EFFECT OF AGE ON CYTOKINE PRODUCTION IN HUMANS

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ABSTRACT

Aging is accompanied by many changes in immune response, with the most consistent and dramatic alterations occurring within the T cell compartment. Since cytokines are central to immune cell communications, age-associated changes in cytokine production may contribute to these alterations. While data from murine studies suggest a switch from a Th1 (IL-2, IFN γ) to a Th2 (IL-4, IL-6, IL-10) cytokine response, this model has not been as clearly established in humans. In addition, this current review of over 50 studies in humans suggests that age-associated changes in cytokine production are not consistent.

INTRODUCTION

The exquisite orchestration of the immune response is dependent on functional regulation at many levels. Although disruption at some levels may still allow for adequate responses, alteration of a key regulator or dysfunction of several regulatory steps often results in an inadequate immune response, such as lack of protection after vaccination or, alternatively, in an inappropriate immune response, such as autoimmune reactivity. Aging is accompanied by many changes in immune response, including decreased lymphocyte proliferative responses to both mitogens and antigens, decreased delayed type hypersensitivity reactions, and decreased antibody responses to vaccination and infection. The most consistent and dramatic alterations are observed in the T cell compartment. The mechanism(s) responsible for the age-associated alterations in immune function have not been established. However, since cytokines are central to immune cell communication and effector activity, many researchers have investigated the contribution of changes in cytokine production to the age-associated changes in immune response.

Data from murine studies suggest an age-related dysregulation of cytokine production. The consistently observed decline in interleukin-2 (IL-2) [1], and the generally observed rise in interleukin-4 (IL-4) in aged mice [2,3] have led many researchers to propose that in the murine system aging is accompanied by a switch from a predominant production of cytokines that induce and support cell-mediated immune responses (type 1 cytokines: IL-2, IFN γ , TNF α , IL-12, IL-15) in young animals, to a predominant production of cytokines that induce humoral responses (type 2 cytokines: IL-4, IL-5,

IL-6, IL-10, and IL-13) [1,4]. This model for altered cytokine production with aging has not been as clearly established in humans. It is unclear whether this difference between humans and mice reflects the genetic heterogeneity of humans or the environmental factors (e.g. general health status, nutrition, smoking, exercise) which confound human studies.

The purpose of this review is to summarize published studies on age-related changes in cytokine production in humans. However, several criteria have been selected as requirements for inclusion of data so that appropriate comparisons can be made. First, young and elderly subjects must be evaluated in the same study. This is important due to the inherent interassay variability of measurements of immune function, including cytokine production. Second, young subjects must be between 18 and 45, while elderly subjects must be >60. The 45-60 age group is commonly omitted from age-related studies since fluctuations in hormone levels that occur during this timeframe, particularly in women, can greatly skew the results obtained. Third, the primary focus will be data obtained from short-term cultures of peripheral blood mononuclear cells (PBMC) or isolated T cells. However, since the goal of short-term *in vitro* culture is to reflect the *in vivo* potential of the individual to respond to antigenic stimuli, plasma and serum levels of cytokines are also included. Results from long-term lymphocyte cultures are not included since phenotypic changes occur with long-term culture that may alter cytokine profiles [5].

In addition to the above criteria, several other parameters, including the health status of the subjects and the methods used to induce and quantitate cytokines, can greatly influence the outcome of studies of immune response. Differences among studies, therefore, can reflect the population or a technical variable, rather than inconsistencies regarding the effect of age. Finally, the reader must be cognizant of the heterogeneity that exists among the responses of the elderly. The influence of these parameters on results of immune studies will be reviewed first. Then the studies will be described in light of these considerations.

Parameters that Influence Results of Immune Studies

Health Status of Subjects

It is imperative that the characteristics of the subject population participating in any given study be recognized. Conclusions drawn from research involving nursing home elderly may not be applicable to healthy,

ambulatory elderly, and vice versa. Most investigators exclude subjects who report any disease of immune dysfunction, malignancy, or use of medications known to affect the immune system, such as steroids or cytotoxic drugs. Other investigators have adopted more strict selection criteria (e.g. the Senieur protocol) [6] to dissociate the effects of disease processes common in the elderly from alterations in immune responses that are specifically age-related. As seen in Table 1, the Senieur protocol employs clinical and laboratory data to exclude subjects with documented or underlying disease processes. The major drawback of the Senieur protocol, however, is that while controlling for the effect of disease, it delineates a group of exceptional individuals who have escaped major age-related illnesses. Since the Senieur protocol excludes the majority of the community-dwelling elderly, studies employing this protocol may underestimate the magnitude of immunosenescence generally found in the geriatric population. Since nutrition or exercise can also influence immune responses in both young and elderly [7,8], the most stringent investigation of the effect of age on immune function should also consider nutritional status and level of physical activity among the elderly and between young and elderly. Few, if any of the studies, including those using the Senieur protocol, have included these parameters. However, all these issues should be considered in evaluating results among studies. If the goal is to decipher the direct effects of age on immune function, then studies with the most stringent health criteria should be used as the benchmark. However, if the purpose is to define age-associated alterations in immune function so that strategies for more effective immunizations or cancer immunotherapies for the elderly can be developed, then results from a research subject population that resembles the general elderly population should be the standard. For the purposes of this review, studies are classified as Senieur (Table 1), healthy (i.e. subjects without evidence of diseases known to alter immune function nor taking medication affecting immune function) or frail (i.e. institutionalized or elderly with multiple chronic or debilitating diseases).

Table 1: Senieur Protocol

The exclusion criteria for admission to immunogerontological studies as developed by the EURAGE Concerted Action Programme on Ageing of the European Community:

1. Clinical information (including follow-up 2 weeks)
 - Infection
 - Inflammation
 - Malignancy
 - Other conditions which influence the immune system
2. Laboratory data (findings outside age-dependent reference range)
 - Erythrocyte sedimentation rate, hemoglobin, mean corpuscular volume, leukocyte count with differentiation
 - Urea, alkaline phosphatase, glucose, SGOT, SGPT
 - Protein and immunoelectrophoresis to exclude B cell malignancies
 - Urinalysis: protein, glucose, sediment
3. Pharmacological interference
 - Prescribed medication for treatment of defined disorder
 - Medication with known influence on the immune system

Heterogeneity of the Immune Response

It is critical to understand that even within subpopulations of the elderly classified by health status, there is considerable heterogeneity of immune response [9,10]. In any given sampling of elderly subjects there will be a certain percentage with immune responses similar to young controls, a percentage that respond at a very low level, with the majority responding at 50-75% of the level of young controls [10]. Whether the heterogeneity reflects health status, genetic variability, or behaviors such as smoking or level of physical activity, is not fully understood. However, it is quite possible that a sample of ten elderly will randomly reflect either the high or low responders. In order to assess definitively whether the results of a small study are skewed, the results of individual subjects need to be evaluated. However, only a limited number of studies report data in this manner. The implication for research is that large sample sizes are necessary in order to reflect the general elderly population.

Variation in Induction and Measurement of Cytokines

Age-related changes in cytokine production have been reported in many different experimental systems, in which different cell populations, stimulation protocols, and techniques for measuring cytokine production have been utilized. In humans, the majority of the studies evaluate cytokine production by PBMC. For obvious reasons, investigation of cells from spleens, lymph nodes, bone marrow, or thymus is not performed unless the tissue is being removed for a clinical reason. In these cases, cytokine production of these cells usually reflects the underlying disease process. Therefore, the current data regarding age-related changes in cytokine production in humans primarily reflect the capacity of the circulating immune cell pool.

Stimulation protocols fall into three categories: 1) non-specific stimulation via cell surface receptors (e.g. PHA, ConA, anti-CD2, anti-CD3, or anti-CD28 antibodies); 2) specific stimulation via the T cell receptor (e.g. tetanus toxoid, influenza antigens, mixed lymphocyte reactions); or 3) stimulation with agents that bypass the cell membrane (e.g. phorbol esters and calcium ionophore). The stimulus used in a study has important implications for evaluation of the data. The nature of the interaction of the stimulus with the cell indicates the level at which age-related alterations, if any, exists. For example, an age-related alteration in cytokine production observed when cells are stimulated via the cell membrane, but not with stimuli that bypass the membrane, suggests that the defect involves the membrane interaction and/or associated transduction mechanism(s).

In addition to the type of stimuli, the concentration of the stimuli and the time of assessment of cytokine production in response to the stimuli can also significantly affect the results. In our experience, supraoptimal concentrations of stimuli tend to mask any age-related changes, while suboptimal concentrations may magnify

these changes. Further, age-related differences in the kinetics of cytokine production may confound comparisons. For example, while peak lymphocyte proliferation or cytokine production of young and elderly subjects occurs on the same day after stimulation, the young response peaks and falls sharply, while the elderly response peaks and slowly declines (i.e. flattened curve) [10]. Since most studies measure proliferation and cytokine production at one time point and one concentration of stimulus, differences among studies may reflect subtle shifts in these conditions.

Comparison of reports on age-related alterations in cytokine production is made more difficult by the variation in techniques used to measure cytokines. The techniques employed to measure cytokines in supernatants from stimulated cells are either bioassays or antibody assays, generally the enzyme linked immunosorbent assay (ELISA). Bioassays assess the functional activity of a given sample in comparison to reference standards. Although many of these assays utilize growth or inhibition of growth of cell lines specifically reactive with the cytokine in question, other functional activities can also be assessed, such as the inhibition of viral cytopathology by interferon (IFN). In contrast, ELISA measures the total amount of a cytokine using antibodies specific for one epitope of the cytokine. Although highly sensitive and specific in measuring cytokine concentration, ELISA does not provide any information about the function of the cytokines measured. Comparison of bioassays and ELISA using samples spiked with recombinant $\text{TNF}\alpha$, IL-2, IL-6, and $\text{IFN}\gamma$ have shown that these two techniques do not produce comparable results [11]. In addition, there is considerable variability among the measurements determined with commercial ELISA kits from different companies. Our own data indicate that the concentrations of $\text{IFN}\gamma$ in supernatants of PHA-induced PBMC were not only different when assessed by ELISA and bioassay, but also the levels obtained by ELISA were not correlated with the levels detected in bioassays ($r^2=0.02$; unpublished data). The lack of correlation between measurements by ELISA and by bioassay is particularly important in aging research, since it is not yet determined whether cytokines produced by the elderly are equally functional to those of young.

Some investigators have attempted to bypass the problems of measuring protein in supernatants by evaluating the mRNA for cytokines produced in young and elderly cells. While levels of mRNA provide valuable information about age-related alterations in the transcription of cytokine genes, mRNA results do not always reflect cytokine production and secretion. For example, Holbrook and colleagues [12] measured splenocyte IL-2 production and IL-2 mRNA in young and aged rats and found that in 25% of the rats protein and mRNA levels were mismatched (i.e. an age-related decline in protein was accompanied by an age-related increase in mRNA or vice-versa). We found similar discordance in humans between PHA-induced $\text{IFN}\gamma$ mRNA and $\text{IFN}\gamma$ production

as assayed by bioassay [Ryan and Murasko, submitted].

A new approach has been to measure cytokines after translation, but before secretion from the cell. In this approach, lymphocytes are chemically treated to inhibit protein secretion. Cells are then permeabilized so that fluoresceinated antibodies specific for the cytokines of interest enter the cell and bind to the cytokine. The percent of cells producing the cytokine are quantitated using flow cytometry. This technique, while not yet commonly employed, allows not only assessment of the percent of cells producing any particular cytokine, but also identification of the cell types that have been induced to produce the cytokines. This is accomplished by labeling both surface membrane markers and intracellular cytokines with specific antibodies. This technique, however, gives no indication of the amount of cytokine produced per cell. In addition, in our experience, normal mitogenic stimulation of PBMCs induces a fairly low percentage (<10%) of positive cells in both young and elderly, making evaluation of age-related changes difficult. Potentiation of the signal by hyperstimulating cells is possible, but may result in responses that do not reflect *in vivo* age-related alterations.

In summary, in order to critically evaluate the current literature on changes in cytokine production with age, the health of the subjects, the cell types and stimulus used, and the techniques employed to measure cytokines must be carefully considered. While it is important not to generalize about information gained from one system to another, the findings of various systems can be used to develop an overall picture of the age-related differences in immune cell regulation of cytokine production. In this review, comparisons will be made among studies using the same cell types and stimuli. Efforts will be made to draw general conclusions from reports of age-related changes and, where possible, to explain conflicting reports.

Type 1 cytokines

IL-2

Interleukin-2 (IL-2), previously known as T-cell growth factor (TCGF), is produced by T-cells and is necessary for the activation and proliferation of T-lymphocytes [13]. IL-2 also stimulates B-cells and activates NK-cells [13]. Since the decline in lymphoproliferative response is one of the most consistent age-related changes in immune function, investigators have focused on IL-2 because of its inherent role in T-cell proliferation. The first report of age-related changes in IL-2 production in humans demonstrated that PHA and ConA stimulated PBMC from elderly subjects produced less IL-2 than young controls as measured by bioassay [14]. Since that sentinel article, 20 studies that meet our criteria have evaluated IL-2 production in young and elderly subjects (Table 2). Of these studies, 16 evaluated IL-2 production, while four assessed both IL-2 and IL-2 mRNA. Seventeen studies employed PBMC and three used purified T-cells.

Table 2. Type 1 Cytokines

Cytokine	Specimen	Culture Conditions: Stimulus	Time	Result	Assay	Subjects: Number (age range or mean $\pm$ SD) Elderly	Subjects: Number (age range or mean $\pm$ SD) Young	Health Status	Reference
IL-2	PBMC	PHA	24 hrs	O<Y	BIOASSAY	10 (76)	10 (25.4)	Senieur	[20]
	PBMC	PHA or ConA	48 hrs	O<Y	BIOASSAY	10 (67-81)	7 (21-30)	NR	[14]
	PBMC	PHA	48 hrs	O<Y	BIOASSAY	10 (70-105)	10 (20-39)	healthy	[10]
	PBMC	PHA	48 hrs	O<Y	BIOASSAY	28 (61-79)	145 (18-30)	healthy	[39]
	PBMC	PHA	72 hrs	O<Y	BIOASSAY	12 (>60)	12 (<40)	healthy	[19]
	PBMC	PHA	72 hrs	O<Y	BIOASSAY	13 (>65)	14 (<39)	healthy	[21]
	PBMC	PHA	72 hrs	O<Y	BIOASSAY	17 (>60)	13 (<40)	healthy	[22]
	PBMC	PHA	48 hrs	O<Y	ELISA	16 (79.6 $\pm$ 7.5)	16 (24.6 $\pm$ 3.1)	Senieur	[18]
	PBMC	PHA or ConA	48 hrs	O=Y	BIOASSAY	6 (65-84)	7 (25-35)	healthy	[54]
	PBMC	PHA	48 hrs	O=Y	BIOASSAY	31 (71-85)	54 (23-39)	Senieur	[9]
	WHOLE BLOOD	PHA	48 hrs	O=Y	ELISA	41 (>65)	44 (25-34)	Senieur	[38]
	PBMC	PHA	48&72 hrs	O=Y	ELISA	46 (65-93)	19 (21-32)	healthy	[A]
	PBMC	PHA or anti-CD2 & CD28	72 hr	O=Y	BIOASSAY	41 (75-84)	23 (19-28)	Senieur	[23]
	T CELLS	anti-CD2	48 hrs	O=Y	ELISA	9 (>60)	8 (<40)	healthy	[15]
	T CELLS	anti-CD3	48 hrs	O<Y	ELISA	9 (>60)	8 (<40)	healthy	[15]
IL-2 mRNA	T CELLS	anti-CD3	72 hrs	O<Y	BIOASSAY	12 (65-85)	12 (20-29)	healthy	[16]
	PBMC	PMA/A23187	72 hrs	O=Y	BIOASSAY	8 (>60)	8 (<40)	healthy	[19]
	T CELLS	PMA/A23187	24 hrs	O=Y	BIOASSAY	12 (61-85)	8 (20-38)	healthy	[17]
	PBMC	Trivalent influenza vaccine	24 hrs	O=Y	BIOASSAY	12 (61-85)	8 (20-38)	healthy	[17]
	PBMC	post-vaccination	3 days	O<Y	BIOASSAY	26 (62-100)	9 (<35)	Senieur	[24]
	PBMC	A/Texas (H1N1)	2 days	O<Y	BIOASSAY	31 (62-85)	29 (22-39)	healthy	[26]
	PBMC	pre-vaccination		O=Y					
	PBMC	post-vaccination		O=Y					
	PBMC	A/Beijing (H3N2)	2 days	O=Y	BIOASSAY	13 (60-84)	13 (22-32)	healthy	[25]
	PBMC	pre & post-vaccination		O<Y					
	PBMC	A/Shanghai (H3N2)		O=Y					
	PBMC	pre-vaccination		O=Y					
	PBMC	post-vaccination		O=Y					
	PBMC	A/Philippines (H3N2)		O=Y					
	PBMC	pre & post-vaccination		O=Y					
IL-2R	PBMC	MLC	5 days	O=Y	RIA	16 (62-81)	17 (23-43)	healthy	[27]
	PBMC	PHA	6 hrs	O<Y	NORTHERN	12 (>60)	12 (<40)	healthy	[19]
	PBMC	PHA	6 hrs	O<Y	NORTHERN	17 (>60)	13 (<40)	healthy	[22]
	PBMC	PHA	24 hrs	O<Y	ISH-VIA	6 (76)	6 (25.4)	Senieur	[20]
	PBMC	PMA/A23187	24 hrs	O=Y	NORTHERN	8 (>60)	8 (<40)	healthy	[19]
	T CELLS	PMA/A23187	24 hrs	O=Y	NORTHERN	12 (61-85)	8 (20-38)	healthy	[17]
	PBMC	ConA	24 hrs	O=Y	FLOW CYTOMETRY	10 (21-40)	10 (65-96)	healthy	[32]
	PBMC	PHA	48 hrs	O<Y	FLOW CYTOMETRY	14 (72-91)	14 (24-29)	Senieur	[33]
	PBMC	PHA	48 hrs	O<Y	IFA	50 (65-85)	50 (23-37)	healthy	[58]
	PBMC	PHA	72 hrs	O<Y	FLOW	12 (>60)	12 (<40)	healthy	[19]
	PBMC	PHA	72 hrs	O<Y	FLOW	13 (>65)	14 (<39)	healthy	[21]
	PBMC	PHA	72 hrs	O<Y	ELISA	15 (>60)	13 (<40)	healthy	[22]
	PBMC	PHA	72 hrs	O=Y	FLOW	10 (>60)	10 (<40)	healthy	[59]
	PBMC	PHA	72 hrs	O=Y	IMMUNOGOLD	15 (>70)	14 (<38)	healthy	[34]
	PBMC	PHA	72 hrs	O<Y	¹²⁵ I-L-2 BINDING	5 (76)	5 (25.4)	Senieur	[20]
	PBMC	PMA/A23187	72 hrs	O=Y	FLOW CYTOMETRY	8 (>60)	8 (<40)	healthy	[19]
	T CELLS	PHA & IL-2	3 days	O<Y	SDS-PAGE	10 (70-91)	10 (22-29)	Senieur	[60]
	T CELLS	anti-CD3	48 hrs	O<Y	¹²⁵ I-L-2 BINDING	2 (65-85)	12 (20-29)	healthy	[16]

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Table 2. Type 1 Cytokines (Continued)

Cytokine	Specimen	Stimulus	Culture Conditions:	Time	Result	Assay	Subjects: Number (age range or mean $\pm$ SD) Elderly Young		Health Status	Reference
IL-2R mRNA	PBMC	PHA		14 hrs	O<Y	NORTHERN	12 (>60)	12 (<40)	healthy	[19]
	PBMC	PHA		14 hrs	O<Y	NORTHERN	17 (>60)	13 (<40)	healthy	[22]
	PBMC	PMA/A23187		24 hrs	O=Y	NORTHERN	8 (>60)	8 (<40)	healthy	[19]
IFN γ	T CELLS	anti-CD3		6 hrs	O=Y	NORTHERN	9 (>60)	7 (<40)	healthy	[15]
	T CELLS	anti-CD2		6 hrs	O=Y	NORTHERN	9 (>60)	5 (<40)	healthy	[15]
	T CELLS	PMA/A23187		24 hrs	O=Y	NORTHERN	12 (61-85)	8 (20-38)	healthy	[17]
	PBMC	PHA		24 hrs	O<Y	RIA	28 (66-87)	71 (20-64)	healthy	[35]
	PBMC	PHA		48 hrs	O<Y	ELISA	16 (79.6 $\pm$ 7.5)	16 (24.6 $\pm$ 3.1)	Senieur	[18]
	PBMC	PHA		48 hrs	O<Y	BIOASSAY	28 (61-79)	145 (18-30)	healthy	[39]
	PBMC	PHA		72 hrs	O=Y	BIOASSAY	58 (>65)	17 (22-38)	healthy	[31]
	PBMC	PHA		72 hrs	O>Y	BIOASSAY	46 (65-93)	19 (27.5 $\pm$ 0.8)	healthy	[A]
	WHOLE BLOOD	PHA		96 hrs	O>Y	BIOASSAY	40 (>65)	44 (25-34)	Senieur	[38]
	PBMC	ConA		24 hrs	O>Y	RIA	15 (>60)	14 (15-30)	Senieur	[37]
	PBMC	PMA/ionomycin		24 hrs	O=Y					
	PBMC	anti-CD3		5 days	O=Y	ELISA	16 (>65)	16 (<30)	Senieur	[40]
	PBMC	PHA/PMA		72 hrs	O=Y	ELISA	13 (80.8 $\pm$ 2.1)	13 (26.8 $\pm$ 0.8)	Senieur	[46]
	PBMC	TSST		5 days	O<Y	ELISA	6 (70-90)	6 (20-35)	Senieur	[36]
	PBMC	PPD, tetanus toxoid, or trivalent influenza vaccine		5 days	O=Y	ELISA	16 (>65)	16 (<30)	Senieur	[40]
IFN γ mRNA	PBMC	trivalent influenza vaccine pre & post-vaccination		5 days	O<Y	BIOASSAY	270 (65-97)	30 (23-38)	healthy	[41]
	PBMC	MLC		120 hrs	O=Y	RIA	16 (62-81)	17 (23-43)	healthy	[27]
	T CELLS	PMA/A23187		24 hrs	O=Y	RIA	12 (61-85)	8 (20-38)	healthy	[17]
	T CELLS	A/AA/Matton (H1N1)		72 hrs	O<Y	FLOW CYTOMETRY	2 (65-86)	2 (23-35)	healthy	[42]
	T CELLS	PMA/A23187		24 hrs	O>Y	NORTHERN	12 (61-85)	8 (20-38)	healthy	[18]
	PBMC	PHA		24 hrs	O>Y	RT-PCR	58 (65-92)	35 (20-36)	healthy	[B]
	PBMC	PHA		3 days	O=Y	ELISA	9 (77-99)	20 (NR)	frail	[43]
		SAC		24 hrs	O=Y					

A = Bernstein E, Snyder S, Otubu O, Murasko DM. Mitogen-induced cytokine production by elderly subjects, submitted.

B = Ryan Q and Murasko DM. Effect of age on mitogen induced lymphokine production: Differential alteration of IL-2 and IFN γ gene expression in elderly humans.

NR = not reported

RIA = radioimmunoassay

ISH-VIA = in situ immunohistochemistry-video image analysis

IFA = Immunofluorescence Assay

Twelve of the studies stimulated PBMC non-specifically: all of the twelve utilized the mitogens PHA or ConA, but one also stimulated with a combination of anti-CD2 and anti-CD28 antibodies. Of these 12 studies stimulating PBMC with mitogen, 8 reported an age-related decline in IL-2 production and 4 reported no change. The health status of subjects, culture conditions, or measurement techniques did not vary significantly between studies that reported an age-related, or no decline in mitogen-induced IL-2 production. The majority of the studies (10/12) measured IL-2 production by bioassay using CTLL cells. The two studies that employed ELISA [18, Bernstein et al. submitted] showed contrasting results, with one reporting no age-related difference in IL-2 production [Bernstein et al. submitted] and the other reporting a decline [18]. Differences in sample size appear to distinguish studies that found an age-related difference in IL-2 production from those that did not. All but one of the studies that found a difference evaluated 10-17 elderly [10, 14, 18, 20-22], while 3 of the 4 studies reporting comparable IL-2 production between young and elderly subjects evaluated 31-46 elderly [9, 23, Bernstein et al. submitted]. Interestingly, one of the larger studies that demonstrated no age-related difference in IL-2 production by mitogen-induced PBMC also evaluated IL-2 production by PBMC induced with anti-CD2/CD28 antibodies [23]. In both cases IL-2 production and proliferative responses were comparable between the 23 young and 41 elderly subjects [23]. It is possible that the discrepant results obtained in studies using similar experimental techniques (mitogen-stimulated PBMC production of IL-2 measured by bioassay) are due to inclusion of too few subjects to account for the inherent heterogeneity among human subjects. Thus, it is possible that the difference in outcomes may reflect a sampling error, rather than a true biological difference.

The four studies in which PBMC were stimulated with specific antigen also demonstrated highly variable results. In three separate studies by McElhaney and colleagues, PBMC were stimulated with influenza antigen (i.e. live influenza virus or trivalent subvirion influenza vaccine) [24-26]. When PBMC were stimulated with a trivalent whole inactivated influenza vaccine, IL-2 was significantly reduced in the elderly 8 weeks after vaccination relative to young controls [24]. However, age-related differences in IL-2 production appeared to be strain dependent when specific influenza strains were used as stimuli. When PBMC were evaluated before immunization, stimulation with live A/Texas virus or A/Shanghai virus resulted in decreased IL-2 production with age, while stimulation with A/Beijing virus or A/Philippines virus showed no age-related change [25, 26]. After influenza vaccination, no age-related differences in IL-2 production were seen, regardless of viral strain utilized [25, 26].

One of the difficulties in measuring responses to specific antigens is the low precursor frequency of lymphocytes specific for a particular antigen. The consequence of this low frequency is that *in vitro* proliferative responses and cytokine production in short term cultures may be near or below the level of detection of available assays.

One study attempted to control for this problem by using alloantigen responses in a one way mixed lymphocyte reaction which induces strong proliferative and cytokine responses [27]. In this study, there was an age-related decline in proliferation of alloactivated PBMC, but an age-related increase in IL-2 production, as measured by a radioimmunoassay (RIA). The discordance between increased IL-2 production and decreased proliferative responses in the elderly of this study could not be explained by altered kinetics of production or increased utilization of IL-2 by PBMC of young subjects. Interestingly, in their analysis of individual subjects, IL-2 production and proliferation were positively correlated in the elderly ($r=.64$; $p<0.008$), but not in the young. This finding concurs with other studies that have not shown strong correlations between IL-2 production and proliferation of PBMC in response to ConA stimulation [28].

Of all the studies using PBMC, only one [19] evaluated IL-2 production in response to PMA/Ca⁺⁺ ionophore. This stimulus, as mentioned earlier, can induce cytokine production and proliferation without interacting with the cell membrane. Song and colleagues evaluated PBMC of 8 elderly and 8 young subjects and found no difference in IL-2 production in response to PMA/Ca⁺⁺ ionophore. However, an age-related decline in IL-2 production was observed after PHA stimulation of PBMC [19]. These findings suggest that an age-related defect in IL-2 signaling pathways occurs before pKC localization and Ca⁺⁺ mobilization (i.e. PIP₂ metabolism pathway). Unfortunately, it seems that the responses to PHA and to PMA/Ca⁺⁺ ionophore were not measured in the same subjects. Therefore, it is impossible to definitively determine whether these results reflect a true disparity between activation pathways or rather reflect the heterogeneity between two small subject populations (PHA: 12 young and 12 elderly subjects; PMA/Ca⁺⁺ ionophore: 8 young and 8 elderly).

To our knowledge, there are no other studies evaluating age-related changes in IL-2 production by PBMC stimulated with PMA/Ca⁺⁺ ionophore. However, several studies using T cells purified from PBMC support the conclusion that the age-associated decrease in IL-2 production is a membrane mediated alteration. One study demonstrated an age-related decline in IL-2 production by T cells in response to anti-CD3 [16], while in another study stimulation of T-cells with PMA/Ca⁺⁺ ionophore revealed no difference in IL-2 production [17]. Song and colleagues also measured T cell production of IL-2 in response to either immobilized anti-CD3 or anti-CD2. This study showed that while stimulation with anti-CD3 revealed age-related declines in T-cell proliferation and IL-2 production, stimulation with anti-CD2 showed no such decline [15]. In this study, the difference between stimulation through the TCR/CD3 complex or CD2 activation pathways could not be explained by differences in the number of cells positive for CD2⁺ or CD3⁺. It should be noted, however, age-related increases

in the expression of CD2 molecules per cell and decreases in CD3 molecules per cell have been reported in another study [30].

In summary, of the 19 reports investigating IL-2 protein levels after stimulation with mitogen or antigen, 11 showed an age-related decline in IL-2 production, five showed no significant difference ($p>0.05$), and one showed an age-related increase. Two studies using live influenza virus demonstrated varying results: one strain in each paper showed a decrease, while another strain resulted in no age-associated change. Although it is fairly well accepted that aging in mice and humans is accompanied by a decline in IL-2 production, our review of the current literature indicates that the decrease in IL-2 is seen in many, but not all, studies. Therefore, a decrease in IL-2 production cannot be presented as a definitive age-associated alteration in humans. Even with strict comparisons of studies, such that only studies employing the same cell type from subjects of comparable health, with the same stimulus, culture conditions, and measuring techniques are evaluated, disparate results are obtained.

It is possible that the difference in results reflects experimental systems that were not optimal for assessing cytokine production (e.g. concentration of stimulus or time of evaluation). Another possibility for the discrepancy among studies is the heterogeneity of IL-2 production in the elderly [31], such that subject sampling may randomly skew for one result or another. This has been demonstrated in studies evaluating IL-2 production and mRNA in parallel using cells stimulated under the exact same conditions [17,20,21,22]. Those studies showing age-related declines in IL-2 production also found declines in IL-2 mRNA [20,21,22], while those showing no difference in IL-2 production also found no difference in IL-2 mRNA [17,20]. Although mRNA and protein levels are not directly correlated, the finding of a defect, or no difference, in both parameters may suggest that the finding reflects biological heterogeneity between the two study populations. The composite data of the reviewed data clearly suggests that while some elderly produce less IL-2 than the young, not all elderly demonstrate an age-related decrease in IL-2 production. A question for future investigation is to determine the factors that make those elderly who produce IL-2 at the level of young different from those who do not.

If IL-2 is the major factor responsible for the age-associated decrease in lymphoproliferation, then supplementation with exogenous IL-2 should rescue the decreased lymphoproliferative responses of the elderly. Although addition of IL-2 to PBMC cultures from elderly increased the proliferative response, it did not restore lymphoproliferation to the level of PBMC from young [14]. Similarly, our laboratory has reported that among elderly subjects selected for lower proliferative responses to mitogen relative to young controls, addition of IL-2 was able to increase proliferative responses to the level of young only in about 30% of these elderly [10,31]. Investigators have suggested that these results suggest

a defect in the expression of IL-2R. Eight of the 11 studies that have assessed mitogen-induced expression of IL-2R on lymphocytes have shown a significantly reduced level of IL-2R in the elderly. While the lack of agreement among all studies may reflect population heterogeneity, it is possible that the kinetics of IL-2R expression during experimental protocols may explain these inconsistent results. Recent data suggest that activation-induced early expression of IL-2R on PBMC of elderly subjects is decreased, while later expression may be equivalent to that of PBMC from young subjects [32]. However, of the three studies demonstrating no difference between young and elderly, one was performed early (24 hr) and two late (72 hr). The one study that stimulated PBMC with phorbol esters and ionomycin did not reveal any difference in IL-2R expression between young and elderly. While these results suggest that changes in IL-2R expression may reflect early alterations in membrane activation, it should be noted that when PBMC from elderly individuals were selected for expression of IL-2R comparable to young controls, exogenous IL-2 was still unable to restore the proliferative response to the level of the young [33]. Therefore, while IL-2 and IL-2R induction are essential for lymphoproliferation, the age-associated decrease in proliferation may not be a direct consequence of changes in IL-2 or IL-2R.

IFN γ

Another type 1 cytokine, IFN γ , is secreted mainly by T-lymphocytes and NK cells, and has many effector functions including anti-viral activity, induction of MHC class I and II antigens, activation of macrophages, and upregulation of nitric oxide synthetase [13]. Since IFN γ , like IL-2, is a strong inducer of cell-mediated immune (CMI) responses, investigators have hypothesized that age-related changes in IFN γ may play a role in the decline of CMI with age. As seen in Table 2, the number of studies that addressed IFN γ expression are more limited than those that have examined IL-2. In addition, the stimuli used for induction, the time of assessment and the methods of quantitation are more varied. It is, therefore, not surprising that IFN γ has been shown to increase, decrease, or remain unchanged with age. These inconsistencies may very well be due to the experimental stimulus and/or the phenotype of the responding cells.

Of the twelve studies utilizing PBMC or whole blood, six stimulated with PHA, one with ConA, one with anti-CD3 (OKT3), one with PHA and PMA, one with a superantigen, (toxic shock syndrome toxin, TSST), two with specific antigens (influenza vaccine, PPD, or tetanus toxoid), and one by mixed lymphocyte reaction. Stimulation of PBMCs with non-specific stimuli such as mitogens, anti-CD3, or the superantigen TSST resulted in age-related decreased IFN γ production in three cases [18,35,36], no difference in another four cases [31,38,39,40] and an increase in two cases [37, Bernstein, et al. submitted]. The four studies using the largest

number of subjects [31,35,38, Bernstein et al. submitted] all used PBMCs from healthy elderly subjects that were stimulated with PHA, and still reported three different outcomes. The largest of these studies (58 elderly), as well as a study with 28 elderly, showed no age-associated change in IFN γ production by PBMCs in response to PHA stimulation [31,39], another with 46 elderly showed an age-related increase [Bernstein, et al. submitted], while the fourth, also with 28 elderly, showed an age-related decline [35]. These studies varied both in the incubation time prior to harvesting culture supernatants and in the techniques used to measure IFN γ . In the study that demonstrated an age-related decline, supernatants were harvested at 24 hours and IFN γ measured by RIA [35], while in the other three studies IFN γ levels were measured by bioassay and supernatants were obtained either 48 hours [39] or 72 hours [31, Bernstein, et al. submitted]. It is unclear whether the discrepant results of these studies are due to altered kinetics of IFN γ production or variations in experimental techniques.

In three of the studies evaluating PBMC stimulation with specific stimuli, two reported no significant age-related changes in IFN γ production [27,40]. The largest study, in which 270 elderly were evaluated for IFN γ production in response to trivalent subvirion influenza vaccine before and after vaccination, indicated that the elderly produced significantly lower IFN γ levels both pre- and post-vaccination [41]. Similar, but non-significant, trends have been reported in the smaller studies. For example, Bodnar and colleagues [40] stimulated PBMC from 16 young and 16 elderly subjects with trivalent whole inactivated influenza vaccine, PPD, or tetanus toxoid and found in all cases non-significantly lower IFN γ production in the elderly compared to the young. In our experience, a significantly greater number of elderly than young do not produce detectable levels of IFN γ after stimulating with specific antigen such as influenza vaccine. Thus, when evaluating mean IFN γ production, the responses are skewed in the elderly, but not in the young. This poses a particularly difficult problem in interpreting data from studies with small samples. Yet in the case of Bodnar's study, there appeared to be equal numbers of young and elderly who did not produce detectable levels of IFN γ . It is unclear whether the lack of significant differences seen in two of the three studies evaluating PBMCs specifically stimulated with antigen, represents a true finding or reflects the small numbers of subjects (13-16) used in these studies.

Since IFN γ can be produced by NK cells as well as T cells, some of the discrepancies in IFN γ production using PBMC could reflect differential activation of NK cells by the various activation protocols. Two studies utilized purified T cells to evaluate age-related changes in IFN γ production. One study evaluated T-cell production in response to stimulation with live influenza virus and showed a 2-fold, but non-significant decrease, in the number of IFN γ producing T-cells when stimulated with live influenza virus as measured by immunofluorescent

intracellular cytokine staining [42]. These results are consistent with the two studies of influenza stimulation of PBMC in which both studies showed a decrease, but only one was significant. The results from the T cells stimulated with influenza also suggest that the differences in IFN γ production by PBMC of the elderly are due, at least in part, to alterations in T cells. The other study which evaluated T-cells, stimulated with PMA/Ca⁺⁺ ionophore. This study showed an age-related increase in IFN γ mRNA but no difference in IFN γ protein [17]. Since stimulation with PMA/Ca⁺⁺-ionophore does not require any specific membrane interactions, it is very difficult to compare these results to those of T cells or PBMC stimulated with mitogen or antigen, since these latter events require membrane activation. The one other study that assessed whether or not age-associated changes in IFN γ production was a membrane associated phenomenon observed that PBMC stimulated with ConA demonstrated an age-associated increase in IFN γ production, while stimulation of the same PBMC with PMA and ionomycin resulted in comparable IFN γ production by young and elderly [37].

In summary, no definitive conclusion can be drawn regarding the effect of age on IFN γ production in humans with the currently available data. Non-specific stimulation with PHA, anti-CD3, or superantigen TSST, demonstrated the full range of results from increased to decreased IFN γ production with age. However, there may be a trend for decreased IFN γ production in response to antigen specific stimulation.

IL-12

Although IL-12 is not produced by T cells, and therefore is not classically considered a type 1 cytokine, it is still an important regulator of the type 1 cytokines. It is secreted by macrophages and B-cells and enhances CMI by upregulating IFN γ production [13]. As seen in Table 2, only one study has reported on the effect of age on IL-12 production [43]. This study of 9 frail institutionalized elderly demonstrated no age-related difference in IL-12 production when PBMC were stimulated with either PHA or superantigen (*Staphylococcus aureus* Cowan). Given the key role of IL-12 in regulating IFN γ production and thus cell-mediated immune responses, further research into age-related changes in IL-12 is necessary. Fortunately, commercially available techniques for measuring IL-12 (e.g. ELISA) are becoming more readily available.

Type 2 Cytokines

IL-4

IL-4 is considered the prototypic type 2 cytokine in that it strongly supports humoral immune responses. It is secreted by T-cells, B-cells, macrophages, mast cells, and basophils. It induces B-cell differentiation and antibody class switching to IgE and IgG₄ [13]. In addition, it plays an important regulatory role in inhibiting the production of Type 1 cytokines and stimulating the production of Type 2 cytokines [1]. Since in mice, the

age-related switch from Type 1 to Type 2 cytokines is associated with decreased cell-mediated immunity and increased humoral responses [44], many researchers have attempted to look for an age-related change in IL-4 production in humans that could explain the diminished cell-mediated immune responses, such as decreased delayed type hypersensitivity. Table 3 summarizes the four studies evaluating IL-4 production in young and elderly.

Three of the studies stimulated PBMC with PHA, the fourth stimulated PBMC with ConA. Three studies found no age-related difference in IL-4 production [35,37, Bernstein et al. submitted], while one could not detect any IL-4 after stimulation of either young or elderly after PHA or anti-CD3 stimulation [23]. The fourth study, however, did find an age-related increase in IL-4 production after PBMC stimulation with anti-CD2 and anti-CD28 together [23]. These authors argue that the pattern of IL-4 production they observed mirrored the proliferative responses to the stimuli; i.e., there was no age-related difference in proliferative responses or IL-4 production by PBMCs to PHA or anti-CD3, but there was an age-related increase in both proliferation and IL-4 production after stimulation with anti-CD2 and anti-CD28 together [23]. There are two concerns with this conclusion. First, since no IL-4 was detectable after PHA or anti-CD3, it is difficult to state whether there is no difference between groups, or, if there is a difference, but only in a range below the limits of the assay being utilized. Second, they did not observe an age-related decrease in PHA and anti-CD3 induced proliferation, that is seen in most studies. They argue that this is due to their use of the Senieur criteria for subject selection. Although other studies have used the Senieur criteria and have observed decreased proliferation, it is possible that their results reflect a very healthy elderly population.

Several laboratories have attempted to evaluate the age-related changes in IL-4 production of PBMC stimulated with various antigens. Bodnar and colleagues were unable to induce detectable levels of IL-4 production by PBMC from young or elderly after stimulation with trivalent whole inactivated influenza vaccine, PPD, or tetanus toxoid [40]. Similarly in our laboratory, IL-4 was not detectable (<8 pg/ml) after stimulation of PBMC with trivalent influenza subviral vaccine, although the same PBMC produced detectable amounts of IL-4 in response to PHA.

In summary, although no data are currently available on changes in IL-4 production upon antigen stimulation, the two fairly large studies in which IL-4 could be detected after induction with PHA indicate that both young and elderly produce comparable amounts of IL-4. Studies with additional stimuli are necessary, however, before the full potential of PBMC from elderly subjects to produce IL-4 can be ascertained.

IL-6

IL-6 is a type 2 cytokine that is as equally important to T and B cell responses, as it is to the acute phase inflammatory responses. It is secreted by T cells, monocytes, macrophages, fibroblasts, epithelial cells, mast cells, endothelial cells and hepatocytes [13]. As well as inducing acute phase proteins, IL-6 induces T-cell activation and differentiation, B cell differentiation and mucosal IgA responses. Table 3 summarizes the available literature evaluating IL-6 production between young and elderly.

Four studies have evaluated age-associated changes in PBMC or whole blood production of IL-6 as measured by ELISA. Candore and colleagues stimulated PBMC from 5 elderly and 9 young subjects with PHA and found comparable IL-6 production after 24 hours of incubation

Table 3. Type 2 Cytokines

Cytokine	Specimen	Culture Conditions: Stimulus	Time	Result	Assay	Subjects: Number (age range or mean $\pm$ SD)		Health Status	Reference
						Elderly	Young		
IL-4	PBMC	PHA	24 hrs	O=Y	ELISA	31 (66-87)	70 (20-64)	healthy	[35]
	PBMC	PHA	48 & 72 hrs	O=Y	ELISA	46 (65-93)	19 (21-32)	healthy	[A]
	PBMC	anti-CD2&CD28	72 hrs	O>Y	ELISA	41 (75-84)	23 (19-28)	Senieur	[23]
	PBMC	ConA	24 hr	O=Y	ELISA	15 (>60)	14 (15-30)	Senieur	[37]
		PMA/ionomycin		O=Y					
IL-6	PBMC	PHA	24 hrs	O=Y	ELISA	5 (66-87)	9 (20-64)	healthy	[35]
	PBMC	PHA/PMA	24 hrs	O=Y	ELISA	13 (80.8 $\pm$ 2.1)	13 (26.8 $\pm$ 0.8)	Senieur	[46]
			48 & 72 hrs	O>Y					
	WHOLE BLOOD	PHA	96 hrs	O=Y	ELISA	40 ($\geq$ 65)	39 (25-34)	Senieur	[38]
	PBMC	trivalent influenza vaccine pre & post-vaccination	5 days	O=Y	ELISA	270 (65-97)	30 (23-38)	healthy	[41]
	PLASMA	none	NA	O=Y	ELISA	21 (61-70)	34 (21-40)	healthy	[51]
	PLASMA	none	NA	O>Y	ELISA	13 (60-74)	20 (25-40)	healthy	[49]
	PLASMA	none	NA	O>Y	BIOASSAY	18 (67-99)	20 (21-35)	healthy	[50]
	SERA	none	NA	O=Y	BIOASSAY	26 (75-92)	13 (22-35)	healthy	[52]
IL-10	PBMC	none	24 hrs	O=Y	ELISA	12 (75-102)	12 (19-34)	Senieur	[53]
	PBMC	PHA	48 & 72 hrs	O=Y	ELISA	46 (65-93)	19 (21-32)	healthy	[A]
	PBMC	TSST	5 days	O=Y	ELISA	6 (70-90)	6 (20-35)	Senieur	[36]
	PBMC	SAC	24 hrs	O=Y	ELISA	9 (77-99)	20 (NR)	frail	[43]
	PBMC	trivalent influenza vaccine pre-vaccination	5 days	O=Y	ELISA	270 (65-97)	30 (23-38)	healthy	[41]
		none	NA	O=Y	BIOASSAY	26 (75-92)	13 (22-35)	healthy	[52]
	SERA								

[35]. Fagiolo and colleagues stimulated PBMCs from 13 young and 13 Senieur elderly with a combination of PHA and PMA and measured IL-6 production at 24, 48, and 72 hours [46]. In agreement with the study of Candore, et al, Fagiolo and colleagues found comparable IL-6 production between young and elderly when measured at 24 hours. In contrast, PBMCs from the elderly produced increasing levels of IL-6 over the next 48 and 72 hours of incubation with PHA and PMA, while PBMC from the young maintained the level produced at 24 hours [46]. This age-related alteration in kinetics of the IL-6 response resulted in significantly increased mean IL-6 production by elderly PBMC, compared to the mean production by young PBMC, at 48 and 72 hours. Since PHA is a T cell mitogen and PMA induces the activation of monocytes in the PBMC culture, as well as activation of pKC without specific membranes interaction, the cell type and the mechanism contributing to the altered kinetics of IL-6 production by PBMC of elderly subjects cannot be distinguished in this study. Interestingly, assessment of IL-6 production by whole blood incubated with PHA for 96 hour indicated no difference between young and elderly subjects. Therefore, if kinetics of IL-6 production is an important age-associated change, alterations between young and elderly may only be apparent at intermediate timepoints.

The effect of age on IL-6 production by PBMC stimulated with specific antigen has only been evaluated in response to influenza vaccine [41]. No age-related differences in IL-6 production were observed when PBMCs from 30 young and 270 elderly subjects were stimulated *in vitro* with trivalent influenza vaccine before and after influenza immunization. Interestingly, there was considerable heterogeneity in IL-6 responses in both the young and elderly before and after influenza vaccination with concentrations ranging from 0 pg/ml to 2661 pg/ml.

In the absence of inflammation, IL-6 is usually undetectable in the serum or plasma [47]. Although they included subjects younger than 60 in the "elderly" group when comparing with young controls, Wei and colleagues were the first to demonstrate an age-related increase in levels of IL-6 in sera [48]. Since then, four studies have evaluated IL-6 in plasma or sera with varying results. Two studies measured IL-6 in plasma and found an age-related increase [49,50]. One measured IL-6 by an ELISA [49], while the other used bioassay [50]. Two other studies found no difference in IL-6 levels in plasma by ELISA [51] or in sera by bioassay [52].

The variability in results seen in plasma/sera may be due to a number of factors. First, although all of the subjects included in these four studies were reported to be healthy, they were not screened specifically for inflammatory diseases (e.g. arthritis) which are known to result in higher levels of circulating IL-6 [47]. Second, the concentration of IL-6 in plasma/sera is so low that it is usually below the level of detection of most assays. James and colleagues reported that IL-6 was not detectable in the plasma of half of the subjects, while in the

other half it varied from 1-10 pg/ml, with only three of 21 subjects having IL-6 concentration greater than 70 pg/ml [51]. In this study, the presence or absence of detectable levels of IL-6 in the plasma was unrelated to age. Kania and colleagues also reported very low levels of IL-6 in plasma with the maximum concentration measured being 3.6 pg/ml [49]. However, they were still able to show an age-associated increase in IL-6 concentration ($p < 0.0001$).

Overall in the human system, the data for an age-related increase in IL-6 is not convincing. Studies using similar techniques and subject populations have contrasting results. Elevated levels of IL-6 have been clearly demonstrated in disease states [47] that are associated with aging. However, in the absence of disease, this review does not support an age-associated change in IL-6 production.

IL-10

IL-10 is produced by T and B cells, monocytes, and macrophages and acts to inhibit macrophage activity by inhibiting cytokine production and downregulating MHC class II antigen expression [13]. Like IL-4, IL-10 also plays a key regulatory role in inhibiting production of type 1 cytokines [1], thus downregulating cell-mediated responses. Investigators have hypothesized that an age-related increase in IL-10 production may influence the age-related decrease in cell-mediated immunity. A seminal article in mice showed an age-related increase in IL-10 production by ConA stimulated CD4⁺ cells [4]. Similar increases, however, have not been observed in humans as summarized in Table 3.

A number of stimuli have been utilized to evaluate PBMC production of IL-10. Llorente and colleagues evaluated basal IL-10 production by PBMC cultured for 24 hours without stimulation and showed comparable levels in young and elderly [53]. Stimulation of PBMCs with PHA [Bernstein, et al. submitted] or with superantigens TSST [36] or *Staphylococcus aureus* Cowan (SAC) [43] also demonstrated no age-related differences. Only one study in which PBMC were stimulated with influenza vaccine showed an age-related difference IL-10 production [41]. PBMC production of IL-10 after stimulation with trivalent influenza vaccine was measured by ELISA before and after *in vivo* influenza immunization in 270 elderly and 30 young subjects. Prior to vaccination, young and elderly produced comparable amounts of influenza vaccine-induced IL-10 *in vitro*. Vaccination resulted in significantly increased IL-10 production in the young, but not in the elderly. After vaccination, PBMC from elderly individuals produced less IL-10 after restimulation in culture with trivalent influenza vaccine than did PBMC from young controls [41]. In the only published study reporting on serum levels of IL-10, concentrations of IL-10 were found comparable in young and elderly subjects [52].

The data suggest that there is no overall difference in IL-10 production with age. The age-related decline in influenza vaccine-induced IL-10 production that is ob-

served only after influenza vaccination may indicate that an age-associated decline in IL-10 production in response to specific stimuli is subtle and may be unmasked only by *in vivo* immune challenges, such as illness or vaccination.

Proinflammatory Cytokines

IL-1- β

IL-1 β is secreted by monocytes, macrophages, neutrophils, epithelial cells, NK cells, and T and B cells [13]. It promotes B and T cell differentiation, activates NK cells, and increases expression of surface adhesion molecules like ICAM on endothelial cells. Table 4 summarizes the available studies on the effect of age on IL-1 β production.

Five studies evaluated IL-1 β production by PBMCs: three stimulated with LPS a potent inducer of monocytes/macrophages, one with PHA/PMA, and one by a mixed lymphocyte reaction. Of the three studies in which PBMC were stimulated non-specifically with LPS, one demonstrated an age-related increase in IL-1 β [18], one showed no difference [54], and one showed an age-related decrease in IL-1 β production [55]. These studies measured IL-1 β with ELISA and RIA. Although it might seem reasonable that different experimental techniques could explain the discrepant results, two of the studies used ELISA to measure IL-1 β and still had opposite results [18,55]. The small sample sizes of each study, coupled with the limited number of studies, makes it difficult to draw any conclusions about IL-1 β production by LPS-induced PBMCs.

Two studies evaluated IL-1 β production by isolated monocytes stimulated with LPS [56,57]. One demonstrated an age-related decline in IL-1 β production as

measured by bioassay in 5 young and 5 elderly [56]. The other demonstrated comparable IL-1 β production between 18 young and 16 elderly by ELISA [57]. Since LPS preferentially activates monocytes/macrophages, the findings of these studies using purified monocytes can be compared to those using PBMC. Thus of the five studies stimulating with LPS, two showed an age-related decline, two showed no difference, and one showed an age-related increase in IL-1 production. Analysis of various parameters of these studies revealed no factor which could explain the differences in results.

In accord with the above study that found age-related increases in IL-1 β production [18], Fagiolo and colleagues found that PBMC from Senieur elderly stimulated with a combination of PHA and PMA secreted greater IL-1 β than did PBMC from young subjects [46]. Although IL-1 β is produced mostly by monocytes, cytokines from T cells (e.g. IFN γ) can enhance IL-1 β production by monocytes. Since PHA/PMA activates both T cells and monocytes/macrophages, it is difficult to determine the contribution of T cells to IL-1 β production in this study.

Molteni and colleagues activated PBMCs specifically by a mixed lymphocyte reaction and found an age-related increase in IL-1 β production [27]. This age-associated increase in IL-1 β production was accompanied by an age-related decline in proliferative response to allogeneic stimulation. Therefore, in this system, there was a discordance between decreased proliferative response and increased IL-1 β production in the elderly.

In summary, considerable variation is observed among the small number of studies that have evaluated IL-1 production. Since the two studies that stimulated both T

Table 4. Proinflammatory Cytokines

Cytokine	Specimen	Culture Conditions:		Result	Assay	Subjects: Number (age range or mean $\pm$ SD)		Health Status	Reference
		Stimulus	Time			Elderly	Young		
IL-1 β	PBMC	LPS	24 hrs	O=Y	RIA	10 (65-84)	10 (25-35)	healthy	[54]
	PBMC	LPS	24 hrs	O<Y	ELISA	10 (80-88)	10 (22-39)	healthy	[55]
	PBMC	LPS	48 hrs	O>Y	ELISA	16 (79.6 $\pm$ 7.5)	16 (24.6 $\pm$ 3.1)	Senieur	[18]
	PBMC	PHA/PMA	72 hrs	O>Y	ELISA	13 (80.8 $\pm$ 2.1)	13 (26.8 $\pm$ 0.8)	Senieur	[46]
	PBMC	MLC	48 hrs	O>Y	BIOASSAY	16 (62-81)	17 (23-43)	healthy	[27]
	MONOCYTES	LPS	16 hrs	O<Y	BIOASSAY	5 (>65)	5 (=25)	healthy	[56]
	MONOCYTES	LPS	24 hrs	O=Y	ELISA	16 (65-79)	18 (20-27)	NR ²	[57]
IL-8	PBMC	LPS	24 hrs	O<Y	ELISA	10 (80-88)	10 (22-39)	healthy	[55]
	MONOCYTES	none	24 hrs	O<Y	ELISA	16 (65-79)	18 (20-27)	NR	[57]
		none	24 hrs	O=Y					
		LPS	24 hrs	O>Y					
		LPS	24 hrs	O=Y					
	SERA	none	NA	O<Y	ELISA	15 (76-82)	22 (26-42)	all w/ pneumonia	[55]
TNF- α	PBMC	LPS	24 hrs	O<Y	ELISA	10 (80-88)	10 (22-39)	healthy	[55]
	PBMC	LPS	48 hrs	O>Y	ELISA	16 (79.6 $\pm$ 7.5)	16 (24.6 $\pm$ 3.1)	Senieur	[18]
	PBMC	PHA/PMA	72 hrs	O>Y	ELISA	13 (80.8 $\pm$ 2.1)	13 (26.8 $\pm$ 0.8)	Senieur	[46]
	PBMC	MLC	48 hrs	O>Y	RIA	16 (62-81)	17 (23-43)	healthy	[27]
	MONOCYTES	LPS	24 hrs	O=Y	ELISA	16 (65-79)	18 (20-27)	NR	[57]
	SERA	none	0 hrs	O<Y	ELISA	15 (76-82)	22 (26-42)	all w/ pneumonia	[55]
	SERA	none	0 hrs	O=Y	BIOASSAY	26 (75-92)	13 (22-35)	healthy	[52]

cells and monocytes demonstrated increased production of IL-1, while the five studies that stimulated monocytes directly reported variable results, it is possible that the effect of age on monocyte production of IL-1 is highly dependent on the stimulus and the level of interaction with other cells. However, more studies utilizing various stimuli to induce the same monocyte or PBMC preparations are needed to assess the validity of this statement.

IL-8

IL-8 is secreted by monocytes, macrophages, and neutrophils and acts as a chemoattractant to areas of inflammation [13]. The two studies addressing age-related changes in IL-8 production are summarized in Table 3. Gon and colleagues observed an age-related decline in IL-8 production LPS-stimulated PBMCs from healthy subjects [55]. In the same study, IL-8 concentration in sera was significantly lower in elderly, compared to young, subjects with both groups having pneumonia [55]. Although Clark and colleagues observed age-associated changes in IL-8 production after 24 hours of culture of monocytes with or without LPS, this age effect was gender specific. IL-8 production was decreased in unstimulated monocytes from elderly males, while IL-8 production by LPS-stimulated monocytes from the same subjects was significantly higher, than comparably treated monocytes from young males. Interestingly, there was no age-related difference among female subjects under either condition [57]. Since only two studies address IL-8 production in the elderly, no general conclusions can be made, even though both of these studies suggest that aging is accompanied by a decline in IL-8 production.

TNF α

Tumor necrosis factor α (TNF α) is secreted by macrophages, monocytes, T and B cells, NK cells, neutrophils, and endothelial cells [13]. As well as having antitumor and antiviral activity, TNF α supports neutrophil activity and phagocytosis. Also known as cachexin, TNF α is responsible for the cachexia that accompanies chronic inflammation. Studies addressing TNF α are summarized in Table 4.

Two studies measured TNF α levels present in the sera of young and elderly subjects [52,55]. Gon and colleagues measure TNF α from young and elderly subjects with pneumonia by ELISA and found an age-related decline [55]. Peterson and colleagues measured TNF α in healthy young and elderly subjects by bioassay and found no age-related difference in TNF α [52]. The contrasting results from these two laboratories could be due to either the health of the subject population or the assay used for measuring TNF α .

Two studies evaluated LPS-induced PBMC production of TNF α in healthy elderly and young subjects, with one demonstrating an increase [18] and the other a decrease in the elderly subjects [55]. The study that found an age-related increase in TNF α production used PBMC from Senieur elderly that were incubated for 48 hours [18], while the study demonstrating a decrease

incubated PBMC from healthy elderly for 24 hours [55]. A third study assessed monocyte production of TNF α upon 24 hour stimulation with LPS; no difference in TNF α levels was observed.

Two other studies employing PBMC showed age-related increases in TNF α production. Fagiolo and colleagues stimulated PBMC with PHA and PMA together [46], while Molteni et al. utilized a mixed lymphocyte reaction [27]. Since both systems stimulated both monocytes and T cells, and since T cells can produce TNF α , it is impossible to compare these results to the above results which preferentially stimulated only monocytes.

It is important to note that in the same study using allogenic stimulation, Molteni and colleagues found a similar age-related increase in IL-1 β production [27]. In addition, they demonstrated that in the elderly, IL-1 β production was correlated with TNF α production ($r=0.745$; $p=0.003$) [27], but that neither cytokine was correlated with decreased proliferative responses to the allogeneic stimuli. This study suggests complex age-associated changes in immune function upon antigenic stimulation. However, since no other studies have addressed these issues, particularly with respect to TNF α , IL-1 and proliferation, generalizations about changes that may occur with age cannot be made.

In summary, similar to the case with IL-1 β , studies of similar experimental conditions demonstrate conflicting data as to the association of age with TNF α production. Further, the majority of studies include small numbers of subjects, so it is difficult to determine whether or not differing results represent sampling error. Unfortunately, there are not enough studies of TNF α production using similar experimental conditions to reveal consistent patterns of age-associated changes in TNF production.

TGF β

Transforming growth factor β (TGF β) is secreted by platelets, macrophages, and lymphocytes and acts to chemotactically attract monocytes and macrophages, induce activated macrophages to secrete IL-1 β , and induce class switching of B cells to IgA [13]. Only one study reports on the relationship between age and TGF β levels [52]. Peterson and colleagues evaluated TGF β levels in sera in 26 healthy elderly and 13 young using a bioassay and found no age-related differences in TGF β concentrations. Obviously more studies are needed to evaluate the role, if any, TGF β has in immunosenescence.

DISCUSSION

Given the key role of cytokines in cross-regulatory communication between immune cells, alterations in their production or use are quite likely responsible for at least some of the changes seen in immunosenescence. Efforts to elucidate the mechanisms of the age-related decline in cell-mediated immunity in humans have resulted in many papers focused on the association between age and cytokine production. Unfortunately, a

clear picture of age-related changes in cytokine production in humans has yet to be elucidated. Our review of the published data has not found evidence for consistent age-related changes in any of the type 1, type 2 or proinflammatory cytokines. This conclusion is in direct contrast to the assumptions presented in much of the current literature on human immunosenescence indicating an age-related decline in IL-2 production and a concomitant increase in IL-4. While the reports included in this review suggest that IL-2 is decreased in more than half of the studies, the studies on IL-4 are too limited in number and varied in outcome to make any definitive statement.

Even when employing the strictest comparisons between studies with similar subject populations and culture conditions, conflicting results were observed in almost all cases. The most likely reason for the inconsistencies, taking into account the possibility of experimental error, is that the small numbers of subjects employed by most researchers cannot account for the heterogeneity of the age-related alterations in immune response. In our own experience in using sample sizes over 250, we see subpopulations within the total healthy elderly subject population such that about 15% produce no cytokines and about 12% produce cytokines at the level of young controls [41]. In order to control for this variability, not only must large sample sizes be employed, but individual responses, as well as mean population responses, must be analyzed and presented. Based on these observations, the ideal situation would be to document age-associated changes within healthy elderly individuals both relative to themselves and to established normative values for young. Since "normal" values for cytokine production in response to various stimuli have not been established, the best approach may be to identify specific immune stimuli (e.g. vaccination) and to evaluate age-associated changes in cytokine production in the context of other parameters of the immune response (e.g. antibody production, lymphocyte proliferation). Without such investigations of associations between age, cytokine production, and immune response, it is not possible to definitively state that dysregulation of cytokine production is an important mechanism for an age-related decline in immune function.

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