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LECTIN-MEDIATED INDUCTION OF IL-4-PRODUCING CD4+ T CELLS[1]

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Cultured murine CD4+ T cells have been shown to differentiate into IL-2- or IL-4-producing subsets. The factors responsible for the development of CD4+ T cells which produce IL-2 but not IL-4 and cells capable of producing IL-4 but not IL-2 are unknown. Here we describe a system that allows the controlled induction of IL-2- or IL-4-producing T cells after one single round of activation. Freshly isolated CD8-depleted T cells were activated with various polyclonal T cell activators for 48 h, washed, and then expanded under different conditions. IL-2 and IL-4 production were induced by restimulation of T cells and were measured with CTLL cells that respond to both cytokines and mAb to IL-2 and IL-4. T cells produced mainly IL-2 and small amounts of IL-4 when restimulated after expansion culture for 12 days with rIL-2 alone. However, after expansion for 12 days in the presence of rIL-2 plus Con A, we observed a 30- to 100-fold up-regulation of IL-4 activity and a 100-fold down-regulation of IL-2 when assessed by responses of CTLL cells incubated with the supernatant of restimulated T cells and by responses of CTLL cells cocultured with restimulated cells. An increase of IL-4 and decrease of IL-2 was also observed when the results were based on the cell numbers at the beginning of the expansion culture. The induction of IL-4 and the down-regulation of IL-2 1) were not reproduced with α-methyl-mannoside-treated supernatant of Con A-stimulated spleen cells, 2) were not dependent on the presence of large numbers of APC, 3) did not result from differential consumption of lymphokines after restimulation, 4) were not due to a difference in the time course of IL-2 or IL-4 release in either T cell population, and 5) were obtained regardless of the agents used to activate or to restimulate the T cells. Because Con A remained detectable on the T cell surface and because expansion of activated T cells with IL-2 plus Con A for several days was necessary, our results indicate that mainly IL-4-producing CD4+ T cells can be induced by prolonged engagement of T cell surface molecules.

CD4+ helper/inducer T lymphocytes are considered to be the principal regulatory cells of the immune system (1). Their function seems to be closely related to the pattern of lymphokines they secrete upon activation. Among these lymphokines, IL-2 and IL-4 are of particular interest, as they critically modulate a large number of immune-mediated reactions. IL-2 promotes the growth of both B and T lymphocytes and plays an essential role in the expression of T lymphocyte effector function (2, 3). IL-4 modulates lymphocyte function by promoting B and T cell growth, regulating Ig isotype expression, and promoting cytotoxicity (4–7).

Experiments with long term cultured and cloned murine CD4+ T cells revealed heterogeneity with respect to lymphokine production: many IL-2-producing CD4+ T cell clones also secrete IFN-γ and lymphotoxin, whereas many IL-4-producing CD4+ T cell clones also release IL-5, IL-6, and the cytokine synthesis inhibitor upon stimulation (8–10).

In vitro and in vivo experiments have shown that the lymphokine pattern produced by such cultured CD4+ T cells (clones and lines) is closely related to functional heterogeneity: IL-2-producing T cell clones and lines are reported to mediate delayed type hypersensitivity and contact sensitivity in vivo, macrophage activation in vitro and B cell help for IgG2A production in cognate T-B cell culture systems (1–14). IL-4-producing CD4+ T cell clones and lines are capable of stimulating in vitro IgG1 and IgE production by B cells in cognate interaction (14, 15). More importantly, IL-2/IFN-γ- or IL-4-producing cells that differentially regulate the pattern of immune responses seem also to be generated in vivo (16–18).

Reports on the lymphokine production of freshly isolated and stimulated CD4+ T cells from nonimmunized mice are conflicting. These cells were found by most investigators to produce high levels of IL-2, low levels of IFN-γ, and no IL-4 (19–22) (M. Röcken and C. Hauser, unpublished data). However, Bottomly et al. (23) found an IL-4-producing subpopulation of CD4+ T cells characterized by its weak staining with the anti-CD45 mAb YCD45R-1. The reasons for these diverging results are not yet elucidated. Variations in the previous exposure to pathogens, the methods for cell preparation, and the conditions for culture and cell activation might contribute to the conflicting results.

IL-2 and no IL-4 is detected by most authors in freshly isolated and in vitro activated murine CD4+ T cells. Although IL-4 may become detectable a few days after T cell activation, CD4+ T cells producing IL-4 but little or no IL-2 were found after repetitive stimulations of cells expanded either in bulk or clonal culture systems (15, 21, 22, 24, 25). As the generation of CD4+ T cell lines producing IL-4 but little or no IL-2 was observed in various Ag-specific and polyclonal systems and with the use

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of different types of APC (M. Röcken and C. Hauser, unpublished data), the seemingly common requirement for the induction of CD4+ T cells producing IL-4 but little or no IL-2 in these systems was multiple stimulation. Results by Gajewski et al. (25), who obtained IL-2-producing clones with the SN[4] of a secondary MLC or rIL-2 plus IFN-γ- and IL-4-producing clones with Con A SN or rIL-2, suggest that, in addition to restimulation, cytokines might play a role for the generation of either IL-2- or IL-4-producing clones.

However, precise signals required for the induction of IL-4- but not IL-2-producing T cells have not been identified. We attempted to induce CD4+ T cells producing IL-4 but little or no IL-2 after one single round of activation in vitro.

We describe a system that allows the controlled induction of either mainly IL-2- or mainly IL-4-producing T cells within 2 wk of culture and after one single round of T cell activation. We report that expansion of freshly isolated and activated CD4+ T cells in the presence of rIL-2 plus Con A or PHA induces up-regulation of IL-4 and down-regulation of IL-2 release in response to restimulation compared with cells expanded with rIL-2 alone.

MATERIALS AND METHODS

Animals. Female BALB/c, C3H and male CBA mice, 5 to 12 wk of age, were purchased from IFFA-Credo (Arbresle, France) or KFM (Füllinsdorf, Switzerland).

Culture medium. Culture medium consisted of 50% RPMI 1640 with L-glutamine, 20% Click's EHAA, 20% NCTC-135, 1% MEM nonessential amino acids, 100 U/ml penicillin, 100 μg/ml streptomycine, and 2 mM L-glutamine (all from GIBCO, Grand Island, NY), 10% heat-inactivated FCS and 0.015% $NaHCO_3$ (Seromed, Munich, FRG), 1 mM sodium pyruvate (Fluka, Buchs, Switzerland), and 2×10^{-5} M 2-ME (Sigma, St. Louis, MO).

Preparation of CD4+ T cells. Inguinal, axillary, subscapular, and mesenteric lymph node cells were purified by passage over nylon wool columns and incubated at 4°C for 30 min with mAb to MHC class II (M5/114.15.2) and CD8 (5.3-6.72) (both from the American Type Culture Collection (ATCC), Rockville, MD). The cells were washed and then incubated with mouse anti-rat κ-chain mAb (MAR-18.5, ATCC) and rabbit C (1:10; Cedarlane, Hornby, Ontario, Canada) for 45 min at 37°C. After washing, these cells were >97% CD4+, <1% CD8+ and <1% MHC class II+ as assessed by FMF. In 4-day proliferation assays at 10^5 cells/100 μl, no increased incorporation of [3H]methyl-thymidine (DuPont de Nemours, Regensdorf, Switzerland) was observed in the presence of Con A (2 μg/ml; P-L Biochemicals, Milwaukee, IL) when compared with the spontaneous [3H] thymidine uptake.

Preparation of Con A SN. Con A SN was prepared by stimulation of BALB/c SC (10^7 cells/ml) for 24 h with Con A (5 to 10 μg/ml). The SN was filtered and stored at −20°C. α-mm (20 mg/ml; Sigma) was added to the Con A SN where indicated.

Activation, expansion, and restimulation of T cells. CD8-depleted T cells (10^6) were activated in 2 ml culture medium in 24-well tissue culture plates (Costar, Cambridge, MA) with either 3000 rad (^{137}Cs source, 10.3 rad/s) irradiated SC (1.5×10^6 cells/ml) plus Con A (2 μg/ml) or PMA (1 ng/ml; Sigma) plus Con A (2 μg/ml) or PMA (10 ng/ml) plus ionomycine (100 ng/ml; Calbiochem, La Jolla, CA). After 2 days, the cells were washed three times, once with α-mm (20 mg/ml) when cells were activated with Con A.

The washed cells were resuspended for expansion culture in fresh culture medium containing human rIL-2 (50 U/ml; a generous gift from Dr. C. R. Franks, Eurocetus, Amsterdam, Netherlands) or rIL-2 plus agents to be tested (Con A, PHA (Pharmacia, Uppsala, Sweden), mAb 145-2C11 (1% culture supernatant of the hybridoma 145-2C11) (26), Con A SN, α-mm). Cells were expanded in 25-ml flasks (Falcon, Becton Dickinson, Mountain View, CA) with 5 to 10 ml culture medium (2 to 4×10^6 cells) or in 24-well tissue culture plates with 2 ml medium (1 to 2×10^6 cells) containing human rIL-2. Alternatively, we expanded the cells in 96-well round-bottomed tissue culture plates (Costar); each well was seeded with 5×10^3 cells in 100 to 200 μl of culture medium containing 50 U/ml rIL-2. The medium (50 to 90%) was replaced three times a week with fresh culture medium containing rIL-2 (50 U/ml). The agents we tested (lectins, mAb 145-2C11, Con A SN, α-mm) were changed together with the medium. When reanalyzed by FMF after 12 days of expansion culture, about 90% of the cells were CD4+.

After 12 days (if not otherwise stated), cells were washed three times with medium and restimulated. Cells expanded in tissue culture flasks or 24-well plates were restimulated at 0.25×10^6 cells/ml in 24-well plates, unless otherwise stated. Cultures expanded in 96-well plates were split into 2 wells, washed, and restimulated in 200 μl culture medium. After the expansion culture, cells previously activated with SC plus Con A were restimulated with 900-rad irradiated SC-T (1.5×10^6 cells/ml; SC treated with mAb to CD4 (GK1.5; from ATCC), CD8 (5.3-6.72) and Thy-1.2 (HO-13-4; from ATCC) followed by MAR-18.5 plus C) plus Con A (2 μg/ml), with SC-T plus PHA (10 μg/ml) or with PMA (1 ng/ml) plus Con A (2 μg/ml). CD4+ T cells previously activated with PMA (1 ng/ml) plus Con A (2 μg/ml) or with PMA (10 ng/ml) plus ionomycin (100 ng/ml) were restimulated with the same activators, respectively. SN were harvested 48 h after restimulation unless otherwise stated.

Antibodies for FMF analysis. For cell phenotyping, we used mAb GK1.5 phycoerythrin conjugate (Becton Dickinson) or mAb 5.3-6.72 and mAb M5/114.15.2 followed by a goat F(ab')2 anti-mouse IgG-FITC (Tago, Inc., Burlingame, CA). Cell-bound Con A was revealed with an affinity-purified goat antibody to Con A followed by a biotinylated antibody to goat IgG (both from Vector Laboratories, Inc., Burlingame, CA) and fluorescein-streptavidin (Amersham International, Amersham, UK).

Lymphokine assays. To measure the production of IL-2 and IL-4 a specific lymphokine assay was used (27). Briefly, 5×10^3 CTLL cells responding to both IL-2 and IL-4 (a kind gift of Dr. E. M. Shevach, LI, NIAID, NIH, Bethesda, MD) or, where mentioned, 5×10^3 CTLL cells responding only to IL-2 (a kind gift of Dr. H. R. MacDonald, Ludwig Institute for Cancer Research, Epalinges, Switzerland) were incubated in full size or half-area flat-bottomed 96-well tissue culture plates (Costar) with the dilute lymphokine-containing culture SN in the presence of either S4B6 (kindly provided by Dr. T. Mosmann, DNAX, Palo Alto, CA; ascites 1/1000), a neutralizing mAb to mouse IL-2 (28), 11B11 (kindly provided by Dr. W. E. Paul, LI, NIAID, NIH, Bethesda, MD; ascites 1/1000), a neutralizing mAb to mouse IL-4 (29), both mAb or no mAb. Internal standards for IL-2 and IL-4 calibrated with human rIL-2 or with murine rIL-4 (obtained from Dr. W. E. Paul NIH), respectively, were incubated in parallel. Proliferation was assessed by [3H]methyl-thymidine incorporation after pulsing the cultures (0.5 μCi/well) for the final 6 to 8 h of the 28 h culture period. Maximal [3H]thymidine incorporation induced by optimal IL-4 concentrations was between 40 and 80% of that for IL-2.

Alternatively, after expansion culture, washed T cells were transferred at varying numbers into flat-bottomed 96-well half-area tissue culture plates and were restimulated with SC-T (1.5×10^6/ml) plus Con A (2 μg/ml). The cultures were immediately irradiated (3000 rad) and 5000 CTLL cells were added to each well. IL-2 and IL-4 were determined with mAb (ascites 1/500) as described above. [3H]Thymidine incorporation was assessed as above.

All data shown represent the mean of triplicate or duplicate cultures; SD are indicated.

RESULTS

Controlled induction of IL-2 or IL-4 producing CD4+ T cells after one single round of activation. Freshly isolated CD8− T cells (>97% CD4+) from unprimed mice were activated for 48 h with SC plus Con A. The cells were then extensively washed and expanded in fresh culture medium containing 50 U/ml human rIL-2 plus, when mentioned, the agents to be tested (Fig. 1). After 12 days, the cells were washed and restimulated with PMA plus Con A. Culture SN were harvested 48 h later and analyzed for the presence of IL-2 and IL-4 with a specific T cell growth factor assay. Cells expanded in culture medium containing rIL-2 alone released CTLL growth-promoting activity that was blocked to 95% by a mAb to IL-2 (Table I). However, when 20% Con A SN was added together with rIL-2, the entire CTLL growth promoting activity

[4] Abbreviations used in this paper: SN, supernatant of stimulated cells; Con A SN, SN of Con A-stimulated SC; SC, spleen cells; SC-T, T cell-depleted SC; α-mm, α-methyl-D-mannopyranoside; FMF, flow microfluorimetry.

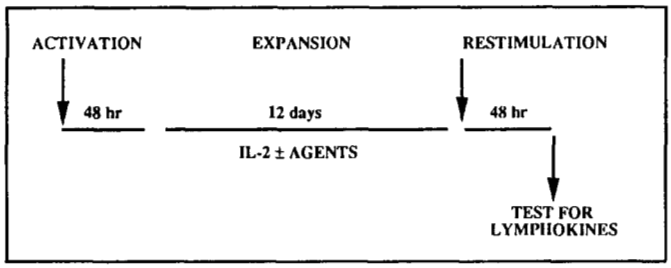

Figure 1. Experimental protocol for activation, expansion and restimulation. CD8-depleted T cells were activated with polyclonal T cell activators. After 2 days, cells were washed and resuspended in fresh culture medium containing rIL-2 plus the agents we tested. The cells were fed three times a week with fresh culture medium, rIL-2 plus the agents we tested. At day 12 the cells were washed again and restimulated for lymphokine production with the indicated T cell activators. If not otherwise stated, the 2-day SN was harvested and tested for lymphokine activity.

was blocked by a mAb to IL-4 (Table I). These results suggest that, after restimulation, $CD4^+$ T cells release mainly IL-2 when expanded in culture medium containing rIL-2 and mainly IL-4 when expanded in culture medium supplemented with 20% Con A SN.

Induction of IL-4 and loss of IL-2 release is due to the presence of Con A during the expansion culture. The previous results indicated that Con A SN was critically involved in the increase of IL-4 and the decrease of IL-2 in the SN of restimulated T cells. The effect of Con A SN on IL-2 and IL-4 production could be completely inhibited by α-mm (Table I). This suggested that not a single cytokine present in Con A SN but Con A was responsible for the induction of $CD4^+$ T cells producing IL-4. Indeed, the addition of Con A alone to T cells expanding with IL-2 led to increased levels of IL-4 in the SN of restimulated T cells. The effect of Con A on the IL-2/IL-4 pattern was inhibited by α-mm and was dose-dependent (Table I). The dose-response curve was steep in all titration experiments. Concentrations of Con A at which release of both cytokines was detected ranged from 0.25 to 1.0 μg/ml depending on the experiment. FMF analysis with an antibody to Con A revealed that the lectin was still bound to the cells 3 to 5 days after the last medium change (performed three times a week).

To determine the minimal duration of the expansion culture that was required to obtain T cells producing IL-4 but no detectable IL-2, we either added Con A at various times of the expansion culture to the IL-2-containing culture medium or α-mm to cultures containing IL-2 plus Con A. Cells producing detectable IL-4 alone were obtained when Con A (2 μg/ml) was present from day 8 or earlier through day 12 of the expansion culture. The effect of Con A (present in the expansion culture since day 0) was inhibited when α-mm was added at day 4 or earlier (Table II). Cells expanded during the first 6 days with IL-2 plus Con A and thereafter with IL-2 alone for another 6 days (α-mm was added at day 6) showed the same lymphokine pattern upon restimulation as cells expanded for the entire 12-day period with IL-2 plus Con A. The IL-2/IL-4 pattern from cells that had been expanded with IL-2 plus Con A, washed, and allowed to rest for 24 h before restimulation did not differ from cells immediately restimulated after expansion culture (data not shown). Therefore, it is unlikely that the IL-2 release was only transiently inhibited by Con A.

To investigate whether the induction of IL-4-producing T cells with Con A was restricted to BALB/c mice, we tested the IL-2 and IL-4 release of restimulated cells from CBA and C3H mice. Restimulated CD8-depleted T cells from these strains also produced IL-4 and no measurable IL-2 after expansion with IL-2 plus Con A but mainly IL-2 after expansion in the absence of Con A (Table III).

Quantification of IL-2 and IL-4. A quantitative estimate of IL-2 and IL-4 present in the SN of restimulated T cells expanded with IL-2 alone or with IL-2 plus Con A was obtained by testing the SN at serial dilutions in the CTLL assay and comparing the curves obtained in the presence of one or the other anti-cytokine mAb to rIL-2 and rIL-4 standards of known activity (27). Upon restimulation with PMA plus Con A, the 48-h SN of T cells expanded for 12 days with IL-2 plus Con A (2 μg/ml) contained about 26×10^3 U IL-4 per ml and per 10^6 restimulated cells (U/ml $\times$ 10^6 restimulated cells) but no measurable IL-2 (<6 U/ml $\times$ 10^6 restimulated cells), whereas the SN of cells expanded in IL-2 alone contained about 470 U/ml $\times$ 10^6 restimulated cells IL-2 and 0.8×10^3 U/ml $\times$ 10^6 restimulated cells IL-4 (Fig. 2). The addition of Con A during the expansion culture thus led to a 30-fold increase of IL-4 and a ≥80-fold reduction of IL-2, when based on the number of restimulated cells.

Neither the size nor the geometry of the culture vessels (25-ml flasks, 24-well plates, 96-well plates) nor the cell density either at the beginning of the expansion culture (5×10^4 cells/ml to 1×10^6 cells/ml) or for the restimu-

TABLE I

Induction of IL-4-producing CD8-depleted T cells is dependent on Con A and inhibited by α-mm[a]

Expt.	Culture Conditions	[^{3}H]Thymidine Incorporation (cpm $\times$ 10^{-3}) in Presence of[b]			
		No antibody	Anti-IL-2	Anti-IL-4	Both antibodies
1	IL-2	32.9 ± 1.0	1.7 ± 0.2	30.1 ± 0.6	1.5 ± 0.2
	IL-2 + Con A SN 20%	21.1 ± 2.7	20.1 ± 0.7	3.2 ± 0.1	1.1 ± 0.0
	IL-2 + Con A SN 20% + α-mm 20 mg/ml	31.2 ± 1.6	3.1 ± 0.1	31.0 ± 1.8	1.1 ± 0.3
2	IL-2	74.3 ± 3.7	6.5 ± 0.3	73.1 ± 5.0	3.6 ± 0.4
	IL-2 + Con A 0.25 μg/ml	77.5 ± 2.7	4.5 ± 0.1	72.4 ± 6.8	2.9 ± 0.2
	IL-2 + Con A 0.50 μg/ml	75.7 ± 2.6	7.5 ± 0.1	70.4 ± 3.5	2.9 ± 0.2
	IL-2 + Con A 1.00 μg/ml	46.7 ± 0.5	45.8 ± 0.8	2.9 ± 0.3	3.2 ± 0.3
	IL-2 + Con A 2.00 μg/ml	50.0 ± 3.0	47.4 ± 0.6	3.3 ± 0.1	3.1 ± 0.2
	IL-2 + Con A 2.00 μg/ml + α-mm 20 mg/ml	76.1 ± 3.1	8.3 ± 0.3	76.6 ± 4.6	3.2 ± 0.3

[a] CD8-depleted T cells activated with SC plus Con A (Expt. 1) or PMA plus Con A (Expt. 2) were expanded for 12 days under the culture conditions indicated and thereafter restimulated with PMA plus Con A.

[b] IL-2/IL-4 were assessed in the 48-h culture SN (dilution 1/40) as described in *Materials and Methods*. Data shown are mean ± SD of triplicate cultures.

TABLE II

Presence of Con A for several days is required for the induction of T cells that release IL-4 but no measurable IL-2[a]

Con A Present in Culture	[^{3}H]Thymidine Incorporation (cpm × 10^{-3}) in Presence of[b]			
	No antibody	Anti-IL-2	Anti-IL-4	Both antibodies
None[c]	22.4 ± 1.1	3.7 ± 0.2	21.5 ± 1.1	1.3 ± 0.1
Day 0–12[d]	9.0 ± 0.3	7.7 ± 1.0	1.4 ± 0.2	1.4 ± 0.1
2–12[d]	8.1 ± 0.1	7.7 ± 1.2	1.3 ± 0.0	1.4 ± 0.2
4–12[d]	8.5 ± 0.8	7.9 ± 0.3	1.4 ± 0.0	1.2 ± 0.1
6–12[d]	8.1 ± 0.6	9.0 ± 0.5	1.4 ± 0.1	1.5 ± 0.2
8–12[d]	6.5 ± 0.4	6.2 ± 1.1	1.3 ± 0.2	1.3 ± 0.0
10–12[d]	14.1 ± 0.9	9.0 ± 0.5	15.7 ± 0.8	1.4 ± 0.1
Day 0–10[e]	8.7 ± 0.1	8.9 ± 1.4	1.4 ± 0.1	1.3 ± 0.1
0–8[e]	9.7 ± 0.4	9.7 ± 0.3	1.4 ± 0.4	1.1 ± 0.0
0–6[e]	9.6 ± 0.4	9.7 ± 0.7	1.2 ± 0.1	1.2 ± 0.1
0–4[e]	12.9 ± 1.2	9.3 ± 0.8	10.7 ± 0.1	1.2 ± 0.3
0–2[e]	17.9 ± 0.9	8.9 ± 0.3	19.1 ± 1.1	1.1 ± 0.3

[a] CD8-depleted T cells activated with SC plus Con A were expanded for 12 days under the culture conditions indicated, and thereafter restimulated with PMA plus Con A.

[b] IL-2/IL-4 were assessed in the 48-h culture SN (dilution 1/40) as described in *Materials and Methods*. Data shown are mean ± SD of triplicate cultures.

[c] Cells were expanded in the presence of IL-2 (50 U/ml).

[d] Con A was first added at various days of the expansion culture with IL-2 (50 U/ml) and thereafter until day 12 (as described in *Materials and Methods*).

[e] α-mm was added at various days to cells expanding with IL-2 plus Con A from day 0 on.

TABLE III

IL-2 plus Con A also induce IL-4 in CD8-depleted T cells from CBA and C3H mice[a]

Mouse Strain	Culture Conditions	[^{3}H]Thymidine Incorporation (cpm × 10^{-3}) in Presence of[b]			
		No antibody	Anti-IL-2	Anti-IL-4	Both antibodies
C3H	IL-2	26.6 ± 0.5	4.4 ± 0.5	26.1 ± 2.3	2.2 ± 0.2
	IL-2 + Con A	11.6 ± 1.0	10.5 ± 0.4	1.7 ± 0.1	1.8 ± 0.2
CBA	IL-2	26.9 ± 1.3	4.4 ± 0.0	24.9 ± 0.3	2.2 ± 0.3
	IL-2 + Con A	13.5 ± 0.4	13.0 ± 0.4	1.9 ± 0.1	2.0 ± 0.3

[a] CD8-depleted T cells from CBA and C3H mice were activated with SC plus Con A, expanded for 12 days with IL-2 (50 U/ml) or IL-2 plus Con A (2 μg/ml) and thereafter restimulated with PMA plus Con A.

[b] IL-2/IL-4 were assessed in the 48-h culture SN (dilution 1/40) as described in *Materials and Methods*. Data shown are mean ± SD of triplicate cultures.

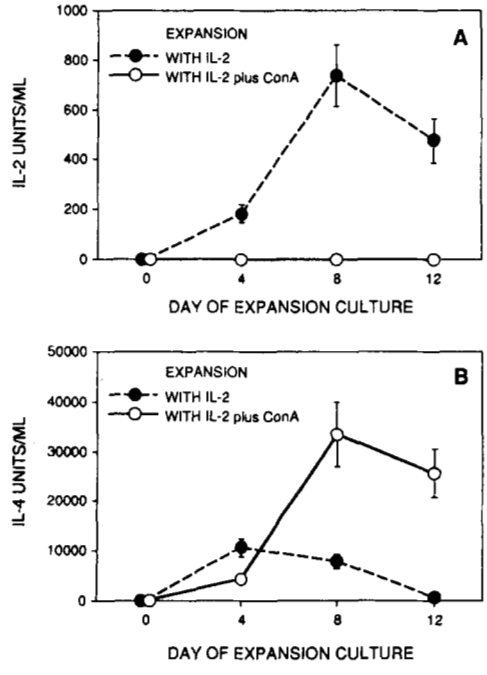

Figure 2. CD8-depleted T cells expanding with IL-2 plus Con A release no measurable IL-2 but increasing amounts of IL-4. CD8-depleted T cells were activated with SC plus Con A and expanded either with IL-2 or with IL-2 plus 2 μg/ml Con A. The cells were restimulated with PMA plus Con A at a density of 0.25 × 10^6 cells/ml on the indicated days of the expansion culture. The IL-2 activities (*A*) and IL-4 activities (*B*) in the 48-h culture SN were assessed as described in *Materials and Methods* and normalized to lymphokine release per 10^6 cells.

lation (0.2 × 10^6 cells/ml to 2 × 10^6 cells/ml) had any influence on the IL-2/IL-4 pattern measured after 12 days of expansion culture (data not shown).

The cell yields differed depending on the culture conditions. The number of cells expanded for 12 days with IL-2 alone increased 5 to 10 times in tissue culture flasks and 20 to 40 times in 96-well plates. The number of cells expanded with IL-2 plus lectin or mAb 145-2C11 increased 1.5 to 4 times in tissue culture flasks and 10 to 20 times in 96-well plates. Thus, the presence of Con A during the expansion culture resulted in about two to four times lower cell yields when compared with cells expanded with IL-2 alone.

In 96-well plates, 5 × 10^3 activated cells were seeded and expanded for 12 days with IL-2 plus lectin (Con A, PHA), with IL-2 plus mAb 145-2C11, or with IL-2 alone. The cultures were then washed and restimulated for lymphokine production. The IL-2/IL-4 pattern in these SN therefore represents cytokines produced after expansion and restimulation with respect to the same starting cell number (5 × 10^3/well). IL-2 activities in SN (1/40) from cells expanded with IL-2 alone and IL-4 activities in SN (1/40) from cells expanded with IL-2 plus lectins (Con A, PHA) induced consistently maximal CTLL responses (Tables I, III, and IV). SN from cells expanded with IL-2 plus lectins contained no measurable IL-2. SN from cells expanded with IL-2 alone contained no or little measurable IL-4. Because the span of maximal to minimal response of our CTLL cells to SN-derived IL-2 and IL-4 covered at least 1.5 orders of magnitude, the differences of IL-2 and IL-4 in SN from cells expanded in IL-2 plus

TABLE IV

IL-4-producing T cells are induced in a APC-independent system when expanded with IL-2 plus Con A, PHA, or an antibody to CD3ε(145-2C11)[a]

Expt.	Culture Conditions	[^{3}H]Thymidine Incorporation (cpm × 10^{-3}) in Presence of[b]			
		No antibody	Anti-IL-2	Anti-IL-4	Both antibodies
1	IL-2	74.3 ± 3.7	6.5 ± 0.3	73.1 ± 0.5	3.6 ± 0.4
	IL-2 + Con A	50.0 ± 3.0	47.4 ± 0.6	3.3 ± 0.1	3.1 ± 0.2
	IL-2 + PHA	47.1 ± 0.5	47.3 ± 1.9	3.3 ± 0.1	3.3 ± 0.2
	IL-2 + 145-2C11	47.3 ± 2.9	41.9 ± 2.6	4.1 ± 0.1	3.4 ± 0.1
2	IL-2	42.3 ± 0.9	3.8 ± 0.2	38.2 ± 3.1	3.3 ± 0.5
	IL-2 + Con A	31.2 ± 5.3	28.0 ± 2.5	4.2 ± 0.1	3.7 ± 0.1

[a] CD8-depleted T cells were activated either with PMA plus Con A (Expt. 1) or with PMA plus ionomycine (Expt. 2). After expansion for 12 days with IL-2, IL-2 plus Con A (2 μg/ml), IL-2 plus PHA (10 μg/ml) or IL-2 plus 145-2C11 culture SN (1%), the cells were restimulated with the agents used for activation.

[b] IL-2/IL-4 were assessed in the 48-h culture SN (dilution 1/40) as described in *Materials and Methods*. Data shown are mean ± SD of triplicate cultures.

Con A and in IL-2 alone (shown in Tables I, III, and IV) represent differences of a factor of at least 10. Thus, when the assessment is based on the starting cell number, expansion with IL-2 plus lectins induced an up-regulation of IL-4 and a down-regulation of IL-2 by a factor of ≥10. This was confirmed by titration of some selected SN (data not shown).

Time course of IL-2 and IL-4 production by cells expanded in IL-2 plus Con A or in IL-2 alone. To investigate whether the ratio of IL-2 and IL-4 released into the SN varied over the time, we analyzed SN harvested at different time points after restimulation with PMA plus Con A. During 72 h, the pattern of IL-2 and IL-4 released by the cells remained unchanged (Fig. 3). Hence, the IL-2/IL-4 pattern of both CD4+ T cell populations was not simply due to a difference in the time courses of IL-2 and IL-4 production.

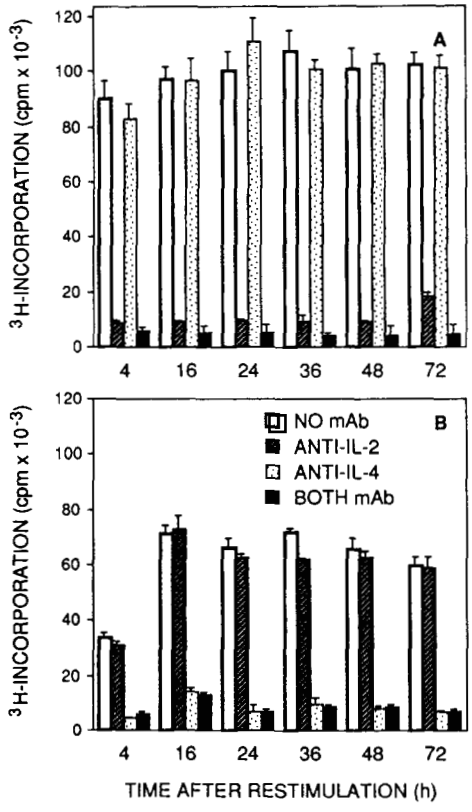

Figure 3. Pattern of IL-2 and IL-4 released by CD8-depleted T cells expanded with either IL-2 or with IL-2 plus Con A (2 μg/ml) is constant for at least 3 days. The cells were activated with SC plus Con A, expanded with either IL-2 (*A*) or IL-2 plus Con A (*B*) and restimulated at 0.25 × 10^6 cells/ml with PMA plus Con A. The SN were harvested at the indicated time after the restimulation. IL-2/IL-4 in the culture SN were tested as described in *Materials and Methods* (SN dilution 1/40).

To study the IL-2/IL-4 pattern in function of the expansion time, aliquots of cells expanded either with IL-2 alone or with IL-2 plus Con A were washed and restimulated for lymphokine production after various periods of expansion culture (Fig. 2). The SN of cells expanded for 4 days with IL-2 contained both IL-2 and IL-4. The SN of cells restimulated on day 8 or 12 of expansion culture contained two to three times more IL-2 (based on the number of restimulated cells), whereas the capacity of the cells to release IL-4 decreased by more than 10-fold when compared with cells restimulated on day 4 (Fig. 2).

Cells expanded with IL-2 plus Con A never released measurable quantities of IL-2 into the SN after restimulation. However, IL-4 in the SN of restimulated cells increased 8- to 10-fold between day 4 and day 8 of expansion culture (Fig. 2).

The effect of APC, PHA, and polyclonal T cell-activating agents. In the cultures described above, APC were used for the initial activation of T cells. To determine whether their presence in large numbers was critical for the induction of IL-2 or IL-4 producing T cells, the cells were activated and restimulated with either PMA plus Con A or with PMA plus ionomycin. Under both conditions, T cells produced IL-4 but no detectable IL-2 when expanded with IL-2 plus Con A, whereas they produced mainly IL-2 when expanded in the absence of Con A (Table IV). As the freshly prepared cells were >97% CD4+ and did not proliferate in response to Con A, these results indicated that the modulation of the IL-2/IL-4 pattern was independent of the presence of large numbers of APC during the activation, expansion and restimulation of the cells.

Because the content of IL-2 or IL-4 measured in the SN may depend on the type of agents used to stimulate the T cells (30), we used several different agents to restimulate T cells expanded with IL-2 or IL-2 plus Con A. In the SN of CD4+ T cells activated with SC plus Con A and expanded with IL-2 plus Con A (2 μg/ml) an increase of IL-4 accompanied by a decrease of IL-2 was detected whether the cells were restimulated with SC-T plus Con A, SC-T plus PHA, PMA plus Con A, or PMA plus ionomycin. T cells from the same origin that had been expanded in medium without Con A released mainly IL-2 when restimulated under the same conditions (data not shown). The results further demonstrate that the effect of the lectins was largely independent of the conditions used to restimulate T cells.

As the effect of Con A could also be reproduced with PHA and with a mitogenic mAb to the CD3 ε-chain (145-2C11; Table IV), our results suggest that the modulation of the IL-2/IL-4 pattern was due to T cell receptor complex mediated triggering of the expanding T cells.

IL-2 and IL-4 release assessed by a coculture assay. The pattern of IL-2 and IL-4 secreted by restimulated T cells into their close environment may differ from the pattern found in the culture SN. Therefore, we incubated cells at graded cell numbers with SC-T plus Con A in 96-well plates, irradiated them immediately and added the CTLL indicator cells (Fig. 4). The growth-promoting effect of 10^4 T cells expanded in IL-2 was blocked by a mAb to IL-2, whereas it was blocked by a mAb to IL-4 in the case of 10^4 $CD4^+$ T cells expanded with IL-2 plus Con A (Fig. 4, *A* and *B*). When IL-2 and IL-4 production of either cell population was estimated by means of the relative [^{3}H] thymidine incorporation, 10^4 cells expanded with IL-2 alone released about as much IL-4 as 10^2 cells expanded with IL-2 plus Con A, indicating that IL-4 was about 100-fold up-regulated by Con A (Fig. 4, *A* and *B*). Inversely, 10^4 cells expanded with IL-2 plus Con A released as much IL-2 as 10^2 cells expanded with IL-2 alone (Fig. 4, *A* and *B*). The IL-2 data were confirmed with CTLL cells that respond only to IL-2 (Fig. 4, *C* and *D*). Together, these results suggest that IL-2 was about 100-fold down-regulated by Con A. As addition of the CTLL 24 h after restimulation revealed similar results (data not shown), it is unlikely that large amounts of IL-2 or IL-4 were released but remained undetected. Therefore, the results obtained with the coculture assay confirmed our findings obtained with the culture SN.

DISCUSSION

In this paper we present a system that allows the controlled induction of IL-2- or IL-4-producing T cells in vitro within 12 days of expansion culture in freshly prepared, CD8-depleted and activated T cells from nonsensitized mice. IL-2 was required for the generation of mainly IL-2-producing T cells, whereas IL-2 plus Con A or PHA was required for the induction of mainly IL-4-producing T cells. IL-4 up-regulation and IL-2 down-regulation was observed whether the results were based on the number of seeded cells at the beginning of expansion culture (Tables I, III, and IV) or on the number of cells restimulated at the end of expansion culture (Figs. 2–4). Hence, IL-4 was induced and IL-2 down-regulated independently of the different cell yields resulting from expansion with IL-2 plus lectin or with IL-2 alone.

Although activated T cells expanded in rIL-2 alone produced only little IL-4 and large amounts of IL-2 after 12 days of expansion (Figs. 2 and 4), a different IL-2/IL-4 pattern was observed when the expansion culture was shortened. At day 4 of expansion culture (6 days after primary in vitro activation), T cells released mainly IL-4 and little IL-2 when restimulated (Fig. 2). Similar results were published by Swain et al. (21). When restimulated at day 8, cells expanded in medium plus rIL-2 alone produced both IL-2 and IL-4. However, from day 12 on (Fig. 2) and for at least 8 weeks (data not shown), cells expanded in rIL-2 alone produced mainly IL-2 upon restimulation. It thus appears that the ability of $CD4^+$ T cells to produce IL-2 after primary in vitro activation and expansion culture in IL-2 alone was markedly diminished during the first days (day 0 to 4 of expansion), but was recovered after prolonged culture (≥8 days) in IL-2 alone. The capacity of the cells to produce IL-4 behaved reversely: for a short period after primary activation, the cells were capable of producing IL-4 upon restimulation. However, after 12 days of expansion culture with IL-2 alone, the cells released very little IL-4 (Fig. 2).

In view of these results, we speculated that a cytokine produced during the APC-T cell interaction could account for the up-regulation of IL-4 and the loss of the capacity to produce IL-2. To provide putative soluble factors, we added the SN of Con A-stimulated SC to the IL-2 containing medium during the expansion culture. T cells expanded in the presence of rIL-2 plus Con A SN produced

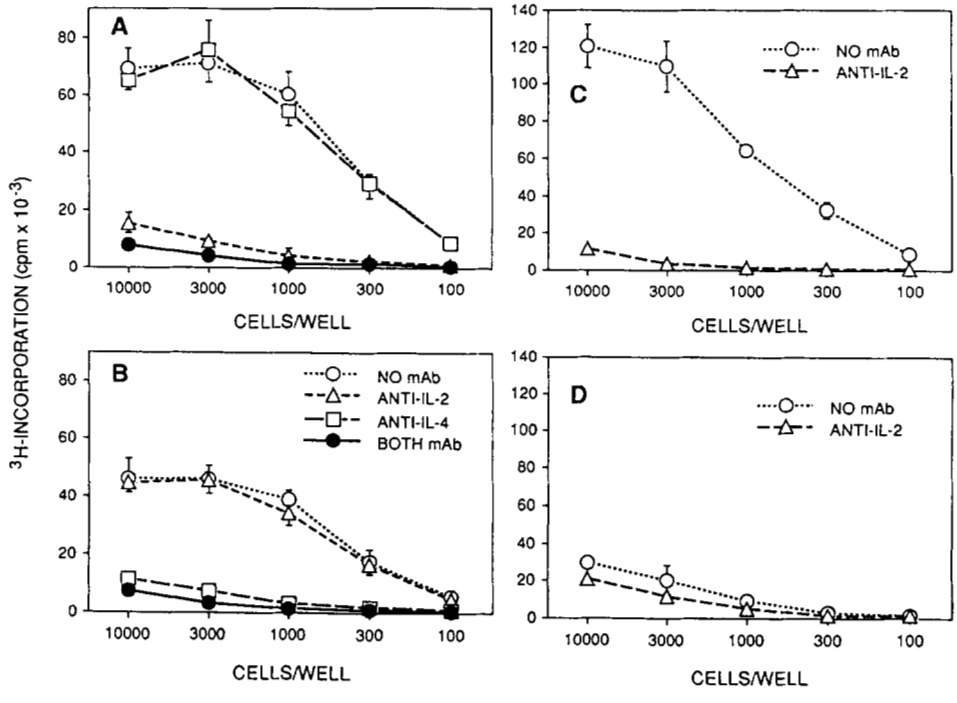

Figure 4. Coculture assay confirms that Con A induces IL-4 but not IL-2 producing T cells. CD8-depleted T cells previously activated with SC plus Con A and expanded either with IL-2 or with IL-2 plus Con A (2 μg/ml) were added to SC-T plus Con A in 96-well plates. After irradiation of the plates, mAb and 5×10^3 CTLL were added immediately. Proliferation was assessed by [^{3}H]thymidine incorporation after 28 h. *A* and *B* show data with CTLL cells that respond to IL-2 and IL-4; *C* and *D* show data with CTLL cells that respond to IL-2 but not to IL-4.

IL-4 and no measurable IL-2 in response to restimulation (Table I). As Con A SN is a rich source of biologically active factors, cytokines could have been responsible for the generation of IL-4-producing T cells. However, in our system, the IL-4 up-regulation and the IL-2 down-regulation observed in the presence of rIL-2 plus Con A SN could be reproduced with rIL-2 plus Con A alone (Table I), but not when we replaced Con A SN with single defined cytokines (IL-1β, IL-3, IL-4, IL-5, IL-6, macrophage CSF, granulocyte-macrophage CSF, TNF-α, IFN-γ; data not shown). In addition, the effect of the Con A SN and of Con A was completely blocked by α-mm and could be reproduced with PHA (Tables I and IV).

FMF analysis showed that Con A was bound to the cells 3 days and more after the addition of IL-2 plus Con A (2 μg/ml). Because we changed the culture medium including rIL-2 and Con A every 2 to 3 days, it is very likely that Con A was continuously bound to the majority of the expanding T cells. The effect of Con A on the IL-2/IL-4 pattern in response to restimulation was strongly diminished when Con A was added only after day 8 of the expansion culture or when α-mm was added at day 4 or earlier to cells expanding with rIL-2 plus Con A. When Con A was continuously present during the entire expansion culture, IL-4 was 30- to 100-fold up-regulated and IL-2 down-regulated about 100-fold as assessed in the SN of cells restimulated at equal numbers and by a coculture assay (Figs. 2 and 4). Thus, signals required for the induction of IL-4- but not IL-2-producing T cells were provided by the continuous presence of rIL-2 plus Con A or PHA during the expansion culture preceding restimulation. This suggests that prolonged binding of lectins to surface molecules mediates a critical signal for the induction of IL-4 and the loss of IL-2 release. However, these experiments did not show whether the binding of Con A triggered the induction of IL-4 directly or through an autocrine or paracrine mechanism. We are presently addressing this issue in our laboratory.

Lectins bind to a number of glycoproteins expressed on the plasma membrane of T cells. The activation of T cells with Con A and other lectins depends on the expression of the TCR complex (31), which hence is possibly involved in the modulation of IL-2 and IL-4 production. Binding of Con A to the TCR complex during the expansion culture might trigger the observed enhancement of IL-4 and the loss of IL-2 release in response to restimulation. This is supported by the observation that the effect of Con A could be reproduced with a mAb that activates T cells via binding to the CD3 ϵ-chain. We have studied the expansion of T cells in the presence of various mAb that activate T cells and were able to identify distinct surface molecules involved in the IL-2/IL-4 regulation, including the variable chains of the TCR (M. Röcken, K. M. Müller, J.-H. Saurat, and C. Hauser, manuscript in preparation).

Although the number of contaminating MHC class II$^+$ cells was clearly less than 1%, as assessed by FMF, it cannot be excluded that remaining MHC class II$^+$ or MHC class II$^-$ APC were involved in the modulation of IL-2 and IL-4 production by lectins. Attempts to study the influence of remaining non-T cells with limiting numbers of CD4$^+$ T cells failed because of the exquisitely low cloning efficiency of CD4$^+$ T cells cultured in the absence of feeder cells. It is therefore possible that a small number of remaining APC may have provided additional signals necessary for the induction of IL-4 by lectins. Alternatively, the binding of Con A could replace at least a part of natural signals that may be provided by APC.

Our data also raise the question of how the binding of Con A to activated T cells influenced the regulation of IL-2 and IL-4. Although Con A alone is not capable of activating T cells for lymphokine production and proliferation, it can elicit T cell responses such as an increase of cytosolic free Ca^{2+}, induction of phosphatidyl inositol hydrolysis and activation of protein kinase C in T cell lines (31). Thus, the binding of Con A alone could cause transduction of signal(s) responsible for the induction of IL-4 and the down-regulation of IL-2 release. Experiments with Ag-specific T cell lines revealed first evidence that the transduction of "incomplete" signals, as it might have occurred with Con A alone, can profoundly influence the subsequent T cell responsiveness, including the pattern of lymphokine release. Investigating the induction of Ag-specific tolerance, Jenkins and co-workers showed that exposure of IL-2-producing (type 1) Th cell clones to chemically fixed APC plus peptide Ag completely inhibited the IL-2 but not the IL-3 release in response to APC plus antigen. This response pattern persisted for several days when the clones were restimulated with Ag plus intact APC (32, 33). Quill and Schwartz (34) reported similar results with purified MHC class II molecules in planar lipid membranes, antigenic peptide fragments, and IL-2 producing (type 1) Th cell clones. In our hands, expansion of IL-2-producing CD4$^+$ T cell clones (kindly provided by Dr. J. Louis, Institute of Biochemistry, University of Lausanne, Epalinges, Switzerland) in IL-2 plus Con A resulted in a profound down-regulation of IL-2 release in response to restimulation with APC plus Ag or PMA plus Con A. This was also observed when EL 4 cells (from an IL-2- but not IL-4-producing subclone; kindly provided by Dr. R. Zubler, University Hospital, Geneva, Switzerland) were expanded in the presence of Con A (our unpublished data). However, IL-4 was not induced in these cell lines. These results are compatible with the view that the signal(s) mediated by Con A may be capable of suppressing IL-2 production in T cell lines even in the absence of APC.

The failure to induce IL-4 in these long term cultured T cell clones might be related to the stability of their lymphokine pattern (8). However, freshly isolated and once activated CD4$^+$ T cells (as used here) may differ from long term cultured T cells with regard to the inducibility of IL-4 production. Thus, a common precursor T cell that changes its lymphokine response pattern according to previous cell surface molecule engagement could account for our results. Alternatively, T cell surface molecule engagement during the expansion culture with IL-2 may select for progenies of mutually exclusive precursor cells. We are currently addressing this topic.

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