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Signal 3 Tolerant CD8 T Cells Degranulate in Response to Antigen but Lack Granzyme B to Mediate Cytolysis

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Naive CD8 T cells that respond in vivo to Ag and costimulation in the absence of a third signal, such as IL-12, fail to develop cytolytic function and become tolerated. We show in this study that CD8 T cells purified from TCR transgenic mice and stimulated in vitro in the presence or absence of IL-12 form conjugates with specific target cells, increase intracellular Ca2+, and undergo degranulation to comparable extents. Perforin is also expressed at comparable levels in the absence or presence of a third signal, but expression of granzyme B depends upon IL-12. Levels of granzyme B also correlate strongly with the cytolytic activity of cells responding in vivo. In contrast, an increase in CD107a (lysosomal-associated membrane protein 1) expression resulting from degranulation cannot distinguish in vivo generated lytic effector cells from tolerated, noncytolytic cells. Thus, it appears that cells rendered tolerant as a result of stimulation in the absence of a third signal fail to lyse target cells because they are “shooting blanks.” The Journal of Immunology, 2005, 175: 4392–4399.

R ecent reports have proposed that CD8 T cells undergo a programmed response after brief exposure to Ag and costimulatory ligands leading to multiple rounds of cell division, acquisition of effector function, and development of a stable memory population (1–4). However, there are multiple descriptions of CD8 T cells that divide in response to Ag but fail to produce effector cytokines or become cytolytic (5–10) and often these cells are tolerated, suggesting a more complex model of CD8 T cell activation. Using an in vitro system of highly purified naive CD8 T cells responding to defined stimulatory ligands and recombinant cytokines, we have shown that when provided with Ag and costimulation, naive CD8 T cells will proliferate vigorously but will not differentiate into lytic effectors unless they also receive a third signal that can be provided by IL-12 (11, 12). Recently we have shown that type I IFNs can also provide the third signal (13). Further studies demonstrated that although brief exposure to Ag and costimulatory ligands resulted in some “programmed” cell division, prolonged exposure to both Ag and IL-12 were required for optimal clonal expansion and differentiation of effector function (14). The dissociation of proliferation and differentiation was also demonstrated in vivo models of CD8 T cell activation (5, 11, 15, 16), and it was shown that rIL-12 could substitute for adjuvant in promoting an effective CD8 T cell response (15, 16). We also observed that CD8 T cells which were activated to expand in the absence of IL-12, were unable to develop effector function even when restimulated in vivo with a fully activating stimulus of peptide and adjuvant, i.e., the CD8 T cells were functionally tolerant (11, 16). These results are consistent with a model in which resting dendritic cells can stimulate naive CD8 T cells to divide, but the cells do not accumulate or develop effector functions, and ultimately become tolerant. In contrast, dendritic cells activated through TLR or other receptors increase expression of MHC class I and costimulatory ligands and can also provide a third signal in the form of IL-12 that will stimulate strong clonal expansion of CD8 T cells and cause differentiation into lytic effectors and eventually a stable memory population.

It has been known for some time that IL-12 stimulates increased production of IFN-γ by Ag-activated CD8 T cells (reviewed in Refs. 17, 18). We have identified several differences between CD8 T cells activated in vitro with two vs three signals, including IL-12-dependent up-regulation of the transcription factor Bcl-3, which promotes cell survival (19), and a higher and more prolonged expression of CD25, which may also promote clonal expansion in response to IL-2 (20). These effects do not have an obvious connection with development of lytic activity. To begin to understand how IL-12 promotes effector function, we have examined the phenotype of the nonlytic CD8 T cells activated in vitro with only two signals and the fully lytic CD8 T cells activated with three signals, focusing on various components of granule-mediated lysis. In this pathway, CD8 effector cells form conjugates with target cells expressing cognate Ag, and via signaling through the TCR release granules into the extracellular space between the effector and target cells (reviewed in Ref. 21). One of the granule proteins, perforin, is critical for entry of granule contents into the target cell, although the exact mechanism for this is unclear (reviewed in Ref. 22). Upon entry into the target cell, granule-associated serine proteases, in particular granzyme B, cause caspase activation and apoptosis in the target (reviewed in Ref. 23). When we compared the nonlytic with lytic CD8 cells we found that the only significant difference between them was a much higher granzyme B content in the lytic cells, and granzyme B levels correlated strongly with lytic activity. In many respects the cells that are activated in vitro with two signals can be seen as in vitro counterparts to cells that are tolerated in the absence of a third signal in vivo. A phenotypic analysis of adoptively transferred CD8 T cells stimulated in vivo using tolerizing (peptide alone) or activating (peptide and IL-12) conditions again revealed a strong correlation between granzyme B expression and lytic effector function. These results suggest that when stimulated under tolerizing conditions CD8 T cells acquire many of the characteristics of effector cells, including the ability to release lysosomal granules in response to Ag on target cells.
However, these granules are deficient in granzyme B, a critical mediator of apoptosis.

Materials and Methods

**Mice, cell lines, and reagents**

OT-I mice having a transgenic TCR specific for H-2Kb and OVA257–264 (24) were a gift from Dr. F. Carbone, University of Melbourne (Melbourne, Australia). OT-I mice were also crossed with Thy-1-congenic B6.PL-Thy1a/Cy (Thy1.1) mice (The Jackson Laboratory) and bred to homozygosity. The OT-I, OT-I/PL, and 2C TCR transgenic mice (25) were maintained under specific pathogen-free conditions at the University of Minnesota (Minneapolis, MN). C57BL/6Nc mice were purchased from the Jackson Laboratory Institute (Frederick, MD). Experiments were performed in compliance with relevant laws and institutional guidelines and with the approval of the Institutional Animal Care and Use Committee at the University of Minnesota. E.G7 tumor cells (EL-4 thymoma transfected with OVA) or P815 tumor cells were used as targets in in vitro cytotoxicity assays and EL-4 cells were used as controls for specificity. Anti-lyrophosomal-associated membrane protein 1 (anti-LAMP-1, anti-CD107a) FITC-conjugated Ab was purchased from BD Pharmingen. Anti-human granocyte B PE-conjugated Ab, which cross-reacts with mouse granocyte B, was purchased from Caltag Laboratories. Anti-mouse perforin Ab (clone KM585) was purchased from Research Diagnostics. Anti-rat Ig PE secondary Ab was purchased from Caltag Laboratories. Anti-mouse perforin Ab (clone KM585) was purchased from Caltag Laboratories. Anti-rat Ig PE secondary Ab was purchased from Jackson ImmunoResearch Laboratories. Unconjugated 2C11 (anti-CD3ε) Ab and all other directly conjugated fluorescent Abs were purchased from BD Pharmingen, eBioscience, or BioLegend. Monochromatic Ab 1B2 that recognizes the 2C transgenic TCR (26), a gift from Dr. H. Eisen (Massachusetts Institute of Technology, Cambridge, MA), was purified from hybridoma culture supernatant.

**Naive T cell purification**

Inguinal, axillary, brachial, cervical, and mesenteric lymph nodes (LN)3 were harvested from OT-I, OT-I/PL, or 2C mice, pooled, disrupted to obtain a single cell suspension, and passed over Cellex CD8 enrichment columns (Cedarlane Laboratories). Cells were stained with anti-CD44 FITC and anti-CD8 PE mAbs and sorted using a FACSVantage flow cytometer (BD Biosciences). This procedure yielded a population of naive CD8+ cells that were >99% CD8+ and >98% CD44low. Alternatively, LN cells were enriched for CD8+CD44low cells by negative selection using MACS magnetic cells sorting (Miltenyi Biotecn). Briefly, cells were coated with FITC-labeled Abs specific for CD4, B220, I-A*, CD11c, and CD44. Anti-FITC magnetic microbeads were added to cells that were then passed over separation columns attached to the MACS magnet. The cells that did not bind to the column were collected and were >95% CD8+ and <0.5% CD44high.

**In vitro proliferation and cytotoxicity assays**

A total of 5 × 10^4 purified CD8+ T cells and 2 × 10^5 artificial APCs were placed in flat-bottom microtiter wells in 200 μl of RPMI 1640 medium supplemented with 10% FCS, 4 mM L-glutamine, 0.1 mM nonessential amino acids, 1 mM sodium pyruvate, 100 U/ml penicillin and streptomycin, 10 mM HEPES, and 5 μM 2-mercaptoethanol (MP). Alternatively, LN cells were enriched for CD8+CD44low cells by negative selection using MACS magnetic cells sorting (Miltenyi Biotecn). Briefly, cells were coated with FITC-labeled Abs specific for CD4, B220, I-A*, CD11c, and CD44. Anti-FITC magnetic microbeads were added to cells that were then passed over separation columns attached to the MACS magnet. The cells that did not bind to the column were collected and were >95% CD8+ and <0.5% CD44high.

**Intracellular staining**

Intracellular staining

Cells were collected from cultures and resuspended in HBSS with 1% FCS. Indo 1-AM (Molecular Probes) was added at a final concentration of 5 μg/ml and cells were incubated at 37°C for 45 min. After washing, cells were resuspended in HBSS with 1% FCS, and warmed prior to a FACScalibur flow cytometer (BD Biosciences) for 1 min to generate a baseline. Either Indo-EY or Indo-4 cells were then added at a 4:1 ratio to the Indo-1 AM labeled cells. After incubating for 30 s at 37°C, the mixture of cells was returned to the flow cytometer and fluorescence was monitored for an additional 4 min in real time to measure release of intracellular Ca2+.

**Results**

Results were analyzed using FlowJo software (Tree Star).

**Ab, Ag, and B7-1 immobilization on microspheres and microtiter wells**

Methods for generating artificial APCs by immobilizing Abs, MHC Ags, and costimulatory ligands on 5-micron diameter latex microspheres have been previously described in detail (27). Microspheres were coated with Abs to CD3ε or the 2C TCR (1B2) using 2.5 μg of Ab/10^7 microspheres. For coating with Ag, microbeads were mixed with DimerX H-2Kb fusion protein (BD Pharmingen) using 2.5 μg of DimerX H-2Kb Ig/10^7 latex microspheres. Peptide was loaded onto the H-2Kb portion of the fusion protein by incubating the coated microspheres with 0.1 μM OVA257–264 for 2 h at 37°C, followed by extensive washing to remove free peptide. When used, B7-1 in the form of a recombinant mouse B7-1/Fc chimera (R&D Systems) was immobilized on the microspheres along with Ab using 0.6 μg/10^7 microspheres. For all artificial APC preparations, immobilization of proteins was verified by staining with fluorescent Abs and analysis with flow cytometry.

The method for immobilizing Ag and B7-1 on microtiter wells has been described in detail previously (14). Briefly, 50 μl of DimerX H-2Kb fusion protein diluted to 2 μg/ml in PBS and 50 μl of recombinant B7-1/Fc chimicrome protein diluted to 0.4 μg/ml in PBS were added to wells of flat-bottom microtiter plates for 1.5 h at room temperature. After washing with PBS, peptide was loaded onto H-2Kb by adding OVA257–264 peptide at 0.02 μM in 100 μl of RP-10 and incubating for 2 h at 37°C. Wells were washed three times in RP-10 to remove unbound peptide before adding CD8 T cells. These wells are referred to as Ag-B7-coated wells.

Adoptive transfer and immunization of OT-I/PL transgenic cells

Pooled LN from OT-I/PL mice were disrupted to yield single cell suspensions and washed with PBS. Before transfer, the cells were analyzed by flow cytometry to determine the percentage of CD8+ cells. Their CD8+CD469, and CD44 phenotypes were determined to confirm that the cells that were transfected were not activated. A total of 1.5 × 10^6 CD8+ cells in 0.3 ml of PBS was transferred via tail vein injection into age- and sex-matched naive 4- to 8-wk-old C57BL/6 recipients. Recipient mice were then rested for at least 24 h before immunization. For immunization, the OVA257–264 synthetic peptide (SIINFEKL, ResGen; Invitrogen Life Technologies) was dissolved in PBS and injected via tail vein in a volume of 0.2 ml. Where indicated, animals received 1 μg of murine rIL-12 (Genetics Institute) or 50 μg of LPS in the same injection.

Conjugate formation

Effector cells from day 3 in vitro cultures were collected and washed in HBSS to remove serum proteins. Cells were resuspended to 4 × 10^7/ml in HBSS, and CFSE (Molecular Probes) was added to a final concentration of 2.5 μM to PBS was transferred via tail vein injection into age- and sex-matched naive 4- to 8-wk-old C57BL/6 recipients. Recipient mice were then rested for at least 24 h before immunization. For immunization, the OVA257–264 synthetic peptide (SIINFEKL, ResGen; Invitrogen Life Technologies) was dissolved in PBS and injected via tail vein in a volume of 0.2 ml. Where indicated, animals received 1 μg of murine rIL-12 (Genetics Institute) or 50 μg of LPS in the same injection.

Calcium flux

Cells were collected from cultures and resuspended in HBSS with 1% FCS. Indo 1-AM (Molecular Probes) was added at a final concentration of 5 μg/ml and cells were incubated at 37°C for 45 min. After washing, cells were resuspended in HBSS with 1% FCS, and warmed prior to a FACScalibur flow cytometer (BD Biosciences) for 1 min to generate a baseline. Either Indo-EY or Indo-4 cells were then added at a 4:1 ratio to the Indo-1 AM labeled cells. After incubating for 30 s at 37°C, the mixture of cells was returned to the flow cytometer and fluorescence was monitored for an additional 4 min in real time to measure release of intracellular Ca2+.

Intracellular staining

Cells were fixed in Cytofix buffer (BD Pharmingen) for 15 min at 4°C, and permeabilized in saponin-containing Perm/Wash buffer (BD Pharmingen) for 15 min at 4°C before staining with PE-conjugated anti-granzyme B Ab or with

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3 Abbreviations used in this paper: LN, lymph node; MFL, mean fluorescence intensity; LAMP, lysosomal-associated membrane protein; LU, lytic unit;
anti-mouse perforin Ab followed by PE-conjugated anti-rat Ig Ab. LN cells harvested from adoptively transferred mice were also stained with Ab to CD8 and Thy1.1 to mark the OT-I/PL cells. Cells were washed once with Perm/Wash buffer and once with PBS containing 2% FBS. Stained cells were analyzed on a FACSCalibur flow cytometer using CellQuest software.

**Degranulation assay**

Cells from in vitro culture were collected, washed, and placed back in culture both with and without 1 μM OVA257–264 peptide, and with 6 μM/ml of the monensin-containing solution GolgiStop (BD Biosciences), and 5 μg/ml FITC-conjugated anti-LAMP-1 (anti-CD107a) Ab or FITC-conjugated isotype control Ab. After 3 h at 37°C, cells were washed and analyzed on a FACSCalibur flow cytometer using CellQuest software. Degranulation by LN cells harvested from adoptively transferred mice was assayed the same way, except that the samples were also stained with Abs to CD8 and Thy1.1 to mark the OT-I/PL cells before analysis.

**Results**

Artificial APCs were made by coating latex microspheres with mAbs to either CD3ε or the TCR. These artificial APCs could stimulate naive CD8+ T cells from OT-I (Fig. 1A) or 2C (Fig. 1C) transgenic mice to proliferate in vitro if exogenous IL-2 was added to the cultures. The addition of IL-12 to these cultures did not significantly increase proliferation on day 2; however, it was absolutely required for development of lytic effector function by day 3 of culture (Fig. 1, B and D). We also found that production of an effector cytokine, IFN-γ, was dependent on a third signal from IL-12 (data not shown). We have shown previously that exogenous IL-2 was not required for proliferation if costimulatory B7-1 molecules were coinmobilized with Ag on the artificial APCs, but IL-12 was still required to generate lytic effectors (11). Similarly, a requirement for IL-12 could not be replaced by addition of more IL-2 to cultures, because under conditions giving maximal proliferation in response to anti-TCR and IL-2 (Fig. 1E), addition of IL-12 still increased the lytic response in this experiment by >1000-fold (Fig. 1F). Finally, Ag density on artificial APCs was varied by coating microspheres with H-2Kb and pulsing them with OVA257–264 peptide at varying concentrations. Proliferation of the OVA-specific OT-I cells depended on exogenous IL-2 and varied with Ag dose, whereas IL-12 had minimal effect (Fig. 1G). In contrast, effector function remained dependent on exogenous IL-12, even under conditions of maximal Ag-driven proliferation (Fig. 1H). Naïve CD8 T cells from wild-type mice could also be activated to proliferate by exposure to anti-CD3 and B7-1-coated artificial APCs. In this model, too, when lytic function was measured in a redirected lysis assay, we again saw that effector function only developed in the presence of IL-12 (data not shown). Thus, the observation that naïve CD8 T cells require a third signal to develop lytic effector function but not for proliferation was seen for both TCR transgenic and wild-type CD8 T cells and for multiple sources of signals 1 and 2. This requirement could not be bypassed by high Ag concentration, high density of B7-1, high IL-2 concentrations, or by ligation of the TCR with high affinity mAb (Fig. 1 and Ref. 11).

To better understand the effects of IL-12, we examined the phenotype of the nonlytic cells activated with two signals and the lytic CD8 T cells that received three signals. We first tested the ability of the two cell populations to form conjugates with cells expressing the cognate Ag. Purified naïve 2C transgenic CD8 T cells, specific for the H-2Ld alloantigen, were activated with anti-CD3-coated artificial APCs in the presence of IL-2, with or without IL-12, harvested from cultures on day 3, and labeled with CFSE. They were then mixed with PKH-labeled cells that did (P815) or did not (E.G7) express their target Ag. After a brief incubation period the cells were gently resuspended and analyzed by flow cytometry. Conjugates between the effector and target cells were detected as events that were positive for both CFSE and PKH fluorescence. As seen in Fig. 2, A and B, cells stimulated with or without IL-12 formed conjugates with P815 target cells, with a slightly higher percentage of cells activated with two signals found to be in conjugates. This ability to bind to the target cells did not correlate with the ability to lyse the targets, however, because cells stimulated with two signals formed conjugates with P815 cells but lysis of the targets was minimal, whereas cells stimulated with three signals lysed P815 targets effectively (Fig. 2C). Both conjugate formation and lysis were Ag-specific, as cells from both cultures failed to form conjugates with (Fig. 2, D and E) or lyse (Fig. 2F) irrelevant E.G7 target cells. Comparable results were obtained in two separate experiments, using both 2C and OT-I TCR transgenic CD8 T cells (data not shown).
but no Ca\(^{2+}\) with anti-CD3 or with Ag-coated artificial APCs (data not shown), 3). Similar calcium flux was observed when the TCR was activated in OT-I T cells activated with either two or three signals (Fig. 3). After exposure to E.G7 cells, equivalent rapid calcium flux was seen in OT-I T cells activated with either two or three signals (Fig. 3).

Having determined that the nonlytic cells stimulated with only two signals could form Ag-specific conjugates with target cells, we wanted to determine whether TCR-mediated signaling occurred, as measured by an Ag-stimulated rise in intracellular Ca\(^{2+}\). Activated OT-I T cells were mixed with E.G7 cells, which are OVA transfected EL-4 cells that express the Ag recognized by the OT-I TCR. After exposure to E.G7 cells, equivalent rapid calcium flux was seen in OT-I T cells activated with either two or three signals (Fig. 3). Similar calcium flux was observed when the TCR was activated with anti-CD3 or with Ag-coated artificial APCs (data not shown), but no Ca\(^{2+}\) flux was seen in response to EL-4 cells that lack the Ag recognized by OT-I cells (Fig. 3). As expected, only the cells that received three signals lysed E.G7 cells (data not shown). These results show that this early TCR-mediated signaling event is intact in both lytic and nonlytic T cells.

Killing by CD8 T cells can be mediated by Fas ligand or by perforin-containing granules. To confirm that the cytolytic activity being examined in these experiments was perforin-dependent, we examined inhibition by concanamycin A, which causes degradation of perforin in the granules, and therefore specifically inhibits perforin-dependent killing but not Fas ligand-dependent killing (28). Cultures of OT-I T cells activated with artificial APCs, IL-2, and with or without IL-12, were assayed for lytic activity in the presence and absence of concanamycin A. Only those cells cultured with IL-12 were able to lyse target cells, and >90% of the IL-12-dependent lytic activity was blocked in the presence of concanamycin A (data not shown). Thus, the perforin-dependent granule exocytosis pathway is responsible for the majority of lytic activity from these cultures.

Because the lytic activity by cells that received three signals was mediated by perforin-containing granules, it was possible that the failure of cells activated with only two signals to kill targets was due to a failure to degranulate and release the granule contents. This failure has been described for tumor-infiltrating CD8 T cells (29) and for some mutations that lead to defects in cell-mediated lysis (30, 31). In the process of granule exocytosis, the lytic granules move via association with microtubules to the site of contact between the effector and target cells, fuse with the effector cell plasma membrane, and release their contents into the extracellular space between the effector and target (21). When lytic granules fuse with the plasma membrane, LAMPs are transiently exposed on the outer surface of the membrane. A recently described method (32) detects Ag-triggered degranulation by labeling the transiently exposed LAMPs with fluorescent Abs, which are internalized with the LAMPs but can still be quantified by flow cytometry. To determine whether nonlytic CD8 T cells that were stimulated with only two signals were capable of Ag-driven degranulation, OT-I T cells were cultured for 3 days in Ag and B7-1-coated microwells with or without IL-12. The cells were collected, washed, and then placed back in culture in the presence of OVA\(_{257-264}\) peptide and anti-LAMP-1 Ab. As a control, cells were cultured with peptide and isotype control Ab. We found that >70% of the cells from cultures with only two signals bound anti-LAMP-1 Ab during the 4 h incubation with Ag (Fig. 4A), indicating that degranulation had occurred. Cells that had received three signals also stained with
anti-LAMP-1 Ab when restimulated with peptide, at a frequency comparable to that of cells cultured without the third signal (Fig. 4B). The ability to degranulate did not correlate with killing, however, because cells that received two signals lacked lytic activity, whereas cells that also received the third signal efficiently lysed E.G7 targets (Fig. 4C). The detection of LAMP-1 on the cell surface depended on the re-exposure of cells to peptide Ag because no cells from either type of culture could be directly stained with anti-LAMP-1 Ab (data not shown). In this experiment (Fig. 4), the fraction of nonlytic cells that stained with LAMP-1 Ab was ~87% of the fraction of lytic cells that were stained. This fraction varied among experiments from 60 to 100% (Fig. 5A and data not shown). In all experiments, however, the cells cultured without IL-12 lacked lytic function, whereas those cultured with IL-12 were effective killers. Therefore, it appears that although the majority of cells cultured without IL-12 degranulate in response to Ag, they fail to lyse their targets.

We next wanted to confirm, as has been demonstrated previously (33), that the ability of the OT-I CD8 T cells to degranulate was dependent on exposure to Ag and to determine when this ability developed during the course of the response. Naive OT-I T cells were activated with plate-bound Ag-B7-1 without IL-12 or with two different concentrations of IL-12. The ability to degranulate when re-exposed to Ag was assayed over the course of 4 days of culture. As expected, naive (day 0) cells exposed to Ag could not degranulate, as they were not stained with anti-LAMP Abs. Ag-B7-1-stimulated cells acquired this function after the second day of culture, and the ability to degranulate was maintained through the fourth day of culture (Fig. 5A). Cells from cultures that also received IL-12 showed the same time course for developing the ability to degranulate, with slightly higher percentages of these cells stained with LAMP-specific Abs. Lytic effector function developed only in cultures supplemented with IL-12, in a dose-dependent manner, with maximum activity detected after 3 days of culture (Fig. 5B). Thus, CD8 T cells demonstrate Ag-driven release of lysosomal granules only after 48 h of activation, and the kinetics and acquisition of this function were not affected by the addition of IL-12 to cultures. As seen before, degranulation did not correlate with lytic effector function because only cells cultured with IL-12 were able to lyse their targets. For these cells, maximum ability to degranulate was reached by 48 h, whereas lytic activity peaked 24 h later. In addition, lytic activity declined by day 4 but degranulation remained maximal.

Because degranulation failed to result in lytic activity by cells cultured without IL-12, we next asked whether these cells contained the granule-associated proteins involved in causing target cell death. We examined the expression of perforin, the granule protein that is required for the entry of granule contents into the target cell, and granzyme B, one of the granule-associated serine proteases that cause caspase activation and apoptosis in the target cells. OT-I cells activated with or without IL-12 were fixed, permeabilized, and stained

FIGURE 5. Kinetics of acquisition of ability to degranulate or lyse target cells by CD8 T cells in response to varying amounts of IL-12. Purified, naive CD8⁺ OT-I T cells were cultured in Ag-B7-coated microtiter plates, with or without the addition of IL-12 at 2 U/ml (high) or 0.2 U/ml (low). A. At the start of culture (day 0) or after 1, 2, 3, or 4 days of culture, degranulation in response to Ag was measured. B. Lytic activity of cells recovered from each time point was measured; results are expressed as LU per culture.

FIGURE 6. CD8 T cells activated with two signals express less granzyme B but similar amounts of perforin, as compared with cells activated with three signals. Purified naive CD8⁺ OT-I T cells were cultured for 3 days with microspheres coated with anti-CD3 Ab-coated artificial APCs, along with IL-2 and without (A) or with (C) IL-12. Cells were stained with anti-perforin Ab (thick histograms) or with isotype control Ab (thin histograms) followed by anti-rat Ig PE Ab, as described in Materials and Methods. (B & D) Purified naive CD8⁺ OT-I T cells were cultured for 3 days in Ag-B7-coated microtiter plates, without (B) or with (D) the addition of IL-12. Cells were stained with anti-granzyme B PE Ab (thick histograms) or with PE-labeled isotype control Ab (thin histograms), as described in Materials and Methods. MFI of perforin or granzyme B staining for the entire population is indicated; lytic activity was also measured and the LU per 10⁶ effectors generated in each type of culture is also indicated.

FIGURE 7. IL-12 dose dependence for granzyme B expression and degranulation. Purified naive CD8⁺ OT-I T cells were cultured in Ag-B7-coated microtiter plates, with or without the addition of IL-12 at the indicated concentrations. After 3 days, cells were characterized in three ways: lytic activity was measured in a 4 h ⁵¹Cr release assay and results were expressed as LU per 10⁶ effectors; cells were stained with anti-granzyme B Ab, and the MFI of the entire population was determined; and degranulation in response to Ag was also measured. All results were then expressed as the percentage of the maximum value obtained for each parameter. The maximum values were: 58 LU/10⁶ for killing; an MFI of 2056 for granzyme B staining; and 85% LAMP-1-positive for the degranulation assay.
with Abs to perforin or granzyme B. Cells from the two types of cultures contained similar levels of perforin as measured by mean fluorescence intensity (MFI of 179 without IL-12 vs MFI of 239 with IL-12) (Fig. 6, A and C), whereas the level of killing by these cells differed by >10-fold, suggesting that the difference in lytic function could not be accounted for by differences in perforin expression. When granzyme B levels were examined, however, we found large differences in expression of this protein (MFI of 238 without IL-12 vs MFI of 1093 with IL-12) (Fig. 6, B and D) that were correlated with exposure to IL-12 in culture and with lytic effector function. These results suggest that although cells activated with only two signals develop the ability to form conjugates with target cells and release granules following this encounter, these granules are deficient in one of the major serine proteases involved in initiating apoptosis in the target cell (23).

The level of granzyme B expression was further found to be dependent on the amount of IL-12 added to cultures and correlated with the level of killing activity that was generated (Fig. 7). Degranulation, in contrast, did not show a dose-dependence on IL-12 or correlate with effector function. This result is demonstrated in Fig. 8, for which varying amounts of third signal cytokine were added to cultures of OT-I cells and Ag and B7-1-coated artificial APCs, and granzyme B expression and degranulation were measured. When the level of granzyme B expression is plotted vs lytic activity a strong correlation is seen (Fig. 8A). In contrast, degranulation by cells from these cultures, measured by LAMP-1 staining, did not correlate with effector function (Fig. 8B). When plotted as a function of granzyme B expression (Fig. 8A) or degranulation (Fig. 8B), linear regression analysis revealed a much stronger correlation of lytic activity with granzyme B expression ($r^2 = 0.899$) than with degranulation ($r^2 = 0.389$). These results support the conclusion that naive CD8 T cells acquire many of the characteristics of effector cells in response to Ag and B7-1. However, the production of granzyme B appears to be strictly regulated and is greatly increased in the presence of IL-12. Therefore, a major way by which a third signal cytokine regulates the development of effector function in CD8 T cells is through regulating the expression of granzyme B.

Previous work showed that adoptively transferred transgenic CD8 T cells that were activated in vivo with peptide Ag alone proliferated but failed to acquire lytic effector function, as measured by ex vivo $^{51}$Cr release assays and by in vivo killing assays in which target cells pulsed with Ag or unpulsed were differentially labeled with CFSE, transferred into the immunized animals, and their survival measured as an indicator of lytic effector function (11, 15, 16). In addition, the cells that responded to peptide alone in vivo persisted in the host but exhibited functional tolerance in that they were unable to develop lytic effector function when rechallenged with Ag and adjuvant. In addition, as was seen for cells stimulated in vitro with Ag-B7 alone (data not shown), OT-I cells activated in vivo with peptide alone were deficient in production of an effector cytokine IFN-γ (11). This phenotype of expansion without differentiation was similar to that of CD8 T cells activated with only two signals in vitro. We therefore wanted to determine whether the CD8 T cells that were stimulated in vivo with antigenic peptide alone were similar to cells activated with two signals

![FIGURE 8. Granzyme B content, but not degranulation, correlates with lytic effector function. Purified naive CD8$^+$ OT-I T cells were cultured with Ag-B7-coated microspheres, with or without IL-2, IL-12, or type I IFN at various concentrations. After 3 days, lytic function, granzyme B content, and degranulation were measured as described for Fig. 7. A, The MFI of granzyme B staining was plotted vs lytic unit generated in each culture, and a line was fit using linear regression; the value of $r^2$ is shown. B, The percentage of LAMP-1-positive cells was plotted vs lytic units, and a line was fit using linear regression; the value of $r^2$ for the line is shown.](http://www.jimmunol.org/)

![FIGURE 9. Granzyme B content, but not degranulation, correlates with effector function generated in vivo. C57BL/6 mice received OT-I/PL LN cells by adoptive transfer on day −1 and were challenged with 10 μg of OVA257–264 per mouse either alone on day 0 or along with IL-12 on days 0 and 2. A and B, On day 3, LN cells were harvested and incubated in vitro with peptide for 3 h in the presence of anti-LAMP-1 Ab, and further stained with anti-CD8 and anti-Thy1.1 Abs. Histograms are gated on endogenous CD8$^+$ lymphocytes (Thy1.1-negative, shaded histograms) or OT-I transgenic CD8$^+$ lymphocytes (Thy1.1-positive, open histograms). The percentage of OT-I cells that stained with LAMP-1 Ab is shown. C and D, Day 3 LN cells were stained with anti-granzyme B Ab along with anti-CD8 and anti-Thy1.1 Abs. Histograms are gated on endogenous CD8$^+$ lymphocytes (shaded histograms) or transgenic OT-I lymphocytes (open histograms). The percentage of OT-I cells that contained granzyme B is shown. E and F, Lytic activity of day 3 LN cells was measured in a 6 h $^{51}$Cr release assay using E.G7 cells as targets. OT-I to target cell ratios were determined by multiplying the LN to target cell ratio by the percentage of OT-I/PL cells in each LN cell population. The percentage of specific lysis at various OT-I to target cell ratios is shown.](http://www.jimmunol.org/)
in vitro with respect to their ability to degranulate in response to Ag and to produce granzyme B. OT-I cells were adoptively transferred into C57BL/6 mice and recipients were challenged by i.v. injection of OVA257–264 peptide alone or along with recombinant mouse IL-12. After 3 days, the OT-I cells had expanded 20-fold in animals that were immunized with peptide alone, with an additional 5-fold expansion when IL-12 was administered (data not shown). At this time, the OT-I cells were also assayed for the ability to degranulate and their granzyme B content was assessed. Lytic activity against E.G7 targets was measured by a direct ex vivo 51Cr release assay. OT-I cells from animals immunized with peptide alone stained with anti-LAMP Abs when exposed to Ag in vitro, indicating that they had developed the ability to release granules (Fig. 9A). However, very few of these cells contained granzyme B (Fig. 9C), and they were unable to lyse E.G7 targets in a 51Cr release assay (Fig. 9E). In contrast, a majority of the OT-I cells from animals immunized with peptide and IL-12 both degranulated and expressed granzyme B (Fig. 9B and D), and these cells were potent killers of E.G7 targets (Fig. 9F). An in vivo adoptive transfer experiment in which multiple peptide and IL-12 conditions were tested revealed a strong correlation between granzyme B expression by OT-I cells and ex vivo 51Cr release (Fig. 10). These results confirm those shown in Fig. 9, and suggest that in contrast with perforin expression or degranulation, granzyme B expression is dependent on a signal 3 cytokine.

**Discussion**

Naive CD8 T cells responding to Ag and costimulatory molecules require a third signal that can be provided by IL-12 for optimal clonal expansion and development of lytic effector function (12, 16). Recently, type I IFNs have also been shown to provide the third signal (13). CD8 T cells activated in vivo in the absence of a third signal do not develop lytic effector function and instead are tolerized; i.e., they are unable to respond to a secondary stimulation with peptide Ag in the presence of adjuvant (11, 15). The increase in clonal expansion of CD8 T cells seen when IL-12 is present may be a consequence of increased production of Bcl-3, a transcription factor that promotes cell survival. This results presumably from regulating expression of one or more genes that influence cell survival (19) as well as from increased and prolonged expression of CD25 (20).

In the present study, we have investigated the role of IL-12 in promoting lytic effector function, mediated by granule exocytosis. To understand the critical role of IL-12 in development of effector function, we compared the phenotype of the nonlytic CD8 cells activated with only two signals vs the lytic cells activated with three signals with respect to several steps in the granule exocytosis pathway of killing. When we examined the formation of stable conjugates with Ag-bearing target cells (Fig. 2), Ag-dependent calcium flux (Fig. 3), and degranulation in response to TCR signaling (Fig. 4), we found that cells activated with two or three signals responded comparably. In contrast, when we examined the expression of the lytic granule proteins perforin and granzyme B, we found that although both the nonlytic and lytic cells contained comparable levels of perforin, only the lytic cells that had been cultured with IL-12 contained high levels of granzyme B (Fig. 6), and the level of granzyme B correlated with lytic effector function (Figs. 7 and 8).

Similar results were found for adoptively transferred transgenic T cells that were activated in vivo. We compared cells activated in vivo with OVA257–264 peptide alone with those stimulated by peptide Ag along with IL-12 and found that both types of cells were able to degranulate, but only the cells activated by peptide and IL-12 expressed granzyme B and had lytic function (Fig. 9). The in vivo studies revealed an even greater dependence on IL-12 for expression of granzyme B than was seen in the in vitro studies: although adoptively transferred CD8 T cells proliferated in response to challenge with peptide alone, >95% of these cells did not express any detectable granzyme B (Fig. 9C). These studies suggest that Ag and costimulation drive much of the differentiation of naive CD8 T cells to become lytic effectors, but that the expression of granzyme B is strictly controlled, which is consistent with the potential for damage by inappropriately activated CD8 effector cells.

A critical role for the granzymes, particularly granzymes A and B, in CD8 T cell-mediated killing has been shown in several viral and tumor models (reviewed in Ref. 23). Granzyme B appears to cause target cell apoptosis by activation of caspase, whereas granzyme A acts through a caspase-independent mechanism, and its importance for lysis may depend on the target (34–37). Mice with targeted deletions in one or the other of these granzymes have limited deficiencies in effector function, whereas mice deficient in both granzymes A and B have significant defects in clearance of certain viruses (34). Thus, although it is not surprising that granzyme B expression is important for lytic effector function, it had not been recognized that it is expressed in response to a third signal cytokine and appears to be regulated more stringently than perforin. As we reported recently (13), type I IFNs can also provide the third signal for CD8 T cells and, like IL-12, stimulate an increase in granzyme B protein expression as measured by intracellular staining. We have not measured granzyme A protein expression in CD8 T cells in response to two or three signals, although there is some evidence for coexpression of granzymes A and B in cells activated in vitro (38) and in a response to influenza virus in vivo (39). However, gene array studies comparing CD8 T cells activated in vitro with two or three signals suggest that in our in vitro culture system, mRNA for granzymes A and G are not present, whereas mRNA for granzymes B, C, and F are increased when IL-12 is present in cultures (P. Agarwal and M. F. Mescher, unpublished observations). It has been reported that although granzymes A and B are critical for target cell DNA fragmentation to occur, cell lysis, as measured by 51Cr release, may be dependent solely on perforin under some conditions (36). We, however, found that cells that contained perforin but only low amounts of granzyme B were not potent effector cells (Fig. 6).
In earlier studies designed to examine the role of IL-12 in the programmed response of CD8 T cells, we found that brief exposure to Ag and costimulatory ligands was not sufficient for optimal production of effector CD8 cells. Instead, continuous exposure to both Ag and IL-12 was required early in the culture period for optimal cell recovery (14). The early effects of IL-12 may be due to its ability to promote proliferation and enhanced survival of CD8 T cells by causing up-regulation of expression of Bcl-3 (19) and prolongation of expression of CD25 (20). IL-12 also needed to be present during the last 16–24 h of culture for development of full effector function. It is tempting to speculate that one of the “late” effects of IL-12 is promoting expression of granzyme B. Preliminary studies have indeed shown that both lytic function and granzyme B expression develop if IL-12 is added as late as the last 24 h of the culture period (data not shown).

Because the standard 51Cr release assay for cell-mediated killing is cumbersome, lacks sensitivity, and reveals the effector capacity of a population rather than of individual cells, a number of possible substitute markers of effector function have been used. IFN-γ production is one effector function of CD8 T cells, but we (11) and others (5, 40) have found that IFN-γ production does not necessarily correlate with lytic capability. When the degranulation assay measuring LAMP-1 expression was first described, it was proposed as a marker of cytolytic cells (32, 41). Wolint et al. (33) subsequently showed, however, that resting nonlytic memory cells also undergo degranulation in response to Ag, but do not express granzyme B. Our results now demonstrate that tolerant cells that lack cytolytic activity, and do not have the capacity to reacquire activity upon restimulation (11, 16), also undergo Ag-dependent degranulation. Thus, it appears that the ability to degranulate in response to Ag identifies Ag-experienced cells, but cannot distinguish between effector, memory, and tolerant cells.

Disclosures
The authors have no financial conflict of interest.

References