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Generation of IL-2-Dependent Cytolytic T Lymphocytes (CTLs) with Altered TCR Responses Derived from Antigen-Dependent CTL Clones[1]

Charles A. Gullo, Mark T. Esser,[2] Claudette L. Fuller, and Vivian Lam Braciale[3]

Ag-specific CD8$^+$ CTL clones require TCR stimulation to respond to IL-2 for growth. Because IL-2 may be produced in the vicinity of CD8$^+$ CTLs when Ag is limiting at the end of an immune response, we have examined the effect of culturing viral-specific CTL clones in IL-2 in the absence of antigenic stimulation. Limiting dilution analysis revealed a high precursor frequency for CTL clones derived from IL-2 propagation (termed CTL-factor dependent (FD)) that are dependent upon exogenous IL-2 for growth and survival and no longer require TCR stimulation to proliferate. Culturing CTL-FDs with infected splenocytes presenting Ag and IL-2 did not revert the clones but did lead to a TCR-induced inhibition of proliferation. The derived CTL-FDs have lost the ability to kill via the perforin/granule exocytosis mechanism of killing, although they express similar levels of TCR, CD3ϵ, CD8$\alpha\beta$, CD45, and LFA-1 compared with the parental clones. The CTL-FDs retain Fas ligand/Fas-mediated cytotoxicity, and IFN-γ production and regulate the expression of CD69 and IL-2Rα when triggered through the TCR. A parental CTL protected BALB/c mice from a lethal challenge of influenza virus, whereas a CTL-FD did not. These findings represent a novel regulatory function of IL-2 in vitro that, if functional in vivo, may serve to down-regulate cellular immune responses. ***The Journal of Immunology*****, 1999, 162: 6466–6472.**

Cytolytic T lymphocytes (CTLs)[4] play a key role in eradicating virally infected cells during cellular immune responses. Each CTL clone has a unique clonotypic TCR that recognizes a specific Ag in the context of the class I MHC. Once this TCR engages the Ag-MHC complex, the CTL is activated to perform many effector functions. These include killing infected host cells, secreting cytokines, modulating cytokine and adhesion receptors, and eventually cell division and proliferation or apoptosis and death. For CD8$^+$ CTLs, three mechanisms of cytolysis have been defined at the molecular level. The perforin, Fas ligand (FasL)/Fas (CD95 ligand/CD95), and TNF-α mechanisms are all activated after TCR engagement (1). The perforin and FasL/Fas mechanisms of killing account for all of the short-term cytolysis detected in vitro, whereas the TNF mechanism requires $>$24 h (1–3). In general, it appears that the perforin mechanism is required for the clearance of virally infected cells and tumors, whereas the FasL/Fas and TNF mechanisms of killing are required for the elimination of potentially autoreactive T cells (3, 4). The importance of perforin and FasL/Fas in an immune response has been revealed by mice defective in these genes (5–7). Perforin-deficient mice fail to eradicate some viral infections but are capable of mediating a strong humoral response against others (8). The lymphoproliferative disorder and autoimmunity that develop in *lpr* (*fas*$^{-/-}$) and *gld* (*fasL*$^{-/-}$) mice demonstrate the importance of these genes in regulating an immune response and maintaining self tolerance (5, 6).

The discovery of cytokines that influence the growth and differentiation of T lymphocytes has provided important insight into the regulation of cellular immune responses. For CD8$^+$ CTLs to grow in vitro, they require IL-2 (9). IL-2 is the prototypic T cell growth factor, and its ability to induce growth and enhance the generation of CTLs is well documented. Early studies demonstrated that IL-2 is an essential factor for the development of CTLs, as neutralizing Abs to IL-2 virtually eliminate the generation of CTLs (10). Even though IL-2 is clearly required for CTL development and growth, its biology is even more complex than simply being a T cell growth factor. Recent studies have shown that IL-2 is required for the development of CTLs and the regulation of T lymphocyte activation-induced cell death and tolerance in vivo. Lenardo showed that IL-2 could program T cells for apoptosis (11). Other work has also suggested that IL-2 may play a direct role in increasing the susceptibility of T cells to apoptosis (12). In addition, the complex biology of IL-2 in vivo was revealed by genetically engineered IL-2-deficient mice (13). Instead of being immunodeficient, these mice suffer from autoimmune conditions such as hemolytic anemia and colitis (14). From these and other studies, it is apparent that the role of IL-2 in activating and regulating an immune response is not completely understood.

We have a panel of well-characterized influenza virus-specific T cell clones that have been propagated in vitro by weekly restimulation with influenza-infected splenocytes plus exogenous IL-2 (15). The exact specificity of these clones and their propagation have been extensively studied (15–17). Under culture conditions of

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[4] Abbreviations used in this paper: CTL, cytolytic T lymphocyte; FasL, Fas ligand; FD, factor dependent; HIFCS, heat-inactivated FCS; CD62L, CD62 ligand; HA, hemagglutinin; i.n., intranasal(ly); wt, wild type.

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infected syngeneic splenocytes and rIL-2, these CTL clones have maintained a stable level of cytotoxicity for several years (16). Within 24 h of Ag stimulation, the CTL clones increase in volume and surface and increase their IL-2Rα levels to create high-affinity IL-2R$\alpha\beta\gamma$. IL-2Rα levels peak on days 2 or 3 and decrease over the next 5–8 days; by 10–14 days after Ag stimulation, the CTL clones revert to small quiescent cells with low IL-2Rα levels (15, 17). Concomitant with this fall in IL-2Rα expression, the clones become refractory to the IL-2-proliferative signal (17). Although the quiescent clones do not proliferate in response to IL-2, they express the lower affinity IL-2R$\beta\gamma$; in addition, IL-2 maintains the viability of these clones. This intermediate affinity $\beta\gamma$ receptor is capable of transducing a signal (18, 19), but the physiological significance of the $\beta\gamma$ receptor on resting T cells is unknown. Because IL-2 may be produced in the vicinity of $CD8^+$ CTLs at the end of an immune response when Ag is limiting, we decided to examine the effect of culturing influenza-specific CTL clones in IL-2 in the absence of antigenic stimulation. Here, we report that the long-term culturing of CTL clones in IL-2 in the absence of Ag results in CTLs with altered phenotypes and differentiation states. Furthermore, we find that the generation of these IL-2-dependent CTL factor-dependent (FD) clones under these experimental conditions occurs at a high frequency. We characterize the various differences of these cells from their respective parental clones. These data reveal a novel regulatory effect of IL-2 in vitro and may represent a regulatory mechanism in vivo.

Materials and Methods

Cell lines

The L1210Fas$^+$ (H-2^d) (20) and the L1210Fas$^-$ (H-2^d) (21), target cells were maintained in DMEM (Life Technologies, Gaithersburg, MD) (4.5 g/L glucose) supplemented with 10% (v/v) heat-inactivated FCS (HIFCS) (HyClone Laboratories, Logan, UT), 2 mM glutamine, 5×10^{-5} M 2-ME, and antibiotics (10 U/ml penicillin G and 10 μg/ml streptomycin sulfate).

T lymphocyte clones

The T lymphocyte $CD8^+$ T cell clones 11-1, 14-7, and 14-13 (22, 23) were stimulated in vitro with influenza A/JAP/57 virus-infected, γ-irradiated (2000 rad) BALB/c spleen cells every 7 days with 10 U/ml human rIL-2 (Biosource International, Camarillo, CA) in complete media (Iscove's media (Life Technologies), 10% HIFCS, 2 mM glutamine, and 5×10^{-5} M 2-ME). CTL-FDs were seeded every 7 days at 0.5×10^6 cells in 5 ml of complete media plus 30 U/ml rIL-2 and split 1:2 after 3.5 days.

Flow cytometry

Day 8 CTLs or day 4–6 CTL-FDs were collected, separated from dead cells by Fico/Lite-LM (Atlanta Biologicals, Norcross, GA), washed, and stained with various mAbs to lymphocyte receptors. Abs to CD2 (RM2-5), CD8α (53-6.7), CD8β (53-5.8), CD28 (37.51), CD25 (7D4), CD45RB (16A), and CD69 (H1.2F3) were all purchased from PharMingen (San Diego, CA). Hybridomas producing Abs to CD3ϵ (145-2C11), CD49d (RI-2), CD56 (YN1/1.7.4), CD62 ligand (CD62L) (Mel14.D54), and LFA-1 (FD441.8) were purchased from the American Type Culture Collection (Manassas, VA). Anti-CD90, Thy1.2 (5a-8) was purchased from Caltag (Burlingame, CA). Anti-CD95 (RMF6) was purchased from Kamiya Biomedical (Seattle, WA). For the stimulation experiments, 10^6 cells/ml were mock-stimulated or stimulated with plate-bound anti-CD3 at 5 μg/ml for 24 h in DMEM, 1% FCS, 2 mM glutamine, and 50 μM 2-ME. T cells were collected, transferred to a round-bottom microtiter plate, centrifuged, and resuspended in 50 μl of anti-CD25 (7D4) or anti-CD69 (H1.2F3) (1 μg/ml) for 30 min on ice in PBS with 1% serum. T cells were washed twice, incubated for 30 min on ice with streptavidin R-PE (5 μg/ml) (Caltag), washed three times in PBS with 1% serum, and analyzed on a FACScan (Becton Dickinson, Mountain View, CA).

^{51}Cr release cytotoxicity assay

Target cell lysis was measured by the ^{51}Cr release assay as described previously (22). Briefly, target cells were incubated with sodium ^{51}chromate for 3 h at 37°C. Targets were washed three times and mock-treated or sensitized with hemagglutinin (HA)204-212 (LYQNVGTYV), HA529-537 (IYATVAGSL), or nucleoprotein 147-155 (TYQRTRALV) peptide (24, 25) (0.01 μM) before plating at 10^4 target cells/well. In all experiments, CTL and CTL-FD clones were added at an E:T ratio of 5:1 in round-bottom plates, spun for 1 min at 250 $\times$ *g*, and incubated at 37°C in a CO_2 incubator. Supernatants (100 μl) were harvested from each well at 4 h for the perforin-killing experiments and at 6–8 h for the FasL/Fas-killing experiments and were counted on a gamma counter (Isomedic; ICN Biomedicals, Huntsville, AL). All experiments were performed in quadruplicate. Spontaneous release was <10% in all experiments. Percent specific lysis was calculated as follows: % specific ^{51}Cr release = 100 $\times$ ([experimental release cpm − spontaneous release cpm]/[total cpm − spontaneous release cpm]).

Intranasal (i.n.) influenza virus inoculation

The i.n. infection of mice was performed essentially as described by Graham et al (26) and Lukacher et al (27). Mice were anesthetized using metofane (methoxyflurane) (Mallinckrodt Veterinary, Mundelein, IL). The LD_{50} had been determined as described previously (26). Mice were infected i.n. with 50 μl of a 10 LD_{50} of A/JAP/57 in cold Iscove's modified DMEM (Life Technologies) without antibiotics.

CTL adoptive transfer

Adoptive transfer experiments were performed as described previously (26, 27). Briefly, day 6 CTLs were separated from dead cells using Fico/Lite-LM (Atlanta Biologicals) and washed three times with 37°C 2% HIFCS (HyClone Laboratories) in Iscove's modified DMEM without antibiotics. Approximately 10^7 cells were resuspended in media and kept at 37°C. Within 30–40 min of i.n. inoculation, the CTL clones 14-7 or 14-7FD were injected i.v. via the tail vein. Control mice received 2% FCS media i.v. without cells. The 14-7 and control experimental groups consisted of five mice, and the 14-7FD group consisted of nine mice. Mice were monitored for survival for 21 days.

Proliferation assay and viability counts

Proliferation assays were performed as described previously (28). In brief, 10^4 cells were plated in a flat-bottom, tissue culture-treated, 96-well plate for 72 h under various conditions. A total of 10 U/ml of IL-2 was given in indicated conditions at the beginning of the assay. Under some conditions, irradiated and influenza-infected (A/JAP/57) BALB/c splenocytes were provided at 10^5 cells/well. At 6 h before harvest, 1 μCi/ml of [^{3}H]TdR was added to each well. Finally, cells were harvested, and quadruplicate values were calculated in total cpm. Viability counts were performed in the exact same manner, except that cells were removed after 72 h and trypan blue was added at 1:1. Cells were then counted to determine cell concentration. The percentage of recovery was obtained by dividing the final cell number by the input cell number $\times$ 100. Next, quadruplicate values were calculated as concentration (cells/ml) and % recovery (percent).

Results

Generation of CTL-FDs

Historically many laboratories have recognized that CTL clones cultured in vitro for long periods of time could lose their cytolytic capabilities and become less Ag-dependent or even spontaneously cytolytic against certain tumor lines (29–32). In studying influenza-specific clones, we occasionally observed this phenomena and decided to further characterize these types of clones we have termed CTL-FD. We had previously characterized some of the properties of a spontaneous variant, 14-7FD, and we wanted to determine whether we could intentionally derive a panel of CTL-FDs (33). To achieve this, we subcloned CTL-FDs from several CTL clones in IL-2 in the absence of Ag. To determine whether the IL-2-induced generation of CTL-FDs was a result of a selective outgrowth of a minor population of cells within the individual clonal cultures, we performed limiting dilution analysis (34) to clone CTL-FDs and to determine the precursor frequency of a CTL-FD within the parental population. A schematic representation of our experimental design is shown in Fig. 1*a*. Analysis of three representative clones, 11-1, 14-7, and 14-13, revealed that the CTL-FDs were present at a frequency of between 1 in 2 and 1 in 3 (Fig. 1*b*). In these separate limiting dilution analyses, the clones 11-1FD, 14-7FD, and 14-13FD had precursor frequencies

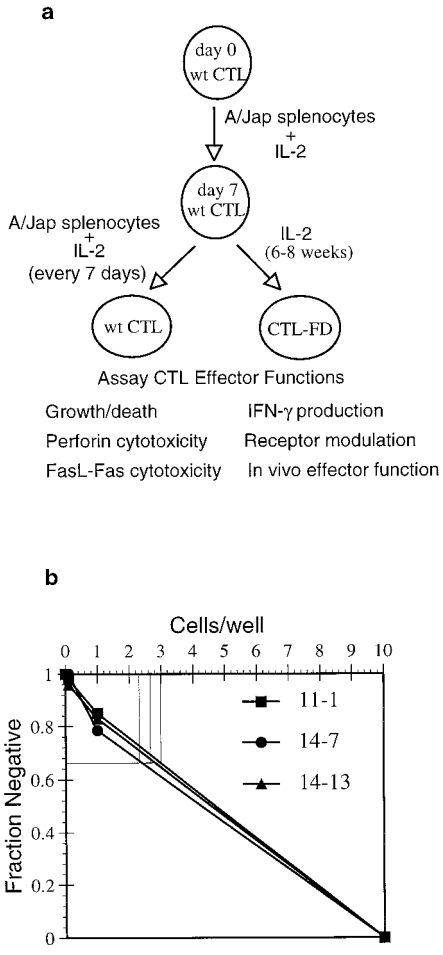

FIGURE 1. Experimental design and precursor frequency of CTL-FDs within a clonal CTL population. *a*, Experimental design. Parental $CD8^+$ (wt CTL) clones normally stimulated with irradiated, syngeneic, influenza-infected splenocytes and 10 U/ml rIL-2 every 7 days were subcloned in rIL-2 (30 U/ml) in the absence of Ag or splenocytes for 6–8 wk. *b*, The precursor frequency of CTL-FDs within a wt CTL population was determined by seeding wt CTLs at 100, 10, 1, and 0.1 cells/well in a 96-well, flat-bottom plate (one plate per dilution) with rhIL-2 alone and allowing them to grow out for 6–8 wk before performing effector function assays. Wells that showed cell growth as seen through an inverted microscope were scored as positive. Precursor frequency analysis was performed as described by Taswell (34).

of 17%, 20%, and 12% at 1 cell/well, respectively. Other influenza-specific T cell clones have been made into FDs as well, with similar precursor frequencies (data not shown). Because there are no APCs in our cultures, IL-2 is acting directly on the CTLs. We find that the frequency is surprisingly high, and these data strongly suggest that there may be a direct CTL to CTL-FD transition rather than outgrowth of a minor population of cells in long-term IL-2 propagation.

Cell surface receptor expression on CTLs and CTL-FDs

The above protocol allows us to reproducibly generate stable IL-2-dependent, Ag-independent clones. Next, we examined a panel of cell surface receptors to identify any gross differences between the CTL and CTL-FD clones serving as a marker for the CTL-FD. Staining for CD3ϵ, CD8$\alpha\beta$, CD45, CD2, CD28, LFA-1, CD56 (ICAM-1), CD62L (Mel14), CD90 (Thy1.2), and CD95 (Fas) did not reveal any gross differences in the CTL-FDs compared with the parental clones, although there were interclone differences and slightly different levels between some parental and FD clones (Table I). The only surface marker that was usually different between all CTL and CTL-FD clones was CD25 (IL-2Rα), which was consistently 2- to 3-fold higher on the CTL-FDs compared with the parental clones. This finding was not surprising, considering that these clones are constantly bathed in IL-2, which likely keeps IL-2Rα expression high (35). Importantly, the levels of the TCR and adhesion molecules were similar between the CTL-FDs and the parental clones.

Growth properties of CTLs and CTL-FDs

Next, we wanted to characterize the growth properties of the parental CTL and CTL-FD clones. From previous work in our laboratory, we knew that at 7–14 days after antigenic stimulation, parental CTL clones require both Ag (influenza-infected splenocytes) and IL-2 to grow in vitro (22); this requirement is in keeping with the competence and progression model of T cell activation. We also had evidence from a spontaneously generated CTL-FD that its proliferative profile was different from the parental clone, 14-7. Fig. 2*a* shows the growth properties of the CTLs and the experimentally derived CTL-FDs. The parental clones 11-1, 14-7, and 14-13 require both Ag and IL-2 to proliferate (Fig. 2*a*). The parental CTLs do not respond strongly to IL-2 alone. Fig. 2*b* shows that the percent recovery from input is poor for 14-7 in the presence of IL-2. However, the percent viability of the remaining cells is ~50% (data not shown). Intriguingly, after 3 days in the presence of Ag alone, there are a significant number of cells remaining, although they do not proliferate and show a low percentage of recovery (30%) (Fig. 2, *a* and *b*). In comparison, the CTL-FDs strongly responded to IL-2 to initiate DNA synthesis and cell division (Fig. 2, *a* and *b*), demonstrating the strong proliferative capacity of IL-2 alone in these clones. Both the parental CTLs and CTL-FDs began to undergo cell death within 24 h in medium without IL-2 (Fig. 2, *a* and *b*). In a reproducible fashion, CTL-FDs incorporated less [^{3}H]thymidine when Ag and IL-2 were present compared with IL-2 alone (Fig. 2*a*). The CTL-FDs have either undergone cell death or are arrested from proliferation within 48–72 h after encounter with Ag (Fig. 2*b*); this observation may explain why we have been unable to revert the CTL-FDs back to

Table I. *Levels of T lymphocyte receptors on CTLs and CTL-FDs*[a]

	CD2	CD3	CD8α	CD8β	CD18a	CD28	CD25	CD45RB	CD49d	CD56	CD62L	Thy1.2
11-1	1[b]	61	47	26	67	69	180	192	0	5	0	41
11-1FD	4	82	57	87	352	55	428	925	2	20	101	151
14-7	16	130	8	28	351	23	50	322	19	64	34	24
14-7FD	8	178	8	61	163	37	477	1137	3	56	5	56
14-13	43	155	20	84	634	35	132	822	13	52	64	434
14-13FD	14	1321	28	136	341	50	1200	493	0	27	112	176

[a] Flow cytometry was performed as described in *Materials and Methods.*
[b] All values are represented as mean fluorescence intensities and are representative of three experiments.

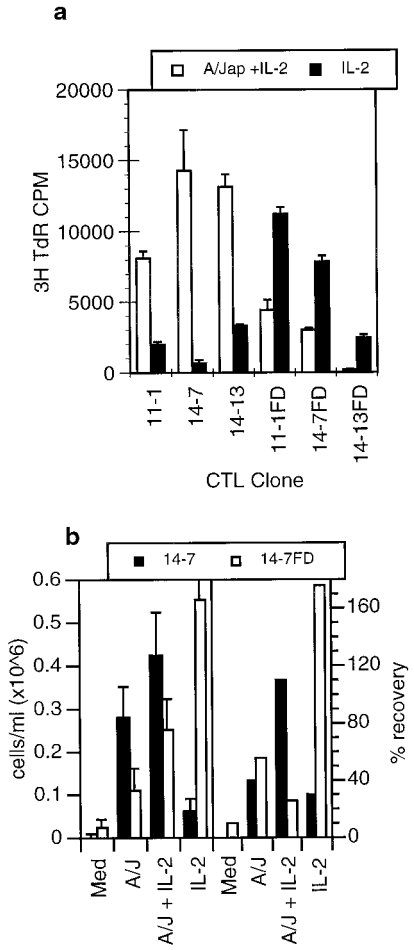

FIGURE 2. Cell growth and death properties of CTL and CTL-FD clones. *a*, A 72-h proliferation assay was performed on day 7 CTLs or day 4 CTL-FDs. Cells were treated with influenza-infected (A/JAP/57) and irradiated BALB/c splenocytes and/or IL-2 at 10 U/ml. The conditions of A/JAP/57 and media alone were <700 total cpm and were omitted. *b*, Cells were treated identically as described in *a* except upon harvest, trypan blue was added (1:1), and cells were counted in a light microscope. Results are representative of more than three independent experiments.

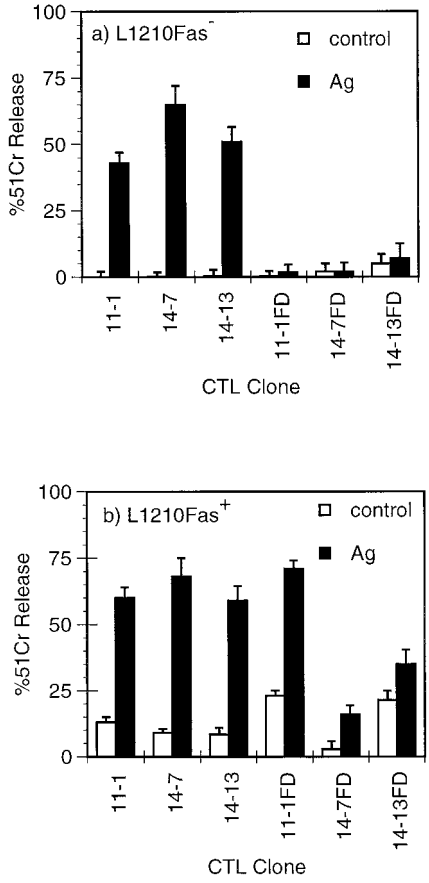

FIGURE 3. Specificity and cytolytic activity of CTL and CTL-FD clones. CTL clones at 6 days after antigenic stimulation or CTL-FD clones were incubated with mock-treated or peptide Ag (0.01 μM) -pulsed L1210Fas$^-$ (*a*) or L1210Fas$^+$ (*b*) targets at an E:T ratio of 5:1 in a 6-h ^{51}Cr cytotoxicity assay. The antigenic peptides for the various clones are as follows: 11-1/11-1FD (HA204–212), 14-7/14-7FD (HA529–537), and 14-13/14-13FD (nucleoprotein 147–155). Results are representative of >10 independent experiments.

the parental phenotype. These data demonstrate that IL-2 is necessary and sufficient to propagate the CTL-FDs. The ability of IL-2 to induce growth, prevent programmed cell death, and eventually differentiate the CTLs into CTL-FDs reveals that IL-2 acts on CTLs to regulate their growth, death, and cytotoxicity.

Specificity and cytolytic activity of CTLs and CTL-FDs

We subsequently examined the specificity and cytolytic activity of the CTL and CTL-FD clones. Historically many laboratories have recognized that culturing CTLs in IL-2 in the absence of Ag resulted in the loss of cytotoxicity (29–32). Because the FasL/Fas mechanism of killing has been described previously (20), we asked whether the CTL-FDs retained perforin and/or FasL/Fas killing. Previously, we reported the loss of perforin-based cytolysis in a spontaneously generated CTL-FD (33) and wanted to determine whether this was generalizable to the CTL-FD clones that were intentionally generated. We tested the ability of CTLs and CTL-FDs to kill Ag-pulsed or influenza-infected syngeneic L1210Fas$^-$ or L1210Fas$^+$ target cells. The parental CTLs killed both the Ag-sensitized Fas$^-$ (Fig. 3*a*) and Fas$^+$ (Fig. 3*b*) target cells, whereas the CTL-FDs killed the Fas$^+$ (Fig. 3*b*) but not the Fas$^-$ target cells (Fig. 3*b*) in an Ag-specific manner. The CTL-FD-induced FasL/Fas killing was inhibited by the Fas.Fc protein or the protein synthesis inhibitor emetine (33). It was also apparent that the CTL-FDs had a higher background killing of uninfected Fas$^+$ target cells (Fig. 3*b*). This is most likely due to the fact that IL-2 induced FasL/Fas killing (36). These data demonstrate that intentionally derived CTL-FDs have selectively lost the ability to kill via the perforin/granule exocytosis mechanism of killing, yet have retained FasL/Fas-mediated cytolytic effector activity.

IFN-γ secretion and receptor expression in CTLs and CTL-FDs

Because it was apparent that these CTL-FDs had lost the ability to kill via the perforin mechanism of killing but had retained FasL/Fas cytotoxicity activity, we examined cytokine secretion and cell surface molecule modulation following TCR stimulation. We have shown previously that a spontaneously generated FD clone retained the ability to produce IFN-γ, and we asked whether the same was true for intentionally derived CTL-FDs (33). CTLs and CTL-FDs were activated with influenza-infected splenocytes, and supernatants were assayed for IFN-γ. Fig. 4*a* shows that both the parental CTLs and CTL-FDs made IFN-γ after specific antigenic stimulation. We also examined whether stimulation through the TCR would induce expression of the activation marker CD69 and the cytokine receptor IL-2Rα (CD25). Using plate-bound anti-

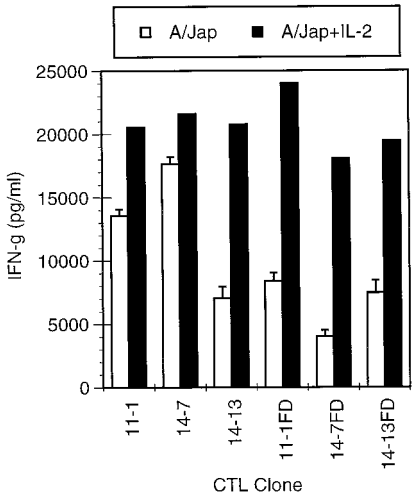

FIGURE 4. CTL and CTL-FD clones secrete IFN-γ in an Ag-specific manner. A total of 10^4 CTL or CTL-FD clones were stimulated with 10^6 influenza-infected (A/JAP/57) splenocytes, A/JAP/57-infected splenocytes plus rIL-2 (10 U/ml), rIL-2 (10 U/ml) alone, or medium alone. After 72 h, supernatants from triplicate wells were collected and pooled, and an IFN-γ ELISA was performed in duplicate. Media alone control groups were <400 pg/ml and were omitted. Results are representative of at least three independent experiments.

CD3ϵ (145-2C11) to stimulate the CTLs, we assayed for the induction of these two surface receptors by flow cytometry. Stimulating through the TCR induced a 10- to 30-fold increase in CD69 on the parental CTLs and a 2- to 8-fold increase on the CTL-FDs; however, the resting levels were higher in the CTL-FD clones (Fig. 5*a*). As mentioned earlier, the resting levels of CD25 on the CTL-FDs were considerably higher than on the parental CTLs (Fig. 5*b* and Table I). Nevertheless, in both the parental CTLs and CTL-FDs, activating through the TCR induced a 2- to 3-fold increase in CD25 expression (Fig. 5*b*). Although the CTL-FD clones have lost the ability to kill via the perforin mechanism, they have retained several CTL effector functions, including FasL/Fas killing, IFN-γ secretion, and CD69 and CD25 up-regulation following antigenic stimulation.

CTL-FD does not protect from a lethal challenge of influenza virus

Our laboratory has shown previously that influenza-specific CTL clones could protect mice from a lethal challenge of influenza virus

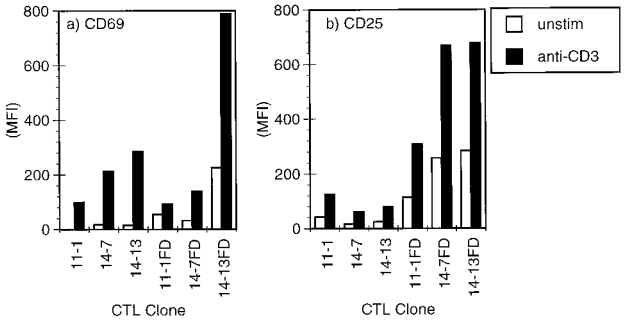

FIGURE 5. CTL and CTL-FD clones up-regulate the early activation marker CD69 and IL-2Rα after TCR stimulation. Day 8 CTLs and day 4–6 CTL-FDs were separated by Fico/Lite, washed, and stimulated with 5 μg/ml plate-bound anti-CD3 for 24 h. Cells were collected, washed, stained for CD69 (H1.2F3) (*a*) or CD25 (7D4) (*b*), and analyzed by flow cytometry. Results are expressed as mean fluorescence intensities and are representative of two independent experiments.

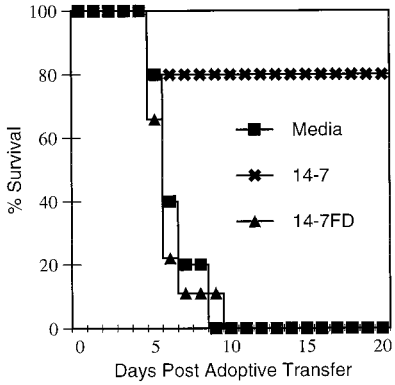

FIGURE 6. CTL-FD does not protect mice from a lethal challenge of influenza virus. Syngeneic BALB/c mice were infected i.n. with $10LD_{50}$ of A/JAP/57 influenza virus and received 10^7 cells of either the CTL clone 14-7 (day 6) or CTL 14-7FD (day 6) within 30 min postinfection. Control mice received media. Experimental groups consisted of nine mice for the 14-7FD group and five mice each for the 14-7 and control groups. Results are representative of two independent experiments.

(27, 37). The antiviral activity of the CTL clones 14-7 and 14-7FD was evaluated by adoptive transfer into lethally infected syngeneic BALB/c mice. The mice were infected i.n. with a 10 LD_{50} dose of A/JAP/57, which would allow the assessment of viral clearance by either clone as a result of protection from death (Fig. 6). Each experimental group received 10^7 CTLs, and the mice were monitored for survival for 21 days. The ability of the parental clone to protect against a lethal infection has been reported previously (27) and is clearly evident in the survival of 80% of the mice receiving 14-7. Despite the ability to kill via the FasL/Fas mechanism and secrete IFN-γ following antigenic stimulation (Figs. 3*b* and 4*a*), 14-7FD was unable to clear the virus and promote recovery. The lack of in vivo protection by 14-7FD was apparent by the fact that the time to death for the 14-7FD adoptively transferred mice was almost identical with the control mice, with >80% mortality in the first 7 days postinfection (Fig. 6). These data reveal that the CTL-FDs did not protect in vivo and suggest that FasL/Fas cytotoxicity and IFN-γ production are not sufficient for viral clearance and that perforin cytotoxicity is required for protection in vivo against influenza.

Discussion

The role of IL-2 in T cell activation and expansion is well documented (9), and our previous work (15, 17, 23, 28) and the results presented here support the idea that $CD8^+$ CTL clones require Ag and exogenous IL-2 to proliferate optimally. We are not the first to observe that the long-term culturing of CTLs in IL-2 in the absence of Ag results in a loss of cytotoxicity (29–31). However, we present the first instance of an intentional derivation of IL-2-dependent CTLs with high frequency, which may suggest the physiological significance of these CTLs. These previous studies focused on continuously growing alloreactive or influenza-reactive CTL lines that had been unintentionally generated. We show here that long-term IL-2 treatment of CTLs results in CTL-FDs that lose Ag-specific perforin- but not FasL/Fas-based cytolysis. However, we find that other TCR triggered events are still maintained. Another property of culturing CTLs in IL-2 for long periods of time is that they become spontaneously cytotoxic toward certain tumor cells (32). Fig. 3*b* shows that clone 14-13FD lysed Fas^+ tumor targets in an Ag-independent manner, most likely due to the ability of IL-2 to induce FasL/Fas killing in $CD8^+$ and $CD4^+$

clones (36). We suggest that this property may be a generalizable phenomena of murine $CD8^+$ T cell clones.

IL-2 is not the only cytokine that can maintain viability and induce the proliferation of CTL clones. Both IL-4 (38) and IL-7 (39) have been reported to induce the survival and expansion of clones in vitro in the absence of Ag, and IL-12 has been shown to drive the expansion of activated T cells and NK cells in the absence of IL-2 (40). It is of interest that CTLs cultured in IL-4 maintain both perforin and FasL/Fas cytotoxicity (41) but lose the ability to produce certain cytokines, whereas CTLs cultured in IL-7 lose at least one mechanism of killing (39). Like our CTL-FD clones, CTLs grown in IL-4 cannot revert to parental effector activity, whereas CTLs cultured in IL-7 (39) or T cell growth factor (29) were capable of reverting to wild-type (wt) activity in vivo and in vitro. The most likely reason why the CTL-FDs could not revert to parental CTL activity when recultured with influenza-infected splenocytes and IL-2 is that they are inhibited in their proliferation and may eventually undergo cell death upon encounter with Ag/MHC or anti-CD3 (Fig. 2*b*). There is evidence that the ability to program T cells for apoptosis is a unique property of the IL-2R, because neither IL-4, IL-7, nor IL-15 programs T lymphocytes for apoptosis (42, 43). This finding is surprising, because IL-4 and IL-7 share the IL-2Rγ_c chain and IL-15 shares both the β_c- and γ_c-chains (44). Recently, work from the laboratory of Abbas et al. demonstrated that IL-2 can augment TCR-induced FasL and decrease FLIP (FLICE-like inhibitor protein) expression of $CD4^+$ T cells (12). It is tempting to speculate that the types of cytokines remaining in an infected site or lymph node once Ag has been cleared could regulate the development of memory CTLs or the deletion of potentially autoreactive CTLs. Thus, this unique function of IL-2 may explain why IL-2-, IL-2Rα-, and IL-2Rβ-deficient mice go on to develop autoimmune conditions similar to colitis and hemolytic anemia (14, 43).

Intentionally generated CTL-FDs can be used in TCR signaling studies. It appears that the CTL-FDs have a block early in the TCR signaling cascade upstream of protein kinase C activation and Ca^{2+} mobilization (45). The three CTL-FDs described here all have a noncharacteristic Ca^{2+} signaling response (45). This block can be overcome with PMA and ionomycin to induce perforin/granule exocytosis and killing (45). From our work and the work of others (33) regarding $CD8^+$ T cells and the work of F. Fitch and colleagues (46) with $CD4^+$ T cells, it appears that the ability of IL-2 to partially desensitize a T cell may be a generalizable phenomena. How IL-2 regulates T cell activation via this mechanism remains to be elucidated. A new paradigm is emerging in T cell activation and differentiation, one involving the alteration of stimulation conditions to subtly change TCR signaling pathways so that T cells become differentially regulated as to activate only a subset of effector activities.

We find that the frequency of in vitro generation of cloned CTL-FDs is quite high. Furthermore, the amount of IL-2 (30 U/ml) needed to generate these FDs is much less than that used previously to generate lymphokine-activated killer and NK cells and may be similar to physiological levels. Our results show that 14-7FD did not protect lethally challenged BALB/c mice in vivo. Although one explanation is that this may be a result of a lack of perforin-mediated cytolysis by the CTL-FD clone, other possibilities exist. These include the inability to traffic to the lungs, a lack of FasL or IFN-γ induction in vivo, or even potential death of the cells. These possibilities are the subject of ongoing investigations in our laboratory. The most intriguing question posed by our work is the possibility that CTL-FDs or CTL-FD-like cells may exist in vivo. Although we have no direct evidence, we can envision a situation in vivo where CTL-FDs may evolve. After a viral infection has been resolved by a CTL response, many CTLs would still be present in the previously infected organ. If another pathogen infected the same organ within a few days, the IL-2 from the newly stimulated CD4 T cells could drive the expansion of CTLs from the previous immune response and differentiate them into CTL-FDs.

In summary, we have shown that the long-term culturing of CTLs in IL-2 in the absence of Ag differentiates CTLs such that they display altered effector responses. We have deliberately derived these CTL-FDs and find that they occur at high frequencies (Fig. 1). Very similar to the originally described natural variant 14-7FD (33), these directed CTL-FD clones lose the ability to kill via the perforin/granule exocytosis mechanism of killing, but retain FasL/Fas cytotoxicity, IFN-γ production, CD69 up-regulation, and IL-2Rα regulation capabilities. Furthermore, the clones retain their Ag specificity. The CTL-FDs have a TCR-signaling defect that can be overcome with phorbol ester and Ca^{2+} ionophore (33). In addition, CTL-FDs were unable to protect BALB/c mice from a lethal challenge of influenza virus, whereas the parental CTLs resolved the infection. We speculate that this novel IL-2 regulatory mechanism may represent a strategy used by the immune system to down-regulate a CTL response in vivo once Ag has been cleared.

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