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Age-Associated Decline in IL-4 Production by Murine T Lymphocytes in Extended Culture

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Aging leads to an increase in the proportion of cells that have the surface phenotype ($CD45RB^{lo}$, $CD44^{hi}$) characteristic of memory T lymphocytes and also to a decline in both the production of IL-2 and the response to this lymphokine. Several groups have reported an increase, with age, in the secretion of IL-4 in short-term T cell cultures and have suggested that this increase could reflect the age-dependent accumulation of memory T cells, which are thought to be principally responsible for IL-4 production in young mice. Because the response to IL-2 declines with age, we hypothesized that IL-4 production would also decline with age if tested under conditions that promoted IL-2-driven expansion and maturation of IL-4-secreting effectors. Using a culture system in which T cells are first activated by immobilized anti-CD3 antibody for 2 days, and then cultured with anti-CD3 plus IL-2 for an additional 9–11 days, we found a 3-fold decline with age in IL-4 production by murine splenic CD4 T cells. Under these conditions memory ($CD45RB^{lo}$) CD4 T cells from young mice produced 22-fold more IL-4 than the reciprocal naive ($CD44^{lo}$) subset. Production of IL-4 by old T cells was also largely attributable to memory T cells, but memory cells from these old donors generated 6-fold less IL-4 in extended cultures than memory cells from young donors. Cultured memory (but not naive) T cells increase in number over a 9-day interval, but the amount of expansion by young memory cells is 4-fold higher than that for old cells. We conclude that the production of IL-4 by memory T cells declines with age under conditions that promote IL-2-driven proliferation and differentiation. © 1993 Academic Press, Inc.

INTRODUCTION

IL-4, produced largely by class II-restricted $CD4^+$ helper T lymphocytes, plays an important role in modulating the quality of the immune response through effects on T and B lymphocytes (1–5), and indeed interstrain differences in IL-4 production can determine the outcome of infection by potentially lethal pathogens (6). Many reports have suggested that not all helper T cells are equally able to secrete IL-4 and in particular that memory T cells are more efficient producers of IL-4 than naive T cells (2, 7, 8), although certain culture conditions seem able to generate IL-4-secreting effectors from naive precursors (9–11).

The immune deficiency of aging involves deficits both in the production of IL-2 and in the response to this lymphokine (12–14), but information about the effects of age on production of other lymphokines has only recently begun to accumulate. Three groups (15–17) have now reported an increase with age in the production of IL-4 by murine helper T cells in short-term *in vitro* cultures, although two disparate reports have also appeared (18, 19). Since aging in mice (15, 16, 20) and humans (21, 22) is

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accompanied by an increase in the proportion of memory T cells at the expense of naive T cells, it has seemed plausible that an age-dependent increase in IL-4 production might reflect memory cell accumulation, although no direct tests of the ability of memory T cells from old mice to produce IL-4 have been reported. Indeed, several lines of argument suggest that memory T cells from old mice may not be as easily activated as memory T cells in young mice. These comparisons have included limit dilution analyses of helper, cytotoxic, and proliferative capacity (20); studies of calcium signal generation (23); and analyses of protein kinase activation pathways (24). Further, the requirement for an IL-2 signal to promote the differentiation, maturation, or proliferation of IL-4-secreting effectors (11, 25), together with the age-associated loss in responsiveness to IL-2 (26–30), would predict an age-dependent decline in IL-4 secretion at least under conditions that promote the IL-2-dependent generation of IL-4 secretors.

Röcken and his colleagues have recently developed and characterized an IL-2-dependent culture system that supports the *in vitro* maturation of IL-4-producing murine T cells (9, 31). In this system resting T cells are first activated for 2 days by concanavalin A (Con A) or anti-CD3 antibody, then expanded for an additional period in the presence of exogenous IL-2 with or without continued stimulation by mitogen or anti-CD3, and finally tested by restimulation (e.g., with phorbol myristic acetate (PMA) and ionomycin) for production of IL-2 or IL-4. These investigators have shown that continued stimulation during this expansion phase, e.g., through the T cell receptor or CD3 complex, is required for generation of IL-4-producing effector cells.

We have used a modification of this extended culture approach to reexamine the question of IL-4 secretion by T cells from aged mice. We report here that when T cells are briefly activated and then further cultured in the presence of IL-2, accessory cells, and immobilized anti-CD3 antibody, IL-4 accumulation is dependent on the presence of memory T cells with the $CD44^{hi}$, $CD45RB^{lo}$ phenotype. Unfractionated CD4 T cells and CD4 memory T cells from old mice are less able to produce IL-4 under these conditions than are T cells from younger animals. Production of IL-4 under conditions where IL-2 is present at growth-promoting levels may decline with age because of an inability of T cells from aged donors to respond appropriately to IL-2 signals.

MATERIALS AND METHODS

Mice. B6D2F1 male mice were purchased either from the Jackson Laboratories (Bar Harbor, ME) or from the aging colony maintained at the Charles River Breeding Laboratories (Wilmington, MA). Mice were housed after receipt for at least 2 weeks in a specific-pathogen-free colony at the University of Michigan's Core Facility for Aged Rodents. "Young" mice were 3–4 months old, and "old" mice were 18–22 months old.

Antibodies and reagents. Ascites fluid was generated from hybridoma cells secreting hamster antibody to the mouse CD3 ϵ chain (clone 145-2C11, the gift of Jeffrey Bluestone), rat IgG2b antibody to mouse CD44 (clone IM7.8.1, the gift of Robert Hyman), rat IgG2a antibody to mouse CD8 (clone 53-6.72), rat IgG1 anti-mouse IL-4 (clone 11B11), and mouse IgM anti-mouse Thy-1.2 (clone HO-13-4); the latter three clones were obtained from the American Type Culture Collection (ATCC; Rockville, MD). CTLL cells were also from ATCC. Rat IgG2a anti-mouse IL-2 and rat IgG2a anti-

mouse CD45RB (16A) were purchased from Genzyme (Cambridge, MA) and GIBCO (Grand Island, NY), respectively. Fluorescein isothiocyanate-conjugated anti-mouse CD3 and anti-mouse CD45RB were purchased from GIBCO, and phycoerythrin-conjugated anti-mouse CD4 and anti-mouse CD8 from Becton–Dickinson (Sunnyvale, CA). Anti-rat IgG- and streptavidin-coated magnetic beads were purchased from Advanced Magnetics (Cambridge, MA). Biotinylated anti-CD44 antibodies were produced in our laboratory from purified Ig. Recombinant mouse IL-2 and IL-4 were purchased from Genzyme; Con A, RPMI 1640, and Sephadex G-10 from Sigma (St. Louis, MO); [^{3}H]thymidine, rabbit anti-mouse Ig, and anti-rat IgG from ICN (Irving, CA); rabbit complement from Pel-Freez (Brown Deer, WI); fetal bovine serum (FBS) from Hyclone (Logan, UT); 24-well and 96-well flat-bottomed culture plates from Costar (Cambridge, MA).

Culture medium. RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum plus antibiotics, L-glutamine, and 5×10^{-5} *M* 2-mercaptethanol was used for all cell cultures.

Cell preparations. Spleens were removed aseptically, and a single-cell suspension was prepared by gently rubbing and pressing the tissue between two frosted glass slides. Cell clumps and tissue debris were removed by passing through nylon mesh. The splenocytes were depleted of sIg$^+$ cells by panning, and then depleted of macrophages and other "adherent" accessory cells by passing through a Sephadex G-10 column. Collected cells were divided into three parts and incubated for 30 min on ice either (a) with 53-6.72 ascites (anti-CD8, 1:200) alone, or (b) together with biotinyl anti-CD44 (1:50), or (c) together with 16A (anti-CD45RB, 1:1,000). The cells were then further incubated for 20 min either with anti-rat IgG-coated magnetic beads alone or together with streptavidin-coated magnetic beads on ice, after being washed. Bead-adherent cells were removed using a magnetic separator. Cells thus obtained were refered to as (a) CD4 T cells, (b) CD4 CD44^{lo} T cells, or (c) CD4 CD45RB^{lo} T cells. CD4 T cells prepared in this way were found to be dependent on added accessory cells for proliferation (95% decline in Con A-stimulated thymidine incorporation in two experiments). Unfractionated CD4 cells from young mice were 74 ± 1% (mean ± standard error, $N = 4$) positive for CD4; 71% of the CD4 cells were CD45RB^{hi}, and 30% were CD44^{hi} ($N = 8$). Preparations of CD44^{lo} CD4 cells were found to be 83% CD45RB^{hi} and 14% CD44^{hi}, while preparations of CD45RB^{lo} cells were found to be 21% CD45RB^{hi} and 80% CD44^{hi} (means of eight determinations). Similar degrees of purity were obtained for fractionations of old mice, except that as expected older donors had higher initial levels of memory cells (old CD4 cells were 49% CD45RB^{hi} and 55% CD44^{hi}).

T cell-depleted residential peritoneal cells (RPC) were prepared by treating mouse peritoneal cells with anti-Thy 1.2 and complement as previously described (27).

IL-4 production in expansion cultures of preactivated T cells. Ninety-six-well flat-bottomed microculture plates were first incubated with anti-rat IgG (4 μg/ml) at 4°C overnight and then washed and incubated with protein G-purified anti-CD3 (0.3 μg/ml) for 2 hr at room temperature. The plates were then washed thoroughly. RPC from young mice were then added (10^4 cells/well), followed by 10^4 responder cells (CD4 or subsets as indicated in the text). These cultures were then incubated at 37°C in a CO_2 incubator for 48 hr; cells so treated are referred to as "preactivated" cells. Next, the culture medium was carefully removed and replaced with fresh culture medium containing rIL-2 at 5 units/ml, and the cultures were returned for further incubation

("Day 0"). At intervals of 2–3 days, culture fluids were removed and replaced by fresh medium containing rIL-2; harvested culture supernatant was pooled from groups of 3–5 wells and stored for up to 2 weeks before IL-4 determination.

IL-4 assay. Each sample of conditioned medium was diluted 1:10 and divided into two aliquots. One aliquot was mixed with anti-IL-2 antibody (1:2000) and the other with both anti-IL-2 and anti-IL-4 (1:1000). Each sample was then tested for ability to support proliferation of CTLL cells (5000 cells/microwell, 18 hr incubation followed by addition of [^{3}H]thymidine for 6 hr). Net proliferation for each sample attributable to IL-4 was calculated as the difference between the two aliquots and converted to nanograms/milliliter IL-4 using a standard curve run simultaneously. Pilot experiments using rIL-2 and rIL-4 established that determination of IL-4 using this combination of antibodies was insensitive to IL-2 concentrations up to 4 units/ml, for IL-4 concentrations from 0.25 to 4 ng/ml.

RESULTS

Decreased production of IL-4 by T cells from aged mice in extended culture. Röcken and his colleagues have recently shown that extended contact with anti-CD3 antibody, in the presence of IL-2, can induce T cells to differentiate into IL-4-producing cells (9, 31). Three groups (15–17) have reported an increase with age in IL-4 production in short-term *in vitro* cultures and have interpreted their data as consistent with the age-dependent increase in the proportion of memory T cells, but none of these reports has included a direct test of the effect of aging on the ability of memory and naive T cells to generate IL-4. We therefore used a modification of the Röcken method to examine the effect of donor age on IL-4 production by unseparated, naive (CD44^{lo}), and memory (CD45RB^{lo}) CD4 T cell subsets. Cells were activated with immobilized anti-CD3 antibody for 2 days, after which IL-2 was added. Culture fluids were replaced with fresh IL-2-containing medium at 2- to 3-day intervals, and the conditioned media assayed for IL-4. In a series of 16 such experiments, each of which compared CD4 cells from young to those from old donors, IL-4 production during the 9- to 11-day cultures was 16.9 ± 4.5 (mean ± SEM) for CD4 cells from young mice and 6.2 ± 2.2 for those from old donors. The young donor produced more IL-4 than the old donor in 15 of these 16 experiments. The effect of age was found to be statistically significant by Student's t test at $P < 0.002$.

IL-4 production in extended culture is generated principally by memory T cells. To see if our culture conditions, like those used in many other laboratories, elict IL-4 production preferentially from cells with the surface phenotype of memory T cells, we tested the ability of separated naive (i.e., CD44^{lo}) and memory (i.e., CD45RB^{lo}) T cells from young mice to generate IL-4 in extended cultures. The data from a series of 18 experiments are presented in Table 1. As expected, IL-4 production from cultured memory cells during 9 days of culture was found to be 22-fold higher ($P < 0.001$) than IL-4 production from naive T cells and 4-fold higher ($P < 0.005$) than that from unseparated CD4$^+$ T cells. The 5-fold difference between unseparated CD4$^+$ and naive T cells was also highly significant ($P < 0.01$).

Age-related decline in IL-4 production by memory CD4 cells. In a further series of seven experiments we compared naive, memory, and unseparated CD4$^+$ T cells from young and old mice for IL-4 production. The results are shown in Fig. 1 as cumulative IL-4 production over the first 3, 6, and 9 days of culture in the presence of IL-2-

TABLE 1

IL-4 Production by T Cell Subsets from Young Donors

T cell subset	IL-4 production, ng/ml
All CD4	9.36 ± 2.5
$CD44^{lo}$ CD4	1.72 ± 0.6
$CD45RB^{lo}$ CD4	39.43 ± 8.8

Note. T cells of the indicated subsets were activated by immobilized anti-CD3 antibody for 2 days, after which IL-2 was added for an additional 9- to 11-day culture period. Supernatants were removed and assayed for IL-4 every 2–3 days, and the cumulative IL-4 production was calculated. The values tabulated are means ($\pm$ SEM) for $N = 18$ experiments.

containing medium. Purified memory T cells from young mice were found to generate 6- to 14-fold more IL-4 under these conditions than memory T cells from old mice ($P < 0.005$ for each of the three culture intervals). Memory T cells from old mice generated more IL-4 than naive T cells or unfractioned CD4 cells from old donors, but the differences were not statistically significant. Differences between young and old memory CD4 cells were apparent and statistically significant by the third day of culture and increased progressively at the 6- and 9-day intervals.

Cell expansion induced by IL-2 and anti-CD3. The data above showed that IL-4 production by T cells from old mice was lower than production by young T cells in extended cultures stimulated by IL-2 and immobilized anti-CD3. To see if the effect of age might be attributable to differential expansion under these conditions, we counted

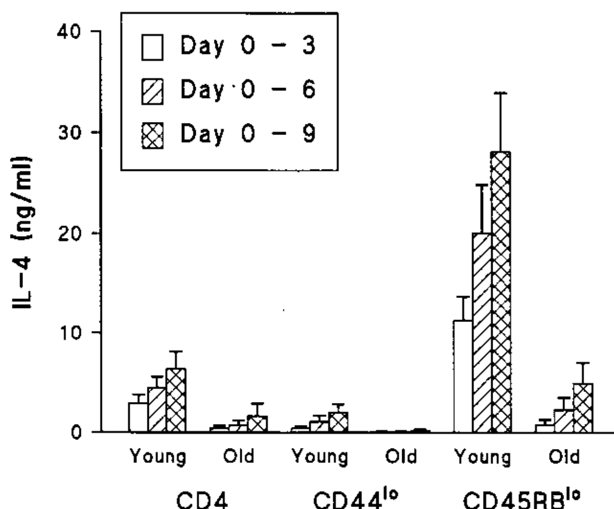

FIG. 1. IL-4 secretion by unfractionated, naive, and memory CD4 T cells from young and old mice in 9-day cultures. Each 0.2-ml well contained 10^4 T cells of the indicated subset from young or old donors, as well as 10^4 young RPC as accessory cells. Cells were preactivated by incubation with anti-CD3 antibody for 2 days and then further incubated with IL-2 plus anti-CD3 for the indicated expansion time. The level of IL-4 produced during each indicated culture interval is plotted as mean $\pm$ SEM for $N = 7$ independent experiments.

the number of viable cells present at the end of 9 days of culture in each of six experiments. The results are shown in Table 2. CD45RB^{lo} memory T cells from young mice expanded from 10^4 cells/well to 5.5×10^4 cells/well under these culture conditions. Expansion of memory cells from old mice was significantly lower (to 1.5×10^4 cells/well, $P < 0.001$). It is thus possible that the relatively poor IL-4 production seen in cultured memory T cells from old mice might reflect, at least in part, impaired proliferation or survival of IL-4-producing effector cells.

DISCUSSION

Our data show that preactivated CD4 memory T cells from young or old mice are able to produce IL-4 when cultured in the presence of immobilized anti-CD3 antibody together with IL-2 and accessory cells, but that both unfractionated CD4 cells and enriched memory T cells from young mice make substantially more IL-4 than do cells from older donors.

There is a good deal of evidence to suggest that variations in CD44 and CD45RB expression distinguish memory from naive T cells within the CD4 pool (7, 32, 33), though not among CD8 cells (34). The claim that alterations in CD45 isoform expression may in some cases be reversible (35) has been disputed (36), but in any case does not alter the strength of the evidence that most CD4 memory cells express high levels of CD44 and low levels of CD45RB. Even if it should eventually be established that CD44 and CD45RB expression do not distinguish cells strictly on the basis of their past developmental history, our data still show substantial differences in IL-4 production between CD44^{lo} and CD45RB^{lo} T cells and show that the accumulation of CD44^{hi}, CD45RB^{lo} T cells in aging mice does not automatically convey an improved IL-4 response in all circumstances.

Our data on aged mice stand in contrast to reports from other groups (15–17) in which aging was said to increase IL-4 secretion in culture. A study of IL-4 production by human mononuclear cells, however, found an age-related decline in IL-4 production, in agreement with our own findings (18). Another report on mouse IL-4 production (19) used a flow cytometric method to determine the proportion of murine splenic cells that contained cytoplasmic IL-4 after 5 days of culture in PHA followed by an additional 16 hr of secondary stimulation by PHA. Although approximately 20% of the spleen cells of 4-month-old mice were found to be IL-4$^+$, fewer than 3% of stimulated spleen cells from 30-month-old mice could be stained by the anti-IL-4 reagent. Mice 12 and 20 months of age showed intermediate levels of IL-4 response. Only two to five animals were used at each age, however, and the age effect was not statistically

TABLE 2

Cell Recovery in 9-Day Cultures of T cells from Young and Old Donors

T cells	CD4	CD44^{lo}	CD45RB^{lo}
Young	1.6 ± 0.2	0.3 ± 0.1	5.5 ± 1.5
Old	0.8 ± 0.2	0.1 ± 0.02	1.5 ± 0.2

Note. Each culture initially contained 10^4 preactivated cells from the indicated T cell subset. Values shown are cell recoveries at the end of 9 days of culture ($\times 10^{-4}$), as means ± SEM for $N = 6$ independent experiments.

significant, nor were naive and memory cells discriminated in this set of experiments. Although preliminary, these data are consistent with our own findings of a loss with age in IL-4 production by unfractionated and memory CD4 T cells. Taken together, these published studies and our own current data suggest that it may be premature to draw any firm conclusions about the effect of age on IL-4 generation and that T cells from old mice may be good producers of IL-4 under some conditions and poor producers in other circumstances.

The groups that reported an increase in IL-4 production by T cells from old mice (15–17) have employed a variety of stimuli (Con A, soluble or immobilized anti-CD3, PMA plus calcium ionophore) and have used either serum-supplemented (15, 16) or serum-free (17) culture media. Each of these studies used relatively high cell densities (not lower than 5×10^4 cells/0.2 ml culture) and short culture intervals (not longer than 96 hr) and did not provide supplementary IL-2 to promote cell expansion or maturation. Although several of these groups noted an increase with age in the proportion of memory T cells (15, 16) and attributed the increase in IL-4 production to this subset shift, none actually measured IL-4 production from naive or memory T cells of old and young mice. Our own experimental strategy was to compare T cells from old and young donors using culture conditions that have been shown, in studies using young mice (9), to promote the differentiation of both naive and memory T cells into IL-4-secreting effectors. These extended cultures are initiated at low cell densities (10^4/0.2 ml culture) and include IL-2 supplementation, in the light of reports (11, 25) that have demonstrated a need for an IL-2 signal to promote the development of IL-4-secreting effectors. Under these conditions IL-4 accumulation was detectable as early as the 3rd day of culture (starting with cells preactivated, without IL-2, for a 2-day period) and continued to increase through at least the 11th day of culture. CD4 memory T cells were found to produce more IL-4 than unfractionated CD4 cells, and these were in turn more potent than naive T cells, which generated detectable levels of IL-4 in some but not in all experiments. Young CD4 populations, and memory cells prepared from them, were found to be more potent than corresponding cells from older donors.

Most published studies suggest that IL-4 is produced principally by memory T cells (2, 7, 8), although long-term culture or repeated stimulation can produce IL-4-secreting effectors from either naive or memory T cells (9–11). The ability of freshly isolated T cells to generate IL-4-secreting effectors *in vitro* depends not only on their original state of differentiation (as monitored, for example, by differential expression of CD44 and CD45RB determinants), but also on the availability of continued T cell receptor ligation (9) and of cytokines including IL-4, TGFβ, and IL-10 (6, 37–39). It seems plausible that the differences between naive and memory T cells, and between T cells from young and old donors, may reflect not merely intrinsic differences in lymphokine production capacity, but also differences in the ability to respond to lymphokines produced endogenously in the first few days of culture. Since IL-2 is known to be required for IL-4 production by freshly isolated T cells (11, 25), differences between young and old mice in the ability to respond to IL-2 signals might include an age-dependent decline in IL-2-dependent proliferation and/or maturation into IL-4-secreting effectors. While the high proportion of memory T cells in aged mice might lead to an increase in initial production of IL-4 generation in high-density short-term cultures, longer culture intervals and lower densities of responding cells seem likely

to favor cell populations that can undergo expansion prior to the production of lymphokine-secreting effector cells, as in the current situation.

Each of these *in vitro* culture systems is highly artificial, and none can be claimed to provide a perfect analogue to the *in vivo* immune response. Nonetheless, our data suggest that it may be premature to assume that the increase, with age, in memory T cell number will necessarily lead to a corresponding increase in IL-4 and other products of the memory subset during the course of natural immune responses, and suggest further that immune defenses that require the production of IL-4 over extended intervals of continuous antigenic stimulation may well be impaired with age as dramatically as are IL-2-dependent responses. Further work using *in vivo* indices of IL-4 activity, including shifts in antibody isotype, will help to throw further light on this controversy.

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