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Progressive Differentiation and Commitment of CD8+ T Cells to a Poorly Cytolytic CD8low Phenotype in the Presence of IL-4

Norbert Kienzle,² Stuart Olver, Kathy Buttigieg, Penny Groves, Michelle L. Janas, Adriana Baz, and Anne Kelso

Exposure to IL-4 during activation of naive murine CD8+ T cells leads to generation of IL-4-producing effector cells with reduced surface CD8, low perforin, granzyme B and granzyme C mRNA, and poor cytolytic function. We show in this study that maximal development of these cells depended on exposure to IL-4 for the first 5 days of activation. Although IL-4 was not required at later times, CD8 T cell clones continued to lose surface CD8 expression with prolonged culture, suggesting commitment to the CD8low phenotype. This state was reversible in early differentiation. When single CD8low cells from 4-day cultures were cultured without IL-4, 65% gave rise to clones that partly or wholly comprised CD8high cells; the proportion of reverted clones was reduced or increased when the cells were cloned in the presence of IL-4 or anti-IL-4 Ab, respectively. CD8 expression positively correlated with perforin and granzyme A, B, and C mRNA, and negatively correlated with IL-4 mRNA levels among these clones. By contrast, most CD8low cells isolated at later time points maintained their phenotype, produced IL-4, and exhibited poor cytolytic function after many weeks in the absence of exogenous IL-4. We conclude that IL-4-dependent down-regulation of CD8 is associated with progressive differentiation and commitment to yield IL-4-producing cells with little cytolytic activity. These data suggest that the CD4+CD8low cells identified in some disease states may be the product of a previously unrecognized pathway of effector differentiation from conventional CD8+ T cells. The Journal of Immunology, 2005, 174: 2021–2029.

Naive CD8+ T cells differentiate into activated CD8+ T cells that have two major Ag-dependent effector functions, cytokine expression and cytolytic activity. CD8+ T cells can lyse target cells by two independent mechanisms, via Fas-Fas ligand interaction or granule exocytosis. In the latter pathway, CTL exocytose perforin, granzymes, and other mediators that are endocytosed by the target cell; perforin enables the release of endosomal contents into the target cell cytoplasm, where granzymes A and B (and potentially C) are known to trigger apoptosis (1, 2). CTL typically display a type 1 cytokine profile (IL-2 and IFN-γ), but the presence of IL-4 during primary activation promotes the development of effector cells that produce IL-4 and other type 2 cytokines (IL-5, IL-6, IL-10, IL-13) (3–6).

The effect of IL-4 on the development of cytolytic function appears to be variable, both in vitro and in models of tumor or viral immunity in vivo. Some work in which IL-4 has been ablated by gene targeting or delivered exogenously, for example using recombinant viruses, indicated that IL-4 reduced CTL activity and/or viral clearance (7–15). Adoptive transfer of type 1- or 2-polarized CD8+ T cells (termed Tc1 or Tc2, respectively) also showed that IL-4-producing Tc2 cells were less efficient than Tc1 cells in tumor rejection (16–18). By contrast, other studies have suggested that IL-4 either had no effect or enhanced the CTL response and viral or tumor clearance in vivo (19–21). Variable results were also reported in vitro: while several groups reported that type 2-polarized CD8+ T cell populations displayed strong CTL activity and CD8 expression (5, 22–24), Erard et al. (4) showed that IL-4-exposed CD8+ populations secreted type 2 cytokines without IFN-γ, were deficient in cytolytic activity and perforin expression, provided noncognate help for B cells, and down-regulated surface CD8 expression.

The last observation raises the possibility that low CD8 expression could serve as a surrogate marker for type 2-polarized CD8 T cells with poor cytolytic activity, which might be masked in populations that also contain CTL. Support for this idea comes from the observation that in vitro stimulation of PBMC from HIV-1-infected patients gave rise to CD4+CD8+ T cell clones that were poor CTL, expressed IL-4 but no IFN-γ, and provided B cell helper function (25). Moreover, we recently found that activation of naive (CD44low) CD8+ T cell populations with anti-CD3 and other anti-receptor Ab, or in MLR, in the presence of IL-4 gave rise to heterogeneous populations of CD8high and CD8low CD4- effector cells. Whereas the CD8high cells produced IL-4 and IFN-γ, were cytolytic, and expressed high perforin and granzyme mRNA levels, the CD8low cells produced IL-4 and IFN-γ, but were poorly cytolytic and expressed low levels of perforin and granzyme B and C mRNAs (26, 27). CD8α expression was also down-regulated at the mRNA level in the latter cells (26). Single cell stimulation showed extensive heterogeneity within individual clones, with their content of CD8low cells ranging from 0 to 100%; these clonal studies indicated that essentially every naive CD8+ T cell could give rise to CD8low cells in the presence of IL-4.
CD8\textsuperscript{low} cells could arise by two, not mutually exclusive, mechanisms. First, loss of CD8 could be a transient phenomenon. For example, previous work has shown that activated CD8\textsuperscript{T} cells transiently down-regulate surface CD8 (and the TCR) in response to peptide/MHC recognition (28–30). Second, the CD8\textsuperscript{high} phenotype could define a population of differentiated effector cells. To distinguish these two possibilities, we have now undertaken kinetic and clonal studies in which we have tracked CD8 expression and cytolytic function in the progeny of individual CD8\textsuperscript{T} cells activated in the presence of IL-4. We show in this study that early and sustained IL-4 exposure is needed for optimal induction of IL-4-producing CD8\textsuperscript{low} cells that then progressively lose the capacity to re-express CD8 and give rise to a stable, IL-4-independent population of poorly cytolytic effector cells. These data suggest that the activated CD4\textsuperscript{+} CD8\textsuperscript{−} cells identified in some disease states, including HIV infection, may be the product of a previously unrecognized pathway of effector differentiation for CD8\textsuperscript{T} cells.

Materials and Methods

**T cell preparation and analysis**

All animal studies were approved by the Queensland Institute of Medical Research Animal Ethics Committee. Spleen cell preparations from C57BL/6 mice from the Animal Resources Centre (Western Australia) were used at 6–9 wk. Cell suspensions from brachial, axillary, inguinal, and lumbar lymph nodes were passed through stainless steel mesh, followed by Ficoll-Paque (Amersham Biosciences) separation. Cells were incubated with PE- or FITC-conjugated anti-CD3e Ab (53.6; BD Pharmingen) and biotinylated anti-CD44 Ab (IM7.81; BD Pharmingen), followed by FITC- or PE-conjugated streptavidin (CalTag Laboratories), respectively, then resuspended in balanced salt solution with 5% heat-inactivated FCS (CSL) and 1 μg/ml propidium iodide (Calbiochem). Viable naive cells were purified using a FACSVantage with Lysis II software (BD Biosciences) based on CD8\textsuperscript{−} and CD44\textsuperscript{high} (lowest 30%) expression; cells were >97% CD8\textsuperscript{−} on reanalysis. For cloning, individual cells were sorted twice onto a glass slide, and on the second sort were deposited in 96-well round-bottom plates (Falcon; BD Biosciences) using an automated cell deposition unit twice, and on the second sort were deposited in 96-well round-bottom plates (Falcon; BD Biosciences) using an automated cell deposition unit. For purification of activated CD8\textsuperscript{high} and CD8\textsuperscript{low} cells, live cells were sorted for high or low CD8 expression, respectively, then resuspended in balanced salt solution with 5% heat-inactivated FCS (CSL) and 1 μg/ml propidium iodide (Calbiochem). Viable naive cells were purified using a FACSVantage with Lysis II software (BD Biosciences) based on CD8\textsuperscript{−} and CD44\textsuperscript{high} expression; cells were >97% CD8\textsuperscript{−} on reanalysis. For cloning, individual cells were sorted onto a glass slide, and on the second sort were deposited in 96-well round-bottom plates (Falcon; BD Biosciences) using an automated cell deposition unit attached to the FACSVantage. For purification of activated CD8\textsuperscript{high} and CD8\textsuperscript{low} cells, live cells were sorted for high (>80) or low or nil (<10) fluorescence intensity of CD8 expression. For analysis without sorting, a FACScalibur was used with CellQuest V3.1f software (BD Biosciences).

**Activation of CD8\textsuperscript{T} T cells with anti-CD3/8/11a Ab**

Single or 1–5 × 10\textsuperscript{5} CD8\textsuperscript{44}\textsuperscript{low} T cells were cultured either in 200 μl in 96-well plates or in 2 ml in 24-well plates previously coated with protein G-purified hamster anti-mouse CD3e (10 μg/ml; 145-2C11), rat anti-mouse CD8α (10 μg/ml; 53.6), and rat anti-mouse CD11a (5 μg/ml; I21/7.7) Ab (26, 31, 32). Cultures were established in growth medium (modified RPMI 1640 containing 10% heat-inactivated FCS (CSL) and 1 μg/ml propidium iodide (Calbiochem)). Viable naive cells were purified using a FACSVantage with Lysis II software (BD Biosciences) based on CD8\textsuperscript{−} and CD44\textsuperscript{high} (lowest 30%) expression; cells were >97% CD8\textsuperscript{−} on reanalysis. For cloning, individual cells were sorted twice, and on the second sort were deposited in 96-well round-bottom plates (Falcon; BD Biosciences) using an automated cell deposition unit attached to the FACSVantage. For purification of activated CD8\textsuperscript{high} and CD8\textsuperscript{low} cells, viable T cells were sorted for high (>80) or low or nil (<10) fluorescence intensity of CD8 expression. For analysis without sorting, a FACScalibur was used with CellQuest V3.1f software (BD Biosciences).

**Activation of CD8\textsuperscript{T} T cells in MLR**

CD8\textsuperscript{44}\textsuperscript{low} T cells (1.5–10 × 10\textsuperscript{5}) from C57BL/6 mice were incubated with 1–2 × 10\textsuperscript{5} allogeneic spleen cells (gamma-irradiated with 3000 rad) from DBA/2 mice in 2 ml of growth medium in neutral or type 2 culture conditions in 24-well plates for 8–9 days. For long-term MLR, T cells were restimulated with irradiated DBA/2 spleen cells weekly.

**Cytokine induction assays**

For clonal assays, cells were washed three times in situ and incubated overnight in the same wells containing immobilized anti-CD3/8/11a Ab with 200 μl of growth medium and 20 IU/ml IL-2. After 18–22 h, supernatants were assayed for cytokines at a single concentration (100%). For bulk assays, T cell populations (5–10 × 10\textsuperscript{5}) were incubated for 22–29 h in 200 μl with IL-2 and 10 μg/ml immobilized anti-CD3 Ab, 5 × 10\textsuperscript{4} allogeneic target cells, or medium only. Duplicate or triplicate serial dilutions of supernatant were assayed for IL-4 and IFN-γ by ELISA using the anti-IL-4 Ab BV4D and biotinylated BVD6 (34) or the anti-IFN-γ Ab R4-6A2 and biotinylated XM1G2, respectively. In Fig. 1, IFN-γ was measured by growth inhibition of the IFN-γ-sensitive cell line WEHI-279 in a colorimetric assay (35). IL-4 and IFN-γ activities were standardized by reference to titrations of baculovirus-derived murine rIL-4 or purified murine IFN-γ (Sigma-Aldrich). For IL-4 neutralization, anti-IL-4 (clone 11B11 or BV4D) or control Ab were added at 10 μg/ml at the start of incubation; these concentrations completely neutralized the secreted IL-4.

**51Cr release assay for cytolytic T cells**

Cells of the Flr\textsuperscript{r} mastocytoma line P815 were labeled with Na\textsuperscript{14}CrO\textsubscript{4} (Amersham Biosciences) for 60 min at 37°C and washed twice in growth medium. Labeled target cells (2–5 × 10\textsuperscript{4}) were incubated for 4–5 h at 37°C with duplicate serial dilutions of T cells in 200 μl in round-bottom 96-well plates. In the case of anti-CD3/8/11a Ab-stimulated T cells, 1 μg/ml anti-CD3 Ab was added to bridge T cells with target cells (redirected assay). Harvested supernatants were dried onto 96-well solid Lumaplates (Packard Instrument), and radioactivity was counted in a Topcount Microplate Scintillation Counter (Packard Instrument).

**RT-PCR and quantitative competitive PCR (QC-PCR)**

Primer sequences and protocols for mRNA preparation, cDNA conversion, and amplification were published recently (26, 36, 37). Briefly, RNA was extracted by Nonident P-40 hypotonic lysis of 1–5 × 10\textsuperscript{5} cells, and cDNA was amplified using avian myeloblastosis virus reverse transcriptase (Promega), oligo(dT) primers, and RNase inhibitors. QC-PCR of single or duplicate cDNA samples was performed with Red Hot polymerase (Advanced Biotechnologies), dNTPs, competitor plasmid, and one of the primer pairs specific for cDNA of perforin, granzyme A, granzyme B, granzyme C, IL-4, and IFN-γ, with the reverse primers being biotinylated at their 5′ ends. Each competitor plasmid encoded the corresponding PCR product sequence with a small deletion and was tested in 5-fold dilutions against a fixed amount of cDNA. PCR products were captured on streptavidin-coated plates, hybridized with FITC-labeled probes specific for either the cDNA or competitor product, and quantitated with an alkaline phosphatase-conjugated anti-FITC Ab and 4-nitrophenylphosphate (Roche Diagnostics Australia). PCR amplification (25 μl assay in 96-well plates) was performed using an Omnigene thermal cycler (Thermo Hybaid), as follows: 1 cycle of 96°C for 4 min, then 60°C for 1 min and 72°C for 1.5 min, followed by 39 cycles of 94°C for 0.5 min, then 60°C for 1 min and 72°C for 1.5 min. For expression controls, CD3e or β\textsubscript{2}-microglobulin cDNA was amplified by QC-PCR or by PCR using serial dilutions of cDNA, respectively.

**Statistical analyses**

The Prism 4.0 software package (GraphPad) was used for statistical analyses.

**Results**

**Requirement for early and sustained exposure to IL-4 for the development of poorly cytolytic IL-4-producing CD8\textsuperscript{low} cells**

We have previously reported that activation of naive CD8\textsuperscript{T} cells in the presence of IL-4 led to development of a subpopulation of IL-4-producing effector cells with poor cytolytic activity and low CD8 expression (26). To determine whether this response required exposure to IL-4 early in primary activation, we studied the effects of delayed IL-4 addition on development of cytolytic and cytotoxic-producing clones from individual CD8\textsuperscript{T} cells. As in our earlier studies (26), a single cell, accessory cell-free culture system was used to allow tracking of the progeny of individual cells and to limit the influence of endogenous cytokines on cell differentiation. Single CD8\textsuperscript{low} cells of naive CD8\textsuperscript{44}\textsuperscript{low} phenotype were purified from lymph nodes of C57BL/6 mice and cultured with IL-2 and immobilized Ab to CD3, CD8, and CD11a, either in the absence of other cytokines (neutral conditions) or in the presence of IL-4 and neutralizing anti-IFN-γ Ab (type 2-polarizing conditions)
added at various times after culture initiation. Cultures were washed after 8 days, then assayed on day 9 for cytokine production and cytolytic activity in a redirected 51Cr release assay. For the latter assay, FcR-bearing P815 tumor cells were used as target cells with bridging anti-CD3 Ab to bypass the requirement for peptide/MHC recognition.

Analysis of individual clones grown in neutral conditions showed that most secreted IFN-γ and very few secreted IL-4 (Fig. 1A). Addition of IL-4 and anti-IFN-γ Ab at day 0 did significantly affect cloning efficiency, but caused a modest reduction in the frequency of IFN-γ-secreting clones and markedly increased the frequency that secreted IL-4. When addition of type 2 stimuli was delayed by 3 or 6 days, the frequency of IL-4-secreting clones was reduced by 21 and 84%, respectively. Fig. 1B shows the cytolytic activity of pools of clones from these cultures. Whereas those grown in neutral conditions were highly cytolytic in the presence of bridging anti-CD3 Ab, pooled clones grown in type 2 conditions from day 0 displayed little lytic activity. CTL activity was higher when addition of type 2 stimuli was delayed to day 3 and was equivalent to that observed in neutral conditions when delayed to day 6. Other experiments showed that the effect of type 2 conditions on both IL-4 production and cytotoxic activity was mainly due to exogenous IL-4, not the anti-IFN-γ Ab (26) (data not shown).

Similar results were obtained in two other experiments. Analysis of a total of 1181 clones derived from CD8+/CD44low cells in the three experiments showed that exposure to type 2 stimuli from day 0 significantly reduced the frequency of CTL-positive clones and increased the frequency of IL-4 producers compared with neutral conditions (p < 0.001; z test). Compared with addition of type 2 stimuli at day 0, delayed addition by 1 or more days progressively increased the frequency of cytolytic clones and/or reduced the frequency of IL-4 producers; when addition was delayed to day 6, frequencies of all parameters were equivalent to those observed in neutral conditions.

These data support the idea that early exposure to type 2 conditions is required for maximal induction of IL-4-producing noncytolytic T cells in this system. However, an alternative interpretation is that late addition of type 2 stimuli reduced the time available for cells to acquire the noncytolytic IL-4-producing phenotype. To test the latter possibility and to analyze CD8 expression in parallel with effector function, CD8+/CD44low cells were activated in bulk cultures in neutral conditions or with addition of IL-4 and anti-IFN-γ Ab at various times between days 0 and 6; after 7 days, cells were harvested and cultured for 1 day in neutral conditions to allow IL-4 secretion, or for 7 days in type 2 conditions before culture for 1 day in neutral conditions. At day 8, most cells cultured in neutral conditions retained high levels of surface CD8α, whereas most cells exposed to type 2 stimuli from day 0 expressed low or undetectable CD8α levels (Fig. 2A). As in our previous study (26), CTL activity was markedly lower in the CD8high population compared with the CD8high population tested in a redirected cytotoxicity assay at day 7. Other experiments indicated that these CD8high cells were first detectable at day 3–4 and reached maximal frequencies at day 7–8 (data not shown). Delayed addition of the type 2 stimuli by 1–4 days progressively increased the proportion of CD8high cells and decreased IL-4 titers in the culture supernatants at day 8 (Fig. 2B). Addition of type 2 stimuli at day 5 or 6, however, had no detectable effect on CD8α expression or IL-4 titers. Similar results were obtained in three other experiments; although the proportion of CD8high cells generated in type 2-polarized cultures varied between experiments, viable cell recoveries were similar under all culture conditions within each experiment, and no loss of CD3ε or TCRαβ and no induction of CD4 expression were detected (data not shown). Progressive diminution of the effects on CD8 expression and IL-4 titers with longer delay in the addition of type 2 stimuli was also seen when the duration of exposure was extended by 7 days (Fig. 2A and B). The data show that the effects of delayed

![FIGURE 1. Effect of delayed IL-4 addition on the development of cytokine-producing and cytolytic T cell clones. Single CD8+/CD44low T cells were cultured with IL-2 and immobilized Ab to CD3, CD8, and CD11a. IL-4 and neutralizing anti-IFN-γ Ab (type 2 conditions) were added to some cultures on day 0, 3, or 6, as indicated. After 8 days, cells were washed in situ and incubated in the same wells in medium with IL-2 for 1 day before functional analysis. Cloning efficiencies at day 8 were 89.6% (neutral), 86.5% (type 2 stimuli from day 0), 88.5% (type 2 stimuli from day 3), and 92.7% (type 2 stimuli from day 6). A, Culture supernatants were assayed for IL-4 by ELISA and for IFN-γ (lower figures) in a colorimetric assay measuring inhibition of WEHI-26 straight cell growth. The scale of the IFN-γ assay is inverted because low OD reflects high activity. Each dot represents a single clone. The broken line indicates the detection threshold is 3 SD of supernatants from control wells in which no clone had developed. The upper panels in each panel indicate the percentage of clones positive for IFN-γ and IL-4, respectively. B, Clones from the upper panels estimated to contain at least 10⁴ cells were pooled and titrated in a redirected 51Cr release assay using P815 target cells in the absence (○) or presence (●) of bridging anti-CD3 Ab.](http://www.jimmunol.org/2002/2023/)
and IL-2 alone (neutral conditions) or in addition with anti-IL-4 Ab, or IL-4 secretion during primary CD8 T cell effector differentiation; en-

Cell surface CD8 and induction of IL-4 synthesis and the CD8 low phenotype. We next examined the duration of IL-4 exposure needed for induction of IL-4 synthesis and the CD8 low phenotype. CD8 CD44 low T cells were activated in bulk cultures in neutral or type 2 conditions from day 0. As in previous experiments, continuous culture with type 2 stimuli showed that, on average, CD8 low cells proliferated at a similar or slightly lower rate (half a division over 3 days) than CD8 high cells. To assess division rates of CD8 high and CD8 low cells, CFSE-labeled cells were cultivated in neutral or type 2 conditions, regardless of whether the clone contained CD8 high cells or only 4 clones contained >50% CD8 high cells at the last time point analyzed. None expressed surface CD4. Other clonal experiments indicated that progressive loss of CD8 continued even when type 2 stimuli were removed and replaced with neutral conditions as early as day 4.

The progressive acquisition of the CD8 low phenotype with continued culture might reflect preferential outgrowth of CD8 high cells. To assess division rates of CD8 high and CD8 low cells, CD8 CD44 low T cells were activated in neutral or type 2 conditions, sorted for high or low CD8 expression at day 4, then labeled with the intracellular dye CFSE and restimulated for a further 3 days with anti-CD3/8/11a Ab in either neutral or type 2 conditions. In two independent experiments, analysis of CFSE levels by FACs showed that, on average, CD8 low cells proliferated at a similar or slightly lower rate (half a division over 3 days) than CD8 high cells from either neutral or type 2 cultures, regardless of whether the CFSE-labeled cells were cultivated in neutral or type 2 conditions (data not shown). We conclude that the progressive accumulation of CD8 low cells in type 2 cultures was not due to a growth advantage over CD8 high cells.

FIGURE 2. Effect of delayed addition or early removal of IL-4 on CD8 expression and IL-4 synthesis during primary CD8 T cell activation. Left panels, CD8 CD44 low T cells were cultured with IL-2 and immobilized anti-CD3/8/11a Ab. IL-4 and anti-IFN-γ Ab (type 2 stimuli) were added to some cultures on day 0 or 1, 2, 3, 4, 5, or 6 days after culture initiation. On day 7, cells were washed and equal numbers were stimulated with either: (A) 1) immobilized anti-CD3 Ab and IL-2 for 1 day, or 2) anti-CD3/8/11a Ab, IL-2, IL-4, and anti-IFN-γ Ab until day 14, then immobilized anti-CD1 Ab and IL-2 for 1 day. Cell surface CD80 expression (A) and IL-4 production (B) were assayed on day 8 (III) or day 14/15 (III) for cells cultured in neutral conditions (nil) or type 2 conditions from the indicated day of culture. The means and SD of triplicate IL-4 determinations are shown; the broken line indicates the detection threshold of the IL-4 assay. Right panels, CD8 CD44 low T cells were cultured with immobilized anti-CD3/8/11a Ab and IL-2 alone (neutral conditions) or in addition with anti-IL-4 Ab, or IL-4 and anti-IFN-γ Ab (type 2 conditions). After 2, 3, 4, or 5 days, cells in type 2 conditions were washed in situ and recultured in the same well in neutral conditions or with anti-IL-4 Ab or type 2 stimuli, as above. On day 8, cells were washed and analyzed for CD8a cell surface expression by FACS (C); equal numbers of cells were recultured with immobilized anti-CD3 Ab and IL-2 for 1 day before IL-4 assay (D).

Addition reflect a requirement for early, rather than prolonged, exposure to type 2 stimuli to achieve maximal down-regulation of CD8 expression and induction of IL-4 synthesis. Marked down-regulation of surface CD8 and induction of IL-4 expression were also seen in the presence of type 2 conditions when the anti-receptor Ab stimulation regimen was altered, either by omitting the immobilized anti-CD8 Ab or by substituting anti-CD11a with anti-CD28 Ab (data not shown).

We next examined the duration of IL-4 exposure needed for induction of IL-4 synthesis and the CD8 low phenotype. CD8 CD44 low T cells were activated in bulk cultures in neutral or type 2 conditions at day 0; some of the former cultures also received neutralizing anti-IL-4 Ab to block endogenous IL-4, or anti-CD4 Ab as a control. After 2, 3, 4, or 5 days, cells were removed from type 2 stimuli, washed, and recultured in neutral conditions, with anti-IL-4 or anti-CD4 Abs, or with type 2 stimuli. At day 8, all cultures were analyzed for CD8 expression and recultured for 1 day in neutral conditions to allow IL-4 secretion (Fig. 2, C and D). As in previous experiments, continuous culture with type 2 stimuli for 8 days caused significant down-regulation of surface CD8 levels and induction of IL-4 synthesis. Cell transfer from type 2 to neutral conditions showed that these effects were maximal after exposure to exogenous type 2 stimuli for 4 and 3 days, respectively. However, when endogenous IL-4 was neutralized with anti-IL-4 Ab in the second culture, the requirement for exposure to exogenous type 2 stimuli increased to 5 days for both parameters. The control anti-CD4 Ab had no significant effect (data not shown). We conclude that the early and continuous presence of IL-4 is required for maximal induction of CD8 low cells and IL-4 secretion during primary CD8 T cell effector differentiation; en-

dogenous IL-4 can replace the requirement for exogenous IL-4 after 3–4 days.

Loss of CD8 expression by type 2-polarized T cells is a progressive process of differentiation

The experiments described above showed that CD8 T cells exposed to type 2 stimuli for 1–2 wk were heterogeneous in their CD8 expression levels and functional activities. To determine whether the loss of CD8 increased with time, we cultured single CD8 CD44 low T cells in neutral or type 2 conditions from day 0. After 7 days, clones were transferred to neutral conditions and sampled sequentially over the next 10 days for analysis of their CD8 expression levels (Fig. 3). Of 24 clones initiated in neutral conditions, all comprised >99.3% CD8 high cells and continued to express CD8 at high frequency (>91.7%) for the remainder of the culture period. In contrast, 23 clones cultured with type 2 stimuli contained variable numbers of CD8 high cells ranging from 0 to 97.9% at day 7. None of these clones regained CD8 high cells with further culture in the absence of type 2 stimuli. Instead, the majority progressively lost CD8 expression such that 16 of the 23 clones contained <15% CD8 high cells and only 4 clones contained >50% CD8 high cells at the last time point analyzed. None expressed surface CD4. Other clonal experiments indicated that progressive loss of CD8 continued even when type 2 stimuli were removed and replaced with neutral conditions as early as day 4 (data not shown).

The progressive acquisition of the CD8 low phenotype with continued culture might reflect preferential outgrowth of CD8 high cells. To assess division rates of CD8 high and CD8 low cells, the experiments described above showed that CD8 T cells exposed to type 2 stimuli for 1–2 wk were heterogeneous in their CD8 expression levels and functional activities. To determine whether the loss of CD8 increased with time, we cultured single CD8 CD44 low T cells in neutral or type 2 conditions from day 0. After 7 days, clones were transferred to neutral conditions and sampled sequentially over the next 10 days for analysis of their CD8 expression levels (Fig. 3). Of 24 clones initiated in neutral conditions, all comprised >99.3% CD8 high cells and continued to express CD8 at high frequency (>91.7%) for the remainder of the culture period. In contrast, 23 clones cultured with type 2 stimuli contained variable numbers of CD8 high cells ranging from 0 to 97.9% at day 7. None of these clones regained CD8 high cells with further culture in the absence of type 2 stimuli. Instead, the majority progressively lost CD8 expression such that 16 of the 23 clones contained <15% CD8 high cells and only 4 clones contained >50% CD8 high cells at the last time point analyzed. None expressed surface CD4. Other clonal experiments indicated that progressive loss of CD8 continued even when type 2 stimuli were removed and replaced with neutral conditions as early as day 4 (data not shown).

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FIGURE 3. Progressive loss of CD8 expression by type 2-polarized T cell clones. Individual CD8 CD44 low T cells were cultured with anti-CD3/ 8/11a Ab and IL-2 in the absence (top panel) or presence of IL-4 and anti-IFN-γ Ab (bottom panel). After 7 days, clones estimated to contain at least 103 cells were washed in situ to remove type 2 stimuli and reincubated in the same wells in medium containing IL-2. Clones were sampled to determine the percentage of CD8 high cells on days 7, 10, 14, and 17. Each data point represents a single clone. Data were pooled from two separate experiments.
The finding that the continuing loss of CD8α expression by clones removed from type 2-polarizing conditions was not due to faster growth of CD8low cells suggested prior commitment of the cells to a continuing process of differentiation initiated by IL-4. To examine the reversibility of this process, CD8low and CD8high cells were purified from polyclonal type 2-polarized cultures at day 4 and restimulated with anti-CD3/8/11a Ab in the presence or absence of IL-4, neutralizing anti-IL-4 Ab (11B11), or a control anti-CD4 Ab. Fig. 4 shows that some cells in the CD8low population regained CD8 expression and, conversely, some cells in the CD8high population lost CD8 expression after 2 days in neutral conditions. Exogenous IL-4 decreased the frequency of CD8high cells in the CD8low population, while anti-IL-4 Ab markedly increased CD8 expression compared with neutral conditions. CD8 expression by the CD8high population was not strongly affected by any of the culture conditions, although the modest effect of IL-4 in decreasing CD8 levels may indicate the persistence of some residual IL-4-responsive cells. Similar results were obtained in two experiments using a different anti-IL-4 Ab (BVD4); both 11B11 and BVD4 increased CD8 expression in the CD8low population, while control anti-IFN-γ or anti-CD4 Ab had no significant effect (data not shown). The data suggested that some CD8low cells had the potential to regain CD8 expression when endogenous IL-4 was neutralized at days 4–6 after primary activation. However, because these experiments were performed in bulk cultures, it remained a formal possibility that rare contaminating CD8high cells accumulated because of their slight growth advantage over CD8low cells and/or preferential apoptosis of the CD8low cells; neither possibility was likely given the high purity of the day 4 FACS-purified CD8low cells (>99%) and the culture time of 2 days.

To determine definitively whether individual CD8low or CD8high cells or their progeny can change their CD8 phenotype during early type 2 polarization, we undertook a clonal analysis of cells that had been FACS purified twice to minimize contamination with cells of the opposite phenotype. CD8 T cells of naive phenotype were activated in neutral or type 2 conditions for 4 days; CD8low and CD8high cells were then sorted, yielding purities of 99.8 and 98.8%, respectively, before single cell cloning in the presence or absence of IL-4 or neutralizing anti-IL-4 Ab (Fig. 5). As detailed in the legend of Fig. 5, secondary culture conditions did not significantly affect cloning efficiencies. Clones generated in neutral conditions retained high CD8 levels regardless of secondary stimulation conditions. Clones derived from type 2-polarized cells that were CD8low or CD8high on day 4, then cultured in neutral conditions contained highly variable percentages of CD8high cells, although the median and mean values were biased toward the phenotype of the founder cell. Fewer cells of either phenotype gave rise to CD8high clones when cultured with IL-4, while neutralization of endogenous IL-4 with anti-IL-4 Ab favored re-expression of CD8 by CD8low cells; for both starting phenotypes, the median percentages of CD8high cells were significantly different between clones grown in neutral conditions and with IL-4, and between clones grown with IL-4 and with anti-IL-4 Ab (p < 0.02, two-tailed Mann-Whitney U test). Among clones derived from CD8low

FIGURE 4. Reversibility of CD8low phenotype early in effector cell differentiation. CD8low T cells were cultured with anti-CD3/8/11a Ab, IL-2, IL-4, and anti-IFN-γ Ab (type 2 conditions). A, On day 4, cells were separated into CD8low and CD8high cells. CD8 expression is shown before (thick line in upper panel; thin line is negative control binding of anti-rat IgG) and immediately after purification of the lowest 10% and highest 18% of cells (lower panels). B, CD8 expression is shown after reculture of the purified populations with anti-CD3/8/11a Ab and IL-2 (neutral) or, in addition, IL-4 or anti-IL-4 Ab (clone 11B11) for 2 days.

FIGURE 5. Clonal analysis of the reversibility of the CD8low phenotype early in effector cell differentiation. A, CD8low CD44low T cells were cultured with anti-CD3/8/11a Ab and IL-2 alone (neutral) or in addition IL-4 and anti-IFN-γ Ab (type 2). On day 4, CD8low cells from neutral cultures and CD8low and CD8high cells from type 2 cultures were sorted twice and cloned by single cell deposition into wells with anti-CD3/8/11a Ab and IL-2 (neutral) or, in addition, IL-4 or anti-IL-4 Ab (clone 11B11). B, The percentage of CD8high cells in each clone was determined 8–9 days after cloning. Each dot represents a clone estimated to contain at least 100 cells. The broken line indicates the median percentage of CD8high cells in each set of clones. Data were pooled from six independent experiments with the following average cloning efficiencies: CD8low cells from neutral cultures, neutral 5.8%, IL-4 3.2%, anti-IL-4 Ab 5.3%; CD8high cells from type 2 cultures, neutral 11.5%, IL-4 9.8%, anti-IL-4 Ab 14.1%; CD8low from type 2 cultures, neutral 11.1%, IL-4 11.6%, anti-IL-4 Ab 16.9%.
cells, 65% of those grown in neutral conditions, 41% grown with IL-4, and 72% grown with anti-IL-4 Ab contained >10% CD8^high cells. Most of the informative clones were tested for surface expression of CD4 and/or CD3; all cells were negative for CD4 and positive for CD3 expression (data not shown). We conclude that, at day 4, many CD8^low cells retained the potential to give rise to CD8^high cells, while some CD8^high cells could lose CD8. Continuous exposure to IL-4 amplified the development of CD8^low cells, while removal of IL-4 promoted reversion to a CD8^high phenotype.

We have previously reported that CD8^low cells isolated from type 2-polarized cultures at day 8 displayed markedly lower cytolytic activity and expressed lower levels of perforin, granzyme B, and granzyme C mRNAs than CD8^high cells from either neutral or type 2 cultures; granzyme A expression was lower in both CD8^low type 2-polarized cells and CD8^high cells from neutral cultures than in CD8^high type 2-polarized cells (26). To determine whether these functional parameters were also correlated with CD8 expression in individual clones, gene expression was examined in random selections of large clones from the experiment in Fig. 5. Analysis of 32 clones for expression of cytokine, perforin, and granzyme mRNAs revealed that CD8 levels were positively correlated with the expression of perforin and granzymes A, B, and C, and negatively correlated with IL-4 (Fig. 6). Most clones expressed IFN-γ without any obvious relationship to CD8 levels.

The CD8^low phenotype is stable in long-term culture

The strong stimulation provided by immobilized anti-CD3/8/CD11a Ab ultimately leads to apoptosis, limiting the use of this system for long-term studies. The long-term stability of the CD8^low phenotype following removal of type 2 stimuli was therefore analyzed in MLR. CD8^CD44low T cells from lymph nodes of untreated C57BL/6 (H-2b) mice were cultured with irradiated DBA/2 spleen cells in neutral or type 2 conditions for 9 days (Fig. 7A). Fig. 7B shows that ~30% of CD8 T cells had lost CD8 expression in type 2, but not neutral, conditions at this stage. CD8^low and CD8^high cells were then purified and propagated with DBA/2 spleen cells in neutral conditions. The CD8 expression profile of the propagated cells was monitored for another 4 wk (Fig. 7C). CD8^high cells maintained their phenotype regardless of their origin. The CD8^low phenotype was also largely maintained over time despite the early emergence of a subpopulation of CD8^high cells. All cells were CD3^+ CD4^−, and loss of surface CD8

**FIGURE 7.** Long-term stability of the CD8^low phenotype. A, CD8^CD44low T cells from C57BL/6 mice were cultured in MLR with irradiated DBA/2 spleen cells and IL-2 (neutral conditions) or in addition with IL-4 and anti-IFN-γ Ab (type 2 conditions). On day 9, CD8^high and CD8^low cells were purified and recultured weekly with irradiated DBA/2 spleen cells and IL-2 for up to 50 days; where indicated, cells (d) were resorted for CD8^high and CD8^low cells. ○, Represent CD8^high cells; ●, represent CD8^low cells. a–g, Indicate populations analyzed in the following panels. B, FACS histograms are shown for surface CD8α expression in populations a and b at day 9. The percentage of CD8^high cells is indicated above the M1 bar. C, The percentage of CD8^high cells is shown in populations derived from CD8^high cells of neutral culture origin (c) and from CD8^low (d) and CD8^low cells (e) of type 2 culture origin on the indicated days of culture. D, CD8^high cells of neutral (c) or type 2-polarized (e) culture origin, and CD8^low cells (g) or CD8^low cells (j) of type 2-polarized origin repurified on day 36, were assayed on day 42 for cytolytic function against 51Cr-labeled allogeneic P815 target cells. E, In an independent experiment, the indicated populations were incubated on day 50 with allogeneic P815 target cells for 20 h before assay of supernatants for IL-4 and IFN-γ by ELISA. The broken line depicts the detection limit of the assay.
protein correlated with very low CD8α mRNA expression levels in CD8\textsuperscript{low} cells when compared with the corresponding CD8\textsuperscript{high} siblings (data not shown). The CD8\textsuperscript{low} and CD8\textsuperscript{high} subpopulations were isolated at day 36 and recultured in neutral conditions (Fig. 7A, cultures f and g). Subsequent analysis of all populations revealed significant functional differences. The long-term CD8\textsuperscript{low} cells (f) were poor CTL in contrast to the CD8\textsuperscript{high} populations, all of which lysed H-2\textsuperscript{a} target cells in a 51Cr release assay (Fig. 7D). Both CD8\textsuperscript{low} and CD8\textsuperscript{high} cells from type 2 cultures secreted IL-4 in response to P815 cells, whereas CD8\textsuperscript{high} cells from neutral cultures did not; in contrast, the type 2-polarized populations produced lower levels of IFN-γ than CD8\textsuperscript{high} cells from neutral cultures (Fig. 7E). Population g displayed the classical Tc2 phenotype (CD8\textsuperscript{+ IL-4\textsuperscript{+} CTL\textsuperscript{−}}) previously described by others and shown to exert a variety of effects in mouse models (16–18). From this and two other experiments analyzing long-term cultures between days 20 and 73, it was concluded that the CD8\textsuperscript{low} phenotype showed a high degree of stability through many rounds of cell division in MLR and was associated with high IL-4 synthesis, limited IFN-γ synthesis, and negligible CTL function. Additional experiments showed that CD8 expression in long-term clonal and polyclonal cultures of CD8\textsuperscript{low} or CD8\textsuperscript{high} cells in MLR was not influenced by exogenous IL-4 or anti-IL-4 Ab (data not shown), supporting the conclusion that these are stable differentiated phenotypes.

**Discussion**

Exposure to IL-4 during primary activation of conventional naive CD8\textsuperscript{+} T cells leads to the generation of effector cells with reduced expression of surface CD8 and an unusual functional phenotype. Compared with CD8\textsuperscript{high} cells activated in the absence of IL-4, this CD8\textsuperscript{low} T cell population expresses higher IL-4 and lower IFN-γ mRNA and protein levels, markedly lower levels of perforin, granzyme B and granzyme C mRNAs, and negligible cytolytic activity. In this study, we show that the IL-4-dependent down-regulation of CD8 and other gene expression changes are not a transient response to activation, but instead are acquired by progressive differentiation and commitment to yield effector cells with a stable, poorly cytolytic CD8\textsuperscript{low} phenotype.

Several lines of evidence support this conclusion. First, development of these cells depended on early and sustained exposure to IL-4. Delayed addition of IL-4 progressively reduced IL-4 induction and CD8 loss, while removal after 3–4 days indicated that endogenous IL-4 could substitute for exogenous IL-4 until about day 5 when continued exposure had no further effect. Second, although the time window for induction of CD8 loss was short, surface CD8 levels continued to decline after removal of exogenous IL-4, beyond the period when endogenous IL-4 would have a significant effect. This phenomenon was not due to preferential outgrowth of CD8\textsuperscript{low} cells. Instead, it appeared to reflect both prior commitment of CD8\textsuperscript{high} cells to a CD8\textsuperscript{low} phenotype and transmission of the CD8\textsuperscript{low} phenotype to daughter cells as the clone expanded.

Third, reversibility studies showed that commitment to the CD8\textsuperscript{low} phenotype increased with time in culture. The loss of surface CD8 was reversible in some cells if IL-4 was removed during the first week of activation. Single cell cloning experiments showed definitively that individual CD8\textsuperscript{low} cells from 4-day cultures could give rise to CD8\textsuperscript{high} progeny, particularly when exogenous IL-4 was removed and endogenous IL-4 was neutralized. In long-term culture, however, most CD8\textsuperscript{low} cells maintained their phenotype whether or not IL-4 or anti-IL-4 Ab was added. The stability of the CD8\textsuperscript{low} phenotype and its transmission to progeny in single cell studies distinguish it from the transient down-regulation of CD8 (and TCR) expression that can occur following peptide/MHC recognition (28–30).

The progressive commitment to the CD8\textsuperscript{low} phenotype is reminiscent of the observation by Murphy et al. (38) that CD4\textsuperscript{+} T cell populations could convert between Th1 and Th2 cytokine profiles when exposed to counterpolarizing stimuli after the first week of culture, but lost this ability after long-term stimulation. The bulk culture experiments in that study did not show direct conversion of individual Th1 or Th2 cells; they are consistent, however, with our earlier clonal studies demonstrating that the frequency of IL-4-responsive multipotent CD8\textsuperscript{+} T cells (i.e., cells that give rise to progeny with different cytokine profiles in the presence and absence of IL-4) declines with primary activation both in vitro and in vivo (6, 39). The new data reported in this work further show that both the CD8\textsuperscript{low} and CD8\textsuperscript{high} phenotypes are reversible at the single cell level by removal or addition of IL-4 early after activation, but become stable with extended culture.

Loss of CD8 expression was not a technical artifact due to outgrowth of contaminating CD4\textsuperscript{+} T cells. Essentially, every naive CD8\textsuperscript{+} T cell could give rise to CD8\textsuperscript{low} progeny when cloned in the presence of IL-4 (26), and CD4 expression was not detected on any CD8\textsuperscript{low} populations and clones tested. The intraclonal heterogeneity of CD8 expression early in expansion of many of the T cell clones described in this work and the observation that single CD8\textsuperscript{low} cells could give rise to CD8\textsuperscript{+} cells further support the conclusion that this is a property of conventional CD8\textsuperscript{+} T cells exposed to IL-4. By contrast, we found no evidence that clonal or bulk cultures of naive CD4\textsuperscript{+} T cells lost surface CD4 expression when exposed to IL-4, even at concentrations 30-fold higher than those used for CD8\textsuperscript{+} T cells (our unpublished observations).

Naive CD8\textsuperscript{+} T cell activation in the presence of IL-4 led to the generation of effector cell populations that were mixed not only in their CD8 expression, but also in their effector functions, producing IFN-γ and IL-4 and including both cytolytic and noncytolytic cells. In our earlier study (26), both CD8\textsuperscript{high} and CD8\textsuperscript{low} populations from these cultures secreted IFN-γ and IL-4, with some bias toward IL-4 production in the CD8\textsuperscript{low} population. However, loss of CD8 was strongly associated with poor cytolytic function, low levels of perforin, granzyme B and granzyme C mRNA levels, and a reduction in granzyme A expression compared with CD8\textsuperscript{high} cells from the same cultures. One of the striking observations made in this work and in our earlier study was that many individual clones generated under type 2 conditions also contained mixtures of CD8\textsuperscript{high} and CD8\textsuperscript{low} cells. In this study, we show that, among such clones, the percentage of CD8\textsuperscript{high} cells was positively correlated with average numbers of perforin and granzyme A, B, and C mRNA molecules per cell and negatively correlated with average numbers of IL-4 mRNA molecules per cell. These quantitative correlations raise the possibility that clones containing a mixture of CD8\textsuperscript{high} and CD8\textsuperscript{low} cells also contain a mixture of perforin/granzyme-expressing cells. Because most clones with a mixed CD8 phenotype progressively convert to a CD8\textsuperscript{low} phenotype, the data further suggest that perforin/granzyme expression (and therefore presumably cytolytic activity) can be acquired, then lost within a clone as it expands in the presence of IL-4.

The mechanism by which some type 2-polarized cells become committed to a poorly cytolytic CD8\textsuperscript{low} phenotype is not yet known. Two types of mechanism can be envisaged. First, the cells may lose responsiveness to the opposing polarization signals. For example, type 2-polarized CD4\textsuperscript{+} T cells have been reported to lose IL-12Rβ2 expression (40) and to up-regulate expression of the suppressor of cytokine signaling 3 (41). It is unlikely that IL-12 is essential for reversion in the present system, however, because some CD8\textsuperscript{low} cells regained CD8 in the absence of any known
source of IL-12 in anti-receptor Ab-stimulated cultures. Second, down-regulation of CD8, perforin, and granzyme gene expression may become fixed by a heritable gene-silencing mechanism, such as CpG methylation. A site in the CD8α promoter that is unmethylated in double-positive thymocytes has been reported to be methylated in mature CD4+CD8− T cells and in CD8 T cells with poor CD8 mRNA expression (42).

It is not yet clear whether this unusual CD8low T cell subpopulation develops and plays a role in vivo. The dependence on early sustained exposure to IL-4 shown in this study suggests that these cells would most likely be induced when naive CD8+ T cells are recruited to an established IL-4-polarized response, particularly if IL-4 is present at the T cell priming site in lymphoid tissues. Once they have down-regulated surface CD8, further triggering would require a higher affinity or avidity of TCR engagement with peptide/MHC to compensate for the lack of avidity enhancement and p56 lck activation normally mediated by CD8 (reviewed in Ref. 43).

A recent study showed that CD8 expression enhanced T cell sensitivity to peptide by at least 10-fold; whereas CD8 was obligatory for activation of T cells with low-affinity TCR, it was not exerted many of the same effects on B cells, macrophages, and other cells. This is because of the poor cytolysis, CD8low cells would not be expected to kill APCs as effectively as conventional CD8high CTLs. However, this is not for required for T cells with high-affinity TCR (44). Because of their poor cytolysis, CD8low cells might promote type 2 polarization of CD4 and other CD8 T cells and cytokines that make Th1 or Th2 interferon.

Down-regulation of CD8 expression on T cells has been reported in several situations, for example in mice chronically infected with Trypanosoma cruzi or Echinococcus multilocularis (48, 49), in HIV-infected mouse thymus (50), and in CD8 T cells in peripheral blood of HIV-infected and healthy humans (51, 52). Regulatory T cells of CD4+CD8− phenotype exhibit immune regulatory capacity in vitro and in vivo; some of these double-negative cells had a TCRαβNNK1.1 phenotype and specifically suppressed the activity of both CD8+ and CD4+ T cells, thereby preventing graft rejection and attenuating graft-vs-host disease (53, 54). None of these studies discriminated between transient and stable down-regulation of CD8 expression. Our studies raise the possibility that T cells with low or absent CD8 expression detected in these circumstances are derived from conventional CD8 T cells by a previously unrecognized pathway of effector T cell differentiation. Experiments are in progress to track the phenotype and function of CD8+ and CD8low T cells transferred in vivo.

Acknowledgments

We thank Paula Hall and Grace Chojnowski for their skillful assistance with FACS, Dr. Geoff Hill for generously providing Ab, Dr. Miles Davenport for statistical advice, and the National Institutes of Health AIDS Research and Reference Reagent Program for the gift of IL-2.

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