$CD8^+CD122^+CD49d^{low}$ regulatory T cells maintain T-cell homeostasis by killing activated T cells via Fas/FasL-mediated cytotoxicity

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The Fas/FasL (CD95/CD178) system is required for immune regulation; however, it is unclear in which cells, when, and where Fas/FasL molecules act in the immune system. We found that $CD8^+CD122^+$ cells, which are mostly composed of memory T cells in comparison with naïve cells in the $CD8^+CD122^-$ population, were previously shown to include cells with regulatory activity and could be separated into $CD49d^{low}$ cells and $CD49d^{high}$ cells. We established in vitro and in vivo experimental systems to evaluate the regulatory activity of $CD122^+$ cells. Regulatory activity was observed in $CD8^+CD122^+CD49d^{low}$ but not in $CD8^+CD122^+CD49d^{high}$ cells, indicating that the regulatory cells in the $CD8^+CD122^+$ population could be narrowed down to $CD49d^{low}$ cells. $CD8^+CD122^-$ cells taken from lymphoproliferation (*lpr*) mice were resistant to regulation by normal $CD122^+$ Tregs. $CD122^+$ Tregs taken from generalized lymphoproliferative disease (*gld*) mice did not regulate wild-type $CD8^+CD122^-$ cells, indicating that the regulation by $CD122^+$ Tregs is Fas/FasL-dependent. $CD122^+$ Tregs taken from IL-10–deficient mice could regulate $CD8^+CD122^-$ cells as equally as wild-type $CD122^+$ Tregs both in vitro and in vivo. MHC class I-missing T cells were not regulated by $CD122^+$ Tregs in vitro. $CD122^+$ Tregs also regulated $CD4^+$ cells in a Fas/FasL-dependent manner in vitro. These results suggest an essential role of Fas/FasL as a terminal effector of the $CD122^+$ Tregs that kill activated T cells to maintain immune homeostasis.

Tregs | Fas/FasL | cytotoxicity T cells | immune homeostasis | central memory phenotype

Fas (CD95) and its ligand FasL (CD178) compose a unique system that is strongly related to programmed cell death (1). FasL has been considered one of the effector molecules involved in the killing of target cells by cytotoxic T lymphocytes (CTLs) (2). When Fas binds to FasL, it induces downstream signal transduction pathways that subsequently activate cell death induction pathways (3, 4). Thus, the Fas/FasL system appears to act as an effector for CTL-mediated killing of virus-infected or cancer cells, similar to the perforin–granzyme system (5, 6). However, because Fas-mutant (*lpr*) and FasL-deficient (*gld*) mice show lymphoproliferative changes, it has been suggested that the Fas/FasL system is important for suppression/regulation of activated effector T cells (7, 8).

Molecular mechanisms after Fas activation have been thoroughly investigated, and the signal transduction pathway has been largely elucidated (9, 10). However, research on the cellular events that use the Fas/FasL system has progressed comparatively slowly. There are only a few relevant reports in this respect, mostly of human $CD4^+Foxp3^+$ Tregs that use the Fas/FasL system for their regulatory activity. [In this article, we use the term "Treg(s)" for any kind of T cells that show immune regulatory activity.] To complicate matters further, some contradictory reports claim that such Fas/FasL-engaging Tregs do not exist (8, 11, 12). No reliable reports suggesting that $CD8^+$ T cells use the Fas/FasL system for their regulatory action are available. Therefore, it is not clear precisely which subset of T cells express FasL or where Fas/FasL-dependent $CD8^+$ Tregs, if such cells exist, are located and at which point they function. Thus, the ultimate pathophysiological role of the Fas/FasL system is still unknown.

Regulation of the immune reaction is of pivotal importance for maintaining health in multicellular organisms. In highly developed animals, Tregs, a subset of T lymphocytes especially known as $CD4^+CD25^+Foxp3^+$ cells, are the main regulators of the immune response (13–16). However, it is not quite clear whether $CD8^+$ regulatory T cells exist; there are only few and contradictory reports on their existence, in contrast to the reports on $CD4^+$ Tregs. A population of predominantly $CD8^+$ suppressor T cells has been described and debated in the 1980s (17, 18). In the 2000s, we found and reported that the cells of central memory phenotype ($CD8^+CD122^+$) also possess the regulatory function, and some other reports regarding $CD8^+$ Tregs with some contradictory findings have been published. To date, more than 10 $CD8^+$ Treg populations with different markers have been reported (19).

One of the best characterized $CD8^+$ Treg populations is the $CD8^+CD122^+$ Treg (122^+ Treg) population. To confirm the existence of $CD8^+$ Tregs, markers that may be possible to distinguish Tregs from other T cells were examined. We previously found that CD49d can separate $CD8^+CD122^+$ cells into at least two subsets ($CD49d^{low}$ and $CD49d^{high}$) (20). To judge which cells have a stronger regulatory activity, we prepared two experimental systems:

Significance

Our study demonstrated that $CD8^+CD122^+CD49d^{low}$ regulatory T cells induced apoptosis in target T cells depending on death receptor Fas (CD95)–FasL (CD178) interaction. This in vitro phenomenon reflected the regulatory activity of these cells in vivo, which was also reproducible in the in vivo experimental system of adoptive T-cell transfer. By using natural mutant mice of Fas or FasL and the gene-targeted mice of IL-10, we confirmed that the regulatory activity of $CD8^+CD122^+CD49d^{low}$ cells was mediated by Fas–FasL but not by IL-10. In addition, the necessity of MHC class I molecules on the target T cells was also confirmed by analysis using $CD8^+$ T cells that lack β2-microglobulin and consequently class I MHC.

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an in vitro system, based on cell culture, and an in vivo system, based on adoptive transfer of T cells.

Interestingly, $CD8^+$ Tregs express CD122 (IL-2/IL-15 receptor β chain) in contrast to $CD4^+$ Tregs, which express CD25 (IL-2 receptor α chain), indicating the fundamental importance of IL-2 at the development/maturation of both $CD4^+$ and $CD8^+$ Tregs. Suzuki and coworkers generated CD122-deficient mice using gene targeting (21) and used them to identify 122^+ Tregs (22). In this study, which is a follow-up study to our previous report, we performed in vitro and in vivo experiments to gain further understanding of these cells. We found that CD49d might be a good marker for classifying $CD8^+$ T cells into naïve, resting T cells ($CD62L^+CD122^-$), effector memory T cells ($CD62L^-$), and T cells of central memory phenotype ($CD122^+CD49d^{low}$), by using multicolor staining of CD62L, CD122, and CD49d. To our knowledge, this is the first report on the role of the Fas/FasL system in the action of $CD8^+CD122^+$ Tregs (122^+ Tregs) (22–25). Additionally, we show that $CD8^+$ Tregs are included in the $CD49d^{low}$ population, which corresponds to the central memory T-cell population, and that their mechanism of suppression depends on the cytotoxicity mediated by the Fas and FasL interaction.

Results

$CD8^+CD122^+$ Cells Are Divided into Subpopulations Expressing Different Sets of Cell-Surface Markers. Although it has been shown that the $CD8^+CD122^+$ population contains Tregs, $CD8^+CD122^+$ cells are generally considered memory T cells. Thus, we needed to identify a better marker to focus on regulatory cells in the $CD8^+CD122^+$ population. We chose CD62L and CD49d; CD62L allows separation of effector memory T cells. CD122 is also frequently used as well as CD44 and CD62L for the analysis of memory and regulatory cells (26). We examined the expression of these cell-surface markers in murine splenic T cells (Fig. 1 *A* and *B*). At least four different subsets of $CD8^+$ cells could be distinguished by this analysis. Their profiles were $CD62L^+CD122^-$, $CD62L^+CD122^+CD49d^{low}$, $CD62L^+CD122^+CD49d^{high}$, and $CD62L^-$. The first subset is usually referred to as $CD122^-$ cells and consists mostly of naïve, resting $CD8^+$ cells (Fig. 1*B*). The last three subsets could be classified into memory or activated cells and may also contain regulatory cells.

$CD8^+CD122^+CD49d^{low}$ Cells Show Regulatory Activity in Vitro and in Vivo but $CD8^+CD122^+CD49d^{high}$ Cells Do Not. We found that $CD8^+CD122^+$ cells can be divided into two populations by their expression level of CD49d. Then, we performed an in vitro experiment in which candidate Tregs were cocultured with their target cells. As the mechanism of regulation by $CD8^+$ Tregs is thought to involve elimination of the target cells (27), candidate Tregs should reduce the number of target (regulated) cells during coculture. Representative results after 72 h of coculture are presented in Fig. 1 *C* and *D*. When $CD49d^{low}$ cells ($CD45.1^-$) were mixed with $CD8^+CD122^-$ cells ($CD45.1^+$) at a 1:1 ratio at the beginning of coculture, the percentage of $CD8^+CD122^-CD45.1^-$ cells markedly decreased during coculture in a time-dependent manner (Fig. 1*C*). When $CD122^-$ cells were cocultured with $CD49d^{high}$ cells, the decrease in the percentage of $CD122^-$ cells was much less marked than when they were cocultured with $CD49d^{low}$ cells (Fig. 1 *C* and *D*). These results of relative change between two different cells cocultured were the reflection of the absolute number of live cells, which is presented in Fig. S1*A*.

These results indicate that $CD8^+CD122^-$ cells reduce their number by some effect of $CD8^+CD122^+$ cells, and such an effect is much stronger in $CD49d^{low}$ cells than in $CD49d^{high}$ cells. However, this interpretation should be adapted only if the proliferation speed of $CD49d^{low}$ cells and $CD49d^{high}$ cells is identical. Therefore, we examined the growth of separately cultured $CD49d^{low}$ and $CD49d^{high}$ cells. As a result, both cells grew at virtually the same rate (Fig. 1*E*, not significantly different, as determined by Student's *t* test, $P > 0.1$ for any culture duration), supporting that $CD49d^{low}$ cells, but not $CD49^{high}$ cells, possess an activity to reduce the number of cocultured $CD8^+CD122^-$ cells, which is possibly achieved by the elimination of target cells (i.e., cytotoxic activity).

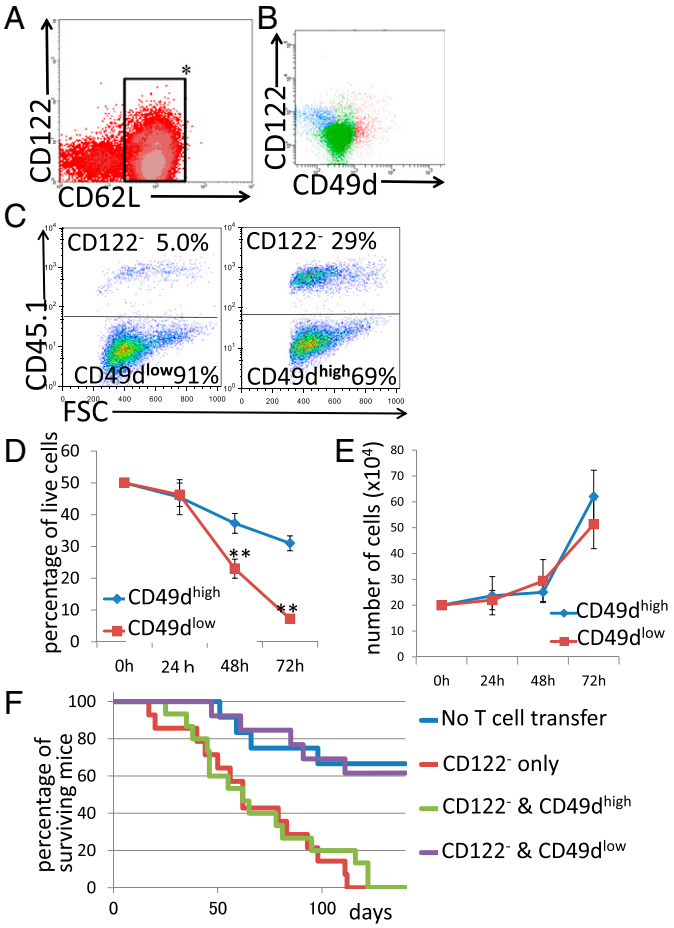

Fig. 1. (*A*) Spleen cells were analyzed for the expression of CD8, CD122, CD62L, and CD49d. (*B*) $CD62L^+$ cells (inside of the rectangle in *A*) were analyzed for the expression of CD122 and CD49d. (*C* and *D*) Analysis of regulatory activity in coculture. (*C*, *Left*) $CD8^+CD122^-CD45.1^+$ cells and ($CD8^+CD122^+$) $CD49d^{low}CD45.1^-$ cells. (*C*, *Right*) $CD8^+CD122^-CD45.1^+$ cells and ($CD8^+CD122^+$) $CD49d^{high}CD45.1^-$ cells. Representative results obtained after 72 h of coculture are shown. (*D*) The percentages of $CD8^+CD122^-$ on total live cells obtained from three independent experiments are shown. **Significantly different at the same time point ($P < 0.01$, Student's *t* test). (*E*) Cell growth analysis of $CD49d^{low}$ and $CD49d^{high}$ T cells. For all time points, the values obtained for $CD49d^{low}$ (red line) and $CD49d^{high}$ T cells (blue line) did not significantly differ ($P > 0.05$, Student's *t* test). (*F*) Analysis of adoptive T-cell transfer to RAG-$2^{-/-}$ mice. Transfer of a mixture of $CD49d^{low}$ and $CD122^-$ T cells (purple line) effectively prolonged survival of the mice; survival of these mice was significantly higher than that of mice receiving $CD122^-$ cells alone (red line; log-rank test, $P = 0.0003$; Wilcoxon test, $P = 0.0014$). In contrast, transfer of a mixture of $CD49d^{high}$ and $CD122^-$ T cells did not significantly affect survival (green line) compared with that of mice receiving $CD122^-$ cells alone (log-rank test, $P = 0.5271$; Wilcoxon test, $P = 0.8964$).

For the in vivo experiment, we essentially followed the method established by Rifa'i et al. (22), with minor modifications. We performed T-cell adoptive transfer into RAG-$2^{-/-}$ mice and followed their survival. The $CD49d^{high}$ cells did not support survival of RAG-2–deficient mice that had received $CD8^+CD122^-$ cells. In contrast, the $CD49d^{low}$ cells clearly reversed the survival of mice to the level of those that had not received T cells (Fig. 1*F*, blue line). Together with the results of the in vitro experiment, these results

confirmed that $CD8^+$ Tregs are encompassed in the $CD49d^{low}$ subset. Thus, in the following text, $CD49d^{low}$ cells are described as "$CD8^+$ Tregs" to reduce the text volume.

$CD8^+CD122^+CD49d^{low}$ Tregs Cannot Eliminate or Suppress Fas-Mutated $CD8^+CD122^-$ Cells from *lpr* Mice. We tested the regulatory activity of $CD8^+$ Tregs under Fas-related conditions. $CD8^+$ Tregs, taken from wild-type mice, were cocultured with $CD8^+CD122^-$ cells, taken from Fas-mutant *lpr* mice, and the percentage of target $CD8^+CD122^-$ cells among total live cells was determined at various time points. The ratio of $CD8^+CD122^-$ cells from *lpr* mice to wild-type $CD8^+$ Tregs decreased as coculture continued (Fig. 2 *A* and *B*). However, the rate and extent of decrease were significantly higher when $CD8^+$ $CD122^-$ cells and $CD8^+$ Tregs, both taken from wild-type mice, were cocultured (Fig. 2 *A* and *B*). These changes in the ratio of cell number were also observed in the data of absolute cell numbers (Fig. S1*B*). The proliferation rate of $CD8^+CD122^-$ cells from *lpr* mice was not different from that of $CD8^+CD122^-$ cells from wild-type mice (Fig. 2*C*). In addition, we examined the proliferation of cocultured cells by the dilution of prelabeled CFSE and further confirmed that any types of cells used in this study show a similar pattern of CFSE peaks (Fig. 2*D*). This result confirmed that the difference observed between $CD8^+CD122^-$ cells from *lpr* mice and those from wild-type mice in the coculture experiment was not due to a difference in proliferation rate but due to resistance to apoptosis of the *lpr* mice-derived $CD8^+CD122^-$ cells. In the in vivo experiment, RAG-2–deficient mice that had received $CD8^+CD122^-$ cells taken from *lpr* mice died, similar to those injected with $CD8^+CD122^-$ cells taken from wild-type mice. Mice that had received a mixture of $CD8^+CD122^-$ cells from *lpr* mice and $CD8^+$ Tregs obtained from wild-type mice died, similar to those that had received $CD8^+CD122^-$ cells from *lpr* mice alone (Fig. 2*E*). Together, these findings indicated that Fas-mutant $CD8^+CD122^-$ cells are resistant to regulation by $CD8^+$ Tregs, which have a marked effect on wild-type $CD8^+CD122^-$ cells and prolong the survival of $CD8^+CD122^-$ cell-recipient mice.

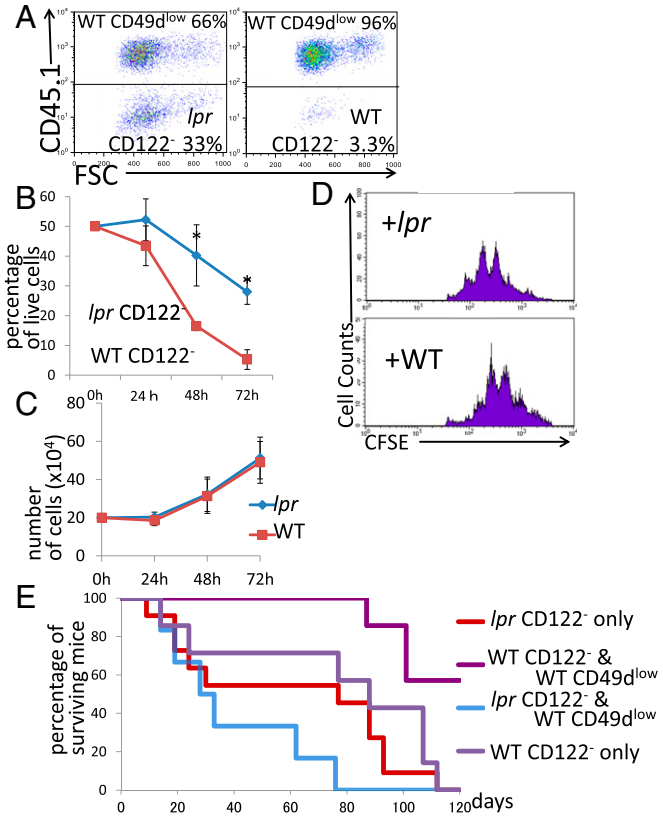

Fig. 2. (*A* and *B*) Analysis of regulatory activity in coculture of $CD49d^{low}$ cells (CD45.1) and $CD8^+CD122^-$ cells (CD45.2) derived from *lpr* mice and wild-type mice. (*A*) Representative results after 72 h of coculture obtained from three independent experiments are shown. (*Left*) $CD122^-$ cells (*lpr*) cocultured with $CD49d^{low}$ cells. (*Right*) $CD122^-$ cells (WT) cocultured with $CD49d^-$ cells. (*B*) The percentages of $CD8^+CD122^-$ cells among total live cells are shown. *Significantly different ($P < 0.05$, Student's *t* test). (*C*) Absolute numbers of $CD122^-$ T cells derived from WT mice (red line) and *lpr* mice (blue line) in simple culture were determined. At all time points, the values obtained for *lpr* mice-derived cells and WT mice-derived cells were not significantly different ($P > 0.05$, Student's *t* test). (*D*) $CD8^+CD122^-$ T cells (WT or *lpr*) labeled with CFSE were cocultured for 48 h and analyzed. The dilution patterns of CFSE are shown as representative results of three independent experiments. (*E*) Analysis of adoptive T-cell transfer of $CD122^-$ T cells from WT or *lpr* mice mixed with $CD49d^{low}$ T cells of WT mice into RAG-2–deficient mice. Addition of WT mice-derived $CD49d^{low}$ T cells to *lpr* mice-derived $CD122^-$ cells (blue line) did not affect survival; survival of these mice was not significantly different from that of mice receiving *lpr* mice-derived $CD122^-$ cells alone (red line; log-rank test, $P = 0.0878$; Wilcoxon test, $P = 0.2537$).

FasL-Mutant $CD8^+$ Tregs Cannot Eliminate or Suppress Conventional $CD8^+CD122^-$ T Cells. First, the expression of FasL in $CD8^+$ Tregs was confirmed by real-time PCR (Fig. 3*A*). Furthermore, the existence of the FasL product on the cell surface was confirmed by flow cytometry (Fig. 3*B*). This result indicated that FasL is a candidate mediator of killing of Fas-expressing target cells.

Next, we examined the activity of FasL-mutant $CD8^+$ Tregs. $CD8^+$ Tregs taken from *gld* mice were cocultured with $CD8^+CD122^-$ cells taken from wild-type mice, and the percentage of target cells ($CD8^+CD122^-$) among $CD8^+$ Tregs was determined at various time points. The percentage of wild-type $CD8^+CD122^-$ cells among $CD8^+$ Tregs from *gld* mice decreased over time; however, the rate and extent of decrease were significantly lower than when both types of cells were taken from wild-type mice (Fig. 3 *C* and *D*). The difference was also observed in the absolute cell number (Fig. S1*C*). The proliferation rate of $CD8^+$ Tregs taken from *gld* mice was not different from that of wild-type $CD8^+$ Tregs (Fig. 3*E*). This result confirmed that an insufficient decrease of cells cocultured with $CD8^+$ Tregs taken from *gld* mice, compared with those cocultured with wild-type $CD8^+$ Tregs, was not due to a relative decrease in the proliferation rate of $CD8^+$ Tregs taken from *gld* mice but rather due to insufficient elimination of $CD8^+CD122^-$ cells by FasL-mutated $CD8^+$ Tregs.

In the in vivo experiment, RAG-2–deficient mice that had received a mixture of wild-type $CD8^+CD122^-$ cells and $CD8^+$ Tregs from *gld* mice died at the same rate as those that had received wild-type $CD8^+CD122^-$ cells alone (Fig. 3*F*). Thus, $CD8^+$ Tregs taken from FasL-mutant *gld* mice demonstrated no ability to control $CD8^+CD122^-$ cells and could not prolong the survival of mice that received these cells.

$CD8^+$ Tregs Induce Apoptosis in Target-Activated $CD8^+CD122^-$ T Cells. To confirm the cytotoxic mechanisms underlying the regulatory action of $CD8^+$ Tregs, we examined cell death during coculture by staining the cells with annexin V and 7-amino actinomycin D (7-AAD) (Fig. 4 *A* and *B*). Although we could not detect annexin V^+7-AAD^- cells, we observed annexin V^+7-AAD^+ cells. $CD8^+CD122^-$ cells cocultured with $CD8^+$ Tregs showed a higher percentage of 7-AAD^+ cells than those cocultured with $CD49^{high}$ cells, indicating that $CD8^+$ Tregs induced apoptosis in $CD8^+CD122^-$ T cells. $CD8^+CD122^-$ cells taken from *lpr* mice did not show an increase in 7-AAD–stained cells after coculture with $CD8^+$ Tregs, indicating that Fas-mutant $CD8^+CD122^-$ cells are resistant to apoptosis induced by $CD8^+$ Tregs (Fig. 4*B*).

$CD8^+CD122^+CD49d^{low}$ T Cells Induce Apoptosis via Caspase 8 Pathway. Judging from the experimental results both in vitro and in vivo, it is strongly suspicious that the regulatory mechanism performed by

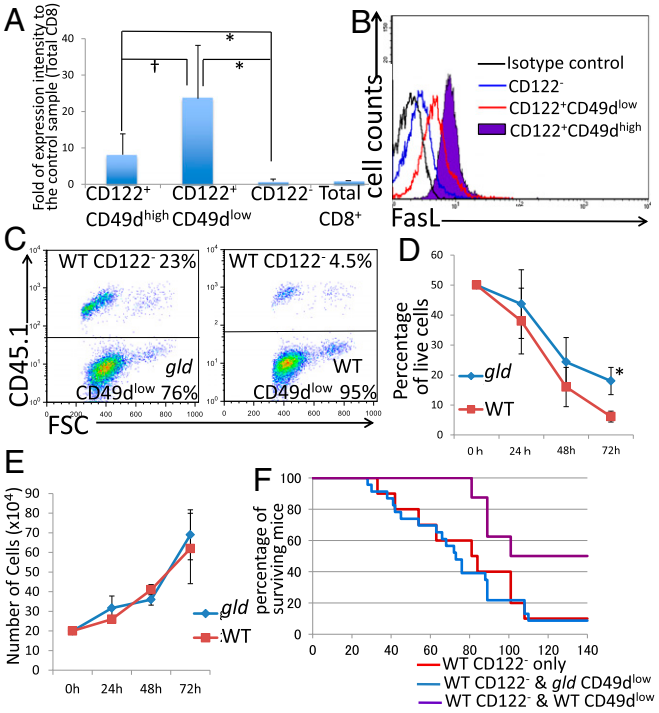

Fig. 3. (*A*) Analysis of RNA expression level of FasL in each population of CD8$^+$ T cells by real-time PCR. For each sample, FasL mRNA levels were normalized to β-actin and are expressed as fold change relative to the control sample (1.00). †Not significantly different ($P > 0.05$, Student's *t* test); **significantly different ($P < 0.01$, Student's *t* test). (*B*) Analysis of FasL expression of flow cytometory. (*C* and *D*) Analysis of coculture of CD8$^+$CD122$^-$CD45.1$^+$ T cells and CD49d^{low} from *gld* or WT mice. (*C*) Representative results obtained from three independent experiments. (*D*) *Significantly different ($P < 0.05$, Student's *t* test). (*E*) Cell growth analysis of CD49d^{low} T cells from *gld* mice and WT mice. At all time points, the results obtained from two different mice were not significantly different ($P > 0.05$, Student's *t* test). (*F*) Analysis of adoptive T-cell transfer of CD122$^-$ T cells (WT) mixed with CD49d^{low} T cells (WT or *gld*) into RAG-2–deficient mice. Addition of *gld* mice-derived CD49d^{low} T cells to CD122$^-$ cells (blue line) had no significant effect on survival compared with transfer of CD122$^-$ cells alone (red line; log-rank test, $P = 0.7608$; Wilcoxon test, $P = 0.6859$).

the CD49d^{low} cells is elimination of cells by using the Fas/FasL system. To prove the involvement of the Fas/FasL system in our T-cell coculture assay, we introduced the assay system of PhiPhiLux and CaspaLux. It is well known that caspases are the key molecule of apoptosis, and caspase 8 is especially directly linked to the Fas-derived signal. When caspase 8 is active, Caspalux-8, a substrate for caspase 8, is digested and becomes a fluorescent substance that can be detected by flow cytometry. As a result, more caspase 8-active cells were observed in the cells cocultured with CD49d^{low} cells (Fig. 4 *C* and *D*), indicating that Fas and its downstream caspase, caspase 8, was activated during the process of apoptosis induction.

CD8$^+$ Tregs Are Increased in Fas/FasL-Mutant Mice but Not in IL-10–Deficient Mice. We demonstrated that CD8$^+$ Tregs, but not CD49d^{high} cells, display regulatory activity that can eliminate cocultured CD8$^+$CD122$^-$ cells in vitro and prolong survival of CD8$^+$CD122$^-$ T-cell–recipient RAG-deficient mice in vivo. The cause of death in CD8$^+$CD122$^-$ T-cell–recipient RAG-deficient mice was not evident but might involve failure of multiple organs, including bone marrow, lung, liver, etc. Additionally, we found that lack of Fas/FasL function affects the regulatory activity of CD8$^+$ Tregs both in vitro and in vivo. Therefore, we expected some changes in CD8$^+$regs of Fas/FasL-mutant mice. The absolute number of CD8$^+$ Tregs and their percentage on total CD8$^+$ cells were significantly increased in *lpr* and *gld* mice but not in IL-10–deficient mice, compared with wild-type mice (Fig. 4 *E* and *F*). This finding indicates the existence of a negative feedback mechanism that controls the activity of CD8$^+$ Tregs.

CD8$^+$ Tregs Cannot Eliminate or Suppress MHC Class I-Deficient CD8$^+$CD122$^-$ Cells. Next, we asked whether recognition of target cells by CD8$^+$ Tregs depends on T-cell receptor (TCR)–MHC class I interaction. To address this question, we used β2-microglobulin–deficient instead of MHC class I-deficient mice; β2-microglobulin–deficient mice have very few CD8$^+$ cells because of a lack of positive selection of CD8$^+$ T cells. This problem can be addressed by generating wild-type mice with bone marrow reconstituted with MHC class I-deficient bone marrow. When CD8$^+$CD122$^-$ cells, taken from the bone marrow-reconstituted mice, were cocultured with CD8$^+$ Tregs (Fig. 5 *A* and *B*). The rate and extent of reduction were lower than when they were cocultured with wild-type CD8$^+$CD122$^-$ cells (Fig. 5 *A* and *B*). The proliferation of CD8$^+$CD122$^-$ cells from mice in which the bone marrow had been reconstituted with that of β2-microglobulin–deficient mice was not significantly different from that of CD8$^+$CD122$^-$ cells

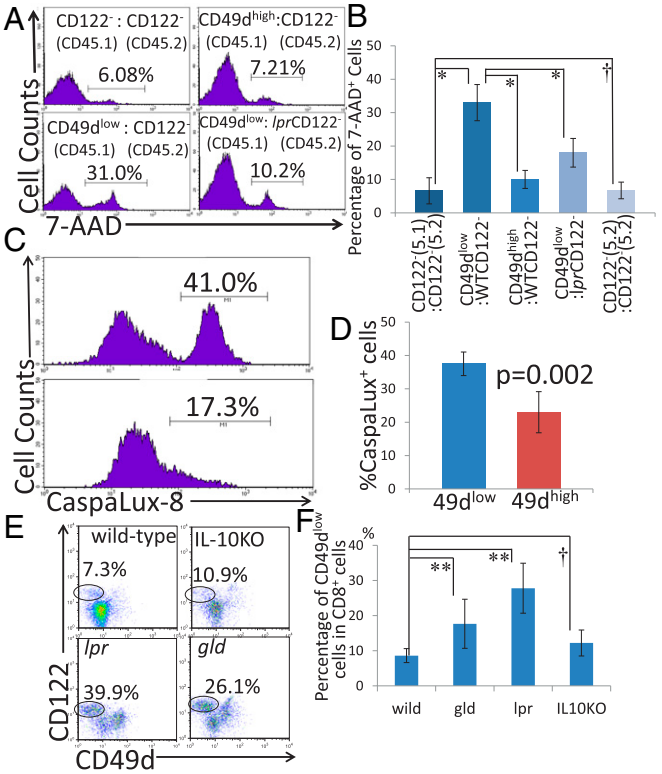

Fig. 4. (*A*) Result of 7-AAD analysis after 48 h of coculture. Percentages of 7-AAD–positive cells among total cultured cells are shown in the histogram. (*B*) Percentages of 7-AAD–positive cells in coculture. *Significantly different ($P < 0.01$, Student's *t* test); †not significantly different ($P > 0.05$, Student's *t* test). (*C* and *D*) Analysis of caspase 8 activity in CD122$^-$ (CD45.1$^+$) cells using CaspaLux8 after coculture with CD49d^{low} or CD49d^{high} (CD45.1$^-$) cells. (*C*) Representative results obtained from three independent experiments. (*D*) Percentages of cells positive for CaspaLux8 in CD122$^-$ CD45.1$^+$ cells are shown. The data are averages of three independent experiments. (*E*) Spleen cells taken from WT, IL-10–deficient, *lpr*, and *gld* mice were analyzed by flow cytometry. Representative results obtained from three independent experiments for each mouse strain are shown. (*F*) Percentages of CD49d^{low} cells in total CD8$^+$ cells in each group are shown. Statistically significant differences between the WT and each mutant strain were determined. **Significantly different ($P < 0.01$, Student's *t* test); †not significantly different ($P > 0.05$, Student's *t* test).

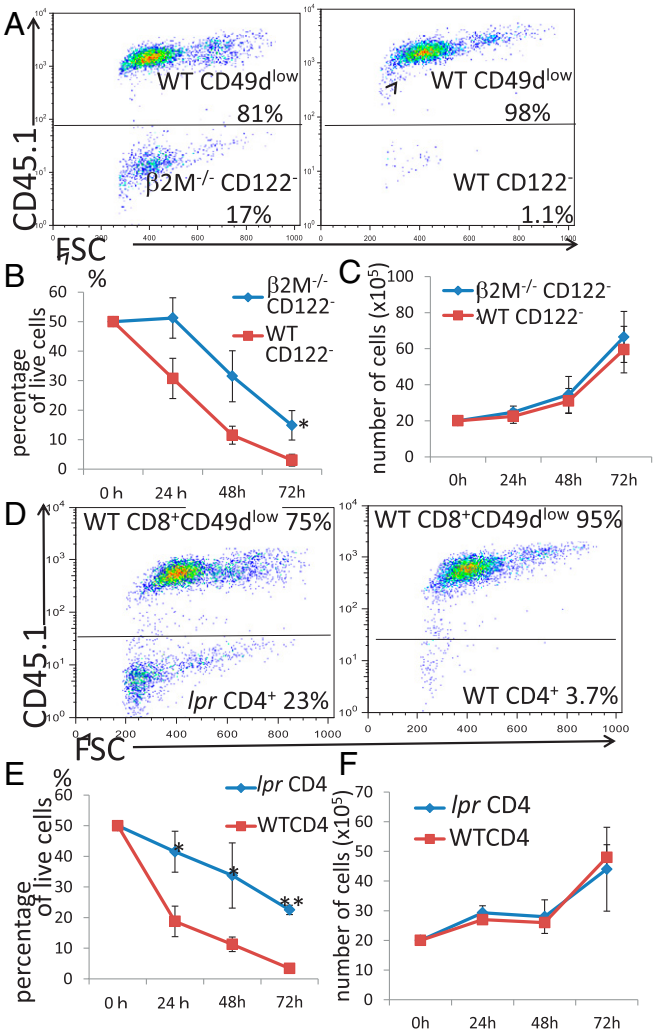

Fig. 5. (*A* and *B*) Analysis of coculture of CD49d^{low} cells (CD45.1) with CD8$^+$CD122$^-$ cells (CD45.2) derived from mice in which the bone marrow had been replaced with that from β2M (β2-microglobulin)-deficient mice. (*A*, *Left*) CD49d^{low} (CD45.1$^+$) cocultured with CD122$^-$(β2MKO) for 72 h. (*A*, *Right*) CD49d^{low} (CD45.1$^+$) cocultured with CD122$^-$ (WT) for 72 h. Representative results after 72 h of coculture obtained from three independent experiments are shown. (*B*) The percentages of CD8$^+$CD122$^-$ cells among total live cells are shown in three independent experiments. *Significantly different ($P < 0.05$, Student's *t* test). (*C*) Absolute numbers of CD8$^+$CD122$^-$ T cells derived from WT mice or from mice (β2MKO) in simple culture. At all time points, no significant differences were found ($P > 0.05$, Student's *t* test). (*D* and *E*) Analysis of coculture of CD49d^{low} T cells (CD45.1$^+$) with CD4$^+$CD25$^-$ T cells derived from *lpr* or WT mice. (*D*, *Left*) CD49d^{low}(CD45.1$^+$) cocultured with CD4$^+$ (*lpr*) for 72 h. (*D*, *Right*) CD49d^{low}(CD45.1$^+$) cocultured with CD4$^+$ (WT) for 72 h. Representative results after 72 h of coculture obtained from three independent experiments are shown. (*E*) Percentages of CD4$^+$ cells among total live cells are shown. Data were obtained from three independent experiments. *$P < 0.05$, Student's *t* test; **$P < 0.01$, Student's *t* test. (*F*) CD4$^+$CD25$^-$ T cells derived from WT and *lpr* mice were harvested, and the absolute numbers of live cells were determined. Average values of three independent experiments are shown. At all time points, the values were not significantly different ($P > 0.05$, Student's *t* test).

taken from wild-type mice (Fig. 5C). This result confirmed that the recovery of the decrease in CD8$^+$CD122$^-$ cells from the bone marrow-reconstituted mice, compared with that in cells from wild-type mice, was not due to a difference in the proliferation rate between the CD8$^+$CD122$^-$ cells taken from the two different types of mice. Taken together, these results strongly indicate that CD8$^+$ Tregs recognize their target cells by interaction between a MHC class I molecule (plus an antigen) and the TCR.

CD8$^+$ Tregs Can Eliminate CD4$^+$ Cells in a Fas-Dependent Manner. One remaining question was whether, besides CD8$^+$ cells, CD8$^+$ Tregs can also suppress and/or eliminate CD4$^+$ cells. CD4$^+$ cells taken from wild-type and *lpr* mice were cocultured with CD8$^+$ Tregs, and the numbers of surviving cells were determined using flow cytometry. CD8$^+$ Tregs were indeed able to reduce the number of CD4$^+$ cells. This activity was evident for wild-type as well as *lpr* mouse-derived CD4$^+$ cells; however, the activity of *lpr*-derived cells was significantly lower than that of wild-type CD4$^+$ cells, indicating that it is Fas-dependent (Fig. 5 *D* and *E*). The proliferation rate of CD4$^+$ cells from *lpr* mice and that of wild-type cells was not significantly different (Fig. 5*F*), indicating that it is Fas-dependent. The proliferation rates of CD4$^+$ cells from *lpr* mice and that of wild-type cells were not significantly different (Fig. 5*F*), indicating that the difference in the reduction speed in coculture was not due to a difference in proliferation rate per se but was due to the elimination of activated CD4$^+$ cells by CD8$^+$ Tregs.

Discussion

The precise cellular events involved in the process of immune regulation related to Fas/FasL systems are largely unknown. Because Fas/FasL-mutated animals are prone to develop autoimmune diseases rather than immunodeficiency and FasL is known to be expressed in CD8$^+$ cytotoxic T cells, it has been predicted that these cells are involved in immune regulation. Additionally, activated CD8$^+$ T cells have been reported to kill clonally established T-cell lines; however, there is no firm evidence that the Fas/FasL system on particular CD8$^+$ T cells in fact regulates the immune response (28). This is the first report describing that CD8$^+$ Treg cells directly control CD8$^+$CD122$^-$ T cells both in vivo and in vitro by using the Fas/FasL system.

CD8$^+$CD122$^+$ Tregs were first reported by Rifa'i et al. (22). However, studies of CD8$^+$CD122$^+$ Tregs have been complicated by the concept of memory T cells marked as CD8$^+$CD122$^+$. Some investigators have tried to separate Tregs from memory T cells by staining the CD8$^+$CD122$^+$ cells with a third marker, such as PD-1 (29), whereas such trials have not succeeded well in persuading other investigators. However, our experimental results obtained in the former studies (20–30) and the present studies have definitely shown the regulatory activity in this T-cell subset. Our current interpretation is that the regulatory and the memory functions are compatible. These cells with regulatory activity may belong to CD8$^+$ memory T cells, possibly recognize self-antigens by their TCR, and induce apoptosis of Fas-expressing activated T cells. Based on our new understanding, it should be possible that cells with memory phenotype (CD8$^+$CD122$^+$) possess the regulatory activity at the same time.

Rifa'i et al. and Endharti et al. (31, 32) reported the regulatory activity of CD8$^+$CD122$^+$ T cells in both in vivo and in vitro experiments. We first examined which subset of the CD8$^+$CD122$^+$ cells possessed regulatory activity. Okuno et al. (20) found that CD8$^+$CD122$^+$ cells in murine peripheral immune organs can be divided into at least two populations according to their expression level of CD49d. The current study indicated that the final suppression mechanism used by the CD8$^+$CD122$^+$ Tregs appears to be killing of the activated T cells, which depends on the Fas/FasL pathway, because Fas-deficient T cells derived from *lpr*/*lpr* mice were resistant to killing (Fig. 2 *A* and *B*) and FasL-deficient Tregs derived from *gld*/*gld* mice could not perform this regulatory activity (Fig. 3 *C* and *D*).

The molecular mechanism underlying immune suppression by CD8$^+$ Tregs and the chemical pathway involved in the regulatory effect of these cells are still largely unknown. We previously reported that CD8$^+$CD122$^+$ Tregs produce IL-10 and that the

cytokine could be the effector molecule of these $CD8^+$ Tregs. Although both the mechanism of recognition of target cells and the effector molecule of regulation by $CD8^+$ Tregs remain to be elucidated, this study yielded some important clues: Activation of Fas via interaction with FasL is largely associated with the killing of T cells that are no longer needed and rather harmful for survival. Another important molecular interaction is that of MHC class I and TCR. β2-microglobulin–deficient cells are neither regulated in the previous study (33) nor in the present study, suggesting the importance of specific recognition between "the regulatory cells" and "the regulated cells." Another important molecule involved in the action of $CD8^+$ Tregs might be Qa-1, a nonclassical MHC class I molecule (MHC-Ib), as H. Cantor and his colleague found and reported a $CD8^+$ T-cell subset that showed a regulatory activity under a Qa-1–restricted manner (30). In their recent publications, however, the Qa-1–restricted $CD8^+$ Tregs they proposed were apparently shown to be $CD122^+CD44^+$ (34, 35). The Qa-1–restricted $CD8^+$ Treg is overlapped with or involved in the $CD8^+CD122^+$ Treg reported by H. Suzuki and his colleagues (22). Two $CD8^+$ Tregs that were formerly thought to be distinct cell populations turned out to be the same one, further confirming the existence of $CD8^+$ regulatory T cells, regardless of whether they are the naturally arising type or the induced type. Although the relation between $CD4^+$ Tregs and $CD8^+$ Tregs has not been clarified in detail, both Tregs are indispensable to maintain T-cell homeostasis, and their cooperative work has been suggested in the prevention and cure of an autoimmune disease (31).

This study forms a basis for continued research of immune suppression by $CD8^+CD122^+$ Tregs. Elucidation of the effector mechanisms of $CD8^+$ and $CD4^+$ Tregs may pave the way to improve immune regulation by Tregs in clinical practice.

Materials and Methods

Mice. Animal care was performed according to the guidelines of Nagoya University (Nagoya, Japan). Experimental protocols were approved by the Ethics Committee of the Nagoya University Graduate School of Medicine (Nos. 27015, 27017, and 27365).

Adoptive T-Cell Transfer to RAG-2$^{-/-}$ Mice. Sorted $CD8^+CD122^-$ T cells (4×10^5 per recipient mouse) simply or mixed with $CD8^+CD122^+$ T cells ($49d^{low}$ or $CD49^{high}$) (1×10^5 per recipient mouse) were i.v. transferred into 8–10-wk-old RAG-2–deficient mice that had received a sublethal dose of irradiation (5.5 Gym). The survival of the T-cell–recipient mice was followed up after adoptive T-cell transfer, and the results were analyzed using Kaplan–Meier survival curves.

Activation of $CD8^+$ T Cells. $CD8^+$ T cells in any desired population were collected from murine spleen and lymph node cells by using a FACS SORP Aria II cell sorter (BD Biosciences) and cultured in an anti-mCD3 antibody-coated 96-well plate for 24 h (1.0×10^5 cells per well).

T-Cell Culture. Activated T cells as described above were either cocultured or cultured alone in RPMI-1640 media containing 10% (vol/vol) FBS and 20 ng/mL of interleukin-2 (PeproTech Inc.) in a round-bottom 96-well plate for 72 h (0.5×10^5 cells per well).

For other information, see *SI Materials and Methods*.

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