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A Clonal Culture System Demonstrates That IL-4 Induces a Subpopulation of Noncytolytic T Cells with Low CD8, Perforin, and Granzyme Expression

Norbert Kienzle, Kathy Buttigieg, Penny Groves, Tom Kawula, and Anne Kelso

Immune deviation of cytolytic T cell function, induced by type 2 cytokines like IL-4, is an attractive concept to explain failure of the immune system in some diseases. However, this concept is challenged by previous conflicting results on whether type 2 cytokine-producing CD8+ T cells are cytolytic. Therefore, we have analyzed the relationship between cytolytic activity and cytokine production among large numbers of primary CD8+ T cell clones. Single murine CD8+ T cells of naive phenotype were activated at high efficiency with immobilized Abs to CD3, CD8, and CD11a in the presence of IL-2 (neutral conditions) or IL-2, IL-4, and anti-IFN-γ Ab (type 2-polarizing conditions) for 8–9 days. Under neutral conditions, most clones produced IFN-γ and were cytolytic. Under type 2-polarizing conditions, most clones produced IFN-γ and IL-4 but displayed variable cytolytic activity and CD8 expression. Separation on the basis of surface CD8 levels revealed that, compared with CD8high cells from the same cultures, CD8low cells were poorly cytolytic and expressed low levels of perforin mRNA and protein and granzyme A, B, and C mRNA. A similar, smaller population of noncytolytic CD8low cells was identified among CD8+ T cells activated in mixed lymphocyte reaction with IL-4. Variable efficiency of generation of the noncytolytic cells may account for the differing results of earlier studies. We conclude that IL-4 promotes the development of a noncytolytic CD8low T cell phenotype that might be important in tumor- or pathogen-induced immune deviation.

heterogeneous cultures provide a means to reconcile the conflicting results of previous studies. Activation of these cells might be one mechanism by which some pathogens and tumors avoid immune destruction.

Materials and Methods

Abs and FACS analysis

The following Abs were obtained: PE- or FITC-conjugated rat anti-mouse CD8α Ab (53.6; BD PharMingen, San Diego, CA); biotinylated anti-mouse CD44 Ab (IM7;81; BD PharMingen); Tri-Color-, FITC-, or PE-conjugated streptavidin (Caltag, Burlingame, CA); FITC-conjugated goat anti-rat Ig (BD PharMingen); FITC-conjugated goat anti-hamster IgG (Kirkgaard & Perry Laboratories, Gaithersburg, MA); rat anti-mouse IFN-γ Abs R4-6A2 and biotinylated XMG1.2 (BD PharMingen); HRP-conjugated rabbit anti-rat Ig (DAKO, Glostrup, Denmark); HRP-conjugated sheep anti-mouse IgG (SILENUS Labs, Boronia, Australia); rat anti-mouse perinfr Ab (KM585 P1-8; Kamiya Biomedical, Seattle, WA); mouse anti-actin Ab (AC-40; Sigma-Aldrich, St. Louis, MO); hamster anti-mouse Fas ligand (FasL)/CD95 ligand (BD PharMingen); and biotinylated mouse anti-hamster IgG (BD PharnMingen). The following mAbs were protein G-purified from cell supernatant: hamster anti-mouse CD3ε (145-2C11), rat anti-mouse CD8α (53.6), rat anti-mouse CD11a (2I1/7.7), rat anti-mouse IFN-γ (XMG1.2), and rat anti-mouse IL-4 Abs BV4D1-1D11 and biotinylated BV6D-24G2. The rat anti-mouse CD3ε Ab (KT3-1.1) and rat anti-mouse L-selectin/CD62L, Ab (Mel-14) were used as diluted hybridoma supernatant. Cells (×10^6) were incubated for 30 min on ice, washed, and resuspended in balanced salt solution supplemented with 5% heat-inactivated FCS (CSL, Parkville, Victoria, Australia) and 1 μg/ml propidium iodide (Calbiochem, San Diego, CA). Cell surface expression was analyzed by FACS (FACSCalibur flow cytometer using CELLQuest V3.1f software, BD Bioscience, San Jose, CA).

T cell preparation

Specific pathogen-free female C57BL/6 mice were obtained from the Animal Resources Center (Perth, Western Australia) and used at 6–9 wk of age. Cell suspensions from brachial, axillary, inguinal, and lumbar lymph nodes were prepared by passing through stainless steel mesh followed by ficoll-paque (Amersham Pharmacia Biotech, Sydney, Australia) separation. Cells were stained for fluorescence-activated cell sorting with PE- or FITC-conjugated anti-CD8α Ab and biotinylated anti-CD44 Ab, followed by FITC- or PE-conjugated streptavidin, respectively. For some experiments, cells were stained with anti-CD62L Ab, followed by FITC-conjugated goat anti-rat Ig Ab, followed by PE-conjugated anti-CD8α Ab and biotinylated anti-CD44 Ab, and finally followed by Tri-Color-conjugated streptavidin. Viable naive cells were sorted using the FACS Vantage with 2 color fluorescence intensity, >200 and low (mean fluorescence intensity, <20) CD8 expression.

Activation of CD8⁺ T cells

Ab-driven stimulation.

Naïve CD8⁺ T cells were stimulated in an accessory cell-free system as outlined in detail previously (27, 28) using immobilized anti-CD3 (2C11), anti-CD8, and anti-CD11a Abs. Briefly, 96-well or 24-well plates (Falcon) were incubated overnight with 40 μl or 250 μl, respectively, of the Ab mix in PBS (anti-CD3 and anti-CD8 Abs, 10 μg/ml; anti-CD11a Ab, 5 μg/ml) and then washed three times in PBS. Single or 500-1000 CD8⁺CD44low T cells were cultured in 200 μl (per 96-well) or in 2 ml (per 24-well) of growth medium (modified DMEM supplemented with 50 μM l-2-ME, 216 mg/l-glutamine and 10% heat-inactivated FCS) containing human rIL-2 (120 IU/ml; Cetus, Emeryville, CA) in the absence or presence of anti-IFN-γ Ab (XMG1.2, 1 μg/ml) and mouse rIL-4 (3.3 ng/ml) supernatant of SF9 insect cells infected with murine IL-4 CDNA-encoding baculovirus (29). One unit per milliliter of IL-4 activity was defined as the amount stimulating half-maximal proliferation of the IL-4-dependent cell line CT-4S (29), and 1 U/ml of baculovirus-derived IL-4 was equivalent to 7.7 ng/ml of rIL-4 (Sigma-Aldrich). For clonal analysis, clone size was assessed microscopically after 8 days of culture using the following scales: 0, no cells; 1, 1–10² cells; 2, 10⁻¹–10⁵ cells; 3, 10⁻⁴–10⁻¹ cells; and 4, >10⁶ cells. Clones were then washed three times in situ in Dulbecco’s balanced salt solution with 2% FCS and once in 96-well medium and incubated in 200 μl of growth medium containing anti-CD3/8/11a Abs overnight with 200 μl of growth medium containing 120 IU/ml IL-2. After 18–22 h, culture supernatants were harvested for cytokine analysis, and clones were assayed for cytolytic and cell surface receptor expression. Bulk cultures were harvested after 7 or 8 days of culture for analysis.

MLR. CD8⁺CD44low T cells (15 to 20) from C57BL/6 mice were incubated with 5×10⁵ allogeneic spleen cells (gamma-irradiated with 2,000 rad) from DBA/2 mice in 200 μl of growth medium supplemented with 300 IU/ml IL-2 (this concentration provided optimal cell growth in the MLR) and with or without 3.3 U/ml IL-4 in 96-well round-bottom plates. After 8 days of culture, activated T cells were purified by Ficoll-Paque separation and analyzed.

Cytokine induction assays

For clonal assays, cytokine induction was induced overnight by the plate-bound anti-CD3/8/11a Abs, and 50 μl of supernatant per well was spot tested without further dilution. To reduce stimulus-independent cytokine synthesis (Fig. 7B), the anti-CD3/8/11a Ab-stimulated T cells were rested in growth medium and IL-2 for at least 4 days after primary activation before the cells were stimulated for cytokine induction. T cell populations (∼10⁶) were incubated for 22–24 h in 200 μl of growth medium containing 120 U/ml IL-2 with the following stimuli: 1) plate-bound anti-CD3/8/11a Abs; 2) allogeneic P815 cells (5×10⁵) with or without soluble anti-CD3 Ab 2C11 (1 μg/ml); and 3) no stimulus. IL-4 and IFN-γ were assayed in duplicate serial titrations of supernatant (50 μl, pooled from at least three replicate samples) by ELISA using the anti-IL-4 Ab BV4D4 and biotinylated BV6D6 (30) or the anti-IFN-γ Ab R4-6A2 and biotinylated XMG1.2, respectively. In some experiments, IFN-γ was measured by the growth inhibition of the IFN-γ-sensitive cell line WEHI-279, as described previously (31). Measured IL-4 and IFN-γ activities were standardized by reference to titrations of baculovirus-derived murine IL-4 or purified murine rIFN-γ (Sigma-Aldrich).

CTL assay

Cells of the H-2d Fcr⁺ mastocytoma line P815 were labeled with Na⁺3CrO₄ (Amersham Pharmacia Biotech) for 60 min at 37°C and then washed twice in growth medium. Labeled target cells (5×10⁵) were incubated for 4.5 h at 37°C with T cells in 200 μl in round-bottom 96-well plates. In the case of anti-CD3/8/11a-stimulated T cells, anti-CD3 Ab (2C11; 1–3 μg/ml) was added to bridge T cells with target cells (redirected CTL assay). Pooled clones or cells from bulk cultures were serially diluted for addition to target cells. In clonal assays, half of each T cell clone was spot tested without further dilution. Harvested supernatant was dried onto 96-well solid Lumaplates (Packard Instrument, Meriden, CT) before the radioactivity was counted in a Topcount microplate scintillation counter (Packard Instrument). For perforin inhibition experiments, T cells (10¹) were preincubated with 91 ng/ml concanamycin A (Sigma-Aldrich; stock dissolved in DMSO at 100 μg/ml) or the DMSO diluent control for 2–3 h at 37°C before addition to the target cells. Spontaneous lysis of target cells was typically <12%, and differences in sample release, performed in duplicate, were within 5%. Total ⁵¹Cr release from target cells was measured by lysis in 1% SDS. The percentage specific lysis was calculated by the following formula: 100 × ([sample cpm – spontaneous release cpm]/total release cpm – spontaneous release cpm).

Immunoblot analysis

Western blot analysis was performed essentially as described (32). Briefly, cells were washed in PBS and lysed in protein sample buffer (2% SDS; 5% e-monothicknesser A (Sigma-Aldrich); 10% glycerol; 60 mM Tris-HCl (pH 6.8); 0.001% bromophenol blue) with sonication and then boiling for 5 min. Total cell protein samples of 3 × 10⁶ cells and a protein standard mix (Kaleidoscope; Bio-Rad, Hercules, CA) were subjected to 10% SDS-PAGE and electro-transferred onto ECL-nitrocellulose filters (Amersham Pharmacia Biotech) using a minigel system (Bio-Rad). Filters were stained with Ponceau S (Sigma-Aldrich) to reveal total protein and were then proteolyzed with Proteinase K (Sigma) for immunoblotting. Filters were washed with 0.1% Tris buffered saline-Triton X-100 and blocked in 5% nonfat milk in PBS. Filters were probed with Abs against mouse β₂-microglobulin, β₂-microglobulin, and β₂-microglobulin.
and the reactions were visualized using the ECL Western blotting detection system (Amersham Pharmacia Biotech). For detection of actin, the same filters were reblotted with anti-actin Ab (0.5 \( \mu \text{g/ml} \)), followed by an HRP-conjugated anti-lgG Ab (1/1000).

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

The detailed protocols for mRNA preparation, cDNA conversion, and amplification were published previously (33, 34). Briefly, RNA was extracted by Nonident P-40 hypotonic lysis of 1000 cells, and cDNA was prepared using avian myeloblastosis virus reverse transcriptase (Promega, Madison, WI), oligo(dT) primers, and RNase inhibitors. For expression analysis of CD8\(\alpha\) and the housekeeping gene \(\beta_2\)-microglobulin (\(\beta_2\)-m), serial dilutions of cDNA (pooled from three replicate preparations) were amplified by PCR using Red Hot polymerase (Advanced Biotechnologies, Leatherhead, U.K.), dNTPs, and the cDNA-specific primer pair CD8\(\alpha\)-F2 (5'-TCTCT TCAGAAGGAATCTTAC), CD8\(\alpha\)-R2 (5'-AGATGTAATAATCA CAGGCG), or \(\beta_2\)-m-F (5'-TGACGGCTTGATGCATC), \(\beta_2\)-m-R (5'- CAGTGTGAGCGAGGATAG), respectively. PCR products were analyzed by agarose gel electrophoresis and sequenced. QC-PCR of three replicate cDNA samples was performed with Red Hot polymerase, dNTPs, competitor plasmid, and one of the primer pairs specific for cDNA of perforin (In-F, 5'-CAACAGCAAGCAACAGGTCT; In-R, 5'-CGTG ATAAATGTCGCTGGCATA), granzyme A (In-F, 5'-CTGAGCCCTGATTAT GCCT; In-R, 5'-CCTGCACCTTTATATGTTGAG), granzyme B (In-F, 5'-ACTTTGGCATCAAGATCAAGCA; In-R, 5'-ACTCTGAGCT CAACCTTCGTG), granzyme C (In-F, 5'-GGCTCACAACATCTAG GCCATA; In-R, 5'-AACCTGCGGATGTTTGTGG), and FasL (In-F, 5'- GTTTCTTCTTCCTGCCAT; In-R, 5'-TCGCTAGACACGTCC). With the reverse primers being biotinylated at their 5' ends. Each of the competitor plasmids 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-IgG Ab (1/1000).

**Results**

**IL-4 depresses cytolytic function in developing CD8\(^{+}\) T cell clones**

We investigated how IL-4 influences cytolytic activity and production of IFN-\(\gamma\) and IL-4 during the development of clones from individual CD8\(^{+}\) T cells of naive phenotype. An accessory cell-free culture system was used to limit the influence of endogenous cytokines on cell differentiation. Single CD8\(^{+}\)CD44\(^{low}\) T cells purified from lymph nodes of normal C57BL/6 mice were stimulated with IL-2 and immobilized Abs 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-\(\gamma\) Ab (type 2-polarizing conditions). After 8 days, cultures were scored microscopically for clone size, washed in situ to remove exogenous IL-4 and soluble Ab, and then incubated for another day in medium containing IL-2 to allow the secretion of cytokines in response to the immobilized anti-receptor Ab. At day 9, supernatants were harvested for cytokine assay, and cytolytic activity was measured by transferring clones into a redirected 51Cr release assay with the Fcr\(^{+}\) tumor P815 cells to which bridging anti-CD3 Ab was added to bypass the requirement for peptide/MHC recognition.

Fig. 1A shows the relationship between clone size and each of the three measured functions. In this and other experiments, cloning efficiencies were routinely in the range 86–97\%, and most clones contained at least 1000 cells and therefore had undergone at least 11 divisions at the time of assay. Cytolytic activity and IL-4 and IFN-\(\gamma\) production were generally undetectable in clones of fewer than 100 cells. Both the frequencies of positive clones and their mean activities in each assay rose with clone size above this 100-cell threshold.

The majority of clones that developed in neutral conditions were cytolytic and secreted IFN-\(\gamma\); very few secreted IL-4. These cytolytic clones and the rare IL-4-producing clones were contained within the IFN-\(\gamma\)-secreting group (Fig. 1B). By contrast, most clones that developed in type 2-polarizing conditions produced IL-4. Preliminary experiments in which IL-4 was included at concentrations from 0.033 to 33 U/ml established that the frequency of IL-4-producing clones was highest when IL-4 was added at 0.33–3.3 U/ml, whereas cloning efficiency was not significantly affected at any dose tested; therefore, 3.3 U/ml was used in all experiments reported here. In general, initial clone growth was slower under type 2-polarized conditions. However, by day 9, there was no significant difference in average clone size between the neutral and type 2-polarizing culture conditions. Addition of a neutralizing anti-IL-4 Ab (BVD4, 10 \( \mu \text{g/ml} \)) to such cultures at day 0 completely inhibited the induction of IL-4 synthesis (data not shown). The frequency and mean activity of IFN-\(\gamma\)-producing clones in type 2-polarizing conditions were slightly reduced compared with clones generated in neutral conditions (Fig. 1A). In contrast, both the frequency and mean activity of cytolytic clones were markedly lower in type 2-polarizing than in neutral conditions, especially among IL-4-producing clones. Clones obtained in type 2-polarizing conditions fell into three main groups, ranked by frequency: IFN-\(\gamma\)^{-}IL-4^{+}CTL\(^{-}\), IFN-\(\gamma\)^{-}IL-4^{+}CTL\(^{+}\), and IFN-\(\gamma\) IL-4^{-}CTL\(^{+}\) (Fig. 1B).

Table 1 summarizes the frequencies of 871 T cell clones derived from CD8\(^{+}\)CD44\(^{low}\) cells in four independent experiments. The type 2-polarizing conditions always significantly reduced the frequency of cytolytic T cell clones and increased the frequency of IL-4 producers compared with neutral conditions. In contrast, the reduction in IFN-\(\gamma\)-producing clones was only statistically significant in two of four experiments. Experiment 4 further showed that the effect of type 2-polarizing conditions on both IL-4 production and cytolytic activity was mainly due to the exogenous IL-4, not the anti-IFN-\(\gamma\) Ab.

To confirm the naive status of the responding cells in this system, CD44\(^{low}\)CD8\(^{+}\) T cells were further sorted for high expression of CD62L (L-selectin) before cloning. Approximately 98% of CD44\(^{low}\)CD8\(^{+}\) T cells were CD62L\(^{high}\). The effects of IL-4 on IL-4 production and cytolytic activity were similar for CD44\(^{low}\) and CD44\(^{low}\)CD62L\(^{high}\)CD8\(^{+}\) T cells (data not shown).

To investigate the mechanisms underlying the effect of IL-4 on cytolytic function, we compared the surface phenotypes of pooled clones grown in neutral and type 2-polarizing conditions (Fig. 2). Whereas CD3 and CD11a expression levels were similar in the two cell populations, many cells in the type 2-polarized population displayed markedly reduced levels of surface CD8\(\alpha\). No significant difference in the viability of pooled clones from the two culture conditions was detected by analysis of propidium iodide uptake or expression of the early apoptotic marker annexin V. In addition, CD44 expression was up-regulated on all cells, regardless of CD8 levels (data not shown). The partial loss of surface CD8 was consistently observed in pools of clones and polyclonal cultures of CD8\(^{+}\) T cells activated with anti-receptor Ab under type 2-polarizing conditions and was detectable as early as day 5 of stimulation. CD8 down-regulation was also seen when anti-IFN-\(\gamma\) Ab or immobilized anti-CD8 Ab were omitted from the cloning cultures and when the usual rIL-4 source was replaced with commercial purified rIL-4 (data not shown).
Loss of surface CD8/H9251 in type 2-polarized clones correlates with their low cytolytic activity

To determine whether the variation in surface CD8 expression among pooled type 2-polarized clones shown in Fig. 2 was due to differences between clones, we analyzed a series of randomly picked clones of similar size (Fig. 3). The majority of clones grown in neutral conditions were CD8(high) cells. Most of these clones were cytolytic and produced little or no IL-4. The remaining four clones showed lower CD8 expression, low cytolytic activity, and negligible IL-4 production. In contrast, most clones grown in type 2-polarizing conditions displayed some reduction in CD8 expression. The profiles of individual clones were extremely variable, ranging from 4 to 98% CD8(high). There was a broad correlation among loss of CD8 expression, low cytolytic activity, and IL-4 secretion. However, the finding that four of the 42 IL-4-producing clones expressed intermediate CD8 levels (between 48 and 65% CD8(high) cells per clone) but showed good cytolytic activity (>28% specific lysis) and two other clones expressed high (>90%) CD8 levels but were noncytolytic (<6.7%) indicated that CD8 expression was not the only parameter affecting cytolytic function. It is noteworthy that there was no difference between CD8 T cells originating from the CD44low/CD62Lhigh and CD44low naive phenotype (Fig. 3B, compare squares with circles).

To directly test the correlation between surface CD8 expression and cytolytic activity, pooled clones generated in neutral or type 2-polarizing conditions were sorted for high or low CD8 expression before assay. Fig. 4A shows that CD8(low) cells from type 2-polarized cultures were at least 25-fold less cytolytic per cell than CD8(high) cells from the same or neutral cultures, confirming a strong association between CD8 levels and cytolytic activity. The quantitative effect of exogenous IL-4 on development of cytolytic function was also seen when clones were pooled and titrated in a redirected 51Cr release assay without prior separation into CD8(high) and CD8(low) cells. Cytolysis required the bridging anti-CD3 Ab, regardless of culture conditions. Based on lytic units per 10⁶ cells, clones obtained in type 2-polarizing conditions were 4.5-fold less lytic per cell than CD8(high) cells from the same or neutral cultures, confirming a strong association between CD8 levels and cytolytic activity.

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**FIGURE 1.** Effect of IL-4 on primary CD8 T cell clones. Single T cells (CD8⁻/CD44⁺) from C57BL/6 mice were stimulated in 96-well plates with IL-2 and immobilized anti-CD3/8/11a Abs in the absence or presence of IL-4 and a neutralizing IFN-γ Ab as indicated. After 8 days, cells were washed in situ and incubated in the same wells in medium with IL-2 only for 1 day. Cytolytic function of the T cell clones was spot tested in a redirected 51Cr release assay using P815 target cells and bridging anti-CD3 Ab. IL-4 secretion was measured by spot test ELISA, and IFN-γ production was measured in a growth inhibition assay using WEHI-279 cells. Each cell clone is represented by a single dot and the threshold for positive assay readings is illustrated by a broken line. A. The relationship between clone size (0, no cells; 1, 1–10² cells; 2, 10²–10³ cells; 3, 10³–10⁴ cells; 4, >10⁴ cells) and cytolysis or cytokine production is shown. The percentage of clones (n = 93–96) positive for the indicated function is given. B. The relationship between cytolysis and cytokine production is shown for clones of size 1–4. Frequencies in each quadrant are given in percentages.
IL-4 and anti-IFN-γ Ab reduced lytic activity by 2.5- to 30-fold (data not shown).

**Loss of CD8α expression is accompanied by low expression of perforin and granzymes**

The lytic activity of CD8+ T cells stimulated in neutral or type 2-polarizing conditions was markedly reduced (to background or at least 21-fold compared with controls) when the T cells were preincubated with concanamycin A before addition to the redirected 51Cr release assay (Fig. 4B), indicating that lysis was mediated by the perforin pathway (35). Therefore, we analyzed CD8high and CD8low T cells from neutral and type 2-polarized cultures for their expression of perforin and the three most prominent granzymes found in activated murine T cells, granzymes A, B, and C (36). The ~70-kDa perforin protein complex was readily detected by immunoblot analysis in the CD8high populations purified from neutral and type 2-polarized cultures but was not detected in the CD8low cells from type 2-polarized cultures or in control P815 cells (Fig. 5A). Subsequent staining with anti-actin Ab showed that protein loading was comparable in all lanes.

Levels of perforin and granzyme A, B, and C mRNA expression were determined by QC-PCR (Fig. 5B). Expression of each product was markedly lower in CD8low cells than in CD8high cells from type 2-polarized cultures: 2.8-fold for perforin (p < 0.03, t test), 4.7-fold for granzyme A (p < 0.07), 8.9-fold for granzyme B (p < 0.04), and 81-fold for granzyme C (p < 0.006). Comparable results were obtained in three independent experiments. Although the product ratios between the three populations varied from experiment to experiment, CD8low cells were consistently found to express lower levels of perforin and granzyme A, B, and C mRNAs than CD8high cells from the same type 2-polarized cultures. This down-regulation of gene expression was selective for perforin and granzymes because levels of β2m mRNA were similar in all three populations and IL-4 mRNA levels were 1.7-fold increased in the CD8low population (data not shown).

To investigate whether the reduction in surface CD8 protein levels was reflected at the mRNA level, expression of CD8α and the control gene β2m was analyzed by RT-PCR (Fig. 5C). CD8α mRNA levels were at least 16-fold lower in the sorted CD8low population from type 2-polarized cultures than in the CD8high populations obtained from these cultures or under neutral conditions. Sequencing of the PCR products confirmed their identity (data not shown). The IL-4-dependent reduction in surface CD8 levels therefore was likely to be due at least partly to lower transcription and/or higher turnover of CD8α mRNA.

We also analyzed mRNA and cell surface protein expression of FasL/CD95 ligand by QC-PCR and FACS, respectively. To amplify the FasL signal detected by FACS, a combination of anti-FasL Ab, biotinylated anti-hamster IgG, and PE-conjugated streptavidin was used. However, FasL mRNA and protein expression was very low in the CD8high and CD8low cell populations, regardless of the culture conditions (data not shown). This result and the data in Fig. 4B were consistent with a previous report indicating that the perforin/granzyme pathway, rather than FasL-Fas interaction, is the major mechanism by which P815 target cells are lysed by activated CD8+ T cells (37). Taken together, the data suggest that the effect of IL-4 on the cytolytic function of primary CD8+ T cell clones is mediated, at least in part, at the level of perforin and granzyme expression.

**The effect of IL-4 on cytolytic function is also observed in allogeneic MLR**

To examine whether IL-4 also affected CD8 expression and cytolytic function in Ag-stimulated T cells, CD8+CD44low T cells

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**Table I. Effect of IL-4 on primary CD8 T cell clones**

<table>
<thead>
<tr>
<th>Expt.</th>
<th>Culture Conditions</th>
<th>Anti-IFN-γ Ab</th>
<th>Cloning Efficiency&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Positive Clones&lt;sup&gt;b&lt;/sup&gt;</th>
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<sup>a</sup> Percent of wells containing a visible clone (n = 93–96).

<sup>b</sup> Percent of clones positive for the indicated function. Asterisks indicate significant difference from the frequency detected in the absence of exogenous IL-4 (*, p ≤ 0.01; **, p < 0.001; z test).

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**FIGURE 2.** IL-4 induces down-regulation of surface CD8α protein. Individual CD8+CD44low T cells were stimulated with anti-CD3/8/11a Abs in the absence (-----) or presence of IL-4 and anti-IFN-γ Ab (---). After 8 days, cells were washed in situ and reincubated as in Fig. 1. Cell clones of sizes 3 and 4 were pooled and analyzed by FACS for cell surface expression of CD8α, CD3ε, and CD11a as indicated. Unlabeled cells (CD8α) or cells stained with secondary Ab only (CD3ε, CD11a). FL1-H, Fluorescence.
from lymph nodes of untreated C57BL/6 (H-2b) mice were stimu-
lated in an MLR with irradiated DBA/2 (H-2d) spleen cells and
IL-2 with or without IL-4 for 7 days. When the unfractionated
populations were then assayed for cytolytic activity in a 51 Cr re-
lease assay with P815 (H-2d) target cells, no difference was ob-
served between cells grown in the presence or absence of IL-4
(Fig. 6A), in contrast to results presented above for cells activated
with anti-receptor Ab. However, FACS analysis revealed signif-
icient down-regulation of surface CD8 expression on a proportion of
cells (18%) from type 2-polarized cultures (Fig. 6B). CD8low and
CD8high cells were therefore purified from neutral and type 2-po-
larized cultures and assayed for cytolytic activity against P815
target cells. Fig. 6C shows that the lytic activity of the CD8low

population generated with IL-4 was ~10-fold lower than that of
either of the two CD8high populations. From this and three other
experiments, it was concluded that IL-4 down-regulated surface
CD8 expression and depressed cytolytic function in a subpopula-
tion of CD8 T cells activated in the MLR.

CD8low cells can synthesize cytokines in response to target cells

Because IL-4-induced CD8low cells displayed reduced lysis of
P815 target cells via bridging anti-CD3 Ab or alloantigens, we
analyzed whether these cells were also impaired in their ability to
respond to target cells by producing cytokines. CD8low/CD44 low
T cells were activated with anti-receptor Ab or allogeneic cells in
the presence or absence of IL-4, then sorted for high or low CD8
expression. The sorted populations were restimulated with P815
cells in the presence or absence of bridging anti-CD3 Ab or with
immobilized Ab to CD3, CD8, and CD11a for 24 h. As shown in
Fig. 7, all populations produced substantial titers of IFN-γ in re-
sponse to both P815 and immobilized Ab; CD8low cells produced

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**FIGURE 3.** IL-4-induced loss of CD8 correlates with lack of cytolysis
in clonal populations. Individual CD8+ T cells of naive phenotype were
stimulated with anti-CD3/8/11a Abs in the absence or presence of IL-4 and
anti-IFN-γ Ab. After 8 days, cells were washed in situ and reincubated as
in Fig. 1. Randomly picked clones of size 3 or 4 were analyzed separately
for the percentage of CD8high cells and T cell function. A, FACS histo-
grams for CD8α expression of eight clones of CD44low origin that were
generated in neutral (left) or type 2-polarizing conditions (right). The per-
centage of CD8high cells per clone is given above the bar markers. B, Thirty-two clones of CD44low origin (●) and 10 clones of CD44low
CD62Llow origin (■) were generated in neutralizing conditions; 33 clones of
CD44low origin (○) and 10 clones of CD44lowCD62Llow origin (□) were generated in type 2-polarizing conditions. The relationship between
CD8 expression and cytolytic function (top) or IL-4 secretion (bottom) is
illustrated. Cytolysis was spot tested in a redirected 51Cr release assay
using P815 target cells and anti-CD3 Ab. IL-4 production was spot tested
by ELISA and standardized by reference to titrated rIL-4. –––, Threshold
for positive assay readings. Data are pooled from three separate
experiments.

**FIGURE 4.** Cytolysis is reduced in CD8low cells and by concanamycin
A. CD8low/CD44low T cells were stimulated for 8 days with anti-CD3/8/11a
Abs in the absence or presence of IL-4 and anti-IFN-γ Ab and assayed in
a redirected 51Cr release assay using P815 target cells and bridging anti-
CD3 Ab. A, Activated T cells from bulk cultures were FACS sorted for
CD8 expression before assay. CD8 high cells activated without IL-4 (●); CD8high cells (○) and CD8low cells (□) activated with IL-4 and anti-IFN-γ
Ab. B, Clones were stimulated under neutral (top) or type 2-polarizing
conditions (bottom). A mixture of size 1–4 cell clones were pooled and
pretreated with concanamycin A (●), medium (○), or DMSO diluent (□)
before assay. The cytolysis of medium-treated T cells was also assayed in
the absence of the bridging anti-CD3 Ab (×).
within the same clones, often led to masking of the noncytolytic CD8low populations generated in response to anti-receptor Ab or different experiments. The results show that the IL-4-induced subpopulation of 10 cells: CD8high cells activated in neutral conditions (average results and SDs obtained from three replicate samples of the equivalent of 10 cells: CD8high cells activated in neutral conditions); CD8low cells (Δ) activated in type 2-polarizing conditions. C, RT-PCR was performed for CD8α and the housekeeping gene β2-M using 4-fold serial dilutions of the cDNAs (left to right) from the sorted cell populations.

5- to 6-fold lower titers than CD8high cells from the same cultures in response to each type of stimulus. Significant IL-4 secretion, in contrast, was only detected in supernatants of cells that had been exposed to exogenous IL-4 during priming and was generally higher in cultures of CD8low cells than in the corresponding CD8high cells. The reciprocal relationship between IL-4 and IFN-γ production by CD8low and CD8high cells was observed in four different experiments. The results show that the IL-4-induced CD8low populations generated in response to anti-receptor Ab or allogeneic cells retained the capacity to produce cytokines in response to target cells against which they displayed a reduced lytic activity.

Discussion

Here we show that exposure to IL-4 during primary activation promotes the development of a subpopulation of CD8+ T cells that down-regulate CD8α, have minimal cytolytic activity, and synthesize both IL-4 and IFN-γ. Cells of this phenotype were identified in cultures activated with Abs to CD3, CD8, and CD11a or with allogeneic APCs. However, the coexistence of variable proportions of CD8high, cytolytic IFN-γ+IL-4+ T cells in the same cultures, and even within the same clones, often led to masking of the noncytolytic phenotype and may explain why some previous studies did not detect these cells. The finding that the noncytolytic phenotype was associated with low expression of surface CD8 protein and of perforin and granzyme mRNAs suggests that the poor cytolytic activity of these cells in 51 Cr release assays reflected the dual impairment of target cell recognition and the lethal hit.

Most of the experiments reported here were performed in clonal cultures stimulated with anti-receptor mAb and IL-2 in which the great majority of single CD8+CD44low T cells give rise to clones over ~8 days. When IL-2 was the only cytokine added, most clones were cytolytic and produced IFN-γ without IL-4. Inclusion of IL-4 with or without anti-IFN-γ mAb in these cultures from day 0 did not interfere with clonal expansion but resulted in the formation of clones of variable functional phenotype. Separation of cells from pooled IL-4-exposed clones based on their surface CD8α levels showed that this marker distinguished a noncytolytic CD8low fraction from the typical cytolytic CD8high population, with both populations secreting IL-4. Similar cells could be identified in bulk cultures stimulated with anti-receptor mAb and in one-way MLR established with purified CD8+CD44low responder cells.
Erard et al. (4) previously found that exposure to IL-4 during activation of murine CD8 T cells with either PMA and ionomycin or with alloantigens led to the development of CD8 cells with low cytolytic activity, perforin expression, and IFN-γ production and elevated production of type 2 cytokines. However, other groups reported no differences in the cytolytic activity or CD8 levels expressed by CD8 T cells activated in the presence or absence of IL-4 in various Ag-driven systems, although IL-4 did induce IL-4 synthesis (5, 24–26). The CD8 marker appears to offer a key to explaining these conflicting results. Data shown in two of these reports reveal some loss of surface CD8 expression, by up to 22% of cells activated in MLR in the study of Sad et al. (24) and a small unspecified number in the study of Carter and Dutton (25). In our hands, the percentage of CD8low cells was affected by several parameters, including the duration of culture and the initial ratio of responder to stimulator cells in MLR (CD8 down-regulation increased with time and lower ratio; data not shown). Later work by Erard et al. (38) showing that TGF-β antagonized the effect of IL-4 on cytolytic and CD8 expression suggests another factor that might contribute to the lesser effect of IL-4 in MLR and other APC-dependent systems compared with the APC-free anti-receptor mAb system used here. We observed in different MLR and anti-CD3/8/11a Ab stimulation experiments (by varying culture time) that the effect of IL-4 on cytolytic activity was generally undetectable in cultures where fewer than ~25% of cells had down-regulated CD8 (data not shown). In these cases, FACS purification of the CD8low cells was necessary to demonstrate the presence of a noncytolytic population. Therefore, the presence of such cells might not have been detected in some earlier studies.

Others have also reported that type 2-polarized CD8 T cells could provide B cell help (4, 23, 24). In our hands, the noncytolytic CD8low fraction induced ~10-fold higher levels of IgM and IgG1 when irradiated and cocultured with splenic B cells in the presence of immobilized anti-CD3 Ab, compared with CD8high cells derived from the same type 2-polarized culture (N.K., unpublished observations).

The noncytolytic CD8low subpopulations we identified in anti-receptor mAb-activated cultures and MLR secreted IFN-γ as well as IL-4, although average IFN-γ titers were lower than in type 2-polarized cultures of CD8high cells. This finding calls into question the value of IFN-γ as a surrogate marker for CTL in systems where IL-4 may be present. In the absence of a single-cell assay for cytolytic activity, ELISpot and FACS analysis of intracellular IFN-γ are widely used to count CD8+ effector cells, based on the strong positive correlation between IFN-γ titers and cytolytic activity observed at the population level in many responses. The results obtained here indicate that such methods would overestimate CTL numbers if they detected cells of the IFN-γ-producing noncytolytic phenotype described here.

Isolation of CD8low cells from IL-4-exposed cultures identified a poorly cytolytic population with significantly lower levels of mRNA for perforin and granzymes A, B, and C than the cytolytic CD8high fraction obtained from the same cultures or from cultures activated under neutral conditions. This suggests at least two mechanisms by which IL-4 may have reduced cytolytic activity.

The first is reduction of target cell recognition due to low CD8 coreceptor expression. Early work showing that cytolytic activity of T cells activated in MLR can be reduced by blocking surface CD8 with soluble Abs or by selective removal of CD8 with trypsin led to the suggestion that CD8 stabilizes low-affinity interactions between TCR and class I MHC complexes (39). Subsequent studies have shown that CD8 reduces the off-rate of this interaction, indicating that it is an active participant in the recognition complex (40). The mechanism of the effect of IL-4 on CD8 levels is not known. We show here that CD8α expression was down-regulated at both the mRNA and surface protein levels, suggesting effects on transcription rate and/or mRNA turnover. Given the role of the GATA-3 transcription factor in promoting IL-4 and IL-5 gene transcription and potentiating type 2 T cell polarization (41, 42), it is notable that the murine CD8α promoter contains three functional GATA-3 binding sites (43). The loss of surface CD8 expression did not depend on CD8 ligation because it was observed in the anti-receptor Ab-driven system when anti-CD8 Ab was omitted, and it persisted for at least 6 days when cells were transferred into IL-4-free medium, with or without immobilized anti-CD3/8/11a Abs, after a week of IL-4 exposure (data not shown).

The second candidate mechanism for the effect of IL-4 on cytolytic activity is a reduction of granule cytotoxicity due to low
perforin and granzyme synthesis. Erard et al. (4) previously showed a correlation between low cytolyis and low perforin mRNA levels by Northern analysis. In the present study, analysis of perforin expression by RT-PCR and immunoblot demonstrated that this mediator was markedly lower at both the mRNA and protein levels in CD8low cells than in the CD8high fraction. Although granzyme synthesis could only be assessed at the mRNA level because of lack of reagents for protein detection, the data presented here suggest a substantial effect of IL-4 on the expression of three of these proteases, granzymes A, B, and C. This effect was most profound for granzyme C. Perforin is known to play a crucial role in the granule exocytic pathway of cytotoxicity (36, 44, 45), whereas granzyme B contributes to the rapid apoptotic death detected in 51Cr release assays (46, 47) and granzyme A has been proposed to act independently of granzyme B (48). The function of granzyme C, however, is enigmatic, and only one report has attributed any role to this mediator in lymphocyte cytotoxicity (49). For the moment, the significance of its inhibition by IL-4 is obscure.

The negligible expression of perforin and granzymes in the CD8low population was not due to failed activation because these cells secreted substantial titers of both IFN-γ and IL-4 in response to anti-CD3 Ab or Ag stimulation, and they expressed high levels of the activation marker CD44. It is difficult to ascertain whether the loss of surface CD8 impaired their recognition of stimulator cells for cytotoxic production, but it is notable that the opposite IFN-γ/IL-4 ratios produced by CD8low and CD8high cells were observed in response to both types of stimulus, suggesting that this reversal was intrinsic to the producing cells rather than a differential response to different stimuli.

Whereas it is clear that IL-4 actively down-regulated expression of CD8 (because this corepressor was expressed on all starting cells), it is not clear whether it prevented the initial onset of perforin and granzyme expression in newly activated CD8+ T cells (for example, by countering signals that make these genes available for transcription through demethylation or other processes) or whether it down-regulated the expression of these genes after they had been activated. Experiments are in progress to attempt to track this pathway in developing clones. Similarly, it remains to be determined whether there is a tight linkage between the loss of CD8 expression and low or absent perforin and granzyme expression. Such a linkage is supported, however, by a study using βm-deficient mice that found that failed CD8β-TCR engagement resulted in down-regulation of CD8 and granzyme B mRNA in CD8+ T cells; CD8αβ expression was silenced by promoter methylation (50). The fact that IL-4 was expressed by both CD8low and CD8high cells indicates that the IL-4-induced loss of CD8 expression was not tightly linked to the induction of IL-4 synthesis.

There are several previous studies that show the signature of IL-4-induced CD8 T cell immune deviation in vivo. For example, human CD8 T cells from HIV-infected patients produce IL-4 (8), and during HIV infection there is a switch of cytolytic type 1-polarized CD8 T cells to cells that make type 2 cytokines and lack cytolysis and/or CD8 expression (8, 51). In some viral immunity models in mice, expression of IL-4 down-regulates cytolytic T cell function with subsequent delay or even breakdown of virus clearance in the challenged animal (11, 12, 15, 17, 18). In light of the results reported here, further investigation on the linkage among CD8 expression, type 2 cytokine expression, and cytolytic function should lead to a deeper understanding of immune deviation of CD8 T cell responses in vivo.

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