CD28, IL-2-Independent Costimulatory Pathways for CD8 T Lymphocyte Activation

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We investigate, here, the mechanism of the costimulatory signals for CD8 T cell activation and confirm that costimulation signals via CD28 do not appear to be required to initiate proliferation, but provide survival signals for CD8 T cells activated by TCR ligation. We show also that IL-6 and TNF-α can provide alternative costimulatory survival signals. IL-6 and TNF-α costimulate naive CD8 T cells cultured on plate-bound anti-CD3 in the absence of CD28 ligation. They act directly on sorted CD8-positive T cells. They also costimulate naive CD8 T cells from Rag-2-deficient mice, bearing transgenic TCRs for HY, which lack memory cells. They also costimulate naive CD8 T cells from Rag-2-deficient mice, bearing transgenic TCRs for HY, which lack memory cells, a potential source of IL-2 secretion upon activation. IL-6 and TNF-α provide costimulation to naive CD8 T cells from CD28, IL-2, or IL-2Rα-deficient mice, and thus function in the absence of the B7-CD28 and IL-2 costimulatory pathways. The CD8 T cell generated via the anti-CD3 plus IL-6 and TNF-α pathway have effector function in that they express strong cytolytic activity on Ag-specific targets. They secrete only very small amounts of any of the cytokines tested upon restimulation with peptide-loaded APC. The ability of the naive CD8 T cells to respond to TCR ligation and costimulatory signals from IL-6 and TNF-α provides a novel pathway that can substitute for signals from CD4 helper cells or professional APC. This may be significant in the response to viral Ags, which can be potentially expressed on the surface of any class I MHC-expressing cell.


A number of candidates have been suggested for the nature of the second costimulatory signal, signal two. These have included B7.1 (8, 9) and B7.2 (10–15), which interact with CD28 (16) and CTLA-4 (17), the CD2 ligand (18–21), the CD30 ligand (22), CD40 (23, 24), the heat stable Ag ligand (25–27), CD137, the 4-1BB ligand (28–30), cytokines IL-1 (31–35), IL-2, and IL-6 (32–36), TNF-α (36, 37), TGF-β (38), and various others, including adhesion molecules, such as ICAM-1 (39–43) and LFA-3 (44).

Interest has focused most strongly on B7.1 and B7.2, which have been shown to interact with CD28 and induce signaling pathways that end in the stabilization of IL-2 message (45–50). Interaction of these same receptors with CTLA-4 provides inhibitory signals (51–56).

The way that most other costimulatory signals function is less well established. Some have been seen as growth factors, others survival factors, while yet others have been presumed to work by increasing the adhesion between T cells and APC (57). In particular, it was not established whether some of these other costimulators acted directly on the responding T cells or whether they induced one of the other known costimulators (such as B7) on the APC (58).

There has also been a gradual appreciation that signal two is involved in survival of the responding cells (36, 37, 59), but, based on the studies with T cell clones, the concept that costimulation is required in addition for the initial activation is still widely accepted. This issue is not easily resolved, and the apparent result depends on the assay used and the interpretation of the result. Thus, absence of thymidine incorporation is taken as evidence that the cells did not divide. However, as we show here, the cells can divide rapidly but die before or during the thymidine uptake period.

There are a number of additional caveats that should be kept in mind when reviewing the experimental basis for these models. First, it is important to remember that the initial proposal of Bretscher and Cohn (1) was a theory designed to provide an explanation for a regulatory step in the activation of naive B cells.
Much of the later work concerned the conditions for the activation of T cell clones, not naive T cells, and it is clear that cells in a T cell clone may be in a very different developmental state from that of the naive T cell immediately ex vivo. It is also very clear that the activation requirements for naive cells are very much more stringent than those for effector cells or resting memory cells. Sagerstrom et al. (60) have shown that naive cells rapidly lose the requirement for a second signal as they progress into their response. Similar findings were seen for stimulation via CD2 (19), and studies by our colleagues have shown that effector cells need much lower numbers of TCR ligand interactions and have little requirement for costimulation (61). It is also to be noted that the costimulatory requirements for proliferation and for T cell functions, such as cytotoxic activity or cytokine secretion, can be very different (H. Sepulveda, unpublished observations).

Finally, most of the studies have concerned the stimulation of CD4 T cells (41), with only a few studies involving CD8 (6, 7, 42, 43, 59, 66). It is not clear how far the conditions for activation of the two lineages may differ.

In the current studies, we have been concerned only with the costimulatory requirements for populations of naive CD8 T cells immediately ex vivo and have utilized extensively purified populations of CD8 T cells from TCR transgenic mice specific for a known peptide, in which most of the T cells are naive. We utilized a number of T cell markers, such as CD44, CD25, and Ly6C, to distinguish between the phenotype of naive, effector, and memory cells. In our initial experiments, we have confirmed that CD8 cells are activated and divide in response to TCR ligation in the apparent absence of costimulation and that the role of costimulation is to provide survival signals. We have stimulated the CD8 T cells with plate-bound Ab to the TCR complex to eliminate the contribution of APC and have examined the effect of costimulation on the responding T cells. We were thus able to show that there are alternate costimulatory molecules to B7.1 and ICAM-1, including IL-6 and TNF-α, that they act directly on the responding CD8 T cells, and that it is naive CD8 cells that respond to these cytokines, and that these cytokines can provide costimulatory signals in the absence of the CD28 and IL-2 pathways. CD8 T cells stimulated with plate-bound anti-CD3 plus IL-6 and TNF-α are physiologically active in that they develop into effector cells that are cytotoxic on Ag-specific targets, but have little ability to secrete cytokines. These costimulatory molecules can thus provide a mechanism for CD8 T cell activation in the absence of professional APC. This may be important in the response to viral infection in which the viral peptide may be expressed on any class I MHC-expressing cell.

Materials and Methods

Mice

Mice were purchased from the Animal Breeding Facility at the Trudeau Institute (Saranac Lake, NY), Clone-4 v8.2/vo10 TCR-transgenic mice were kindly provided by Dr. Linda Sherman (Scripps Research Institute, La Jolla, CA). The Clone-4 TCR-transgenic mice bear the α- and β-chains of the Clone-4 CTL specific for the transmembrane peptide, residues 518–529 (IYSTVASSL) of hemagglutinin (HA)3 2 on H-2Kb. The Clone-4 TCR-transgenic mice were backcrossed for eight generations with B10.D2, CD28-deficient, IL-2-deficient, and IL-2Ra-deficient mice were bred at the Trudeau Institute from stock kindly provided by Drs. Carl June (62), I. Horak (63), and Laurie Davidson (64), respectively. HY-specific TCR-transgenic Rag-2−/− mice were purchased from Taconic Farms (Germantown, NY). Anti-HY TCR-transgenic H-2b mAbs were obtained from Dr. Drew Pardoll (Baltimore, MD) and have been bred in our facility.

Cell preparations

CD8 T cells isolated from the spleen and lymph nodes were enriched by passing through nylon wool and treating with anti-CD4 (RL172.4), anti-CD8 (3.155), mAbs, and complement, and stimulated with LPS (25 μg/ml) and Dextran sulfate (25 μg/ml) for 48 h.

Preparation of Tc1 and Tc2 effector cells

CD8 T cells from the spleens and lymph nodes of Clone-4 TCR-transgenic mice were prepared as above. APC were loaded with the HA peptide (11 μM) at 37°C for 30 min, treated with mitomycin C (50 μg/ml, Sigma) at 37°C for 40 min, and washed three times before use. CD8 T cells were cultured in RPMI 1640 (Irvine Scientific, Santa Ana, CA) supplemented with penicillin, streptomycin, glutamine, 2-ME, HEPEs, and 10% FCS (HyClone Laboratories, Logan, UT). For effector generation, CD8 T cells from the Clone-4 transgenic mice (2 × 10^5 cells/ml) were stimulated with HA peptide-loaded APCs (2 × 10^5 cells/ml) in the presence of IL-2 (20 U/ml, supernatant from the X63Ag-IL-2 murine cell line), IL-12 (9 U/ml, kindly provided by Dr. Stanley Wolf (Genetics Institute, Cambridge, MA)), and anti-IL-4 (10 μg/ml, 11B11) for Tc1 cultures and in the presence of IL-2 (20 U/ml), IL-4 (200 U/ml, X63.Ag-IL-4 supernatant), and anti-IFN-γ mAb (XMG1.2, 20 μg/ml) for Tc2 cultures. On day 4 of culture, effectors, were 99% CD8+ Vβ8+. Flow cytometry

The following mAbs were used for immunofluorescent staining: CyChrome anti-CD8 (PharMingen, San Diego, CA), anti-vβ8 PE (PharMingen; clone MR5-2), FITC anti-CD62L (PharMingen; clone MEL-14), FITC anti-CD44 (PharMingen; clone IM7), FITC anti-CD45RB (PharMingen; clone 2G12), FITC anti-CD25 (PharMingen; IL2Ra-chain, clone 3C7), anti-Ly6C (PharMingen; clone AL-21), PE anti-CD54 (PharMingen; clone 3E2), FITC anti-CD80 (PharMingen; clone 16-10A1), and FITC anti-CD86 (PharMingen; clone GL1). After staining with the appropriate Abs, samples were analyzed using a FACScan (Becton Dickinson, Mountain View, CA), and data processed using CellQuest (Becton Dickinson) software. For the sorting of the CD8 T cells, CyChrome anti-CD8 (PharMingen; clone 53-6.7) was used.

Cell cultures

Cells were cultured in RPMI 1640 (Irvine Scientific), supplemented with penicillin, streptomycin, glutamine, 2-ME, HEPEs, and 10% FCS (HyClone Laboratories). Cultures were set up in 96-well plates (Costar, Cambridge, MA) in triplicate, in 0.2-ml volumes at a concentration of 2 × 10^3/ml. For stimulation with anti-CD3, 96-well plates were coated with 0.1 ml of the hamster Ab 145-2C11 (American Type Culture Collection (ATCC), Manassas, VA) at 10 μg/ml for 2 h at 37°C. Then, plates were washed twice with 0.3 ml of PBS per well. When anti-CD28 is used, the ascites of the cell line 37NS1 (kindly provided by Dr. J. P. Allison, University of California, Berkeley, CA) was added at 1:50, a dilution found to provide optimal costimulation. Cultures were supplemented with recombinant murine IL-6, TNF-α (R&D Systems, Minneapolis, MN), either alone or in combination, and were added at a concentration of 5 ng/ml and 20 ng/ml, respectively. For the cell counts used to determine cell recoveries, cultures were set up in 48-well plates (Costar, Cambridge, MA) in duplicates in 1-ml volumes at 2 × 10^5 cells/ml and counted using trypan blue exclusion to differentiate between live and dead cells.

Proliferation

The proliferation of CD8 T cells was measured by the ~16 h uptake of [3H]thymidine (ICN Biomedicals, Irvine, CA) added at 0.2 μCi/well, 48 h from the start of culture.
Analysis of cytokine production

Enriched CD8 T cells (2 × 10^6 cells/ml) were stimulated with mitomycin-treated P815 cells (1.2 × 10^6/ml) loaded or not with the HA peptide. IFN-γ, IL-4, and IL-5 were measured by specific ELISAs as described (65).

Cytotoxicity assay

P815 cells were used as targets and loaded or not with the indicated concentrations of the HA peptide for 30 min at 37°C. Subsequently, target cells (1 × 10^6 cells/400 μl RPMI medium containing 1% FCS) were incubated with 3.7 mBq ^{31}Cr (sp. act. 1.85 TBq/g; NEN Life Science Products, Boston, MA) for 1 h at 37°C. Labeled targets were washed three times before use. CD8 T cells were set up at the indicated ratios with the labeled targets (10^4 targets/well) at 37°C. Supernatants were collected after 4 h, and radioactivity was detected by γ-counting. Means and SEs of duplicate cultures are shown. The percentage of cytotoxicity was calculated using the formula: 100 × [(cpm experimental − cpm spontaneous)/(cpm total − cpm spontaneous)]. Spontaneous release was typically 10–15% of the maximum release.

Results

Costimulation provides survival signals

The CD8 cells were purified from spleen and lymph nodes of Clone 4 TCR transgenic mice specific for the peptide TVASSL, as described in Materials and Methods. They were phenotypically naive, CD44^{lo}, CD62L^{hi}, CD25^{−}, with low expression of CD86. They were purified from spleen and lymph nodes of Clone 4 TCR transgenic mice specific for the peptide TVASSL, as described in Materials and Methods. They were phenotypically naive, CD44^{lo}, CD62L^{hi}, CD25^{−}, with low expression of CD86. They were phe-

Materials and Methods

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5-(and-6)-Carboxyfluorescein diacetate succinimidyl ester (CFSE) and propidium iodide (PI) analyses of cell proliferation and survival

To get a more comprehensive picture of the response of the naive CD8 cells stimulated under different conditions, we used the following dye-label technique. Cells were first stained with the cytoplasmic vital dye, CFSE, before in vitro stimulation. The intensity of dye label fell rapidly to 50% in the first few hours of culture (data not shown) and then stabilized in the absence of cell division. The subsequent division of the CFSE-labeled cells was monitored by the reduction of dye intensity to half after each cell division. CD8 T cells were cultured either alone, on plate-bound anti-CD3, or on plate-bound anti-CD3 plus anti-CD28. The cells were harvested at day 1, 2, 3, or 4 and stained for CFSE and Vβ8, and PI was added, as described in Materials and Methods. In the subsequent four-color flow cytometric analysis, cells were gated for cells that were CD8^+, Vβ8^+, and were analyzed for the level of PI and CFSE label. Representative data are presented in Fig. 2, as a two-color plot for PI and CFSE (left panels), and as the histogram for CFSE (right panels) for each of the culture conditions, for days 1, 2, 3, and 4 of culture.

This analysis reveals many features of the response not readily discernible by the thymidine incorporation data shown for the same experiment. First, looking only at the CFSE histograms for days 1, 2, 3, and 4 (right panels of each pair, Fig. 2), it can be seen that all of the cells undergo cell division on TCR ligation, in the presence (anti-CD3 plus anti-CD28) or absence of costimulation (anti-CD3 alone). Cells cultured alone do not divide and survive poorly (CD8 only). There are no cells left with the original CFSE intensity at the later time points in the stimulated cultures, even in cultures stimulated with anti-CD3 alone. Second, the cells progress through multiple rounds of division whether the costimulation is strong (with anti-CD28) or absent or weak (plate-bound anti-CD3 alone), and the cells appear to divide at the same rate. Under conditions of strong costimulation, the cells survive (left panel of anti-CD3 plus anti-CD28) and can be seen to undergo as many as eight rounds of division (right panel), and few cells become PI-positive. It is important to note that if all the cells had survived, there would be a much greater increase in total cell number by day 4 than is actually observed, and it is clear that, even under optimal conditions, cells are being lost. The PI CFSE plot for each day showed that a variable number of the CFSE-labeled cells become PI-positive at each time point and after each cell division. In the absence of costimulation, a major fraction of the cells die at each stage (as seen in the left panels for anti CD3 alone). Under conditions of strong costimulation, this number is markedly reduced. It is important to note that cells that are seen as already PI-positive at one time point have disintegrated by the next time point and no longer
score in the gated plot. They can be seen, however, for an extra day in the FS, SS plot (data not shown), but are gated out of the further analysis.

We were concerned that small numbers of memory CD8 T cells derived from cells expressing endogenous TCR chains might contribute IL-2 upon anti-CD3 stimulation and costimulatory signals. We therefore repeated the analysis with CD8 T cells from anti-HY TCR transgenic RAG-2−/− mice, as shown in Fig. 3A. It can be seen that cells still divided in response to anti-CD3 alone, as judged by progressive loss of CFSE stain, as shown in the upper panel, and went through as many divisions as did cells costimulated with anti-CD28 (lower panel). This experiment has been conducted three times with comparable results, twice with CD8 T cells from anti-HY Rag-2-deficient mice, and once with cells from anti-HY SCID mice. Cells stimulated with plate-bound anti-CD3 alone increased in size, and all showed up-regulated expression of CD69 as seen in Fig. 3B.

Costimulation in the absence of anti-CD28

In other experiments (data not shown), we found that the CD8 T cell populations we have used consist of naive cells, require costimulation to proliferate at day 2 and expand in number to day 4, and that they can receive costimulatory signals from ICAM-1 and B7, as shown by others for CD4 (40, 41) and CD8 T cells (42, 43). We found, however, that different APC differ widely in their ability to stimulate proliferation, even though they express the same known costimulatory molecules in comparable amounts. We sought to discover the factors responsible for this difference. We showed that APC, such as P815, A20 B cell line, and LPS/dextran sulfate-stimulated B blasts, made large amounts of message for IL-6, TNF-α, TNF-β, and LT-β (data not shown), which were potential candidates for costimulatory molecules. Of these, we focused on IL-6 and TNF-α, as they had been previously reported to enhance the CD4 T cells response to antigenic stimulation (35–37) and because murine TNF-β was not available and LT-β is not a secreted molecule.

We therefore investigated the ability of IL-6 and TNF-α to substitute for B7 and ICAM-1 costimulation. Purified naive CD8 T cells were cultured on plate-bound anti-CD3 in the presence and absence of varying concentrations of IL-6 or TNF-α. It can be seen that either cytokine provided strong proliferative stimuli in a dose-dependent manner, approaching the maximum at ~3–5 ng/ml for each cytokine (Fig. 4). The cell recoveries at days 3 and 4, for same factors, reflect the cumulative effect of IL-6 and TNF-α on cell proliferation and survival (Table I).

IL-6 and TNF-α act directly on CD8 T cells

Although the CD8 populations are >90% CD8−, Vβ8−, it was possible that the cytokines acted indirectly by inducing costimulatory molecules, such as B7, on some contaminating cell population. We stained the purified CD8 T cells with anti-CD8 and sorted for CD8-positive cells. The resulting population consisted of 99% CD8 T cells, which were CD44 low, CD62L high, CD45RB high, CD25-negative, and showed both low and high staining with anti-Ly6C (data not shown). We cultured three populations: 1) the original purified, but unsorted, CD8 cells; 2) the unsorted CD8 reacted with anti-CD8 but not sorted; and 3) the sorted CD8 cells on plate-bound anti-CD3, in the presence and absence of the same cytokines and measured the [3H]thymidine uptake (Fig. 5). The pattern of the response of sorted and unsorted populations (regardless of whether the latter had been reacted with the anti CD8 or not (data not shown)) to the costimulatory cytokines was very similar, indicating that the effect was directly on the CD8 cells themselves and not via some contaminating population.

IL-6 and TNF-α enhance the anti-CD3 stimulation of CD8 T cells from anti-HY TCR transgenic Rag-2−/− mice

Memory CD8 T cells secrete IL-2 upon restimulation in vitro (65), and it was possible that contaminating memory cells could supply small amounts of this costimulatory factor. To confirm that the IL-6 and TNF-α acted on a totally naive population of CD8 T cells,
we prepared CD8 T cells from anti-HY TCR transgenic Rag-2<sup>−/−</sup> mice, which can be assumed to have zero or much reduced numbers of memory CD8 T cells. It can be seen in Fig. 6 that these cells gave little response to plate-bound anti-CD3 alone, but responded to costimulation with TNF-α, IL-6, or both at levels comparable to that seen with anti-CD28. It was of interest that under these conditions IL-6 and TNF-α act synergistically.

**IL-6 and TNF-α act via a CD28, IL-2-independent pathway**

In other experiments (data not shown) we found that TNF-α, but not IL-6, could up-regulate B7.1, but not B7.2, on anti-CD3-stimulated CD8 T cells, and it was thus possible that TNF-α, at least, could still act via the CD28 pathway, providing costimulation by T cell-T cell interactions. We showed, however, that IL-6 and TNF-α could provide synergistic costimulation to plate-bound anti-CD3-stimulated CD8 T cells from CD28<sup>−/−</sup> mice (Fig. 7). The response to costimulation by IL-6 or TNF-α alone was less marked than with CD8 T cells from the Clone-4 HA mice, and strong synergy was seen when both cytokines were added.

IL-2 provides strong proliferative signals to CD8 T cells activated by plate-bound anti-CD3. In other experiments (data not shown), we found that the IL-6- or TNF-α-induced secretion of small amounts of IL-2 into the supernatants early in the response, but the amount was small (100 U) compared with that induced with anti-CD28 costimulation (550 U). It was possible, however, that the amount secreted was enough to account for the costimulatory effect, and we next examined whether or not IL-6 or TNF-α costimulation was dependent on the production or response to IL-2. It can be seen in Fig. 8 that IL-6 and TNF-α could indeed provide costimulation to plate-bound anti-CD3-stimulated CD8 T cells from either IL-2<sup>−/−</sup> or IL-2Rα<sup>−/−</sup> mice. The proliferative responses were reproducibly lower than those seen with cells from wild-type mice, especially in the case of the IL-2Rα-deficient mice.

**CD8 effectors generated in the presence of IL-6 and TNF-α costimulation have effector function**

Finally, we investigated whether CD8 populations generated with alternate costimulation had effector function. Four-day effectors generated in the presence of IL-6 and TNF-α costimulation had effector function. Four-day effectors...
were prepared by stimulation with plate-bound anti-CD3 and IL-6 and TNF-α as costimulatory molecules. Tc1 and Tc2 populations with type 1 or type 2 profiles of cytokine secretion were prepared as previously described (65) from the same pool of naive CD8 T cells for the purpose of comparison. The resulting cells were tested in a 4-h radiolabeled chromium release assay. The IL-6/TNF-α effectors (which we designate TcIL-6/TNF-α) were shown to have the same level of cytotoxic activity as Tc1 (Fig. 9A). Tc2 effectors were more cytotoxic, as we have observed in our previous studies (65). The same effector cell populations were tested for cytokine production when restimulated with peptide and APC. Tc1 and Tc2 made the cytokines expected, IFN-γ for Tc1 and IL-4 and IL-5 for Tc2. IL-6/TNF-α (TcIL-6/TNF-α) effectors, however, made relatively small amounts of IFN-γ, and no measurable amounts of IL-4 or IL-5 (Fig. 9B). The same supernatants were also negative for IL-10 and IL-2 (data not shown).

Discussion

Our principal findings can be summarized as follows. First, we have confirmed that CD8 T cells respond to TCR ligation alone (or at least when costimulation is reduced to the minimum possible) by cell division, but die rapidly. This is in agreement with the work of others who have also seen proliferative responses in the absence of costimulation when the TCR signal was strong (6, 7, 66). Co-stimulation by the B7/CD28 pathway provides survival signals that keep the cells alive, as previously shown (59). The use of the

FIGURE 5. IL-6 and TNF-α are costimulatory for proliferation of sorted CD8 T cells. Naive HA-CD8 T cells from Clone-4 mice were sorted using anti-CD8 CyChrome (PharMingen) on a FACStar cell sorter (Becton Dickinson). Sorted and not sorted CD8 T cells (2 × 10^5/ml) were placed in 96-well plates coated with anti-CD3 (10 μg/ml). IL-6 (5 ng/ml) and/or TNF-α (10 ng/ml), or anti-CD28 (1:50) were added and cultured for 48 h. Proliferation was measured by the incorporation of [3H]thymidine between 48 and 72 h.

FIGURE 6. IL-6 and TNF-α enhance the proliferation of naive CD8 T cells from anti-male Ag (HY) TCR transgenic Rag-2−/− mice. Naive anti-HY CD8 T cells (2 × 10^5/ml) were cultured with plate-bound anti-CD3 (coated at 10 μg/ml) in the presence or absence of TNF-α (20 ng/ml), IL-6 (5 ng/ml); 2) TNF-α (5 ng/ml), IL-6 (2 ng/ml); 3) TNF-α (2 ng/ml), IL-6 (1 ng/ml); and 4) TNF-α (0.5 ng/ml), IL-6 (0.5 ng/ml). No responses to cytokines were seen in the absence of anti-CD3.

FIGURE 7. IL-6 and TNF-α costimulate the response of CD8 T cells from CD28−/− mice to plate-bound anti-CD3. CD8 T cells from CD28−/− mice were cultured in the presence or absence of plate-bound anti-CD3, IL-6, and TNF-α at four different concentrations: 1) TNF-α (20 ng/ml), IL-6 (5 ng/ml); 2) TNF-α (5 ng/ml), IL-6 (2 ng/ml); 3) TNF-α (2 ng/ml), IL-6 (1 ng/ml); and 4) TNF-α (0.5 ng/ml), IL-6 (0.5 ng/ml). No responses to cytokines were seen in the absence of anti-CD3.

FIGURE 8. IL-6 and TNF-α costimulate the response of CD8 T cells from IL-2 and IL-2Rα−/− mice to plate-bound anti-CD3. CD8 T cells from the cytokine or cytokine receptor-deficient mice were cultured in the presence or absence of plate-bound anti-CD3, IL-6, and TNF-α. No responses to cytokines were seen in the absence of anti-CD3.
tracker dye, CFSE, coupled with PI allowed us to follow the fate of the stimulated cells. In all cases, the CD8 T cells divided in response to T cell ligation, and no cells were left that had not divided. When strong costimulation was provided, a major fraction of the dividing cells remained alive and made it through multiple divisions. When less costimulation was provided, a much higher proportion of cells died after each division. When steps were taken to reduce costimulatory signals to the minimum by using CD8 T cells from TCR transgenic, Rag-2-deficient, or SCID mice, devoid of memory T cells, most of the cells died as judged by PI-positive stain and by low cell recovery. Nevertheless, some cells that had been through multiple division could always be detected. One interpretation, which we favor, is that CD8 T cells divide in response to T cell ligation alone, but die rapidly. In this interpretation, co-stimulatory molecules provide survival signals that keep the responding cells alive. Another interpretation is that the division seen with anti-CD3 alone is due to cells receiving residual co-stimulatory signals from small numbers of contaminating APC in the CD8 T cell preparation. We have repeated this experiment a number of times with similar results, but varying degrees of cell death. We have not pursued this further, since, if all costimulation is removed, all the cells die, either before or after division, and there is no good experimental finding to discriminate conclusively between the two models.

Second, we have shown that there are alternate costimulator molecules to ICAM-1 and B7.1 and B7.2, including IL-6 and TNF-α, that the latter two act directly on the responding CD8 T cells, and that they act on naive CD8 T cells via a pathway that is independent of CD28 and IL-2. The resulting effectors have similar effector functions to cells stimulated with B7-positive APC in that they are strongly cytotoxic but they secret little or no cytokine upon restimulation.

In the studies reported, we set out to define the costimulatory signals that are required for the Ag-driven proliferative response of naive peripheral CD8 T cells. We used purified T cells from “Clone-4” mice bearing the α- and β-chain of the TCR specific for the HA peptide, IYSTVASSL. The majority of the cells have not encountered Ag, and the CD8 cells from these mice are largely naive, as shown by flow cytometric analyses of cell surface markers and by the fact they do not respond to TCR ligation alone, as judged by [3 H]thymidine incorporation in the 48–60 h interval of culture. A small but variable number of memory cells are present, and their presence can affect the response to other cytokines, as will be discussed below.

In earlier studies (our unpublished observations), we had made an analysis of costimulatory action using CL7 fibroblasts transfected with known costimulatory molecules. It was noteworthy, however, that although these transfectants expressed adequate levels of class I MHC, B7.1, and ICAM-1, they were less stimulatory than other APC that we tested. When we screened the various APC for the presence of cytokine mRNA, however, we found that the RNA from the more effective APC contained message for a number of potential stimulatory factors, including IL-6 and TNF-α,
which had been previously shown to enhance T cell response to TCR ligation (36, 37), while the transfected CL7 fibroblast line was negative. These two cytokines were then tested in the presence and absence of stimulation by plate-bound anti-CD3, and it was found that they could deliver these survival signals in the absence of costimulation via CD28. Thus, [3H]thymidine incorporation was enhanced, and cell recovery at day 4 was as good with IL-6 or TNF-α as that with anti-CD28. Titrations of the effect of the cytokines showed that the response became almost maximal by 5 ng/ml. IL-6 and TNF-α together were somewhat more stimulatory than either alone, but, in general, with cells from the Clone-4 mice, there did not seem to be much evidence that the effects of the two cytokines were additive.

Similar experiments with other cytokines (data not shown) showed that IL-1 was without effect and that TGF-β was strongly inhibitory. (Other candidate molecules could not be tested, murine TNF-β/LT-α, because only the human cytokine is available, LTβ because it is not secreted).

We next asked whether these factors acted directly on CD8 T cells or via some intermediate cell present in the purified but unsorted CD8 T cell population. Purified CD8 T cells were reacted with anti-CD8 Ab and positively selected in the cell sorter. We found that the level of [3H]thymidine incorporation and the yield of cells at day four was just as high for sorted CD8 T cells as for the unsorted culture. We concluded that the action of the cytokines did not depend on contaminating cells and that they must act directly on the CD8 T cells.

We have shown elsewhere that IL-2 is produced by memory CD8 T cells when they are restimulated (65) and it was thus possible that anti-CD3 induced IL-2 secretion in the memory cells, which allowed the response of the naive cells to proceed. It was clear, however, that the two cytokines can act directly on naive CD8 T cells, without “help” from memory cells, as the same costimulatory effect was observed when CD8 T cells from TCR transgenic anti-HY Rag-2-/- mice were used. T cells from the anti-HY Rag-2-/- mice are unable to express endogenous α- or β-chains and therefore generate little or no memory T cells in the absence of the male Ag. The response was somewhat lower than that of the wild-type mice, presumably because there was no IL-2 production from memory T cells to enhance the expansion of the responding cells. Joseph et al. (36) have argued that IL-6 and TNF-α are only costimulatory if IL-2 has already been induced. It is still possible that anti-CD3 alone induces some IL-2 in the naive cells, but there was almost no proliferation to anti-CD3 alone. It was noteworthy that, in these experiments and in other experiments in which the number of memory cells and hence the potential for IL-2 production was minimized, that IL-6 alone was barely stimulatory and TNF-α alone was less strongly costimulatory, but that there was now a marked synergy when the two cytokines were added together. We hypothesize that each of the two cytokines are costimulatory in the presence of small amounts of IL-2, but that both must be added when IL-2 is present in very small amounts (as with cells from the Rag-2-/- mice or the CD28-/- mice) or is totally absent, as in the case of the IL-2 knockout). The responses are also larger when IL-2 is present, since IL-2 is a T cell growth factor.

Several investigators (36, 66, 67) have suggested that the requirement for costimulation is reduced when the TCR signal is strong. Other studies have shown requirements for B7 and ICAM-1 costimulation using Drosophila cells (42). Here, the TCR ligand is presumably present at high density, and yet costimulation was required. In the study of Cai and Sprent (66), RMSAs cells were used, which only express cell surface class I MHC when stabilized by peptide. RMSAs is a thymoma and is potentially a source of costimulatory cytokines. Goldstein et al. (6), however, used H-2Ld loaded with peptide at high density, and found the CD8 T cells from 2C mice could respond without any costimulation at all. In our studies, we showed that the costimulatory cytokines were effective at both high (anti-CD3) and low (the CL7 transfectants) TCR ligand cross-linking (data not shown). More recently, Cai and her colleagues (7) have shown that the mobility of the ligand for the TCR is also a factor, providing a much stronger and sufficient signal when the ligand for the TCR is immobilized.

The experiments described in Figs. 5–8 did nothing to reveal the mechanism of the costimulatory effect of the IL-6 nor TNF-α, although the fact that the two cytokines enhance the response to the B7.1-ICAM-1-transfected fibroblasts (data not shown) suggested that they may act by some pathway other than that used by these two costimulatory molecules. We have shown that IL-6 and TNF-α induce only very modest amounts of IL-2, compared with anti-CD28, also suggesting a different pathway. Experiments with CD28 knockout mice revealed that IL-6 and TNF-α costimulation was still observed and must act via some pathway that did not involve CD28. The experiments with CD8 T cells from the IL-2- and IL-2Rα-deficient mice showed that the two cytokines did not require the induction of, or a response to, IL-2 and more probably acted via a signal pathway that led directly to enhanced CD8 survival. It is possible that the IL-6 and TNF-α act directly to elevate survival gene expression or down-regulate death gene expression, but the nature of the signaling pathway has yet to be determined.

The response of the cells from the IL-2-deficient mice, and especially from the IL-2Rα-deficient mice, were much lower than that of cells from the wild-type animal. The significance of the observed lower response is not clear, but CD8 T cells from these mice display a memory phenotype (data not shown) and have been shown to be relatively unresponsive to antigenic stimulation (68).

It was important to determine whether activated CD8 T cells generated with IL-6/TNF-α costimulation, which we designated Tc6/TNF-α, had effector functions. They were indeed as cytotoxic as Tc1 effectors generated with IL-2, IL-12, anti-IL-4, and peptide-loaded APC. They were less cytotoxic than Tc2 effectors generated with IL-2, IL-4, anti-IFN-γ, and peptide-loaded APC and they made only very modest amounts of IFN-γ and no detectable IL-2, IL-4, IL-5, or IL-10. The absence of significant cytokine secretion suggests that different subsets of effectors may arise as a result of different sources of costimulatory signals. We are currently engaged in a more systematic investigation of the range of effector functions.

Finally, it should be noted that the ability of cytokines to substitute for costimulation via B7 may be important in allowing the activation of the class I MHC-restricted CD8 T cells, in the absence of help from IL-2-secreting CD4 T cells and by nonprofessional APC lacking B7 or other cell surface costimulatory molecules. This may be crucial in the response to viruses that do not initially infect APC and that are noncytopathic. Both cytokines can be derived from multiple sources. IL-6 is made by monocytes, fibroblasts, endothelial cells, macrophages, and also by T and B lymphocytes and other cells after activation. TNF-α is secreted by macrophages, neutrophils, and NK cells, following stimulation by IFNs, LPS, or other bacterial products and by activated T cells. The cytokines could also provide an APC-independent alternative to so-called “licensed” APC (69–71) to activate CD8 T cells.

References


