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Cell Proliferation and Cytokine Production by $CD4^+$ Cells From Old Mice

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Abstract Splenocytes from young adult or old C57BL/6NNia mice were stimulated in vitro with the anti-CD3ε mAb, 145-2C11, in either soluble (2C11s) or plate-bound (2C11i) form. In the young group, each mode of cell activation resulted in peak DNA synthesis at ~48 h of culture; at this time point, the old group exhibited response levels to 2C11s or 2C11i that were ~40% of those in the young group. However, in the presence of 2C11i, splenocytes from old donors showed a delayed peak response which approached the peak levels attained in the young group. To analyze the responsiveness of the $CD4^+$ T cell subpopulation, this cell type was isolated from spleens of young or old mice and was stimulated in vitro with 2C11s or 2C11i, in the presence or absence of added accessory cells (T cell–depleted, irradiated splenocytes). The induction of DNA synthesis by 2C11s was accessory cell dependent, and the response in the old group were markedly reduced in comparison to those in the young group. In contrast, stimulation of DNA synthesis with 2C11i was relatively accessory cell independent, resulted in higher response levels in both age groups, and lessened the disparity between age groups. The analysis of IL-2 and IL-4 secretion by stimulated $CD4^+$ cells revealed that, in response to 2C11s and accessory cells, only IL-2 accumulation was detectable and the levels in the young group were ~10-fold higher than the IL-2 levels in the old group. However, stimulation of $CD4^+$ cells with 2C11i and accessory cells yielded improved IL-2 production and a detectable IL-4 response in the old group, whereas the young group exhibited a response profile similar to that induced by 2C11s. Further analysis of the IL-2, IL-4, and IFNγ mRNA levels in 2C11i-stimulated $CD4^+$ cells revealed that old donor cells accumulated similar levels of IL-2 transcripts, but higher levels of IL-4 and IFNγ transcripts, than young donor $CD4^+$ cells. Finally, we analyzed splenic $CD4^+$ cells for membrane expression of four molecules—3G11, 6C10, CD45RB, and CD44—thought to demarcate $CD4^+$ cell subsets with restricted patterns of cytokine production. The $CD4^+$ cell fraction of individual mice contained higher percentages of cell phenotypes associated with increased IL-4:IL-2 production ratios (i.e., $3G11^{lo}$, $CD45RB^{lo}$) and with increased IFNγ synthesis (i.e., $CD44^{hi}$). Taken together, these data show marked alterations in the $CD4^+$ cell subset composition in old mice, detected at the levels of subset marker expression and profiles of cytokine production. Moreover, conclusions regarding $CD4^+$ cell competency in old donors can differ depending on the choices of stimuli and readouts for cell function in the experimental design. Therefore, age-related differences in T cell reactivity in vitro may be partially explained by the shifts in the representation of individual $CD4^+$ subsets, each with potentially unique activation requirements and functional attributes.

Key words: T cells, aging, IL-2, IL-4, IFNγ, CD45RB, 3G11, 6C10, CD44

Mature T cells of the peripheral lymphoid tissues participate in most, if not all, manifestations of antigen-specific immunity. In several mammalian species, advancing age of individuals is thought to be accompanied by diminishing competency of the T cell compartment [1,2]. Much of the existing data on this subject was derived from in vitro systems designed to model

Abbreviations: FCF, flow cytofluorometric; F-H, Ficoll-Hypaque; FL, fluorescein; IF, immunofluorescence; IFN, interferon; IL, interleukin; mAb, monoclonal antibody; PE, phycoerythrin; TCM, tissue culture medium; 2C11s, soluble 145-2C11 mAb; 2C11i, plate-bound 145-2C11 mAB.

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T cell function. For example, in response to various mitogenic or antigenic stimuli, T cells isolated from old humans or rodents generally exhibit decreased DNA synthesis, cell division, and effector function in comparison to cell preparations from younger groups [1–9].

Analyses of pre-S phase events in the T cell response of the aged group have uncovered perturbations in events linked to immediate signal transduction [2,10,11], decreases in early gene expression related to cell cycle entry [12], reductions in the number of cells entering into and progressing through G_1 phase [3,4], decreases in the expression of receptors for growth-promoting factors [2,4,6,10], and, in general, decreases in IL-2 production [2]. Results from studies designed to test responsiveness at the individual cell level [3,4,7,13] suggest that old donor T cells contain a reduced fraction of cells with "youthful" response patterns, rather than a pervasive defect within the cell population. These data have led to the theory that advancing age is paralleled by an increasing accumulation of intrinsically defective T cells, thus contributing to immunological decline in the elderly.

Most age-comparative studies on T cell responsiveness in vitro have utilized total lymphoid cell or T cell preparations, without differential analysis of the $CD4^+$ and $CD8^+$ cell constituents. Moreover, there is now evidence suggesting that functionally distinct cell subsets exist within the $CD4^+$ cell compartment [14–20], and that the $CD4^+$ subset composition is altered in old humans and rodents [21–24]. Thus, it is difficult to extrapolate from past studies on heterogeneous mixtures of cells to the functional status of distinct T cell subpopulations in the elderly. In the present report, we have used the mouse model system to address the question of $CD4^+$ cell competence in aged individuals. Splenic $CD4^+$ cells, isolated from adult mice of young or old age, were monitored for levels of DNA synthesis in response to various modes of cell activation in vitro. In addition we have analyzed $CD4^+$ cells from the two age groups for the expressed levels of four cell membrane determinants—3G11, 6C10, CD45RB, and CD44—thought to define $CD4^+$ cell subsets with restricted patterns of cytokine production. Finally, we have performed age-group comparisons with respect to the production of IL-2, IL-4, and IFNγ in stimulated cultures of $CD4^+$ cells.

MATERIALS AND METHODS

Animals

Specific pathogen-free (SPF), male C57BL/6NNia mice (median life span ~28 mo) were purchased from Charles River Laboratories (Wilmington, MA), under contract with the National Institute of Aging. Cross-sectional studies were performed on mice of young adult (3 to 6 mo) and old (23 to 26 mo) age. All mice were housed in a barrier facility to maintain SPF status, were used within 2 mo of receipt, and were excluded from experiments upon visible evidence of gross internal or external pathology.

Antibodies

Detailed descriptions of the isotype, specificity, and source of AT83 (anti-Thy-1.2), 3.155 (anti-CD8), GK1.5 (anti-CD4), RL172 (anti-CD4), J11d (anti-heat stable Ag), M5/114 (anti-I-$A^{b,d,a}$I-$E^{d,k}$), 11B11 (anti-IL-4), S4B6 (anti-IL-2), SM3G11 (anti-3G11 Ag), SM6C10 (anti-6C10 Ag), C363.16A (anti-CD45RB), IM7.8.1 (anti-CD44), and 145-2C11 (anti-CD3ϵ) monoclonal antibodies (mAb) were presented elsewhere [4,14,23,25,26]. The mAb were purified as described [4,26] and selected mAb were conjugated with fluorescein (FL) [26]. Phycoerythrin (PE)-conjugated YTS 191.1 (anti-CD4; Caltag, San Francisco, CA) and FL-conjugated, rat $F(ab')_2$ anti-mouse IgG (RAMIg; Jackson Immunoresearch, West Grove, PA) were purchased.

Cell Preparations

Spleens were removed from mice and were teased apart in a standard tissue culture medium (TCM) [4,23], and viable mononuclear cells were isolated by using Ficoll-Hypaque density centrifugation (F-H). To prepare T cell–depleted accessory cells, splenocytes were treated with a cocktail of AT83, 3.155, GK1.5, and RL172 mAbs followed by treatment with complement [23]. Viable cells were then isolated by using F-H and were irradiated (^{137}Cs, 2000rad). $CD4^+$ cell–enriched preparations were obtained by treating splenocytes with a cocktail of J11d, M5/114, and 3.155 mAbs, followed by treatment with complement [23]. After the isolation of viable cells by using F-H, the cells were stained with FL-GK1.5 mAb and the $CD4^+$ cells were sorted by using a FACStar flow cytometer (Becton Dickinson, Mountain View, CA) as described [23].

Immunofluorescence (IF) Staining

The methods and formulation of medium for two-color IF staining were described previously [4,23]. For the analysis of 3G11, 6C10, CD45RB, and CD44 expression by $CD4^+$ cells, splenocytes were stained with PE-YTS 191.1 and either FL-C363.16A, FL-IM7.8.1, SM3G11/FL-RAMIg, or SM6C10/FL-RAMIg. Stained cells were analyzed with a FACS IV flow cytometer (Becton Dickinson); the machine settings and optical arrangements for distinguishing PE and FL signals were detailed elsewhere [4]. Fluorescence signals were log amplified (4 decade range) and appropriately compensated, and ~5,000 PE^+ events were acquired for each sample. Data acquisition and analysis were performed with the Consort 30 system (Becton Dickinson). Light scatter- and PE-gated events were redisplayed as frequency distribution histograms (FL versus cell number); marker settings for each histogram were chosen to estimate the percentages of low- and high-expressing cells and to facilitate comparisons between age groups.

Cell Culture

For most experiments, cells were suspended in TCM and were cultured, in triplicate, in 96-well, flat-bottomed microculture plates (Costar, Cambridge, MA) at the following densities: mononuclear splenocytes, 2.5×10^5 cells/0.2 ml/well; $CD4^+$ cells, 10^5 cells/0.2 ml/well; accessory cells, 10^5 cells/0.2 ml/well. For the generation of RNA samples, $CD4^+$ cells were cultured in 12-well cluster plates (Costar; 1.4×10^6 cells/2.7 ml/well). Cell cultures were stimulated with TCM-only, soluble 145-2C11 mAb (0.5 μg/ml), or plate-bound 145-2C11 mAb (wells were precoated with 1.25 μg 145-2C11/0.05 ml/0.32cm^2, 4 h) as described [4,23]. Cultures were then incubated at 37°C in humidified 5% CO_2. For the analysis of DNA synthesis, the replicate cultures were pulsed (2 h) with 1 μCi [^{3}H]TdR (5 Ci/mmol; Amersham/Searle, Arlington Heights, IL) and then were harvested onto glass fiber filters and processed for liquid scintillation spectroscopy.

Assays for IL-2 and IL-4

Supernatant IL-2 and IL-4 levels were measured with the CTLL.6 and 11.6 bioassays, respectively, exactly as described [23]. Human rIL-2 (Biological Response Modifiers program, National Cancer Institute) and mouse rIL-4 (Lilly Research Laboratories, Indianapolis, IN) were used as positive controls in the assays. The 11B11 and S4B6 mAbs were used to render the assays specific for each cytokine [23]. Data are presented as U/ml, with 1 U equal to the reciprocal of the supernatant dilution yielding 33% of maximum, cytokine-specific target cell response [23].

RNA Preparations

Cultured cells were washed in-well with cold PBS, after which total RNA was isolated by acid guanidinium thiocyanate/phenol/chloroform extraction as described [27].

Plasmid Constructs

The full-length cDNA clone for mouse IL-2 (pMuIL-2) was obtained from GeneCell, Inc. (San Diego, CA). The cDNA clones for mouse IL-4 (pcD-SRα-mIL-4) and IFNγ (pcD-mIFNγ) were obtained from Dynax Research Institute (Palo Alto, CA). The mIL-2(A) and mIFNγ(B) subclones were generated by digestion of the original cDNA clones with PvuII/HindIII and EcoRI/PstI (Boehringer Mannheim, Indianapolis, IN), respectively, and ligation of the purified fragments into appropriately digested pGEM-4 vector (Promega, Madison, WI). DNA fragments for the mIL-4(B) subclone were generated by polymerase chain reaction [28] using the original cDNA clone as template, oligonucleotide primers flanked 5′ with either HindIII or EcoRI sites, and reagents from the GeneAmp Kit (Cetus Corp., Emoryville, CA). The amplified fragments were then digested with HindIII/EcoRI, purified, and ligated into pGEM-4. The authenticity of each subclone was verified by dideoxy sequencing (GemSeq K/RT, Promega). The subclones represented the following sequences: mIL-2(A), 198–382 [29]; mIL-4(B), 74–353 [30]; mIFNγ(B), 706–912 [31]. The orientation of each subclone allowed anti-sense RNA synthesis from the T7 promoter of pGEM-4.

RNase Protection Assay

The assay was performed essentially as described by Melton et al. [32]. The anti-sense RNA probes were synthesized by using the Riboprobe Gemini II Core System (Promega), with EcoRI-linearized mIL-2(A), mIL-4(B), and mIFNγ(B) as the templates and 110 μCi [α-^{32}P]UTP (3,000 Ci/mmol; Amersham) as the labeled nucleotide, and were purified as outlined

by Promega. The hybridization reactions (2×10^5 CPM/5 μg target RNA), RNase treatments, and isolations of "protected" RNA-RNA duplexes were as described [32]. Final samples were electrophoresed in standard 5% acrylamide/8M urea sequencing gels. Autoradiography revealed protected probes at 185 nt (IL-2), 280 nt (IL-4), and 206 nt (IFNγ), which were not apparent in control hybridizations (probes plus yeast tRNA). For certain data displays, films were scanned with an Ultroscan XL densitometer (Pharmacia-LKB, Piscataway, NJ) to obtain absorbance units.

RESULTS

Proliferative Capacity of T Cells From Old Mice

Most age-comparative studies on T cell function have reported diminished proliferative responses by T cells isolated from elderly humans and old animals [1–9]. In the experiments depicted in Figure 1, splenocytes from young adult and old C57BL/6NNia mice were stimulated with soluble (2C11s) or plate-bound (2C11i) forms of the anti-CD3ε mAb, 145-2C11, and the levels of DNA synthesis were monitored at several times thereafter. In cultures of young donor splenocytes stimulated with 2C11s, DNA synthesis was above the background level at 18 h, and was progressively increased through 48 h. In contrast, the aged donor splenocytes exhibited significantly reduced DNA synthesis through the early phases of cell culture (24 to 48 h), and a slightly belated peak response. The response of young donor splenocytes to 2C11i was similarly apical at 48 h of culture, but was more vigorously maintained thereafter, in comparison to the 2C11s-stimulated young group. The 2C11i-stimulated old group was again significantly reduced through 48 h of culture, but showed a delayed peak response, relative to the young group. Comparison of the peak responses for all experiments with 2C11i as the stimulus revealed no significant difference ($P = 0.06$, $n = 7$; paired t-test) between the age groups.

Proliferative Capacity of CD4+ Cells From Old Mice

To determine the contribution of CD4+ cells to the response patterns shown in Figure 1, splenic CD4+ cells were isolated from mice of the two age groups and were stimulated in vitro with 2C11s or 2C11i (Fig. 2). The induction of DNA synthesis by 2C11s required the coculture

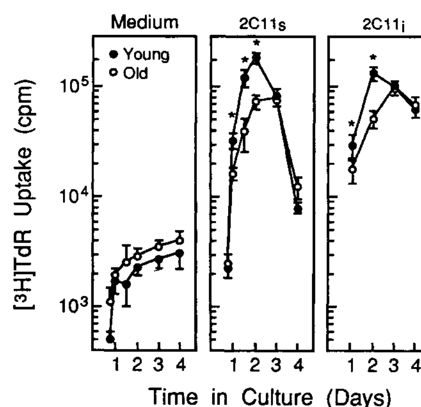

Fig. 1. Induction of DNA synthesis in splenocyte cultures stimulated with 145-2C11 mAb. Splenocyte preparations pooled from multiple young or old C57BL/6NNia mice were cultured in the presence of medium only, 2C11s, or 2C11i. At various times thereafter, cultures were assayed for $[^3H]$TdR uptake. The data are presented as the mean incorporated CPM (± SEM) from replicate experiments. **Left and middle panels:** The data are from 17 experiments, with each mean generated from 3 to 15 values. **Right panel:** The data are from 7 experiments, with each mean generated from 6 values. The asterisks denote significant differences ($P < 0.05$; paired t-test) between age groups.

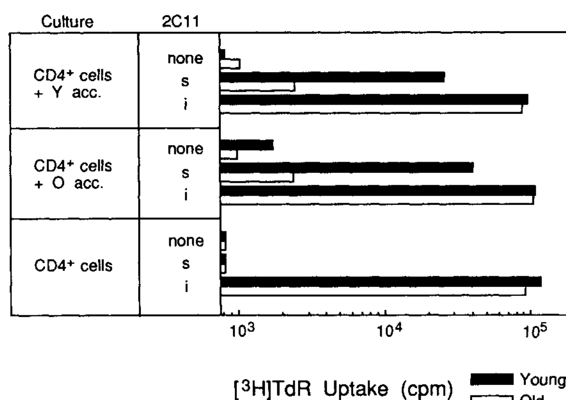

Fig. 2. Induction of DNA synthesis in CD4+ cell cultures stimulated with 145-2C11 mAb. Splenocytes from young or old mice were enriched for CD4+ cells and then further sorted by using FCF procedures (each > 95% CD4+). CD4+ cells were cultured alone or in the presence of T cell–depleted, irradiated splenocytes from young (Y acc) or old (O acc) mice. The cultures were then stimulated with medium only, 2C11s, or 2C11i and, at several times thereafter, were assayed for $[^3H]$TdR uptake. The peak response (mean of replicate wells) for each treatment group is shown.

of CD4+ cells with accessory cells (T cell–depleted, irradiated splenocytes). The peak responses to 2C11s in the old group were ~10-fold reduced in comparison to those in the young group, regardless of whether the accessory cells were derived from spleens of young or old mice. The induction of DNA synthesis by 2C11i was

not dependent on or influenced by the addition of accessory cells. Moreover, 2C11i was a more potent stimulus than 2C11s, and, in this experiment, resulted in similar peak responses occurring at 90 h of culture in both age groups. Over the course of several experiments using 2C11i as the stimulus (data not shown), the average peak response in the old group was ~75% of that in the young group, and this difference was significant ($P < 0.03$, n = 11; paired t-test). It should be noted, however, that at time points prior to the peak responses (i.e., 24 to 48 h), the young group generally exhibited 2- to 3-fold higher levels of DNA synthesis in response to 2C11i (data not shown; ref. 23).

Subset-Associated Determinants on CD4$^+$ Cells

The expression patterns of membrane CD45RB, 3G11, 6C10, and CD44 by mouse CD4$^+$ cells appear to demarcate cell subsets with distinct profiles of cytokine production and differing histories of antigenic exposure [20,25,14–16,33–35]. We used two-color IF staining and FCF analysis to examine the expression patterns of these determinants on splenic CD4$^+$ cells from individual mice of the two age groups (Fig. 3). In the young group, each marker defined a continuum of cells, with distributions that were remarkably consistent between mice. However, parallel analyses of the old group revealed moderate increases in donor-to-donor variablilty, and clearly significant increases in proportions of CD45RB^{lo}, 3G11^{lo}, and CD44^{hi} cells. In contrast, we detected marginal differences in the profiles of 6C10 expression between age groups.

Cytokine Production by CD4$^+$ Cells From Old Mice

Based on past studies on cytokine production by CD4$^+$ cell subsets from young mice [14,15,25,33], the age-associated conversions of 3G11^{hi} → 3G11^{lo} and CD45RB^{hi} → CD45RB^{lo} phenotypes, described in the previous section, would be expected to result in increased capacities for IL-4 production relative to IL-2 production. Accordingly, we isolated CD4$^+$ cells from mice of the two age groups and, following cell stimulation in vitro, monitored the levels of supernatant IL-2 and IL-4 (Fig. 4). Stimulation of young donor CD4$^+$ cells with 2C11s, in the presence of accessory cells, resulted in high levels of peak IL-2 accumulation but undetectable IL-4 levels. Under the same conditions, the old group exhibited marked reductions in peak DNA synthesis (> 10-fold) and IL-2 (> 6-fold) levels and similarly low IL-4 levels. Stimulation of young donor CD4$^+$ cells with 2C11i, in the presence of accessory cells, resulted in peak levels of DNA synthesis and IL-2 and IL-4 accumulation which were similar to those in the 2C11s-stimulated young group. In contrast, activation of old donor CD4$^+$ cells with 2C11i yielded marked improvement in both DNA synthesis

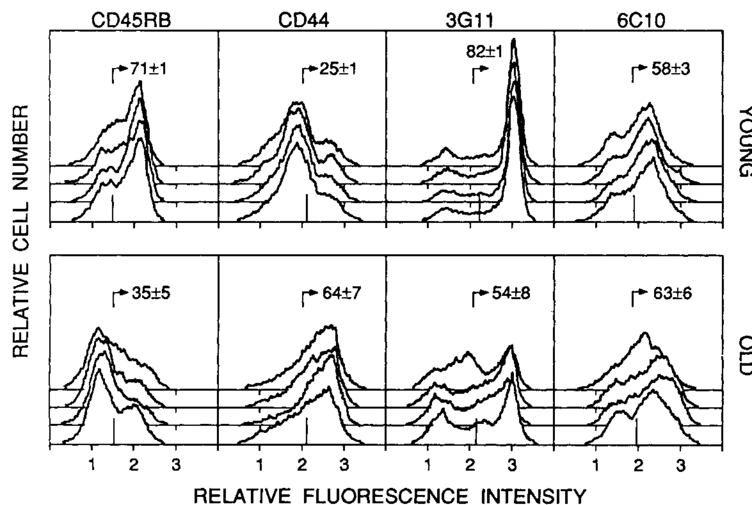

Fig. 3. Expression of membrane 3G11, 6C10, CD45RB, and CD44 by CD4$^+$ cells. Splenocytes from individual *young* (n = 4) and old (n = 4) mice were two-color IF-stained for surface CD4 (PE) and each of the designated membrane molecules (FL). PE-gated events are displayed as FL histograms, with marker settings chosen to demarcate "high" and "low" expressing fractions. The mean % high (± SD) is shown for each staining group (t-test: $P < 10^{-3}$ for age-group comparisons of 3G11, CD45RB, and CD44 expression; $P = 0.11$ for 6C10 expression).

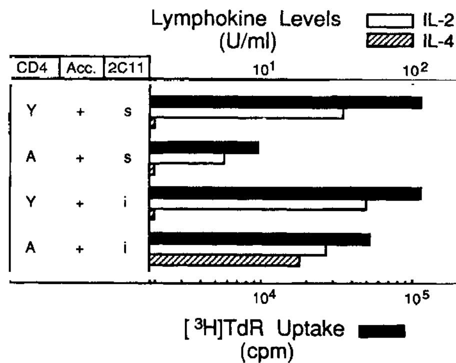

Fig. 4. Induction of IL-2 and IL-4 secretion by CD4+ cells. CD4+ cells were isolated from pooled splenocytes of young (n = 5) or old (n = 5) mice by using FCF procedures (each > 90% CD4+). CD4+ cells were cultured in the presence of accessory cells derived from young mice, and were stimulated with 2C11s or 2C11i. At 24, 48, and 72 h of culture, cells were assayed for [^{3}H]TdR uptake and supernatants were assayed for IL-2 (CTLL.6 assay) and IL-4 (11.6 assay) content. The data represent the peak response for each group. Background responses (no 2C11 or no CD4+ cells) were < 3,100 CPM ([^{3}H]TdR uptake) and < 2 U/ml (both cytokines).

and IL-2 release, and easily detected IL-4 secretion. In all experiments with 2C11i as the stimulus (n = 9), peak IL-2 levels were not significantly different between age groups ($P = 0.09$; paired t-test), whereas peak IL-4 levels were significantly higher in the old group ($P = 0.008$).

We extended these studies by analyzing the steady-state levels of IL-2, IL-4, and IFNγ mRNA in stimulated CD4+ cells of the two age groups (Figs. 5A,B). In cell cultures treated with 2C11i for 24 h, CD4+ cells from old donors accumulated similar levels of IL-2 mRNA, but clearly higher levels of IL-4 and IFNγ transcripts, in comparison to cells from young donors.

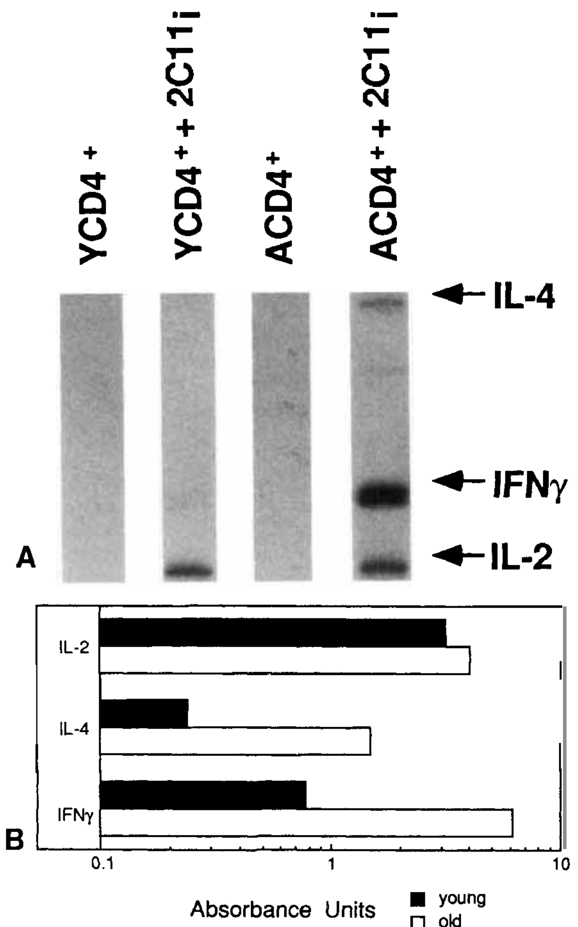

Fig. 5. Analysis of IL-2, IL-4, and IFNγ mRNA levels in stimulated CD4+ cells. CD4+ cells, isolated from young or old mice, were stimulated with medium only or with 2C11i in the absence of added accessory cells. At 24 h of culture, total RNA was extracted from cells, and RNA aliquots originating from 6×10^5 input CD4+ cells were analyzed for IL-2, IL-4, and IFNγ transcripts by using the RNase protection assay. **A:** An autoradiograph from a 20h, −70°C film exposure; **B:** Results from the densitometer scans of lanes representing the 2C11i-stimulated groups.

DISCUSSION

Splenocyte preparations from young adult and old mice differed in their proliferative responses to the T cell activator, 145-2C11 mAb. In response to 2C11s or 2C11i, splenocytes from old donors exhibited ~60% lower levels of DNA synthesis at the time of peak response in the young group (48 h). In addition, the aged group showed a broadened (2C11s) or delayed (2C11i) peak response; in the latter case, the response level of the delayed peak approached the level of the peak response in the young group. In a related report [4], we showed that the age-related differences in responsiveness to 145-2C11 mAb can not be attributed to major differences in the percentages of CD4+ and CD8+ cells in spleens, in the surface levels of CD3ε on T cells, in the dose optima of the 145-2C11 mAb, or in the function of accessory cells. Our findings of reduced S phase activity by old donor T cells are consistent with previous reports of decreased proliferation by T cells from elderly humans or old rodents, stimulated with mitogenic lectins, mAb directed against CD3 or other T cell membrane molecules, alloantigens, or phorbol esters and Ca^{2+} ionophores [1–9]. Age-comparative studies on the kinetics of cell cycle progression by stimulated T cells have revealed that, in the old group, lesser fractions of T cells enter into and progress through G_1 phase, al-

though the rates of progression are similar for the reactive fractions of the two age groups [3,4].

The response profiles of stimulated T cell preparations reflect the sum of the individual response patterns of $CD4^+$ and $CD8^+$ T cells, and provide limited information on the status of T cell subpopulations in the elderly. Thus, we analyzed the proliferative and cytokine-producing capacities of isolated $CD4^+$ cells, under various conditions of cell stimulation. As described by others [36,37], the proliferative response of $CD4^+$ cells to the soluble form of anti-CD3 mAb was accessory cell dependent. Under these conditions, the response of the old group was reduced dramatically relative to the young group. The findings that accessory cells derived from young or old donors behaved comparably in this and other [38] systems would suggest that the age-related differences in cell responsiveness are intrinsic to $CD4^+$ cells. We also showed that the reduced proliferative response in the old group was paralleled by reduced IL-2 production. This finding confirms and extends our previous work on intact splenocyte preparations [4], and is in agreement with several earlier reports of age-related decreases in IL-2 production by lymphoid or T cell preparations, particularly following stimulation with mitogenic lectins [2,7,10,22,39–41].

Plate-bound forms of anti-CD3 mAbs provide a strong, accessory cell–independent stimulus for $CD4^+$ cell proliferation [36,37,42]. We found that the stimulation of $CD4^+$ cells with 2C11i yielded a vigorous response of DNA synthesis in both age groups and, in comparison to stimulation with 2C11s, decreased the disparity between age groups. Concomitant with this increased cell cycle activity, the old group exhibited insignificant differences in peak levels of supernatant IL-2 accumulation, and significantly higher levels of IL-4 accumulation, relative to young controls [23, this report]. These findings were verified by analyzing steady state levels of IL-2 and IL-4 mRNA, and were extended to show increased levels of IFNγ transcripts in the old group. In two studies on $CD4^+$ cells isolated from young and elderly humans, the use of strong T cell stimuli (i.e., plate-bound anti-CD3 mAb plus IL-2 and anti-CD28 mAb [5] or phorbol ester and ionomycin [8]) led to only moderate [8] or no [5] reduction in the proliferative response of the old group, whereas the $CD8^+$ cell population was more profoundly affected by age. In other studies, the stimulation of lymphoid or T cells with phorbol ester and Ca^{2+} ionophore resulted in similar or increased levels of IL-2 production in the old group relative to young controls [9,10,40,43,44]. Reports on the production of other cytokines by old donor T cells are few and sometimes conflicting; however, some studies have shown increased production of IL-3 [43], IL-4 [43], IFNγ [9,45], and a B cell differentiation factor [46] by stimulated T cells from old humans or rodents.

We have shown here that the splenic $CD4^+$ pool of old mice contained significantly higher percentages of $3G11^{lo}$, $CD45RB^{lo}$, and $CD44^{hi}$ cells, and marginally increased percentages of $6C10^{hi}$ cells. These findings are consistent with previous reports by us [23] and others [14,22], with the exception that greater increases in $6C10^{hi}$ cells in older Balb/c mice were noted in one study [14]. In the human system, numbers of $CD45RB^{lo}$ and $CD29^{hi}$ T cells are elevated in elderly individuals [21,24,47]. In both species, these alterations in the phenotypic makeup of the $CD4^+$ cell pool appear to occur gradually with age [21–24,47]. The expressed levels of each of these various membrane molecules distinguish $CD4^+$ cell subsets with distinct functional properties [14–20], and the interrelationships of 3G11 and 6C10 expression [14,15] and 3G11, CD45RB, and CD44 expression [23,48] have recently been reported. The predominant phenotype (> 50%) among $CD4^+$ cells of young adult mice is $3G11^{hi}$ $CD45RB^{hi}$ $CD44^{lo}$ [23]. Based on previous analyses of phenotypic changes in T cells during ontogeny, following thymectomy of mice, or as a consequence of cell activation and memory cell generation [15,16,20,33–35], this combinational phenotype presumably contains the naive $CD4^+$ cell fraction. In old mice, this cell group is markedly reduced [23] with concomitant increases in $CD4^+$ cells expressing high levels of CD44 [22,23], a phenotype associated with activated/memory cells [20,34]. However, within the $CD44^{hi}$ fractions of both age groups exists further heterogeneity, including $3G11^{hi}$ $CD45RB^{lo}$ and $3G11^{lo}$ $CD45RB^{variable}$ cells [23]. The age-related shifts in the $CD4^+$ cell subset compositions defined by membrane phenotypes are consistent with the cytokine profiles induced by 2C11i. For example, $CD4^+$ $CD45RB^{hi}$ and $CD4^+$ $3G11^{hi}$ cells are thought to produce high levels of IL-2 relative to IL-4, whereas increased IL-4 production is associated with the $CD45RB^{lo}$ and $3G11^{lo}$ counterparts [14–16,25,33]. In addi-

tion, the analysis of cytokine production by CD4+ CD44lo and CD4+ CD44hi cells has revealed that stimulated CD44hi cells produce similar or higher levels of IL-2, and higher levels of IL-3, GM-CSF, and IFNγ, compared to CD44lo cells [20,34,35]. Thus, under conditions of efficient cell activation (e.g., 2C11i or phorbol esters and Ca^{2+} ionophores), CD4+ cells from old donors can exhibit a cytokine profile commensurate with the existing subset composition.

The results presented here raise the question of whether the altered responsiveness of CD4+ cells from old mice (and elderly humans) reflects an accumulation of intrinsically defective cells or, rather, an accumulation of intrinsically different cells. Our ability to distinguish between these two possibilities will increase as more data become available on the requirements for cell activation, the capacity for clonal expansion, and the spectrum of displayed effector functions for individual CD4+ cell subsets. Thus, subset-matched cell preparations from young and old donors could be stimulated optimally, and then analyzed for subset-associated functions. Even under the assumption that CD4+ cells in the elderly are not innately defective, shifts in the balance of CD4+ cell subsets could predispose individuals to other health problems. For example, the prevalence of memory cell phenotypes, driven by thymic involution and a longer history of antigen exposure, in combination with the inability to undergo somatic mutation of the TCR genes, would result in a more fixed TCR repertoire. Consequently, the elderly may mount an effective response against previously encountered viral, microbial, or tumor antigens, but may respond poorly to first encounters. In addition, there is evidence suggesting regulatory communications between CD4+ cell subsets [49]; therefore, subset imbalances could lead to poor homeostatic control of the T cell compartment, perhaps disallowing full responsiveness, causing biased responsiveness (e.g., cell-mediated vs. humoral immunity), or allowing the emergence of self-reactive T cells.

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