl-2. These findings provide an insight into a new approach that could be developed to augment an effective immune response in cancer immunotherapy.

2. Materials and methods

2.1. Mice and cell lines

Age- and sex-matched C57BL/6 and gld/gld mice with a B6 background were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Eμ-bcl-2-25 Tg mice, which express Bcl-2 under the control of the 5′ IgH enhancer (Eμ) in T cells, have been described previously (Strasser et al., 1991). All of the mice used for experiments were maintained under specific-pathogen-free conditions in our animal facility and were used at 5–6 weeks of age. EL-4 cells and OVA-transfected EL-4 (E.G7) were derived from C57BL/6 mice ($H\text{-}2K^b$). Tumor cells were cultured in Roswell Park Memorial Institute (RPMI)-1640 containing 10% fetal calf serum (FCS), 100 U/ml penicillin, 100 μg/ml streptomycin, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). Cells were maintained in 75-cm^2 tissue flasks at 37 °C in a humidified 5% CO_2 atmosphere and passaged 2–3 times per week.

2.2. Antibodies and reagents

Fluorescein isothiocyanate (FITC)-conjugated anti-CD44 monoclonal antibody (mAb) (IM7) and anti-CD3 mAb (145-2C11), phycoerythrin (PE)-conjugated anti-NK1.1 mAb (PK136), anti-CD8 mAb (53-6.7), anti-CD95 mAb (Jo2), and anti-γδ TCR mAb (UC7-13D5), and CyChrome-conjugated anti-CD8 mAb (53-6.7), anti-CD4 mAb (H129.19), and anti-TCR-β mAb (H57-597) were purchased from BD PharMingen (San Diego, CA, USA). PE-conjugated anti-CD279 (PD-1) mAb (29 F.1A12), anti-CD62 L mAb (MEL-14), anti-CD152 (CTLA-4) mAb (UC10-4B9), anti-CD122 mAb (5H4), anti-CD127 mAb (A7R34), anti-CD223 (LAG-3) mAb (C9B7W),were purchased from Biolegend (San Diego, CA USA). The PE-conjugated tetramer of the $H\text{-}2K^b$-restricted $OVA_{257-264}$ (SIINFEKL) peptide was purchased from MBL (Nagoya, Japan). Apoptosis was determined by staining the cells with FITC-conjugated anti-active caspase-3 mAb (BD Biosciences) according to the manufacturer's instructions.

2.3. Challenge with tumor cells

EG.7 cells (2×10^5) were placed in 100 μl of phosphate-buffered saline (PBS) and inoculated subcutaneously (s.c.) into the shaved lateral flanks of the mice. The sizes of the primary tumors were then determined every 2–3 days using calipers. The tumor volume (V) was calculated using the formula $V = (A \times B^2)/2$, where A is the longest diameter (mm) and B is the shortest diameter (mm).

2.4. Preparation of cells

Peripheral lymph node (LN) cells and splenocytes were harvested from infected mice, washed, and suspended. To isolate tumor-infiltrating lymphocytes (TILs), tumors were dissected from the mice and minced into 1- to 2-mm pieces. These tumor pieces were then incubated in a mixture of 1 mg/ml collagenase (Invitrogen) and 20 μg/ml DNase (Sigma-Aldrich) in RPMI-1640 containing 10% FCS for 90 min at 37 °C. The lymphocytes were separated by Percoll density gradient centrifugation (Amersham Biosciences) and analyzed using flow cytometry.

2.5. Flow cytometry analysis

The TILs, LN cells, or splenocytes from tumor-bearing mice were preincubated with a culture supernatant from 2.4 G2 to prevent nonspecific staining. After washing, the cells were stained with various combinations of mAbs. Staining with biotin-conjugated mAbs was followed by treatment with streptavidin-CyChrome or -allophycocyanin. In some experiments, the cells were subjected to intracellular staining using a Fast Immune Cytokine System according to the manufacturer's instructions (BD Biosciences), and the fluorescence of the cells was analyzed using a flow cytometer.

2.6. Intracellular active caspase-3 staining

The TILs, LN cells, or splenocytes from tumor-bearing mice were first stained for surface markers in staining buffer with CyChrome-conjugated anti-CD8 mAb and PE-conjugated $OVA_{257–264}$ $H\text{-}2K^b$ tetramers for 30 min at 4 °C. They were then washed, fixed, and permeabilized using the Cytofix/Cytoperm intracellular staining kit (BD Biosciences). Cells were incubated with FITC-conjugated anti-caspase-3 at a 1/100 dilution in perm/wash buffer for 30 min at room temperature. Following intracellular staining, fluorescence of the cells was analyzed using a flow cytometer.

2.7. Statistical analysis

The significance of all data was determined using Student's *t*–test, with a value of $p < 0.05$ considered significant. All analyses were conducted using Stat-View 5.0 (Abacus Concepts).

3. Results

3.1. Fate of Ag-specific activated $CD8^+$ T cells

To elucidate the fate of Ag-specific $CD8^+$ T cells during the tumor

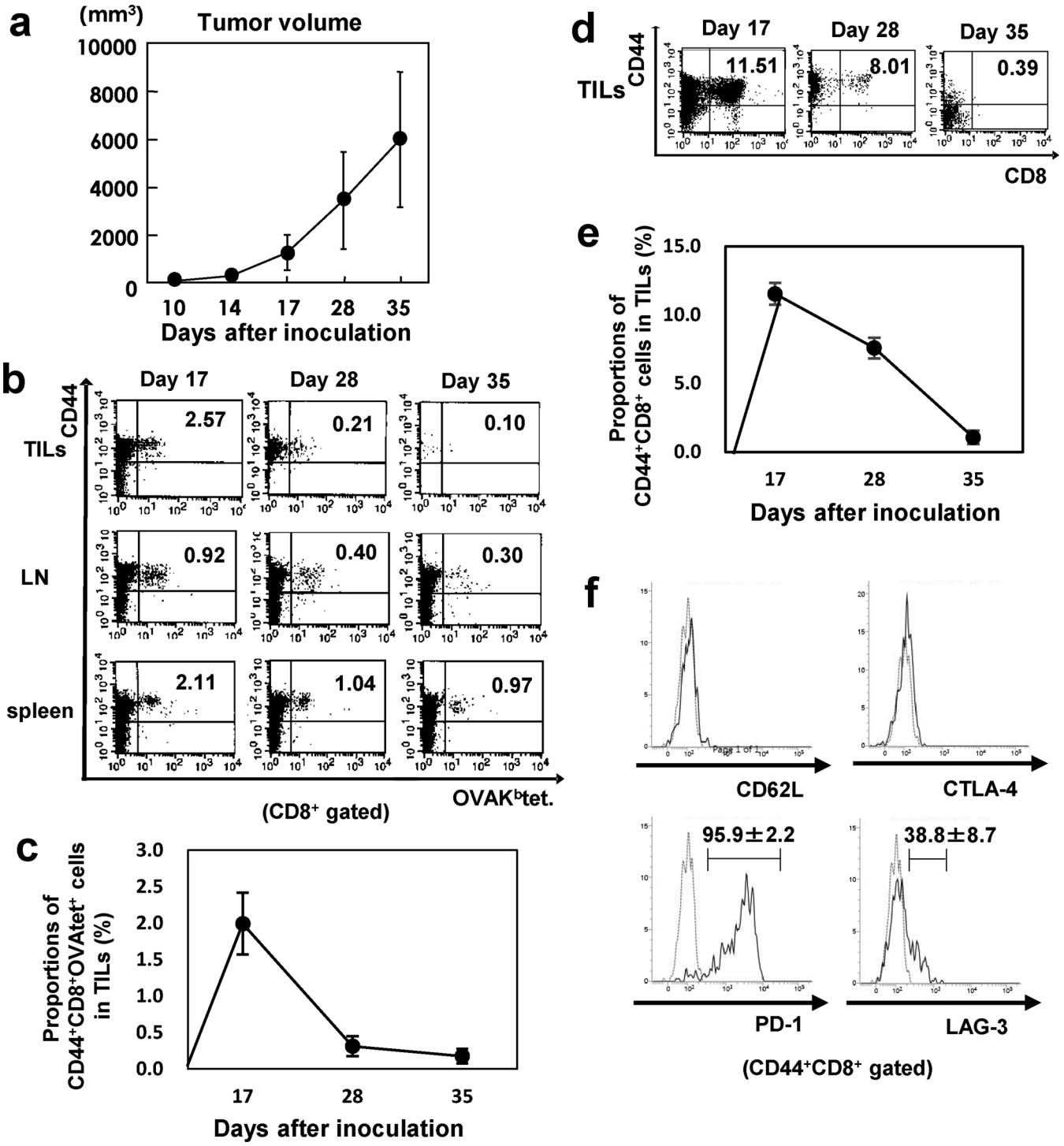

Fig. 1. Number of Ag-specific activated $CD8^+$ T cells in C57BL/6 mice following EG.7 inoculation. (*a*) Tumor volumes in C57BL/6 mice on the indicated days after subcutaneous injection with EG.7 cells (2×10^5). Data were obtained from three separate experiments and are expressed as means ± SD of nine mice at each point. (*b*) Kinetics of $OVA_{257–264}$ specific $CD8^+$ T cells in the tumor-infiltrating lymphocytes (TILs), lymph node (LN) cells, and splenocytes following inoculation with EG.7. Flow cytometry results are presented as typical profiles after an analysis gate had been set on $CD8^+$ cells. Values represent the number of $OVA_{257–264}$ specific tetramer$^+$ $CD44^{high}$ as a percentage of $CD8^+$T cells. Representative results from three separate experiments are shown. (*c*) On days 17, 28, and 35 after inoculation, proportions of $CD44^+$ $CD8^+$ OVA tetramer$^+$ cells in the TILs are shown. Data are presented as means ± SDs for five mice. (*d*) Number of $CD44^+CD8^+$ T cells from TILs on the indicated days after EG.7 inoculation, as determined using flow cytometric analysis. The analysis gate was set on lymphocytes. Five mice per group were analyzed independently, and representative profiles are shown in the figures. (*e*) On days 17, 28, and 35 after inoculation, proportions of $CD44^+CD8^+$ cells in the TILs are shown. Data are presented as means ± SDs for five mice. (f) Representative flow cytometric histograms showing the expression of C62 L, CTLA-4, PD-1, and LAG-3 in $CD44^+CD8^+$ T cells from TILs on day 21 after inoculation. An analysis gate was set on $CD44^+CD8^+$ cells. Data are presented as means ± SDs for three mice. Staining with isotype control Ab was overlaid on each histogram as a dotted line.

immune response, we directly visualized them using MHC class I tetramers coupled with $OVA_{257–264}$ in C57BL/6 mice inoculated with EG.7. A tiny nodule was observed ten days after EG.7 inoculation, but Ag-specific $CD8^+$ T cells were not detected in the TILs, LN cells, or splenocytes at this time using flow cytometry (data not shown). However, at 17 days after inoculation, expansion of $OVA_{257–264}$ specific $CD8^+$ T cells were detected in the TILs, LN cells, and splenocytes, the numbers of which were correlated with the level of tumor growth (Fig. 1a, b, and c). It was also around this time that the peak number of $OVA_{257–264}$ specific $CD8^+$ T cells was observed in each organ during the tumor immune response. Following this peak, the number of $OVA_{257–264}$ specific $CD8^+$ T cells in mice inoculated with EG.7 decreased in the TILs, LN cells, and splenocytes during a prolonged tumor immune response. $CD44^{high}CD8^+$ T cells in TILs also accumulated in the tumor at the peak of the immune response at 17 days after tumor inoculation, thereafter decreasing until disappearing on day 35 after EG.7 inoculation (Fig. 1d and e). $CD44^+CD8^+$ T cells in TILs showed an activation phenotype ($CD62L^-$) and expressed co-inhibitory receptor such as programmed cell death protein 1 (PD-1) and lymphocyte-activation gene 3 (LAG-3) (Fig. 1f). These results suggest that the number of generated Ag-specific $CD8^+$ T cells decreased not only in the locale of the tumor, but also in the peripheral lymphoid organs during a prolonged tumor immune response.

3.2. Apoptosis of activated $CD8^+$ T cells

The number of Ag-specific $CD8^+$ T cells is regulated by the balance between cell survival and apoptosis. To determine whether the reduced number of Ag-specific $CD8^+$ T cells is involved in the apoptosis of effector $CD8^+$ T cells, we next examined the apoptotic potential of $OVA_{257–264}$ specific $CD8^+$ T cells from C7BL/6 mice on days 23 and 30 after EG.7 inoculation during activated T cell exhaustion. The expression of active caspase-3 in $OVA_{257–264}$ specific $CD8^+$ T cells was detected on day 23 after inoculation (Fig. 2a and b), indicating that Ag-specific $CD8^+$ T cells underwent apoptosis during the tumor immune response. Furthermore, the expression level of active caspase-3 in $OVA_{257–264}$ specific $CD8^+$ T cells was higher during a prolonged tumor immune response. A total $CD8^+$ gated analysis showed that only some of the cell population experienced apoptosis during activated T cell exhaustion, indicating that the Ag-specific $CD8^+$ T cells selectively induced apoptosis. These results suggest that exhausted Ag-specific $CD8^+$ T cells underwent apoptosis, resulting in decreased numbers of these cells during the tumor immune response.

3.3. Effect of activated $CD8^+$ T cells on Fas expression

In chronic antigen stimulation, including chronic infection, Fas/FasL signaling is critical for inducing apoptosis in activated $CD8^+$ T cells. Therefore, we examined the expression of Fas in activated $CD8^+$ T cells during the tumor immune response. The expression of Fas was detected on day 21 after EG.7 inoculation in $CD44^{high}CD8^+$ T cells (Fig. 3). Fas expression in activated $CD8^+$ T cells was induced not only in the TILs, but also in the peripheral lymphoid organs, including the peripheral LNs and spleen. These results suggest that activated $CD8^+$ T cells may undergo apoptosis via Fas/FasL signaling during the tumor immune response.

3.4. Role of Fas/FasL signaling in Ag-specific activated $CD8^+$ T cell apoptosis

To determine whether Fas/FasL signaling is critical for the induction of apoptosis in activated $CD8^+$ T cells during the tumor immune response, we examined the fate of $OVA_{257–264}$ specific $CD8^+$ T cells and tumor growth in FasL dysfunctional gld mice following EG.7 inoculation. The frequency of $OVA_{257–264}$ specific $CD8^+$ T cells was markedly higher in gld mice than in control mice in the TILs on day 21 after EG.7 inoculation, and was also higher in the LN cells and splenocytes (Fig. 4a and b). We next examined the kinetics of tumor growth in gld mice following EG.7 inoculation. A tiny nodule was generated in both gld and control mice on day 7 after EG7 inoculation. However, tumor growth was significantly retarded in gld mice compared with control mice on day 28 after EG.7 inoculation, and was inversely correlated with the frequency of $OVA_{257–264}$ specific $CD8^+$ T cells (Fig. 4c). These results suggest that Fas/FasL signaling is critical for the survival of activated $CD8^+$ T cells during the tumor immune response.

3.5. Effect of Bcl-2 expression on activated $CD8^+$ T cell apoptosis

It is well known that apoptosis occurs not only via the death receptor pathway, such as Fas/FasL, but also via the mitochondrial pathway, which involves the Bcl-2 family. Therefore, we next examined the fate of $OVA_{257–264}$ specific $CD8^+$ T cells in Bcl-2 Tg mice following EG.7 inoculation. The frequency of $OVA_{257–264}$ specific $CD8^+$ T cells in the TILs, LN cells, and splenocytes was comparable between Bcl-2 Tg mice and control mice (Fig. 5a and b), indicating that the enforced expression of Bcl-2 failed to rescue apoptosis of the activated $CD8^+$ T cells during the tumor immune response. Furthermore, tumor growth was slightly, but not significantly, retarded in Bcl-2 Tg mice compared with control mice during the tumor immune response (Fig. 5c). Taken together, these findings suggest that it is the Fas/FasL signaling pathway rather than the mitochondrial pathway that is critical for apoptosis of Ag-specific activated $CD8^+$ T cells during the tumor immune response.

4. Discussion

In this study, we examined the fate of Ag-specific activated $CD8^+$ T cells during the tumor immune response using EG.7 cells expressing OVA as a model tumor antigen. We found that Ag-specific activated $CD8^+$ T cells were generated in the TILs and lymphoid organs following EG.7 inoculation, but thereafter decreased by apoptosis in association with the upregulation of Fas in the activated $CD8^+$ T cells, during a prolonged tumor immune response. Ag-specific activated $CD8^+$ T cells protected the majority of activated T cells from death in FasL-dysfunctional gld mice following EG.7 inoculation, whereas the enforced expression of Bcl-2 failed to rescue apoptosis in activated $CD8^+$ T cells. These results suggest that Fas/FasL signaling plays a critical role in the apoptosis of exhausted $CD8^+$ T cells during the tumor immune response.

We initially followed the fate of Ag-specific $CD8^+$ T cells by directly visualizing them using MHC class I tetramers coupled with the TRP-2-SVYDFFVWL-peptide, as a tumor antigen, following inoculation with the B16F10 melanoma cell line. However, although some TRP-2 specific $CD8^+$ T cells were detected in the TILs on day 20 after B16F10 inoculation at the peak of the immune response, it was difficult to analyze the kinetics of tumor-Ag specific $CD8^+$ T cells due to their limited numbers. Therefore, we selected EG.7 cell lines expressing OVA as a model tumor antigen. A tiny nodule was visualized in the lateral flank of mice from day 7 to day 10 after EG.7 inoculation, but we could not detect the $OVA_{257–264}$ specific $CD8^+$ T cells in their TILs using the $OVA_{257–264}$ peptide tetramer system (data not shown), and we were unable to detect $OVA_{257–264}$ specific $CD8^+$ T cells in the LN cells or splenocytes at these times. Furthermore, $CD44^{high}$ $CD8^+$ T cells were also not detected in the tiny tumor nodules in the TILs, indicating that activated $CD8^+$ T cells including $OVA_{257–264}$ specific $CD8^+$ T cells were not infiltrating the tumor site during the generation of the tiny tumor nodules. In contrast, from day 14 to day 21 after tumor inoculation, we found that Ag-specific $CD8^+$ T cells were generated in the TILs and lymphoid organs, the numbers of which were correlated with the level of tumor growth. Thereafter, the number of Ag-specific $CD8^+$ T cells decreased in both the TILs and lymphoid organs during a prolonged tumor immune response. Therefore, we speculate that cell death was

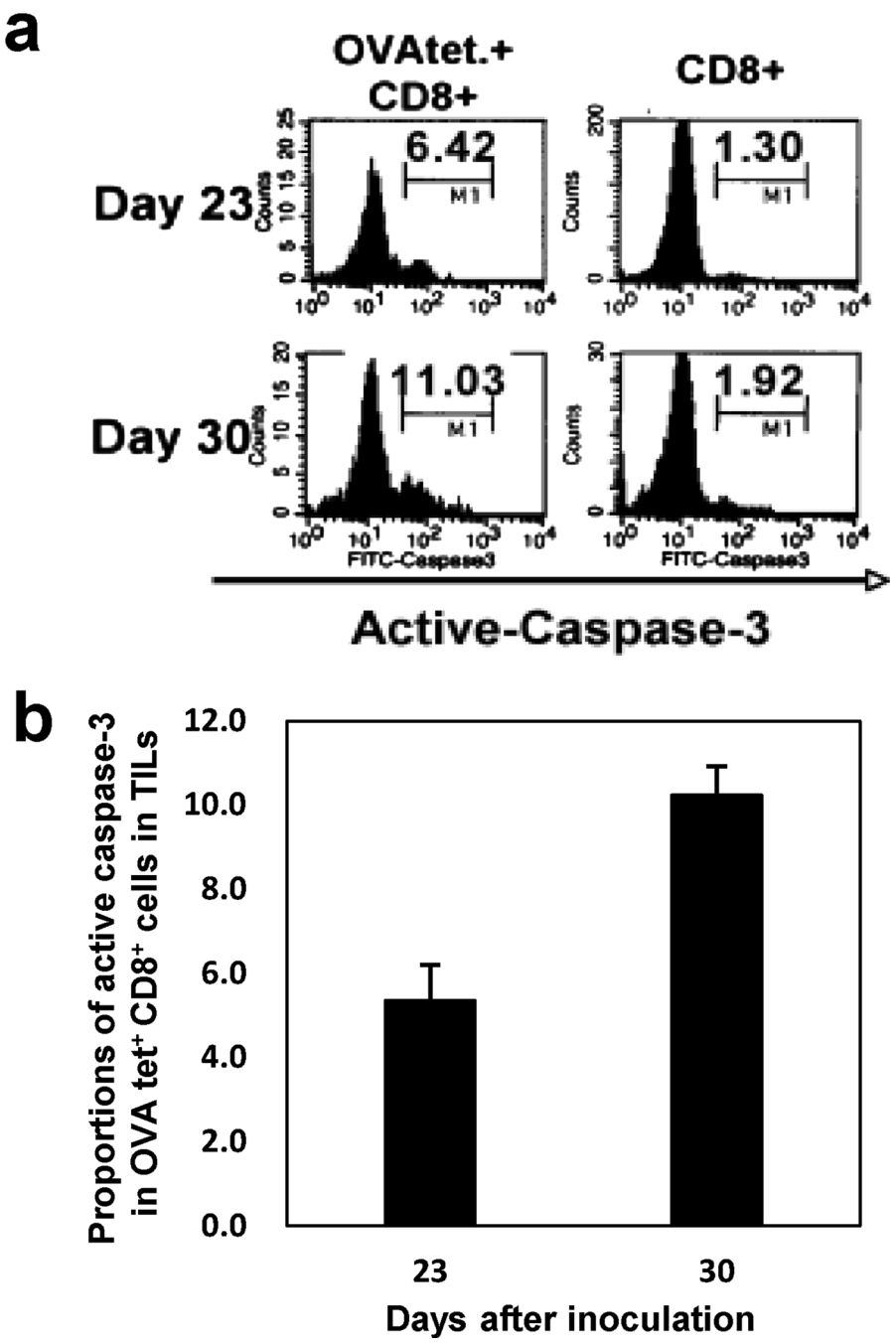

Fig. 2. Apoptosis in exhausted Ag-specific CD8$^+$ T cells during the tumor immune response. (*a*) The apoptosis of activated CD8$^+$ T cells from C57BL/6 mice on days 23 and 30 after EG.7 inoculation was determined using intracellular active caspase-3 staining. An analysis gate was set on CD8$^+$ cells or $OVA_{257–264}$ K^b tetramer + CD8$^+$ cells. Representative results from three separate experiments are shown. (*b*) Proportions of active-caspase-3 expression in CD44$^+$CD8$^+$OVAtet$^+$ cells in the TILs were shown on the indicated days. Data are presented as means ± SDs for five mice at indicated days after inoculation.

induced in these exhausted Ag-specific CD8$^+$ T cells by constitutive TCR stimulation. We also detected the apoptosis of Ag-specific CD8$^+$ T cells following the peak of the immune response with EG.7, indicating that apoptosis was induced in exhausted Ag-specific CD8$^+$ T cells during the tumor immune response in this model. However, it should be noted that we only examined the apoptosis of CD8$^+$ T cells with one immunodominant epitope. Therefore, the generality of this finding and its possible implications awaits further analysis with different epitopes.

AICD is mainly triggered through cell surface proteins of the TNFR family, including Fas (CD95) (Ju et al., 1995; Sytwu et al., 1996; Nagata and Suda, 1995). Fas/FasL signaling has been shown to mediate apoptosis of activated T cells elicited by TCR restimulation *in vitro* (Alderson et al., 1995) and is known to be critical for the apoptosis of Ag-specific effector CD8$^+$ T cells during persistent virus infections *in vivo* (Zhou et al., 2002). Therefore, it is possible that the apoptosis of exhausted Ag-specific CD8$^+$ T cells is induced via Fas/FasL signaling during the tumor immune response under prolonged Ag stimulation. In this study, we found that Fas expression was gradually induced in activated CD8$^+$ T cells following the peak of the immune response against EG.7 and was correlated with the induction of apoptosis. We also found that exhausted Fas-expressing CD8$^+$ T cells could survive in the TILs and lymphoid organs of FasL-dysfunctional gld mice following EG.7 inoculation. These results suggest that the exhausted Fas-expressing CD8$^+$ T cells were susceptible to FasL-mediated apoptosis following

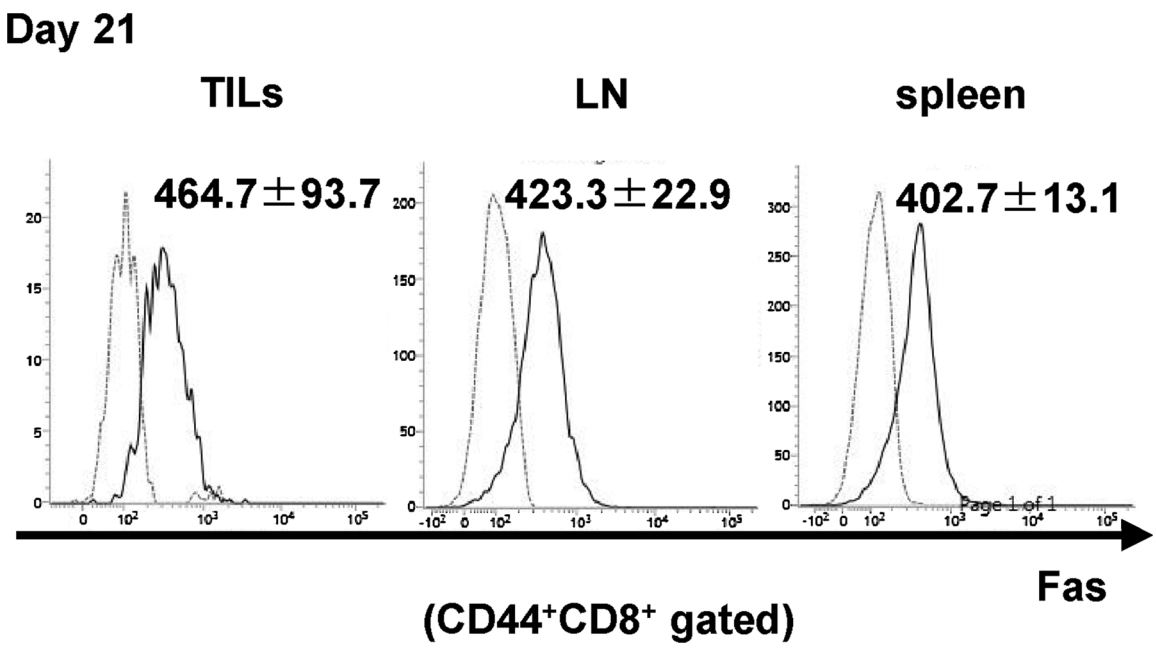

Fig. 3. Fas expression in activated $CD8^+$ T cells during tumor growth. Representative flow cytometric histograms showing the expression of Fas in $CD44^+CD8^+$ T cells from the tumor-infiltrating lymphocytes (TILs), lymph node (LN) cells, and splenocytes on day 21 after inoculation. Histograms are gated on $CD44^+CD8^+$cells. The values in the right corner of each panel represent the mean fluorescence intensity of Fas expression in $CD44^+CD8^+$ T cells. Staining with isotype control Ab was overlaid on each histogram as a dotted line. Data are presented as means ± SDs for three mice.

EG.7 inoculation. It has previously been reported that Fas-expressing T cells are susceptible to apoptosis by FasL in melanoma cells in metastatic lesion, indicating that FasL in tumor cells may contribute to the immune privilege of tumors (Hahne et al., 1996). However, we were unable to detect FasL expression in the EG.7 cell line during the tumor immune response using flow cytometry and immunohistochemistry (data not shown). Therefore, it is most likely that the susceptibility of Fas-expressing exhausted $CD8^+$ T cells to apoptosis is caused by the action of FasL on the host cells. We believe that the induction of apoptosis is a mechanism for preventing a severe inflammatory response during constitutive TCR stimulation, rather than for the tumor to escape the immune system, but further experiments will be required to confirm this.

The other type of cell death that occurs in activated T cells is caused by growth factor withdrawal following TCR-mediated activation, whereby most of the activated T cells die by apoptosis following the eradication of bacteria during an acute infection in order to reduce the level of cytokine production. Several studies have shown that the up-regulation of Bcl-2 in effector T cells via common γ cytokine signaling (including IL-2, IL-7, and IL-15) plays a critical role in preventing activated T cell death during the contraction phase of an acute bacterial infection (Hildeman et al., 2002b; Pellegrini et al., 2003; Van Parijs et al., 1998; Nakajima et al., 1997; Vella et al., 1998; Gett et al., 2003; Yajima et al., 2006). Furthermore, it has previously been reported that rIL-2 administration increases the numbers of exhausted $CD8^+$ T cells during a chronic lymphocytic choriomeningitis virus (LCMV) infection (West et al., 2013) and we have shown that *in vivo* rIL-15 administration increases the number of Ag-specific activated $CD8^+$ T cells during BCG-OVA infection as a model of chronic infection (Tang et al., 2009). Therefore, these mechanisms of growth factor withdrawal-induced apoptosis will also have been involved in the death of the exhausted Ag-specific $CD8^+$ T cells during the tumor immune response. However, the enforced expression of Bcl-2 could not rescue the Ag-specific $CD8^+$ T cells following EG.7 inoculation. Furthermore, it has previously been shown that the expression pattern of IL-7Rα and IL-2Rβ is consistent with the gene-expression data, suggesting that exhausted $CD8^+$ T cells will not efficiently respond to IL-2, IL-4, IL-7, and IL-15 during a chronic infection (Barber et al., 2006; Wherry et al., 2007), and we also failed to detect the expression of IL-7Rα and IL-2Rβ in exhausted $CD8^+$ T cells following EG.7 inoculation (Supplemental Fig.1). We conclude that the upregulation of Bcl-2 is not involved in preventing the apoptosis of exhausted $CD8^+$ T cells during the tumor immune response.

Several previous studies have investigated the anti-tumor immunity of Fas or FasL mutant mice using several tumor cell lines and have found that although the FasL-cytolytic pathway of activated T cells plays a critical role in the eradication of Fas-expressing tumor cells (O'Reilly et al., 2009), the anti-tumor immunity of FasL and Fas mutant mice is comparable to that of wild type mice using both a model of local tumor growth after s.c. inoculation (Hashimoto et al., 1999; Zhang et al., 2009) and lung colonization after intravenous injection (Yang et al., 2012; Winter et al., 1999; Smyth et al., 1999). Moreover, it has been shown that tumor growth is retarded in gld mice compared with control mice (Cao et al., 2015), as corroborated here, and that tumor growth of renal cell adenocarcinoma cell lines expressing the influenza viral hemagglutinin as a model tumor Ag were markedly induced in FasL-dysfunctional gld mice but retarded in Fas-dysfunctional lpr mice compared with control mice (Shanker et al., 2009). Therefore, it is possible that several mechanisms are involved in anti-tumor immunity of Fas and FasL mutant mice depending on the cell lines, hosts, and experimental conditions.

The *in vivo* blockade of Fas signaling of exhausted Ag-specific $CD8^+$ T cells may be useful for cancer immunotherapy. We found that exhausted $CD8^+$ T cells expressed a high level of Fas following EG.7 inoculation, suggesting that antagonistic Fas or FasL antibodies rescue these cells from Fas-induced apoptosis. A previous study reported that intravenous administration of anti-Fas antibodies induces lethal hepatitis in mice (Ogasawara et al., 1993) and a similar toxicity was observed with intravenous administration of anti-FasL antibodies (Rensing-Ehl et al., 1995). However, local injection with anti-Fas Ab has been shown to inhibit tumor growth without inducing hepatitis (Rensing-Ehl et al., 1995). Therefore, the intratumoral administration of anti-FasL or Fas antibodies may be a useful approach for cancer immunotherapy. However, it should be noted that many tumors express Fas at high levels, suggesting that they would be sensitive to Fas-

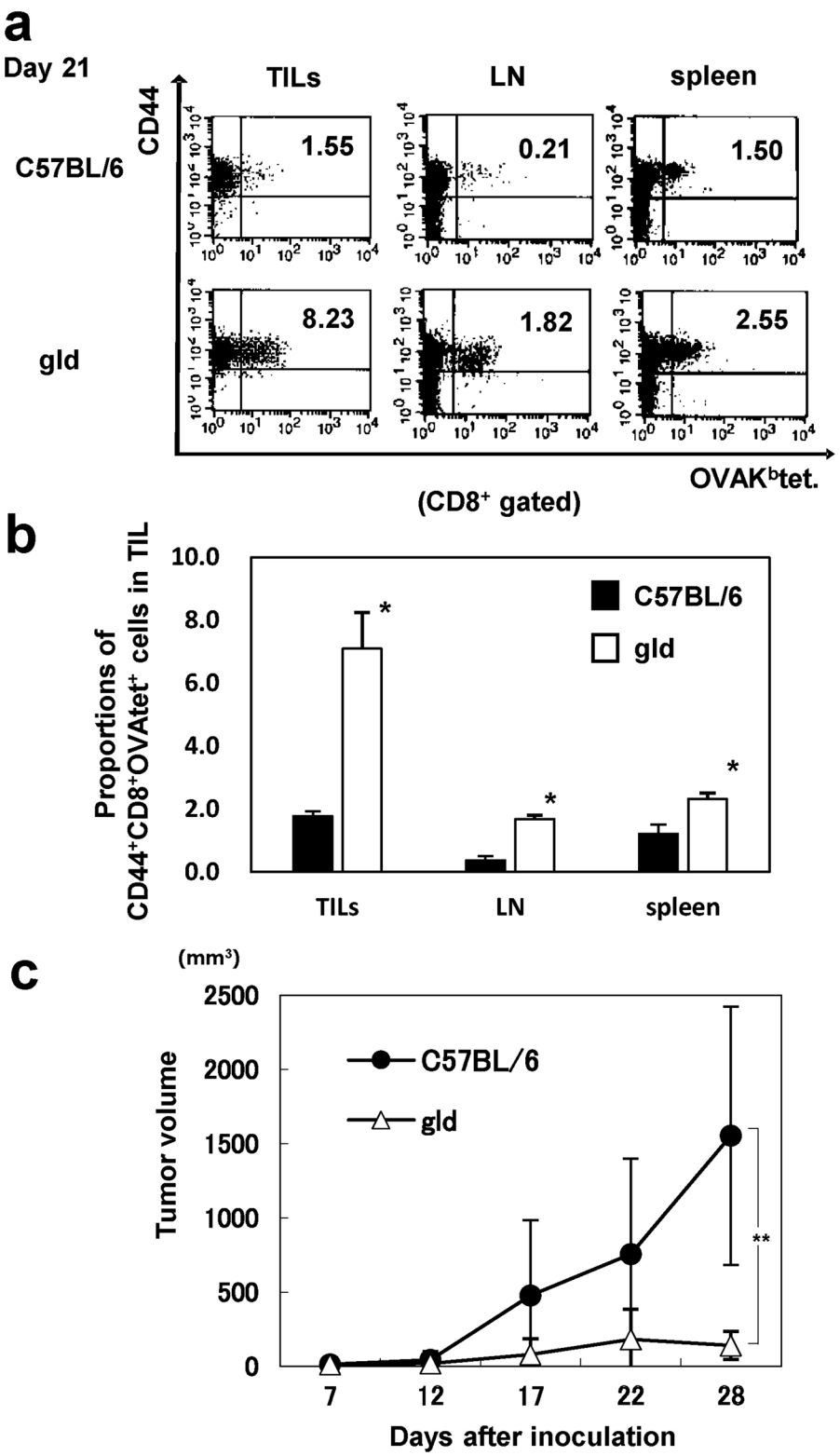

Fig. 4. Activated T cell death in Fas ligand (FasL)-dysfunctional gld mice during the tumor immune response. (*a*) $OVA_{257–264}$ specific $CD8^+$ T cells in the tumor-infiltrating lymphocytes (TILs), lymph node (LN) cells, and splenocytes of C57BL/6 mice or gld mice on day 21 after inoculation with EG.7. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on $CD8^+$ cells. The values represent the number of $OVA_{257–264}$ specific tetramer$^+$ $CD44^{high}$ as a percentage of $CD8^+$T cells. Representative results from three separate experiments are shown. (*b*) On day 21 after inoculation, proportions of $CD44^+$ $CD8^+$ $OVAtet^+$ cells in the TILs were shown. Data are presented as means ± SDs for five mice. ∗, $p < 0.05$, significant different between the values for C57BL/6 mice and these for gld mice. (*c*) The size of tumors in C57BL/6 mice or gld mice on the indicated days after the subcutaneous injection of EG.7. Data were obtained from three separate experiments and are expressed as means ± SD of nine mice at each point. Statistically significant differences between gld mice and C57BL/6 mice are shown (** $p < 0.01$).

induced apoptosis by FasL-expressing T lymphocytes (Trauth et al., 1989). Therefore, antagonistic Fas antibodies may inhibit the eradication of Fas-expressing tumor cells via the FasL-cytolytic pathways of activated T cells.

PD-1/PD-L1 signaling is critical for the induction of exhausted $CD8^+$ T cells during the tumor immune response (Barber et al., 2006; Wherry et al., 2007; Wherry and Kurachi,2015; Pauken and Wherry, 2015), but the relationship between PD-1/PD-L1 signaling and Fas/FasL signaling in exhausted $CD8^+$ T cells remains unclear. PD-1 reduces T cell survival by preventing the expression of the antiapoptotic gene Bcl-xL through the inhibition of PI3K activation during T cell activation (Parry et al., 2005) and by up-regulating the proapoptotic factor Bim (Gibbons et al., 2012). It has also been reported that the expression of PD-L1 in tumor cells increases apoptosis of activated tumor-reactive T cells, resulting in the induction of tumor growth (Dong et al., 2002). Here, we found that the expression of Fas and FasL were up-regulated in

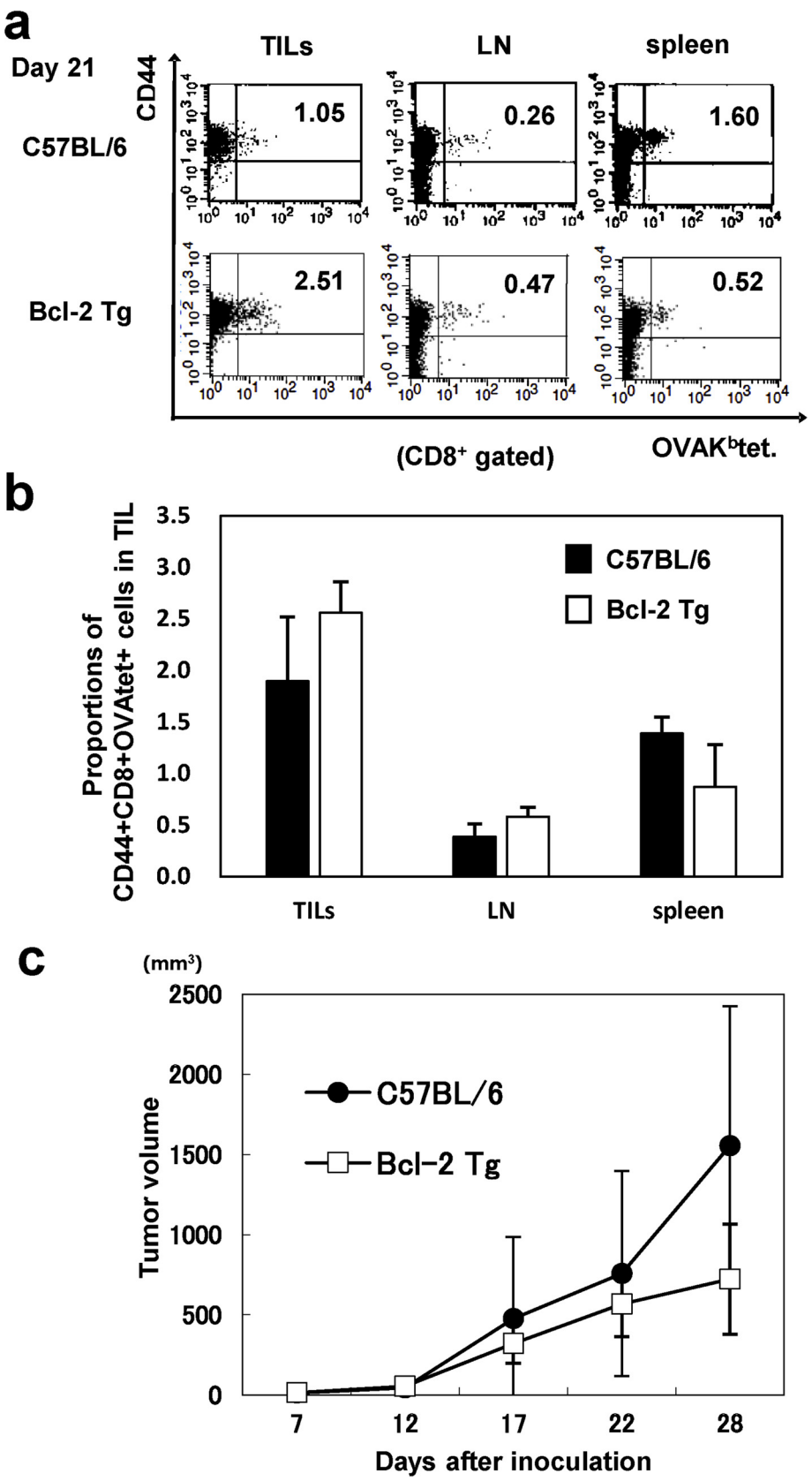

Fig. 5. Effect of the enforced expression of Bcl-2 on the apoptosis of exhausted $CD8^+$ T cells during the tumor immune response. (*a*) Number of $OVA_{257–264}$ specific $CD8^+$ T cells in the tumor-infiltrating lymphocytes (TILs), lymph node (LN) cells, and splenocytes of C57BL/6 mice or Bcl-2 Tg mice on day 21 after inoculation with EG.7. The results of flow cytometry are presented as typical profiles after an analysis gate had been set on $CD8^+$ cells. The values represent the number of $OVA_{257–264}$ specific tetramer$^+$ $CD44^{high}$ as a percentage of $CD8^+$T cells. Representative results from three separate experiments are shown. (*b*) On day 21 after inoculation, proportions of $CD44^+CD8^+OVAtet^+$ cells in the TILs were shown. Data are presented as means ± SDs for five mice. (*c*) The size of tumors in C57BL/6 mice or Bcl-2 Tg mice on the indicated days after subcutaneous injection with EG.7. Data were obtained from three separate experiments and are expressed as means ± SD of nine mice at each point.

activated T cells after TCR-mediated activation upon stimulation of PD-L1 in the tumor cells, indicating that the interaction between Fas and FasL may be a component of the PD-L1-mediated apoptosis of activated T cells. Thus, the combined blockade of both Fas and PD-L1 signaling in the locale of the tumor may induce the survival of exhausted $CD8^+$ T cells and thus prove useful for cancer immunotherapy.

Conflicts of interest

The authors declare that there are no conflicts of interest in relation to this manuscript.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.molimm.2019.01.014.

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