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# IL-4 Enhances Long-Term Survival of CD28-Deficient T Cells[1]

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**CD28 signaling is critical for IL-2 production by established Th1 clones, but CD28 does not appear to play a role in the activation of established Th2 clones. To determine the role of CD28 in the generation of polarized T cells, clones were derived using cells from CD28-deficient ($CD28^{-/-}$ mice, which had been bred with mice that express the DO11.10 transgene, a $CD4^+$ TCR-$\alpha\beta$ receptor that recognizes OVA peptide 323–339 bound to I-$A^d$. Most T cell clones derived from $CD28^{+/+}$ mice survived multiple stimulations, while T cell clones derived from $CD28^{-/-}$ mice survived only if they were derived initially in the presence of IL-4 or both IL-2 and IL-4. Signaling through the CD28 molecule did not appear to be important in the initial activation of T cell clones, as the precursor frequency of clones derived from normal ($CD28^{+/+}$) and $CD28^{-/-}$ mice was similar. Primary stimulation in the presence of IL-4 increased cell number and viability of both $CD28^{+/+}$ and $CD28^{-/-}$ T cells in primary culture. However, the survival of $CD28^{-/-}$ cells is more dependent on IL-4 than is the survival of $CD28^{+/+}$ cells. The continued presence of anti-IL-4 mAb dramatically decreased the number of viable cells in the $CD28^{-/-}$ cultures but had little effect on the viability of the $CD28^{+/+}$ clones. Thus, initial culture with IL-4 allows the isolation of $CD28^{-/-}$ T cell clones that produce IL-4. In these clones, IL-4 acts as both an autocrine growth and survival factor.** ***The Journal of Immunology*****, 1998, 160: 2255–2262.**

Activation of $CD4^+$ T cells is initiated by engagement of the TCR/CD3 complex with a peptide presented by MHC class II molecules on APCs (1–3). In most cases, second or costimulatory signals appear to be delivered by engagement of CD28 on the T cell with B7-1 and/or B7-2 on the APC. The CD28 signaling pathway is thought to augment IL-2 production at both the transcriptional and post-transcriptional stages of gene expression (4). CD28 signal transduction is also important in regulating the response of established Th1 clones to antigenic stimulation. In Th1 clones, TCR stimulation in the absence of CD28 engagement results in little or no IL-2 production or proliferation and causes the T cell to enter an unresponsive state, termed anergy. Anergy is characterized by the inability to produce IL-2 after subsequent restimulation and the absence of a proliferative response, even if both signal 1 and signal 2 are provided. In contrast, the response of established Th2 clones is less dependent on CD28. Ag-induced IL-4 production and proliferation of Th2 clones is not inhibited by stimuli that induce anergy in Th1 clones, and no state analogous to anergy, with respect to IL-4 production, has been observed for Th2 clones (5, 6).

The development of Th1 and Th2 clones is thought to represent divergent immune responses to Ags. Th1 clones produce IFN-$\gamma$ and IL-2 and appear to be responsible for delayed-type hypersensitivity responses and for activation of macrophage killing of intracellular pathogens (7). Th2 clones produce IL-4 and IL-5 and promote humoral immunity through B cell activation, resulting in Ab production and switching to the IgE and IgG1 isotypes (8). In vitro, Th cell subset development is influenced by the cytokine environment. IL-12 and IFN-$\gamma$ skew development toward the Th1 phenotype (9, 10); whereas IL-4 promotes development of the Th2 phenotype (11, 12). Furthermore, the balance of cytokines associated with Th1 and Th2 subsets has been implicated in the outcome of the host response to infections in murine models of leishmaniasis (13), schistosomiasis (14), trichinosis (15), and helminthic infections (16).

More recently, CD28 engagement also has been reported to influence the development of a naive Th cell into a mature Th1 or Th2 effector cell. In both human and murine systems, IL-4 production has been shown to be sensitive to the blockade of CD28 engagement by CTLA4Ig. Studies in vivo have shown that blocking CD28-dependent signaling with CTLA4Ig inhibits Th2-dependent Ab production (17) and also abrogates progressive disease in BALB/c leishmaniasis-susceptible mice by decreasing levels of biologically active IL-4 and increasing levels of IFN-$\gamma$ (18). In addition, transgenic (Tg)[4] mice expressing CTLA4Ig have reduced IL-4 production in response to primary immunization (19).

T cells from $CD28^{-/-}$ mice did not proliferate in response to polyclonal activators, including alloantigen, Con A, and an anti-CD3 mAb (20). Furthermore, IL-2 did not completely restore proliferation to the level observed with wild-type naive T cells (20, 21). T cells from $CD28^{-/-}$ mice bred to DO11.10 mice, which express a $CD4^+$ TCR-$\alpha\beta$ receptor specific for the I-$A^d$-restricted OVA peptide 323–339, proliferated poorly in response to antigenic stimulation and were unable to sustain the response. These results were attributed to substantially lower levels of IL-2 production (22). The addition of the CD28 antagonist (CTLA4Ig) or the genetic disruption of CD28 resulted in decreased viability of DO11.10-Tg $CD4^+$ T cells following Ag-specific T cell activation and an aborted proliferative response (23). However, this decrease in proliferation and viability could not be completely attributed to a decrease in IL-2 production by $CD28^{-/-}$ T cells as the addition

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[4] Abbreviations used in this paper: Tg, transgenic; MACS, magnetic cell separation system; DPBS, Dulbecco's phosphate-buffered saline; PI, propidium iodide.



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of high concentrations of Ag or anti-CD28 mAb enhanced IL-2 production to the same degree, but only cells stimulated with anti-CD28 mAb survived (23).

In the following report, we examined the role of CD28 in T cell survival in response to repeated antigenic stimulation. Most T cell clones derived from $CD28^{+/+}$ mice survived multiple stimulations, regardless of the pattern of cytokine secretion. However, the T cell clones derived from $CD28^{-/-}$ mice survived only if they were derived in the presence of IL-4 or the combination of IL-2 and IL-4. Interestingly, signaling through the CD28 molecule did not appear to be important in the initial activation of T cell clones, as the precursor frequency of clones derived from wild-type ($CD28^{+/+}$) and $CD28^{-/-}$ mice was similar. However, if IL-4 was present during the primary stimulation, increased cell number and viability were observed with both $CD28^{+/+}$ and $CD28^{-/-}$ T cells in primary culture. Furthermore, the continued presence of anti-IL-4 mAb dramatically decreased the number of viable cells in the $CD28^{-/-}$ cultures, but had little effect on the viability of the $CD28^{+/+}$ clones. Additionally, anti-IL-4 mAb inhibited proliferation of $CD28^{-/-}$ clones, but not $CD28^{+/+}$ clones, in a 48-h assay. Thus, initial culture with IL-4 promotes IL-4 production by $CD28^{-/-}$ T cell clones; IL-4 then acts as both an autocrine growth and survival factor.

## Materials and Methods

### *Animals*

Female DBA/2J mice were purchased from The Jackson Laboratory, Bar Harbor, ME. DO.11.10 mice were a gift from Drs. Dennis Loh and Kenneth Murphy (Washington University, St. Louis, MO) (12). The DO11.10 Tg T cells are predominantly $CD4^+$ and express a TCR-$\alpha\beta$ receptor that recognizes OVA peptide 323–339 bound to $I\text{-}A^d$. Mice deficient in CD28 expression ($CD28^{-/-}$) (20) were generated by breeding a male $CD28^{-/-}$ B10.D2 mouse with a female BALB/c $TCR^{+/+}$ mouse, and the offspring were screened for the disrupted CD28 gene by PCR of tail DNA (20) and confirmed by flow cytometry using a CD28-specific mAb, 37.5.1 (24). TCR Tg expression was determined by flow cytometry using a biotinylated TCR-specific mAb, KJ1-26.1. $CD28^{+/-}$ progeny were then back-crossed, and TCR $Tg^+$ mice were screened for CD28 expression. Breeding colonies of $CD28^{-/-}$ $Tg^+$ and $CD28^{+/+}$ $Tg^+$ mice were generated by pairing siblings of appropriate gene expression. Multiple independent mice were used to derive the clones studied in the course of these experiments. The reproducibility of the results confirm that the findings do not result from minor variations in the genetic backgrounds of the mice. All mice were housed in laminar flow units in a barrier facility at the University of Chicago (Chicago, IL) and used between 8 and 12 wk of age.

### *Culture media and reagents*

T cell clones were grown in culture medium consisting of DMEM plus 20% heat-inactivated FCS, 10 mM 3-(*N*-morpholino)propane sulfonic acid, $5 \times 10^{-5}$ M 2-ME, 100 U/ml penicillin, 100 $\mu$g/ml streptomycin, and additional additives (24). OVA peptide 323–339 was synthesized at the University of Chicago Peptide Synthesis Facility. Amino acid determination was performed on the peptide to ensure peptide integrity and purity.

### *Recombinant cytokines and mAbs*

Murine rIL-4 was a generous gift of Immunex Corp. (Seattle, WA). Human rIL-2 was generously provided by Cetus Corp. (Emeryville, CA). All concentrations are expressed in terms of picograms per milliliter or units per milliliter as defined by the supplier of each cytokine. Anti-CD3-$\epsilon$ (145-2C11) (25) was kindly provided by Dr. J. Bluestone (University of Chicago), anti-IL-4 (11B11) (26) by Dr. W. Paul (National Institutes of Health, Bethesda, MD), and anti-IL-2 (S4B6) (27) by Dr. T. Mossman (DNAX, Palo Alto, CA). Purified biotin-conjugated mAb reactive with the DO11.10 TCR (clone KJ1-26.1), derived in the laboratory of Dr. Philippa Marrack (National Jewish Hospital, Denver, CO) (28), was graciously provided by Dr. Terrance Barrett (Northwestern University, Chicago, IL).

### *Purification of $CD4^+$ T cells*

Spleen cells from DO11.10 Tg mice were isolated, and $CD4^+$ cells were positively selected using a MiniMACS Magnetic Separation System. The protocol provided by Miltenyi Biotech (Auburn, CA) was used with some modification. Briefly, adherent cells (dendritic cells and macrophages) were removed by incubating $1 \times 10^8$ spleen cells in tissue culture dishes (Falcon 3003) in complete medium containing 5% FCS for 45 min at 37°C and 5% $CO_2$. Nonadherent cells were then passed twice through Nitex mesh, 60 micron opening (Tetko, Inc., Briarcliff Manor, NY), and suspended in 180 $\mu$l of cold MACs buffer (0.5% BSA in PBS/5 mM EDTA). The cells were then incubated on ice with 20 $\mu$l of magnetic beads conjugated with an anti-CD4 mAb (Miltenyi Biotech 492-01). After 15 min, the cells were passed through a prewashed MiniMACS column attached to a magnet (Miltenyi Biotech 422-01). The column was washed three times with 1-ml aliquots of cold MACs buffer, removed from the magnet, and this procedure was repeated. Following release from the matrix, T cell purity, as determined by flow cytometry using a mAb to the DO11.10 TCR (clone KJ1-26.1), ranged between 96 and 99%.

### *T cell proliferation assays*

Each culture well of a 96-well plate (Costar 3596, Cambridge, MA) received $2 \times 10^4$ cells in 0.2 ml final volume along with the indicated concentrations of various reagents. Supernatants were collected after 21 to 24 h unless otherwise indicated. Cultures were pulsed with 1 $\mu$Ci/well of [$^3$H]TdR (New England Nuclear, Boston, MA) and harvested 12 h later onto glass filters using a Packard Filtermate 196 96-well harvester (Packard Instrument Co., Meriden, CN). Counts were read using a Packard Topcount microplate scintillation counter (Packard Instrument Co.). If lymphokine and proliferation assays were to be performed using the same cultures, 100 $\mu$l of supernatant were removed 21 to 24 h after the initiation of the experiment.

### *Lymphokine ELISAs*

Lymphokines were measured at the times indicated. Commercially available ELISA kits (Endogen, Cambridge, MA) were used to determine IL-2 and IL-4 concentrations. Concentrations were expressed in picograms per milliliter as determined using the standard included in the ELISA kit.

### *Stimulation of T cells with immobilized anti-CD3 mAb*

Culture wells of 96-well plate (Costar 3596) were coated overnight at 0°C with 100 $\mu$l of goat anti-hamster antiserum (Organon Teknika Corp., West Chester, PA) at a dilution of 1:2000 in DPBS. They were then washed twice with 100 $\mu$l of DPBS, 100 $\mu$l of a 1:100 dilution of 145-2C11 hybridoma supernatant in culture medium were added, and the plates were incubated at 37°C for 2 h. After again washing twice, T cells ($5 \times 10^4$) were added to each well in a final volume of 0.2 ml. Supernatant collection and harvesting procedure were as described above.

### *Derivation of new T cell clones/limiting dilution analysis*

$CD4^+$ T cells were isolated as described above, and groups of 24 or 96 replicate microcultures were prepared by doubling dilution in 96-well plates (Costar 3596); wells contained from 500 to 0.24 $CD4^+$ cells/well. These plates were cultured for 14 days in the presence of irradiated (2000 R) T-depleted DBA/2J spleen cells ($2.0 \times 10^5$), OVA peptide (1 $\mu$g/ml), and one or a combination of the following reagents: human rIL-2 (20 U/ml), murine rIL-4 (10 pg/ml), anti-IL-4 (11B11) culture supernatant (50 $\mu$l/well). Between days 10 and 13, wells containing >20 blast cells/well were scored as positive. Minimal estimates of the frequency of proliferating cells, as well as 95% confidence interval, were calculated by the minimum $y^2$ method of Taswell (29).

Wells judged to be clonal were transferred to three microwells after 2 wk along with irradiated DBA/2J spleen cells and OVA peptide. After 1 wk, two of the three positive microwells were transferred to a macrowell of a 24-well plate (Linbro 76-033-055, Flow Laboratories, McLean VA) with irradiated DBA/2J spleen cells and OVA peptide. The third microwell was washed and stimulated with immobilized anti-CD3 mAb as described above, and supernatants were assayed for IL-2 and IL-4 content. After 1 wk, positive macrowells were harvested and cells were counted and then stimulated with irradiated DBA/2J spleen cells and OVA peptide. The cells were maintained by this method for a period of 6 wk. Aliquots of these macrowells ($5 \times 10^4$ T cells) were stimulated with immobilized anti-CD3 mAb as described above, and supernatants were assayed for IL-2 and IL-4.

A total of 300 $CD28^{-/-}$ and 300 $CD28^{+/+}$ clones were selected from four experiments performed as described above. Fifty T cell clones were derived from each of the six initial conditions: no lymphokine addition, IL-2, IL-4, IL-2 with IL-4, anti-IL-4, and IL-2 with anti-IL-4.

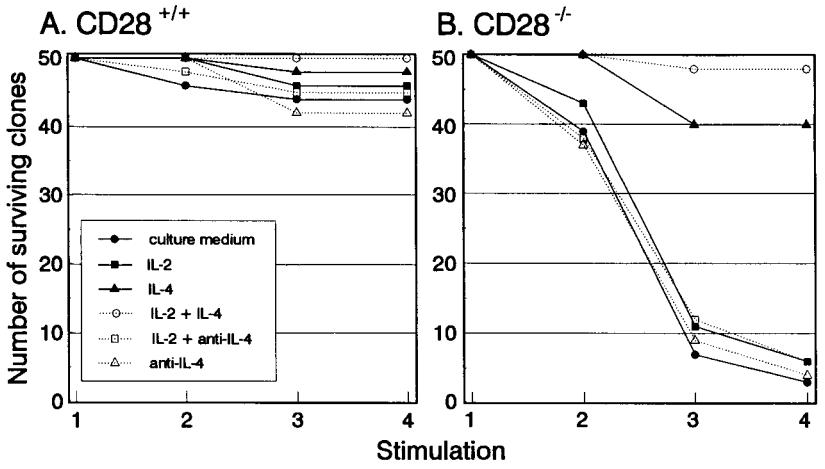


**FIGURE 1.** Initial culture with IL-4 enhances long-term survival of $CD28^{-/-}$ T cell clones. In a total of four experiments, 50 T cell clones were derived by stimulation with irradiated (2000 R) DBA/2J spleen cells ($5 \times 10^5$), OVA peptide (1 $\mu$g/ml), under each of the following conditions: no lymphokine addition, IL-2, IL-4, IL-2 with IL-4, anti-IL-4, and IL-2 with anti-IL-4. The procedure for derivation and maintenance of these T cell clones is described in *Materials and Methods*. In subsequent stimulations, no exogenous lymphokines were added. The viability of $CD28^{+/+}$ T cell clones (*A*) and $CD28^{-/-}$ T cell clones (*B*) are shown after each of four stimulations with DBA/2J spleen cells and OVA peptide. After each stimulation, the number of surviving cells was evaluated. A clone was determined to have "survived" if >20 T cells were visible in the well 7 days after stimulation.

*Viability/growth assay*

Cell cultures, set up at described in the cloning procedure, were analyzed after 14 days using a "no wash" flow cytometric method to identify and count live and dead cells directly out of 96-well culture plates. The direct staining assay was necessary to insure that apoptotic cells were not lost during repeated washes so that accurate live:dead cell ratios could be calculated. Each well was transferred into a 12- × 75-mm Falcon tube (Becton Dickinson Immunocytometry Systems, Mountain View, CA) for staining. Fifty microliters of anti-FcR mAb (2.4G2) was added and incubated for 10 min at room temperature to block nonspecific staining. Directly coupled anti-CD4-FITC was added and incubated at 4°C for 15 min to stain the $CD4^+$ T cells. Ten microliters of propidium iodide (PI) (100 $\mu$g/ml; Sigma Chemical Co., St. Louis, MO) was added immediately before analysis using the FACScan flow cytometer (Becton Dickinson). Fluorescent polystyrene beads (Coulter Corp, Miami, FL) of known concentration were analyzed at the beginning and end of each experiment to insure instrumentation standardization and to permit data normalization; in all experiments the difference between the number of beads analyzed at the beginning and the end of the experiment was <5%. All samples were analyzed for 40 s, and the actual number of cells or beads collected in that time period was used in data evaluation. $CD4^+$ dead and live cells were identified by PI uptake or exclusion, respectively.

## Results

*Primary culture with IL-4 enhances long-term survival of CD28-deficient T cell clones*

Using TCR Tg models, neither $CD4^+$ nor $CD8^+$ $CD28^{-/-}$ T cells were able to sustain proliferation following primary stimulation (22, 23). Furthermore, in the absence of CD28 ligation, T cells mounted an abortive proliferative response and then died (23, 30). To examine the ability of $CD4^+$ $CD28^{-/-}$ T cells to survive multiple stimulations with Ag + APC, T cell clones were derived in the presence of various cytokines. $CD28^{+/+}$ and $CD28^{-/-}$ T cells were cloned in the same experiments and passed on the same days, to minimize the effect of experimental variability. Except following the primary stimulation in which the cells were cultured for 14 days, T cells were expanded by stimulation with OVA peptide and APCs every 7 days. Clones were derived in 96-well plates (stimulation 1). Cells from wells that contained a single clone, based on Poisson distribution, were then expanded into three wells of a 96-well plate (stimulation 2); two of these wells were then placed in a macrowell (stimulation 3). Once in a macrowell, the cells were then expanded into multiple macrowells (stimulation 4). Fifty clones from each of the following initial culture conditions were expanded: no lymphokine addition; IL-2; IL-4; IL-2 with IL-4; anti-IL-4; and IL-2 with anti-IL-4.

The majority of $CD28^{+/+}$ T cell clones survived three stimulations (Fig. 1*A*). Initial culture with IL-2, IL-4, or the combination of these cytokines moderately enhanced the long-term survival $CD28^{+/+}$ T cell clones over those grown in the absence of cytokines: $CD28^{+/+}$ T cell clones initially cultured with IL-2 or IL-4 had approximately the same survival rate, 46/50 and 47/50, respectively; whereas, all clones initially grown in the presence of IL-2 with IL-4 survived four stimulations (Fig. 1*A*). Furthermore, the addition of anti-IL-4 mAb in the initial culture did not decrease long-term survival of the $CD28^{+/+}$ T cell clones, suggesting that if endogenous IL-4 were produced during the initial culture, it is not required for long-term survival of the $CD28^{+/+}$ T cell clones.

If IL-4 was not present in the initial culture, most $CD28^{-/-}$ T cell clones were unable to survive multiple stimulations with OVA peptide and APCs (Fig. 1*B*). All $CD28^{-/-}$ T cell clones initially cultured with IL-4 or IL-2 with IL-4 survived the second stimulation (Fig. 1*B*). Following the third stimulation, 40 of the $CD28^{-/-}$ T cell clones initially grown in IL-4 and 47 of the $CD28^{-/-}$ T cell clones initially cultured with IL-2 plus IL-4 survived; these clones survived the fourth stimulation and several subsequent stimulations (data not shown). Few $CD28^{-/-}$ T cell clones derived initially without added lymphokines or with IL-2 alone survived four stimulations; some died following the second stimulation, most died following the third stimulation, and several more died following the fourth stimulation (Fig. 1*B*). $CD28^{-/-}$ T cell clones that survived subsequent stimulations did not expand nearly as well as the $CD28^{+/+}$ T cell clones (data not shown). With both $CD28^{+/+}$ and $CD28^{-/-}$ T cell clones, the addition of anti-IL-4 mAb in the initial culture did not affect long-term survival of the $CD28^{-/-}$ T cell clones, suggesting that endogenous IL-4 did not enhance long-term survival of either $CD28^{+/+}$ or $CD28^{-/-}$ T cell clones. Thus, the presence of IL-4 in the primary culture increases the likelihood that a $CD28^{-/-}$ T cell clone will survive multiple antigenic stimulations.

*Surviving $CD28^{-/-}$ T cell clones produce IL-2 and IL-4*

It was possible to determine the pattern of lymphokine secretion by individual T cell clones following secondary stimulation with anti-CD3 mAb as early as 14 days after initiating culture. The lymphokine profile of the 50 T cell clones scored for viability is shown in Figure 2. Of $CD28^{+/+}$ T cell clones derived in the absence of IL-4, approximately 50% produced IL-4 in addition to IL-2 (Fig. 2, *panels 1* and *2*). When IL-4 or the combination of IL-2 plus IL-4 was present during the primary stimulation, 48 of 50 $CD28^{+/+}$ clones produced both IL-2 and IL-4 (Fig. 2, *panels 2* and *4*). In contrast, relatively few T cell clones derived from $CD28^{-/-}$ mice in the absence of exogenous cytokines initially produced IL-4,

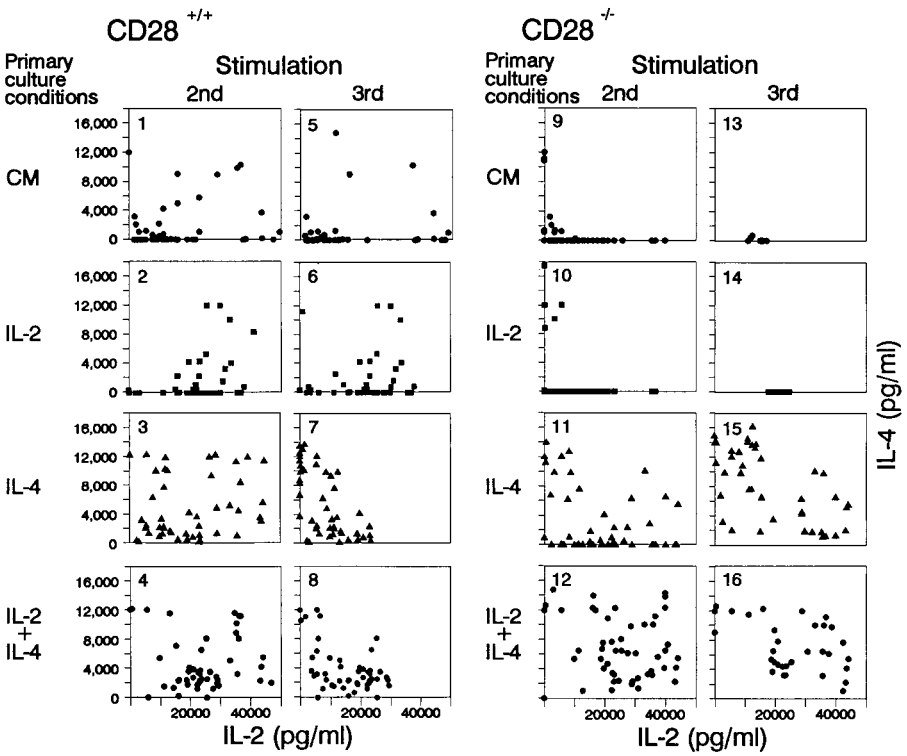


**FIGURE 2.** Surviving $CD28^{-/-}$ clones produce high levels of IL-2 and IL-4. T cell clones were derived by stimulation with irradiated (2000 R) DBA/2J spleen cells ($5 \times 10^5$), OVA peptide (1 $\mu$g/ml), under the following conditions: no lymphokine addition, IL-2, IL-4, and IL-2 with IL-4. Following the second and third stimulations, an aliquot of cells was removed and stimulated with immobilized anti-CD3 mAb. Only lymphokine production by surviving clones is shown, and each data point represents results from an individual clone. Supernatant was collected 24 h after stimulation, and IL-2 and IL-4 production measured using ELISA kits. The lymphokine profile of $CD28^{+/+}$ T cell clones (*A*) and $CD28^{-/-}$ T cell clones (*B*) are shown after the second and third stimulations with DBA/2J spleen cells and OVA peptide. IL-2 production is shown on the *x*-axis and the scale is from 0 to 50,000 pg/ml in 10,000-pg/ml increments. IL-4 production is shown on the *y*-axis and the scale is from 0 to 18,000 pg/ml in 2,000-pg/ml increments. In a total of 4 experiments, the lymphokine profile of 50 clones derived under each condition was measured.

while most produced IL-2 (Fig. 2, *panel 9*). Similar results were obtained when the $CD28^{-/-}$ T cell clones were derived in the presence of exogenous IL-2 (Fig. 2, *panel 10*). $CD28^{-/-}$ T cell clones derived in the presence of IL-4 alone produced lower levels of both IL-2 and IL-4, when compared with the $CD28^{+/+}$ T cell clones derived using comparable conditions (Fig. 2, *panel 3* and Fig. 2, *panel 11*). However, when the combination of IL-2 plus IL-4 was added in the primary culture, the levels of IL-2 and IL-4 production by the $CD28^{-/-}$ T cell clones were within the same range as the $CD28^{+/+}$ T cell clones (Fig. 2, *panel 4*, and Fig. 2, *panel 12*).

As noted above, most of the $CD28^{-/-}$ T cell clones derived in the absence of IL-4 did not survive. The majority (88 of 97) of $CD28^{-/-}$ T cell clones that survived three stimulations produced both IL-2 and IL-4 (Fig. 2, *panels 13* to *16*). A few clones producing only high levels of IL-2 survived initially, but all of these died following the fourth stimulation (data not shown). Most of the $CD28^{+/+}$ T cell clones survived the third stimulation, and the lymphokine profiles of the clones cultured in the absence of IL-4 remained much the same (Fig. 2, *panels 1*, *2*, *5*, and *6*). However, among the populations of clones derived in the initial presence of either IL-4 or the combination of IL-2 plus IL-4, many of the clones that produced IL-2 and IL-4 decreased the levels of IL-2 production (Fig. 2, *panels 3*, *4*, *7*, and *8*). These observations are consistent with previous data, in which the addition of IL-4 skews naive T cells toward the Th0/Th2 phenotype (11, 12).

### *Primary culture with IL-4 enhances precursor frequency of both $CD28^{+/+}$ and $CD28^{-/-}$ T cells*

Theoretically, in a population of TCR Tg T cells, every T cell is capable of replicating and surviving in response to Ag presented by a professional APC; thus, the precursor frequency of responding T cells should be one. However, many factors influence whether that T cell will respond, including the chance encounter with a professional APC, the presence of IL-2 and/or IL-4, and the dose of Ag. In these studies, we compared the effect of exogenous lymphokines on the precursor frequency of $CD28^{+/+}$ and $CD28^{-/-}$ T cells stimulated with T-depleted spleen cells and 1 $\mu$g/ml OVA. Using limiting dilution analysis, the frequency of responding T cells under given culture conditions can be estimated. Varying numbers of $CD4^+$ T cells, ranging from 500 to 0.24 cells/microwell, were cultured with T-depleted irradiated spleen cells ($5 \times 10^5$) and 1 $\mu$g/ml OVA peptide 323–339. In some cases cytokines or mAbs were also added in the concentrations indicated. Between 10 and 14 days wells were scored for T cell growth, and wells containing greater than 20 T cells per well were scored as positive. Statistical analysis of the relationship between the logarithm of the percentage of nonresponding wells and the number of cells cultured per well showed that the data could be fitted to a Poisson distribution, indicating that single T cells gave rise to colonies and that cell-cell interactions did not affect plating efficiency (29).

In the absence of added lymphokines, the apparent frequency of responding T cells from $CD28^{+/+}$ and $CD28^{-/-}$ mice was low, 1/20.4 and 1/53.1, respectively (Table I). The low apparent frequency of responding T cells is probably due to the requirement for a higher concentration of IL-2 and/or IL-4 to sustain multiple rounds of proliferation than the amount produced by an individual T cell in the culture volume of 200 $\mu$l. The addition of lymphokines to the culture (IL-2, IL-4, or IL-2 with IL-4) increased the apparent frequency of responding T cells from both $CD28^{+/+}$ and $CD28^{-/-}$ mice. IL-2 or IL-4 enhanced the apparent frequency of responding T cells to greater than 1 in 10 in both the $CD28^{+/+}$ and $CD28^{-/-}$ cultures (Table I); however, the combination of IL-2 with IL-4 was most effective, increasing the apparent frequency of responding T cells to approximately 1:1. Interestingly, when exogenous lymphokines were added to the cultures, the frequency of responding T cells from $CD28^{+/+}$ and $CD28^{-/-}$ mice was approximately equal, suggesting that in the presence of exogenous lymphokines, the T cells from the $CD28^{+/+}$

Table I. *Primary culture with IL-2, IL-4, or IL-2 plus IL-4 enhances apparent precursor frequency of clones derived from DO11.10 CD28$^{+/+}$ and DO11.10 CD28$^{-/-}$ mice*[a]

| Initial Conditions | CD28$^{+/+}$ Mice | | CD28$^{-/-}$ Mice | |
|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 1 | Expt. 2 |
| No lymphokines | 1/20.4 | NT[b] | 1/53.1 | NT |
| IL-2 | 1/6.9 | 1/4.9 | 1/7.8 | 1/4.5 |
| IL-4 | 1/5.3 | 1/5.6 | 1/1.2 | 1/5.3 |
| IL-2 + IL-4 | 1/1.4 | 1/1 | 1/1.2 | 1/1 |
| IL-2 + anti-IL-4 | 1/7.6 | NT | 1/9.1 | NT |
| Anti-IL-4 | 1/17.2 | NT | 1/41.2 | NT |

[a] Groups of 24 or 96 replicate microcultures were prepared by doubling dilution in 96-well plates. Wells contained from 500 to 0.24 CD4$^+$ cells/well. These plates were cultured for 14 days in the presence of irradiated (2000 R) T-depleted DBA/2J spleen cells ($2.0 \times 10^5$), OVA peptide (1 $\mu$g/ml), and one or a combination of the following reagents: human rIL-2 (20 U/ml), murine rIL-4 (10 pg/ml), anti-IL-4 (11B11) SF (50 $\mu$l/well). Between days 10 and 13, wells containing >20 blast cells/well were scored as positive. Minimal estimates of the frequency of proliferating cells were calculated by the minimum $y^2$ method of Taswell.

[b] NT = not tested.

and CD28$^{-/-}$ mice were responding in similar ways (Table I). Additionally, the frequency of responding CD28$^{+/+}$ and CD28$^{-/-}$ T cells in the presence of anti-IL-4 mAb was approximately the same as in the absence of the mAb (Table I). Thus, endogenous IL-4 did not appear to enhance the frequency of responding T cells.

### *T cell clones derived in the presence of IL-4 have enhanced growth and survival*

To analyze the ability of T cell clones, derived in the presence of various lymphokines, to proliferate and survive, T cells judged to be clonal by Poisson distribution were selected and the number of live and dead T cells was determined by flow cytometric analysis. Fourteen days following initial culture under the conditions described above, wells were harvested and the number of live and dead CD4$^+$ cells were enumerated. As shown in Figure 3, exogenous IL-2 and/or IL-4 increased the number of T cells in both the CD28$^{+/+}$ and CD28$^{-/-}$ cultures. In the cultures containing CD28$^{+/+}$ T cells, the initial presence of IL-2 doubled the number of recovered cells. The initial presence of IL-4 was somewhat more effective; the number of cells increased fourfold. However, the effect of the combination of IL-2 with IL-4 in the initial culture was striking; the number of surviving CD4$^+$ cells increased from <5,000 to >44,000. In contrast, in the CD28$^{-/-}$ cultures, IL-2 had no effect on absolute cell number, while the addition of IL-4 or IL-2 plus IL-4 doubled the number of CD4$^+$ cells.

Interestingly, while the addition of lymphokines at the initiation of the culture enhanced the absolute number of CD28$^{-/-}$ T cells in culture, many of the cells were dead. Without exogenous lymphokines, the ratio of live to dead CD4$^+$ events for CD28$^{+/+}$ and CD28$^{-/-}$ T cells was 0.53 and 0.30, respectively (Fig. 3, *A* and *B*). While all combinations of exogenous IL-2 plus IL-4 enhanced viability, the proportion of viable cells was always much lower in cultures of CD28$^{-/-}$ T cells than in CD28$^{+/+}$ cultures. Furthermore, in both CD28$^{+/+}$ and CD28$^{-/-}$ cultures, IL-4 appeared to have a greater effect than IL-2. In the CD28$^{-/-}$ cultures, the addition of IL-2 enhanced the ratio of live to dead cells to 0.44, while the addition of IL-4 enhanced the ratio to 0.89. In cultures containing CD28$^{+/+}$ T cells, IL-2 alone and IL-4 alone enhanced the viability of CD4$^+$ T cells to 0.98 and 1.96, respectively. Interestingly, in cultures containing CD28$^{+/+}$ T cells, the addition of IL-2 or IL-4, alone or in combination, always resulted in a live:dead ratio close to or greater than 1. In cultures containing CD28$^{-/-}$ T cells, only the combination of IL-2 plus IL-4 at the initiation of the culture enhanced the live:dead ratio to more than 1. Thus, the combination of IL-2 with IL-4 is most effective in enhancing the survival of CD28$^{-/-}$ T cells.

### *IL-4 is necessary for survival of established CD28$^{-/-}$ clones, but not of established CD28$^{+/+}$ clones*

Although CD28$^{-/-}$ T cell clones derived in the presence of IL-4 grew and survived in the absence of additional IL-4 in subsequent stimulations, it is unclear whether IL-4 produced by these cells is required for survival in subsequent cultures. It is possible that IL-4 is not itself a survival factor; initial culture with IL-4 may have activated "survival genes," which enabled CD28$^{-/-}$ T cells to survive multiple stimulations. To determine whether IL-4 is necessary for the long-term survival of CD28$^{-/-}$ Th0 clones, these clones

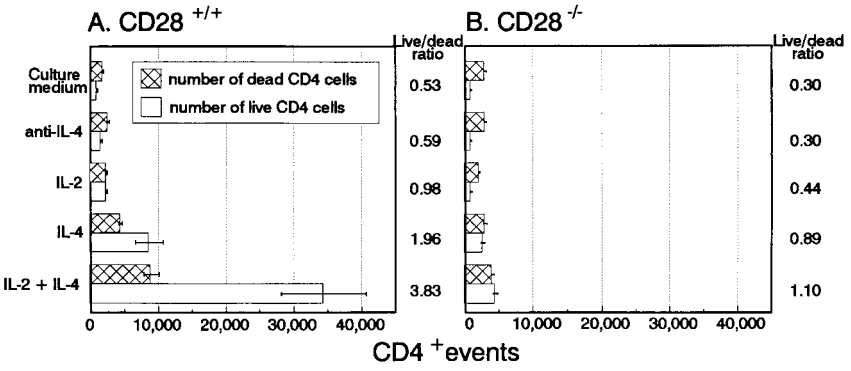


**FIGURE 3.** IL-4 enhances viability and growth of CD28$^{+/+}$ and CD28$^{-/-}$ T cells. In a limit dilution assay, CD28$^{+/+}$ and CD28$^{-/-}$ T cells were cultured for 14 days in the presence of irradiated (2000 R) DBA/2J spleen cells ($5 \times 10^5$), OVA peptide (1 $\mu$g/ml), under each of the following conditions: no lymphokine addition, human rIL-2 (20 U/ml), murine rIL-4 (20 ng/ml), IL-2 with IL-4, and anti-IL-4 (50 ml SF). For each condition, the number of live and dead CD4$^+$ cells was analyzed from 10 wells determined to be clonal by Poisson distribution. The total number of live and dead cells from all 10 wells was calculated and SD determined to be less than 10%. The number of live and dead CD4$^+$ cells was determined by PI exclusion and uptake, respectively. Results are representative of two experiments.

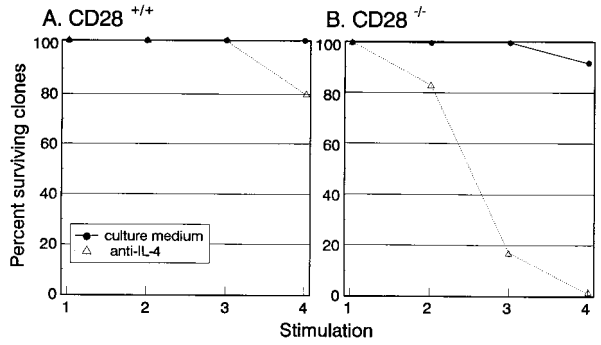


**FIGURE 4.** IL-4 enhances cell survival of $CD28^{+/+}$ and $CD28^{-/-}$ T cells. $CD4^+$ T cells were cultured for 14 days in the presence of irradiated (2000 R) DBA/2J spleen cells ($5.0 \times 10^5$), OVA peptide (1 $\mu$g/ml), and human rIL-2 (20 U/ml). Wells determined to be clonal were analyzed as described in *Materials and Methods*. Each well was stained with a FITC-coupled anti-CD4 mAb in the presence of anti-FcR (2.4G2). Each sample was collected for 40 s. The number of live and dead $CD4^+$ cells was determined by PI exclusion and uptake, respectively. Results are representative of two experiments.

were grown in the presence of anti-IL-4 mAb for several stimulations. A panel of 10 $CD28^{+/+}$ and 12 $CD28^{-/-}$ Th0 clones was used for these experiments. All clones produced high levels of IL-2 (from 1,191 to 28,972 pg/ml) and IL-4 (from 1,020 to 12,887 pg/ml), but the ratio of IL-2 to IL-4 production was highly variable.

Th0 $CD28^{+/+}$ and $CD28^{-/-}$ clones were cultured in 48-well plates with T-depleted irradiated spleen cells, 1 $\mu$g/ml OVA peptide 323–339 in the presence of culture medium, or anti-IL-4 mAb. Seven days following stimulation, wells with visible T cells were harvested and counted. Cells from those wells that contained $>10^5$ cells were washed and restimulated under the same conditions used in the initial culture. Wells that contained $<10^5$ cells were considered not to have survived. This procedure was repeated for three restimulations.

In most cases, the presence of anti-IL-4 mAb did not appear to affect the survival of the $CD28^{+/+}$ clones (Fig. 4*A*). While fewer T cells were recovered from the wells containing anti-IL-4 mAb, there were usually greater than $10^5$ live T cells per well. Only after four stimulations in the presence of anti-IL-4 mAb did a few of the clones fail to survive. This observation suggests that while IL-4 enhances growth of the $CD28^{+/+}$ clones, it is not required for survival. However, for the $CD28^{-/-}$ clones, the presence of IL-4 was necessary for survival (Fig. 4*B*). Several of the $CD28^{-/-}$ clones cultured with anti-IL-4 mAb failed to survive following the first restimulation. The majority of $CD28^{-/-}$ clones failed to survive following the second restimulation in the presence of anti-IL-4, and the remainder did not survive the third restimulation in the presence of anti-IL-4. These observations suggest that IL-4 is required for survival of $CD28^{-/-}$, but not $CD28^{+/+}$ Th0 clones.

### *$CD28^{-/-}$ T cell clones require IL-2 and IL-4 for optimal proliferation*

As IL-4 appeared to play a critical role in the survival of the $CD28^{-/-}$ T cell clones, it was of interest to determine whether IL-4 also contributed to the growth of these clones as measured by short-term (48 h) proliferation. $CD28^{+/+}$ and $CD28^{-/-}$ Th0 clones were stimulated with OVA peptide presented by T-depleted spleen cells, and the ability of anti-IL-2/anti-IL-2R mAb, anti-IL-4 mAb, and the combination to inhibit proliferation was measured. In Table II the average inhibitory affect of the different blocking reagents on 10 $CD28^{+/+}$ and 11 $CD28^{-/-}$ is shown. For $CD28^{+/+}$ Th0 clones, IL-2 appeared to be the predominant growth factor; proliferation was blocked an average of 70.85% in the presence of anti-IL-2/anti-IL-2R mAbs (Table II). Anti-IL-4 mAb had little affect, inhibiting proliferation only an average of 6.25% (Table II) indicating that IL-4 was not required for growth of $CD28^{+/+}$ Th0 clones. Moreover, the combination of anti-IL-2/anti-IL-2R mAbs and anti-IL-4 mAb was not more effective than anti-IL-2/anti-IL-2R mAbs alone.

Table II. *IL-2 and IL-4 are required for optimal proliferation of $CD28^{-/-}$ Th0 clones*[a]

| Clones | % Inhibition of Proliferation | | |
|---|---|---|---|
| | Anti-IL-2 + anti-IL-2R | Anti-IL-4 | Anti-IL-2 + anti-IL-4 |
| $CD28^{+/+}$ | 70.85 ± 9.24 | 6.25 ± 13.58 | 72.45 ± 10.18 |
| $CD28^{-/-}$ | 66.00 ± 9.09 | 41.18 ± 13.51 | 72.53 ± 4.90 |

[a] $CD28^{+/+}$ and $CD28^{-/-}$ clones were cultured with $5 \times 10^5$ syngeneic, irradiated T-depleted spleen cells, OVA peptide (5 $\mu$g/ml), and a 1:100 dilution of anti-IL-4 (11B11) ascites, 50 $\mu$l of a 1:1 ratio of anti-IL-2 (S4B6) and anti-IL-2R (3C7) SF, or both anti-IL-2 and anti-IL-4. [$^3$H]TdR incorporation was measured during the final 12 h of a 48-h incubation. Inhibition is shown as the percent of the proliferative response relative to control (no mAbs) and is the average of 10 $CD28^{+/+}$ and 12 $CD28^{-/-}$ clones. For all values, $p < 0.001$ relative to control values.

As seen with the $CD28^{+/+}$ Th0 clones, anti-IL-2/anti-IL-2R mAbs effectively blocked proliferation of $CD28^{-/-}$ Th0 clones; proliferation was inhibited an average of 66.00% (Table II). Additionally, the anti-IL-4 mAb was also effective at inhibiting proliferation of $CD28^{-/-}$ clones, blocking proliferation an average of 41.18% (Table II). Furthermore, the combination of anti-IL-2/anti-IL-2R mAbs was more effective than the anti-IL-2/anti-IL-2R mAbs alone inhibiting proliferation 72.53% (Table II). These observations suggest that while IL-2 is sufficient to maintain proliferation of $CD28^{-/-}$ Th0 clones; both IL-2 and IL-4 are required for optimal proliferation of $CD28^{-/-}$ Th0 clones.

## Discussion

Previous studies using TCR Tg models have focused on the importance of CD28 signaling in the ability of naive T cells to mount and sustain a primary proliferative response (22, 23). These studies suggested that CD28 engagement both enhances the magnitude and extends the proliferative response following a primary antigenic challenge (22, 23). In the absence of CD28 ligation, T cells mount an abortive proliferative response and then die (23, 30).

While these previous studies described the role of CD28 in a primary response, they did not address the role of CD28 in the development of Th subsets. By using T cell clones derived from DO11.10 TCR $Tg^+$ $CD28^{-/-}$ mice and DO11.10 TCR $Tg^+$ $CD28^{+/+}$ mice, we were able to analyze the ability of $CD28^{-/-}$ T cells to respond to multiple antigenic stimulations over a period of weeks. Under limiting dilution culture conditions, $CD28^{+/+}$ and $CD28^{-/-}$ T cells were initially cultured in the presence of IL-2, IL-4, IL-2 with IL-4, or no lymphokine addition. We found that the majority of $CD28^{+/+}$ T cells clones survived four stimulations with OVA and APCs; however, only those $CD28^{-/-}$ T cell clones initially derived in the presence of IL-4 survived four stimulations. As the presence of IL-4 in a primary culture promotes IL-4 production, it seemed likely that most of the surviving $CD28^{-/-}$ T cell clones would produce IL-4. Almost all $CD28^{-/-}$ T cell clones cultured in the presence of IL-4 or the combination of IL-2 and IL-4 produced both IL-2 and IL-4. Thus, the presence of IL-4 in primary culture promotes IL-4 production, and IL-4 produced upon subsequent stimulations with Ag acts on the T cell to promote survival following TCR stimulation. Thus, IL-4 can act in the

absence of CD28 not only to augment T cell proliferation but also to promote cell survival.

To determine whether IL-4 also enhanced the number of T cells responding to antigenic stimulation or the long-term survival of all T cells, the effect of IL-2 and IL-4 on precursor frequency was examined. Addition of IL-2 or IL-4 to the primary culture enhanced the precursor frequency of T cell clones from both $CD28^{+/+}$ and $CD28^{-/-}$ cultures. Again, IL-4 was most effective if IL-2 was also present at the initiation of the cultures. When analyzed by FACS, the addition of IL-2, IL-4, or IL-2 plus IL-4 increased the number of T cells in both $CD28^{+/+}$ and $CD28^{-/-}$ cultures. However, the combination of IL-2 plus IL-4 was much more effective than either lymphokine alone. Additionally, while exogenous IL-2, IL-4, or IL-2 plus IL-4 was able to reverse the live:dead ratio of $CD28^{+/+}$ T cells to greater than one, only the combination of IL-2 and IL-4 was able to reverse the live:dead ratio of $CD28^{-/-}$ T cells. These observations suggest that the combination of IL-2 and IL-4 enhances growth of $CD28^{-/-}$ T cells at least in part by enhancing survival of $CD28^{-/-}$ T cells following primary stimulation.

Interestingly, once established, $CD28^{-/-}$ clones produce higher levels of IL-4 and IL-2. This suggests that only the clones producing the highest levels of autocrine growth factors survive, or that the absence of CD28 signaling actually promotes cytokine production by established clones. Such a possibility could be the result of failure of $CD28^{-/-}$ clones to up-regulate CTLA-4, since CD28 signal transduction plays an important role in the induction of CTLA-4 expression (31).

The finding that CD28 signaling plays a major role in preventing T cell death following primary antigenic stimulation is consistent with previous observations that CD28 up-regulates at least one gene believed to be involved in the prevention of cell death, *bcl-x* (32). *bcl-*$x_L$ has been shown to protect lymphocytes from apoptosis (32, 33). Boise et al. showed that while CD28 costimulation does not lead to any changes in the levels of bcl-2 found in activated T cells, it does up-regulate *bcl-*$x_L$. Furthermore, the ability of CD28 ligation to prevent TCR-induced death was independent of IL-2, as the addition of high concentrations of IL-2 failed to prevent cell death of cells stimulated with anti-CD3 mAb (23, 30). This finding, taken together with the observation that IL-4, but not IL-2 is capable of preventing cell death of $CD28^{-/-}$ T cells, suggests that in the absence of CD28 engagement, IL-4R signaling is able to trigger survival factors that may partially rescue T cells from death. However, IL-4 does not appear to enhance T cell survival through up-regulation of either *bcl-2* or *bcl-*$x_L$ (L. Boise and C. Thompson, unpublished data). Interestingly, IL-3 signal transduction has been shown to induce survival, inducing phosphorylation and inactivation of the *bcl-*$x_L$ inhibitor BAD (34). Thus, IL-4 signal transduction could work through inhibition of BAD or a related proapoptotic factor.

Furthermore, we also observed that IL-4 was most effective at enhancing T cell survival when high concentrations of exogenous IL-2 were added at the initiation of the culture. This observation suggests that the presence of IL-2 early in culture enhances the ability of IL-4 to promote cell survival. Boise et al. (35) have shown that resting T cell survival is maintained by interaction through the $\beta\gamma$-chains of the IL-2R. The IL-2R $\gamma$-chain is constitutively expressed in lymphoid cells and has been shown to be an important component of IL-2R signaling. Also present in the IL-4R and IL-7R, it is thought to play a role in the signaling of these receptors as well. Perhaps the presence of IL-2 early in T cell activation helps up-regulate expression of the $\gamma$-chain in the IL-4R, making the cell more responsive to IL-4R signaling.

While CD28 and IL-4 play important roles in survival of T cells, they also influence Th subset development. In the current model of Th subset development, the Th1-Th2 decision is influenced by cytokine environment, Ag dose, and CD28 costimulation. Previous studies have suggested that cytokine environment is the dominant factor in Th subset development; stimulation through the TCR and CD28 in the presence of IL-4 promotes Th2 development (12, 36). However, naive T cells must be stimulated to a threshold level of activation, which is dependent on the relative level of CD28 costimulation and Ag dose, to respond to IL-4. Findings by Seder et al. suggest that IL-2 may bypass the requirement for CD28 ligation; naive Tg T cells stimulated initially with Cos cells transfected with MHC class II molecules in the presence of IL-2 and IL-4 produced IL-4 upon restimulation (37). Additionally, our studies suggest that other costimulatory molecules may also enhance IL-2 production, bypassing the need for CD28 engagement. In our studies, when naive $CD28^{-/-}$ T cell were stimulated with T-depleted spleen cells and OVA peptide, IL-4 producing $CD28^{-/-}$ clones were derived. APC populations within the T-depleted spleen cell population express CD44, CD43, CD40, and the ligand for heat-stable Ag, all which have been implicated in providing costimulation in the absence of CD28 ligation (38–41).

Alternatively, high levels of TCR ligation or high levels of CD28 ligation in conjunction with minimal TCR engagement are capable of driving Th2 development. Using DO11.10 naive T cells, it has been reported that increasing TCR ligation in the presence of CD28 costimulation can influence Th subset development (42). Specifically, medium peptide doses (1 mM) gave rise to Th1 like cells producing IFN-$\gamma$, while very high ($>10$ $\mu$M) and low ($<0.05$ $\mu$M) peptide doses drove the development of Th2-like cells producing IL-4 (42). In our studies, for derivation of the $CD28^{+/+}$ and $CD28^{-/-}$ clones we used a medium dose of peptide, 1 $\mu$g/ml, which is equivalent to 3 $\mu$M, and we found that approximately 75% of the $CD28^{+/+}$ clones produced IL-2, but no IL-4 in the absence of any exogenous lymphokines. Thus, our findings are consistent with those of Hosken et al. (42), suggesting that the effect of Ag dose may be extended to derivation of clonal populations. Interestingly, in the absence of exogenous IL-4, few $CD28^{-/-}$ clones (7 of 50) produced IL-4 following secondary stimulation, and only two produced significant levels of IL-4. These findings are consistent with observations by Rulifson et al. who found that bulk cultures of lymph node cells from DO11.10 $CD28^{-/-}$ mice produced little if any IL-4 upon restimulation (43). Furthermore, CD28 stimulation of IL-4 production was dependent on the strength of the CD28 signal. When increasing concentrations of anti-CD28 mAb were added to cultures of DO11.10 lymph node cells and OVA peptide, IL-4 production increased (43).

Collectively, these observations suggest that the functions of CD28 and IL-4 may overlap; both promote IL-4 production and survival. Following primary stimulation, CD28 engagement triggers up-regulation of both IL-4 production and survival gene *bcl-*$x_L$. However, in the absence of CD28 engagement, the addition of IL-4 to the initial culture can promote both IL-4 production and survival. Moreover, once T cells gain the ability to produce IL-4, they can maintain IL-4 production and, thus, survive for multiple stimulations in the absence of CD28 engagement. These findings suggest that CD28 engagement may be important in initiating a Th2 immune response by promoting survival and triggering the initial population of responding T cells to produce IL-4. However, once these cells begin to release IL-4 into the surrounding environment, subsequent responding T cells may survive and differentiate into IL-4-producing cells in the absence of CD28 engagement. Ultimately, these cells become Th2 cells, which utilize a CD28-independent, IL-4-dependent activation pathway.

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