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Fas Ligand Costimulates the In Vivo Proliferation of CD8+ T Cells

Ivy Suzuki, Stefan Martin, Tamar E. Boursalian, Courtney Beers, and Pamela J. Fink

Fas ligand (FasL/CD95L/APO-1L) is one of a growing number of TNF family members whose triggering costimulates maximal proliferation of activated T cells. In this study we show that maximal Ag-dependent accumulation of transferred TCR-transgenic CD8+ T cells requires Fas (CD95/APO-1) expression by the adoptive hosts. Additionally, adoptively transferred FasL+ CD8+ T cells demonstrate a 2-fold advantage in Ag-driven expansion over their FasL− counterparts. This study illustrates the in vivo role of FasL costimulation in the Ag-specific proliferation of both heterogeneous and homogeneous populations of primary CD8+ T cells and long-term CTL lines. Thus, cross-linking FasL on naive and Ag-experienced CD8+ T cells whose Ag-specific TCRs are engaged is required to drive maximal cellular proliferation in vivo. The Journal of Immunology, 2000, 165: 5537–5543.

Members of the TNF and TNF receptor (TNFR) families comprise the majority of activation-induced costimulatory receptor-ligand pairs, which serve both to enhance T cell expansion and differentiation and to mediate T cell-B cell interactions (reviewed in Refs. 1 and 2). The TNFR family includes CD30, CD40, Fas, TNFR1, and TNFR2, all type I membrane proteins whose extracellular ligand-binding region is characterized by repeating cysteine-rich domains. The TNF family includes CD30 ligand (CD30L), CD40L, Fas ligand (FasL), lympho toxin α (LTα), LTβ, TNF-α, and TNF-β. With the exception of LTα, these molecules are type II membrane proteins characterized by a homologous sequence in the extracellular C terminus. The crystallization of TNFR1, LTα, and TNF illustrates that the receptor-ligand interactions between these families involve one homotrimeric ligand binding to three cross-linked receptors organized in a predictable cluster (3, 4).

The TNF family members 4-1BBL, CD27L, CD30L, CD40L, FasL, OX40L, and TNF-α all exhibit the capacity to reverse signal, to transduce a signal inward upon binding specific receptors (2, 5–8). Bipolar signaling may turn out to be the norm for this family of molecules, thereby blurring the distinction between receptor and ligand. Although it is known that these ligands are mainly membrane-bound molecules that primarily interact with their receptors by direct cell-cell contact, little is known about the mechanism of reverse signaling. It is likely that the cytoplasmic domains of these molecules are involved in signal transduction, as the cytoplasmic tails of TNF family members are distinct from each other yet conserved across species (1).

Our work has focused on bipolar signaling by FasL. Previous in vitro studies from our laboratory provide compelling evidence that FasL acts as a costimulatory receptor in CD8+ T cells. From our initial studies (7), three lines of evidence support a role for FasL in positive reverse signaling. First, multiple CD8+ CTL cell lines, independently derived from FasL− gld mice on a C57BL/6 (B6) background, exhibit depressed proliferation upon alloantigenic stimulation compared with CTL lines derived from wild-type B6 mice or Fas+ lpr mice. This was not due to differences in kinetics of proliferation among the cell lines, overexpression of Fas on the gld cell lines (which could result in increased apoptosis of these cells), or a general defect in signaling in the gld cells. Our findings suggest that the presence of FasL is important for inducing maximal proliferation of CTLs. The second major line of evidence implicating FasL in positive signaling used soluble FasLG fusion protein (a chimeric molecule of human IgG and the ligand-binding portion of murine Fas) to block cell surface FasL-Fas interactions. In a dose-dependent manner, soluble FasLG, but not isotype-matched control human IgG, attenuated the proliferation of wild-type B6 CTLs down to the level achieved by Fasl responders. A third line of evidence showed that cross-linking FasL with plate-bound FasLG, in conjunction with suboptimal amounts of anti-CD3, delivered a costimulatory signal for proliferation to FasL+ but not FasL− CTL. We extended these initial studies to examine the role of FasL costimulation in CD8+ vs CD4+ peripheral T cells (9). We found that naive CD4+ T cells can also respond to FasL costimulation when Fas-mediated death is prevented, and that the observed differences between CD4+ and CD8+ T cells stem partly from the differential control of FasL expression in these two cell types. FasL costimulation occurs very early during the course of an MLC, at a time when FasL expression is induced on both CD4+ and CD8+ T cells meeting Ag for the first time. In contrast, Fas-mediated death occurs late in an immune response when high levels of FasL expression are maintained on CD4+ T cells, rendering them sensitive to apoptosis, whereas CD8+ T cells are relatively insensitive to this signal. Taken together, these in vitro data provide evidence that FasL plays dual...
functions in the regulation of CD8+ and CD4+ T cells, having the capacity to both positively and negatively regulate the peripheral T cell compartment.

The in vivo consequence of FasL costimulation is the focus of the current study. In general, costimulation has multiple functions. First, costimulation may regulate the production of cytokines and their receptors that together help regulate cell proliferation. Second, costimulation may strengthen the TCR signal in both amplitude and duration, possibly by enhancing the redistribution and clustering of raft microdomains at the TCR engagement site (10, 11). Third, costimulation may enhance the long-term survival of Ag-activated T lymphocytes (12). Fourth, costimulation may play a role in the differentiation of T cells into mature effector subsets by altering the Th1/Th2 balance (13, 14) and regulating CTL effector development (15).

The following work brings us one step closer to deciphering the function of FasL costimulation by demonstrating that maximizing the Ag-driven in vivo proliferation of both primary and Ag-experienced CD8+ T cells requires FasL costimulation. Thus, proliferative signals delivered to Ag-reactive T cells during the course of an immune response can be further amplified by the engagement of a molecule that can also initiate a death signal through its receptor. With the discovery of increasing numbers of accessory molecules that possess costimulatory function, the activation of T cells is becoming more complex than previously envisioned, involving multiple ligand-receptor interactions that occur at the interface of the T cell and the APC. FasL can now be placed into that group of subtle regulators of T cell proliferation.

Materials and Methods

Mice
B6, B6.MRL-Fas−/− (B6.lpr), B6Smn.CHR-Fas−/− (B6.gld), B6.SJL-Ptp-prec Pep3b/B6.Tg.Bly5.1 (B6.Ly5.1), and C3H. MRL-Fas−/− (CHL.lpr) mice were purchased from The Jackson Laboratory (Bar Harbor, ME) and used at 6–9 wk of age. OT-1 TCR-transgenic mice on a B6 background (OT-1) were derived to express the Vα2+Vβ5+ TCR from a wild-type, but not Fasl gld, lpr gld mice. FasL-deficient OT-1 mice (OT-1.gld) were obtained by cross/backcross breeding of OT-1 and B6.gld mice, screening for Vβ5 expression by flow cytometry and for the gld mutation by a PCR protocol provided by A. Marshak-Rothstein (Boston University, Boston, MA). The PCR using tail DNA was performed (50 cycles: 1 min at 94°C, 1 min at 51°C, and 1 min at 72°C using the specific primers 5′-TCACAACCTCTCACCATTCAAGAACATATTCCTG-3′ and 5′-CATTCAAGACAATATTCCTG-3′) for wild-type and not gld, FasL contains a diagnostic Stul restriction enzyme site. Reaction products digested with Stul (Roche, Indianapolis, IN) were separated on a 6% polyacrylamide gel to reveal a single band of 135 bp for gld FasL, or two bands of 96 and 39 bp for wild-type FasL. Fas-deficient OT-1 mice (OT-1.lpr) were obtained by cross/backcross breeding of OT-1 and B6.lpr mice, screening for Vβ5 expression by flow cytometry and for the lpr mutation by a PCR protocol (17). B6.PL-Thy-1.1 (B6.Thy-1.1) and CHL.B6Fed (C3H) mice were bred and maintained in our animal facility using foundlings obtained from The Jackson Laboratory. Thy-1 congeneric OT-1 mice (OT-1.Thy-1.1) were generated by cross/backcross breeding of OT-1 and B6.Thy-1.1 mice, screening for Vβ5 and Thy-1.1 expression by flow cytometry.

Reagents
PE-conjugated anti-CD8α (53-6.7) mAb, biotin-conjugated anti-Thy-1.2 (30-H12) and anti-Vα2 (B20.2) mAbs, and FITC-conjugated anti-Vβ5 (MR-9.4), anti-Ly-5.1 (A20), and anti-Thy-1.1 (HS151) mAbs were all purchased from PharMingen (San Diego, CA). TriColor-conjugated streptavidin was purchased from Caltag (Burlingame, CA). The H-2Kb−binding peptides, OVA257-264 (OVAp, SIINFEKL) and the vesicular stomatitis virus (VSV)-NP142-149 (VSVp, RGYVYQGL), were prepared as described previously (18).

Flow cytometry

PBLs, splenocytes, and lymph node (LN) cells were stained as described previously (19) and analyzed on a FACScan using CellQuest software (Becton Dickinson, Mountain View, CA). Dead cells were excluded on the basis of forward and side scatter profiles, and at least 104 live-gated events were collected.

Generation and maintenance of Ag-specific CTLs

Alloreactive H-2β2-specific CTLs were generated by incubating naive spleen and LN cells from age-matched B6, B6.lpr, and B6.gld mice with an equal number of irradiated (3000 rad) C3H splenocytes, as previously described (7). Lines were maintained by stimulation every 8–10 days. After the third stimulation, the medium was supplemented with 50 nM α-methyl mannoside and 5% supernatant from rat cells stimulated with 2 μg/ml Con A for 2 days. All CTL lines were routinely monitored by flow cytometry and CTL assay.

Adaptive transfers

For the transfer of wild-type OT-1 cells into B6.wt, B6.lpr, and B6.gld recipients, 20 × 106 unseparated spleen and LN cells (7–10 × 106 CD8+ T cells) were injected i.v. into unirradiated hosts, and recipients were i.p. injected daily on days 1–3 after cellular transfer with 100 nmol of either OVAp or VSVp in PBS. For the transfer of OT-1.lpr cells into B6.wt and B6.lpr recipients, 20 × 106 unseparated spleen and LN cells (4–10 × 106 CD8+ T cells) were injected i.v. into unirradiated hosts, and recipients were i.p. injected daily on days 1–3 after cellular transfer with either PBS or 100 nmol of OVAp. On day 4, the accumulation of the donor population was quantitated by flow cytometry and the cytolytic activity by CTL assay.

For the adoptive transfer of mixed wild-type OT-1 and OT-1.gld donor cells, unseparated spleen and LN cell populations were analyzed before transfer by flow cytometry to ensure the coinjection of 15 × 106 CD8+ Vα2+Vβ5+ T cells of each origin. The Ly5 congenic adoptive hosts were primed daily on days 1–3 of cellular transfer with 25–100 nmol of OVAp, and splenocytes were analyzed on day 4 by flow cytometry. The donor CD8+ T cell populations were distinguished from the host and from each other by Ly5 and Thy-1 expression. In each case, >98% of the recovered donor T cells were found to be Vα2+Vβ5+. For the transfer of Ag-experienced CTL lines into unirradiated B6.Thy-1.1 recipients, 3 × 106 H-2β2-specific CTLs, harvested 8–10 days after their last stimulation, were i.v. injected along with their cell-borne Ag (107 viable anti-Thy-1.2 plus complement-pre-treated CHL splenocytes). Before transfer, CTLs were separated from fragments of antigenic cells over Ficoll (7). Splenocytes and LN cells from the recipients were analyzed for donor cell expansion on day 4 using flow cytometry to distinguish the donor and host C3H8 T cells by Thy-1 expression. All cellular transfers were analyzed over a short time course of 4 days to avoid potential rejection of the donor cells across Ly5 and Thy-1 differences.

Cytotoxicity assay

Cytolytic activity of splenocytes from adoptive transfer recipients was quantitated directly ex vivo by a 51Cr release assay. Serial dilutions of effector cells and 2 × 104 target tumor labeled with 14Ciodide chromo-

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Results
Fas expression by adoptive hosts maximizes the accumulation of Ag-specific CD8\(^+\) T cells in vivo

Cells from OT-1 TCR\(\alpha\beta\)-transgenic mice were used to track the fate of a homogeneous CD8\(^+\) T cell population proliferating to Ag in the presence or the absence of FasL costimulation. Adoptive transfer of wild-type OT-1 cells into B6.wt, B6.lpr, or B6.gld hosts followed by OVAp injections (schematic diagram, Fig. 1A) showed that the OVAp-driven accumulation of OT-1 cells in Fas\(^-\) (B6.wt and B6.gld) mice greatly exceeded that in Fas\(^+\) (B6.lpr) hosts. This difference in Ag-mediated accumulation of the OVA-specific OT-1 cells between the hosts was observed by both flow cytometry (Fig. 1B) and cytotoxic activity (Fig. 1C). The Ag specificity of this accumulation is shown by the lack of donor cell expansion upon injection of VSVp, another K\(^b\)-binding peptide. No OVAp-driven expansion of host cells was measurable (data not shown).

The diminished accumulation of Ag-specific CD8\(^+\) T cells in lpr hosts is not due to Fas-mediated lysis of incoming donor cells by the host

Because B6.lpr mice express increased levels of FasL relative to wild-type B6 mice, it is possible that the lower level of donor cell accumulation seen in the lpr hosts shown in Fig. 1 is due to Fas-mediated cell death of donor OT-1 cells delivered by up-regulated FasL on the lpr host cells. To test this possibility, cells from OT-1.lpr mice, which are incapable of undergoing Fas-mediated lysis, were transferred into adoptive hosts in the presence or absence of FasL costimulation. Adoptive transfer of OT-1.lpr donor cells into B6.wt and B6.lpr hosts followed by OVAp injection (schematic diagram, Fig. 2A) demonstrates that the reduced accumulation of Ag-specific donor cells in Fas\(^-\) hosts (Figs. 1B and 2B) is not a reflection of donor cell elimination by the host, but is more likely due to the lack of FasL costimulation. This difference was observed both by flow cytometry (Fig. 2B and data not shown), where two to four times the number of OT-1.lpr CD8\(^+\) T cells

![A schematic diagram of the cell transfer from wild-type OT-1 donor mice into unirradiated B6.wt, B6.lpr, and B6.gld hosts followed by OVAp injection is shown.](http://www.jimmunol.org/)

**FIGURE 1.** Accumulation of Ag-specific primary CD8\(^+\) T cells requires Fas expression by the adoptive host. A, Schematic diagram of the cell transfer from wild-type OT-1 donor mice into unirradiated B6.wt, B6.lpr, and B6.gld hosts followed by OVAp injection (schematic diagram). B, Flow cytometric analysis of splenocytes from recipients on day 4 after cell transfer. The gates contain the relevant donor T cells identified by their coexpression of CD8 and V\(\alpha\)2. C, CTL assay of the splenocytes analyzed in B, performed directly ex vivo. The x-axis shows the ratio of recovered effectors to target cells, and the y-axis shows the percent specific lysis. Targets for the assay were \(^{51}\)Cr-labeled EL4 (H-2\(^b\)) cells pulsed with either OVAp or VSVp, as indicated; lysis of target cells in the absence of peptide was undetectable. Data are representative of three experiments.

![A schematic diagram of the cell transfer from OT-1.lpr donor mice into unirradiated B6.wt and B6.lpr hosts followed by OVAp injection is shown.](http://www.jimmunol.org/)

**FIGURE 2.** The reduced capacity of Ag-specific primary CD8\(^+\) T cells to accumulate in an lpr host is not due to Fas-mediated elimination of the donor cells by the host. A, Schematic diagram of the cell transfer from OT-1.lpr donor mice into unirradiated B6.wt and B6.lpr hosts followed by injections with OVAp or PBS i.p. daily for 3 days beginning on day 1. B, Flow cytometric analysis of splenocytes from the recipients on day 4 of cell transfer. The gates contain the relevant donor T cells identified by their coexpression of CD8 and V\(\alpha\)2. C, CTL assay of the splenocytes analyzed in B, performed directly ex vivo. Targets for the assay were \(^{51}\)Cr-labeled EL4 (H-2\(^b\)) cells pulsed with either OVAp or VSVp, as indicated; lysis of target cells in the absence of peptide was undetectable. Data are representative of two experiments.
were recovered from wild-type B6 relative to B6.1pr hosts, and by CTL assay (Fig. 2C). No OVAp-driven expansion of host cells was measurable (data not shown). Taken together, these data suggest that FasL costimulation is required to maximize the accumulation of Ag-specific CD8<sup>+</sup> T cells in vivo.

Direct competition of Ag-specific FasL<sup>+</sup> and FasL<sup>-</sup> donor cells in the same adoptive host demonstrates that FasL<sup>-</sup> CD8<sup>+</sup> T cells proliferate better than their FasL<sup>+</sup> counterparts in a primary immune response

To corroborate the previous results, we performed adoptive transfer experiments in which the donor cells, rather than the host cells, differed in expression of functional FasL. To avoid host-to-host variations in comparing the two donor types, the CD8<sup>+</sup> Vα2<sup>+</sup>Vβ5<sup>-</sup> T cells from the spleens and LNs of wild-type OT-1 (Thy-1.1) and OT-1, gld (Thy-1.2) mice were mixed in a 1:1 ratio and injected into unirradiated B6.Ly5.1 recipients (schematic diagram, Fig. 3A). Splenocytes from the recipients were analyzed by flow cytometry (Fig. 3B) on day 4 to quantitate wild-type OT-1 (CD8<sup>+</sup>, Ly5.1<sup>+</sup>, Thy-1.2<sup>-</sup>) and OT-1, gld donor cells (CD8<sup>+</sup>, Ly5.1<sup>+</sup>, Thy-1.2<sup>-</sup>). Expression of the Vα2<sup>+</sup>Vβ5<sup>-</sup>-transgenic TCR by these responders was verified by flow cytometry (data not shown). As shown in Fig. 3, B and C, approximately twice the number of wild-type OT-1 compared with OT-1, gld CD8<sup>+</sup> T cells was recovered from the adoptive hosts. These data further demonstrate the effect of FasL costimulation on the expansion of CD8<sup>+</sup> T cells in a primary immune response.

**FasL costimulation plays a role in the proliferation of oligoclonal Ag-experienced CTLs in vivo**

To determine whether FasL costimulation influences the expansion of previously activated T cells, CTL lines were used as donor cells in an adoptive transfer experiment (Fig. 4). Resting oligoclonal anti-H-2<sup>+</sup> CTL lines derived from B6.wt and B6, gld mice were coinjected with their cell-borne Ag (T cell-depleted C3H or C3H.1pr splenocytes) into unirradiated B6.Thy-1.1 hosts (schematic diagram, Fig. 4). Recipient splenocytes and LN cells were analyzed by flow cytometry 4 days later. As with the previous transfers of naive CD8<sup>+</sup> donor T cells expressing a uniform TCR (Figs. 1–3), the expansion of previously activated oligoclonal wild-type donor cells exceeded that of FasL<sup>-</sup> CTL by a factor of 2–4 (p = 0.03, by Student’s t test; Fig. 4 and data not shown).

**FasL costimulation enhances the burst size of individual polyclonal CTLs**

CTLs can kill target cells through two different lytic pathways: the degranulation pathway, involving perforin and granzymes, and the Fas lytic pathway involving FasL on the CTL and Fas on the target cell (reviewed in Refs. 20 and 21). Our previous studies have determined that the cytolytic activity of B6.wt and B6, gld responders is indistinguishable on a recovered cell basis despite the absence of functional FasL in B6, gld effector cells (7). Furthermore, no traces of EGTA-independent (Fas-dependent) lysis are detectable in our 4-h 51Cr release assay, and alloantigen-specific lysis by wild-type responders is identical on both lpr and wild-type targets (Fig. 5).

The absence of Fas-mediated target cell lysis in these assay conditions allows a comparison of the CTL burst size generated by FasL<sup>+</sup> and FasL<sup>-</sup> responders. To quantitate the influence of FasL costimulation at the level of a single CD8<sup>+</sup> T cell using T lymphocytes that express diverse TCRs, we plated splenocytes from age-matched VSV-primed B6.wt and B6, gld mice in limiting dilution to quantitate the frequency of responding CTL (schematic diagram, Fig. 6, top). The cytolytic activity in each well calculated initially to contain a single resting VSV-specific T cell was used as a measure of CTL burst size. Cultures were maintained for 7 days, and targets were added directly to the wells to assay for specific lysis. The percent specific lysis was significantly higher for the wells plated with FasL<sup>+</sup> anti-VSV CTL than for those plated with FasL<sup>-</sup> anti-VSV CTL (Fig. 6, bottom), revealing the larger burst size for the former compared with the latter responder cells.

**Discussion**

To determine the biological relevance of FasL costimulation, we examined its role in the Ag-specific expansion of CD8<sup>+</sup> T cells in vivo using several different adoptive transfer protocols. In the first set of experiments, TCRαβ-transgenic Ag-specific CD8<sup>+</sup> T cells were transferred into age-matched hosts that could (Fas<sup>-</sup> mice) and could not (Fas<sup>+</sup> mice) provide costimulation to FasL<sup>-</sup> responders (Fig. 1). The limited Ag-specific accumulation of wild-type OT-1 cells in B6, lpr hosts underscores the important role the host plays in providing FasL costimulation to maximize the Ag-specific response by incoming donor cells. The second adoptive transfer protocol (Fig. 2) indicates that this deficit in Ag-specific accumulation in lpr hosts is not due to Fas-mediated killing of the
incoming donor cells, but is more likely a result of the absence of FasL costimulation.

In the third set of experiments, TCR\(\alpha\beta\)-transgenic CD8\(^{+}\) donor T cells with (FasL\(^{+}\) wild-type OT-1) and without (FasL\(^{-}\) OT-1.gld) the capacity to receive FasL costimulation were mixed at a 1:1 ratio before transfer into a common host (Fig. 3). The absence of FasL costimulation leads to a 2-fold decrease in the yield of OT-1.gld donor cells following antigenic challenge. One caveat with these data is the identification of the Ly5.1\(^2\)CD8\(^{+}\)Thy-1.2\(^2\) population as the wild-type OT-1 donor population (Fig. 3B). The presence of any Thy-1\(^{-}\)CD8\(^{+}\) contaminants, such as CD8\(^{+}\) dendritic cells (22), would influence the cell numbers obtained in this category. However, the frequency of these cells is low, and the contaminants are unlikely to be isolated and transferred by the protocol used in this experiment.

Although the inability of donor cells to receive FasL costimulation translates into a 2-fold difference in cell yields (Fig. 3), the inability of lpr hosts to provide FasL costimulation results in a greater difference in donor cell recovery (Figs. 1 and 2). One possible explanation for this difference is that incoming wild-type OT-1 donor cells could be more efficiently eliminated through Fas-mediated death, initiated by the up-regulated FasL in B6.lpr mice (23, 24). The intermediate recoveries observed with the transfer of lpr OT-1 donor cells into lpr hosts shown in Fig. 2 (lower recoveries than wild-type donor cells from wild-type hosts, but higher than wild-type donor cells from lpr hosts, both shown in Fig. 1) suggest some involvement of Fas-mediated donor cell elimination by the host. Therefore, the most likely explanation for the results observed in Fig. 1 is a combination of Fas-mediated cell death and deficient FasL costimulation resulting in reduced wild-type OT-1 donor cell numbers recovered from the B6.lpr adoptive host. The third adoptive transfer experiment (Fig. 3) used a common host, thus allowing a direct comparison of the two donor populations while minimizing the effects of host-to-host variation (including any pre-existing conditions and differences in the efficiency of priming) and potential differences in the two donor populations (including cytokine production and APC number). However, these data could be complicated by a potential increase in Fas-mediated death of gld CD8\(^{+}\) donor cells relative to coinjected wild-type cells as a result of increased Fas expression by gld T cells. This explanation is unlikely, given the relative insensitivity to Fas-mediated death of CD8\(^{+}\) T cells (25) and the fact that four times the

**FIGURE 4.** Ag-experienced FasL\(^{+}\) CTL lines expand more efficiently than do FasL\(^{-}\) CTL lines after Ag exposure in adoptive hosts. Located left of the panels is a schematic diagram of the transfer of FasL\(^{+}\) and FasL\(^{-}\) Thy-1.2\(^{+}\) H-2\(^{k}\)-specific CTL lines into unirradiated B6.Thy-1.1 hosts. Thy-1-depleted C3H splenocytes were injected i.v. with the donor cells. Spleen and LN cells from the adoptive recipients of wild-type (upper panel) or gld (lower panel) CTL donors were analyzed by flow cytometry on day 4 after the transfer. The gates mark the donor CD8\(^{+}\) T cell populations, identified by their Thy-1 expression. The data are representative of four experiments, two of which used C3H/lpr cells as the immunogen (n = 7 for wild type; n = 9 for gld).

**FIGURE 5.** The cytolytic activity of gld CTLs on wild-type and lpr targets in the presence or the absence of extracellular Ca\(^{2+}\) is comparable to that of wild-type responders in a standard \(^{51}\)Cr release assay. Alloreactive anti-H-2\(^{k}\) long-term CTL lines from B6.wt and B6.gld mice were assayed for their ability to lyse \(^{51}\)Cr-labeled Con A-blasted splenocytes from C3H and C3H.lpr mice in a 4-h \(^{51}\)Cr release assay. EGTA was added simultaneously with the targets for the detection of Ca\(^{2+}\)-independent cytolysis.
the competition for Ag recognition from the immune system of the unirradiated host. It is important to note that this outcome was not altered by immunization with C3H/lpr spleen cells (data not shown), demonstrating that Fas-mediated differences in the rate of Ag clearance by FasL+ and FasL− CTLs were not a factor. In this context, it should also be mentioned that the use of C3H/lpr APCs does not affect the ability of FasL+ donor CTLs to receive FasL costimulation, perhaps due to the ability of Fas+ responders and non-APCs to costimulate through FasL (data not shown).

In each of these adoptive transfer experiments, FasL costimulation was measured at the population level using T cells expressing either a uniform or a restricted TCR repertoire. Examination of the burst size of individual resting CTLs in a polyclonal response against VSV demonstrates that FasL costimulation operates at the level of the individual CTL and influences the fate of cells expressing a diverse set of TCRs (Fig. 6). The degree of proliferation, mediated in part by FasL costimulation, ultimately influences the cytolytic activity of the CTL culture. The comparisons made between wild type and gld CTLs in this experiment are validated by the results obtained in Fig. 5, showing that Ca2+-independent, Fas-mediated lysis (27) is not operative in our assay. Neither the addition of EGTA at the time of target cell coincubation (28) nor the use of lpr targets demonstrated any difference in the cytolytic level between wild-type and gld CTLs. Therefore, the difference in cytotoxicity observed for individual wild-type and gld CTLs in Fig. 6 is not due to the presence or the absence of Fas-mediated lysis by the CTLs, but more likely is a result of the differential contribution of FasL costimulation to the burst size of the two responder cell populations.

FasL costimulation affects T cell responses, from monoclonal to polyclonal, regardless of their antigenic history (naive vs Ag experienced), and is measurable at the single-cell level as well as at the population level. It is important to note that this costimulation appears to be Ag specific, being required early after TCR ligation (9) and only in contexts in which T cell stimulation uses the TCR. Stimuli that are subject to FasL costimulation include cell-bound Ag, anti-CD3, and Con A, all of which initiate signaling directly through the TCR. However, FasL costimulation is not demonstrable with stimuli that bypass the TCR, such as PMA plus ionomycin or anti-Thy-1, despite their ability to up-regulate FasL expression (I. Suzuki and P. Fink, unpublished observations). Cells receiving FasL costimulation display an ~2-fold advantage in Ag-mediated expansion compared with cells that have not been costimulated through FasL (Figs. 3 and 4). This finding is consistent with the report of a 2-fold reduction in the expansion of gld compared with wild-type donor cells in a parent → F1 model of acute graft-vs-host disease (29). It remains to be determined whether this difference in expansion in either system reflects a role for FasL costimulation in triggering the entry of Ag-responsive cells into the memory path- way. It is likely, however, that the influence of Ag-specific, FasL-mediated costimulation on CTL burst size will affect the efficiency with which an individual clears an invading pathogen.

In summary, the work described in this paper demonstrates the in vivo function of FasL as a costimulatory receptor for peripheral CD8+ T cells, a function previously defined in vitro for CD8+ (7) and CD4+ T cells (9). Multiple levels of costimulation such as that provided by FasL appear to be a mechanism used by the immune system in fine-tuning T cell reactivity.

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