Impaired Interleukin-12-Dependent T-Cell Functions During Aging: Role of Signal Transducer and Activator of Transcription 4 (STAT4) and Suppressor of Cytokine Signaling 3 (SOCS3)

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Interleukin (IL)-12 is the major inducer of T helper cell (Th) 1-type responses. Despite a higher IL-12 production, phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC), as well as CD4+ or CD8+ T cells from elderly donors released interferon (IFN)-γ amounts similar to those observed in young controls, and underwent only a slight increase in IFN-γ production after IL-12 costimulation. These findings were not due to an age-related reduction in IL-12 receptor expression. Interestingly, no difference in PHA-triggered signal transducer and activator of transcription 4 (STAT4) phosphorylation between young and elderly donors was found, and a significant IL-12-induced STAT4 activation occurred only in PHA-preactivated cells from the younger group. The age-related defect in IL-12 signaling was STAT4-restricted as it did not involve the p38 mitogen-activated protein kinase (MAPK) pathway. Finally, suppressor of cytokine signaling 3 (SOCS3) expression was significantly higher in unstimulated cells from elderly individuals, and it did not diminish after cell stimulation. These results indicate that a defective STAT4 activation, likely dependent on elevated SOCS3 levels, is involved in the impaired IL-12-dependent T-cell functions with aging.

AGING is characterized by an overall imbalance of the immune system, with the most consistent and dramatic alterations occurring within the T-cell compartment. In fact, T cells from elderly individuals show a decreased ability to promote B-cell activation and differentiation, impaired proliferative responses to mitogens and antigens, and a reduced ability to generate allospecific cytotoxic T lymphocytes (CTLs) (1–5). The mechanisms responsible for the age-associated alterations in immune function have not yet been established. However, because 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 responses.

Data from murine studies suggest an age-related dysregulation of cytokine production. The consistently observed decline in interleukin (IL)-2 (6) and the generally observed rise in IL-4 (7) in aged mice have led to the hypothesis that in the murine system aging is accompanied by a switch from a predominant production of cytokines inducing and supporting cell-mediated immune responses (type 1 cytokines: IL-2, interferon [IFN]-γ, tumor necrosis factor [TNF]-β, IL-12, IL-15) to a predominant production of cytokines inducing humoral responses (type 2 cytokines: IL-4, IL-5, IL-6, IL-10, and IL-13). On these grounds, a large volume of articles has been published showing an age-related decline in IL-2 or IFN-γ production (1,8–10) and a concomitant increase in IL-4 (11,12) in human individuals, fostering the assumption that a T helper cell (Th)1/Th2 imbalance featuring a Th2 predominance is a generalized phenomenon occurring with aging.

In an interesting review, Gardner and Murasko (13) recently analyzed more than 60 studies in humans on this topic and came to the conclusion that the data supporting the occurrence of age-related changes in cytokine production are not consistent. Yet, the Th1/Th2 imbalance as a possible cause of T-cell dysfunction in elderly persons continues to be claimed by a number of scientists (14,15), and this topic is still the object of intense debate and investigation.

IL-12 is now considered the key cytokine for the induction of a Th1-type response. One of the most important effects of IL-12 is its striking ability to induce IFN-γ production. In addition, IL-12 enhances the proliferation of activated T cells and increases cytotoxicity (16,17). Therefore, we were recently quite surprised to note that, despite an impaired proliferative capacity and a “normal” IFN-γ production, phytohemagglutinin (PHA)-stimulated peripheral blood mononuclear cells (PBMC) from elderly donors released significantly higher amounts of IL-12 than those found in their younger counterparts (18). In this previous study, we hypothesized that the age-related increase in IL-12 production might represent an attempt to counterbalance a reduced
cytokine biological activity in elderly persons. Herein, we provide experimental support to this hypothesis, demonstrating that the IL-12 ability to enhance T-cell functions is indeed compromised with age as a consequence of changes in postreceptorial IL-12 signaling events involving signal transducer and activator of transcription 4 (STAT4) activation. The role of elevated levels of the suppressor of cytokine signaling 3 (SOCS3) protein in the down-regulation of IL-12/STAT4 signaling with aging is further discussed.

METHODS

Donors

Forty volunteers (18 males and 22 females) older than 65 years (mean age 78 years, range 70–95 years) free of diseases affecting the immune system, cancer, or infections and taking no anti-inflammatory drugs or corticosteroids were selected according to the SENIEUR protocol (19). Forty healthy young blood donors (17 males and 23 females; mean age 30 years, range 25–34 years) were enrolled as controls.

Cell Preparation

PBMC were obtained by centrifugation of heparinized venous blood over Ficoll-Paque gradients (Amersham Pharmacia Biotech, Uppsala, Sweden) (170 g for 45 minutes). The cell suspensions recovered at the interface were washed and resuspended in complete medium (RPMI 1640 supplemented with penicillin [200 IU/ml], streptomycin [100 μg/ml], l-glutamine [2 mM], and 10% heat-inactivated fetal calf serum) or, in the case of further processing, in incubation buffer (phosphate-buffered saline [PBS; pH 7.2], supplemented with 0.5% bovine serum albumin and 2 mM EDTA).

CD4+ and CD8+ T-cell subsets, as well as CD14+ monocytes, were purified by means of magnetic and subsequent flow cytometric cell sorting. Briefly, PBMC were incubated with anti-CD3 monoclonal antibodies (mAbs) conjugated with supermagnetic microbeads (Miltenyi Biotech, Bergisch Gladbach, Germany) in incubation buffer for 15 minutes at 6°C–12°C. After positive selection on a MACS separator (Miltenyi Biotech), CD3+ bead-labeled lymphocytes were double-stained with fluorescein isothiocyanate (FITC)-conjugated anti-CD3 and phycoerythrin (PE)-labeled anti-CD4 or anti-CD8 mAbs (Beckman Coulter Inc., Miami, FL) on ice for 30 minutes. CD3+CD4+ or CD3+CD8+ T cells were finally sorted on a flow cytometer/cell sorter (Coulter Epics Elite; Beckman Coulter Inc.). Analysis of sorted populations indicated that each of the specific cell populations was more than 99.5% pure. Starting with the CD3+ population, negatively selected by magnetic separation, monocytes were isolated by flow cytometric cell sorting as well. Briefly, the cells were incubated with FITC-conjugated anti-CD14 mAbs (Alexis Italia, Florence, Italy) and treated as described above. CD14+ monocytes were sorted on a Coulter Epics Elite, reaching a purity higher than 98%.

Cytokine Production

To assess cytokine production, 2 × 10⁵ PBMC were incubated at 37°C 5% CO₂ in 96-well round-bottom microtiter plates (Corning Life Sciences, Milan, Italy) in the presence of PHA (5 μg/ml) as stimulant. The modulating effects of IL-12 on PHA-induced IFN-γ production was assessed by supplementing cell cultures with recombinant IL-12 (Calbiochem-Novabiochem Co., La Jolla, CA) (2 ng/ml) or anti-IL-12 mAbs (R & D Systems, Minneapolis, MN) (20 μg/ml). Irrelevant isotype-matched mAbs were used as negative controls. At the end of the cultures, the viability of cells from the elderly individuals paralleled in all cases the cell viability observed in the younger counterparts, as determined by the trypan blue assay.

Supernatants were harvested at different lengths of time and kept frozen (~80°C) until assayed. IL-12 p70 and IFN-γ concentrations were determined by commercially available enzyme-linked immunosorbent assay (ELISA) kits (Euroclone, Paignton, U.K.) following the manufacturer’s protocols. The sensitivity of the assays was 3 pg/ml and 5 pg/ml, respectively.

RNA Extraction, Complementary DNA Synthesis, and Semiquantitative Reverse Transcription–Polymerase Chain Reaction

Total RNA was extracted from unstimulated or variously stimulated PBMC using TRIzol Reagent (Invitrogen Srl, Milan, Italy). Complementary DNA (cDNA) was prepared by incubating 1 μg of total RNA with a mixture containing 5X Reaction Buffer, 750 U/ml MultiScribe Reverse Transcriptase, 1 mM deoxynucleoside triphosphate (0.25 mM each), 1.25 mM Random Hexamers, 500 U/ml RNase Inhibitor (all purchased from Applied Biosystems, Milan, Italy), 2.5 mM MgCl₂, and 10 mM dithiothreitol in a final volume of 40 μl at 25°C for 10 minutes, 42°C for 1 hour, and 95°C for 5 minutes. Polymerase chain reaction (PCR) was performed with 4 μl of cDNA (previously diluted to 1 μg/50 μl) in the presence of 10X Reaction Gold Buffer, 25 U/ml AmpliTaq Gold, 0.8 mM deoxynucleoside triphosphate (0.2 mM each) (all purchased from Applied Biosystems), 1.5 mM MgCl₂, and 0.2 μM oligonucleotide primers, in a final volume of 50 μl. PCR products were run on a 15%–25% ThermoCycler (MWG-Biotech, Ebersberg, Germany) for the number of cycles found in preliminary kinetic experiments to be optimal for the individual target cDNA. The times and temperatures used for the annealing step were: 30 seconds at 60°C for β-actin, 25 seconds at 64°C for IL-12 receptor (IL-12R)β1, and 30 seconds at 62°C for IL-12Rβ2. PCR was performed in triplicate for each individual cDNA. To determine the relative concentrations of the specific cDNA in each experimental sample, a control sample of cDNA encoding the gene of interest was diluted over a five log scale and amplified in each PCR experiment (20). Amplified products were then loaded on a 2% agarose gel, visualized by ethidium bromide staining, and quantified by laser densitometry using the ImageMaster 1D Image Analysis Software (Amersham Biosciences, Milan, Italy). Using the values obtained after amplification of the diluted positive controls, we calculated the relative concentration of the initial cDNA in each individual sample. β-actin was taken as the indicator of the actual amount of cDNA used in each PCR. Therefore, the relative concentrations of the IL-12R cDNA in the samples were normalized for β-actin and could be expressed
in relative units. The specificity of amplified fragments was controlled by gel elution of the corresponding band and subsequent analysis by enzymatic digestion with at least two different restriction enzymes.

The primers used (MWG Biotech) were as follows: L-12R\textsuperscript{b}1, 5′-GATGATGATGACTGCTGGTCC-3′ and 3′-TTAATGTCAGCAGCATTTCC-5′; IL-12R\textsuperscript{b}2, 5′-AGG CGATGTCAGCTGGAA-3′ and 3′-CTGAGTGTGATTAG ACAGTACC-5′; and β-actin, 5′-CACAACCTTACAAATG AGCTG-3′ and 3′-TTAATGTCAGCAGCATTTCC-5′.

**Western Blotting**

PBMC (2 × 10\textsuperscript{9}/ml) were stimulated with PHA (5 μg/ml) in 25-cm\textsuperscript{2} tissue culture flasks (Corning Life Sciences). After 24 hours, cells were recovered, washed, and resuspended in fresh complete medium at a concentration of 7 × 10\textsuperscript{5} cells/ml. IL-12 (10 ng/ml) was then added, and the cultures were incubated for a further 20 minutes, on the basis of preliminary time course experiments showing that at this time IL-12 induced the maximal effects in both the young and elderly individuals. After cell recovery and washing, the supernatants were removed and the cell pellets were resuspended in ice-cold lysis buffer containing 10 mM HEPES (pH 7.9), 0.5% Igepal, 10 mM KCl, 0.1 mM EDTA (pH 8), 0.2 mM EGTA (pH 6), 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM sodium orthovanadate, and 1 mM sodium fluoride for 15 minutes at 4°C. After centrifugation, the supernatants were recovered and frozen at −80°C until use.

Samples were mixed 1:1 with 4X sample buffer (8% sodium dodecyl sulfate, 40% glycerol, 5% 2-mercaptoethanol, and a trace amount of bromophenol blue dye in 200 mM Tris-HCl, pH 6.8) and heated at 100°C for 5 minutes before being subjected to 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Proteins were then electrothermically transferred from the gel onto a nitrocellulose membrane in a buffer containing 25 mM Tris-HCl, 192 mM glycine, and 20% methanol at 5.5 mA/cm\textsuperscript{2} for 1 hour at room temperature. Residual binding sites on the membrane were blocked by incubating the membrane in blocking buffer (Tris-buffered saline [pH 7.6] with 0.1% Tween 20 and 5% nonfat dry milk) for 30 minutes at room temperature under gentle agitation. After washing in Tris-buffered saline containing 0.1% Tween 20 (TBST), the membrane was incubated with rabbit polyclonal Abs anti-Thr\textsuperscript{182}/Tyr\textsuperscript{182}-phosphorylated p38 mitogen-activated protein kinase (MAPK) (1:750) (Cell Signaling Technology, Beverly, MA), anti-Ty\textsuperscript{1853}-phosphorylated STAT4 (2 μg/ml) (Zymed Laboratories Inc., South San Francisco, CA), anti-STAT4 (1:1000), or anti-SOC3 (1:200) (both purchased from Santa Cruz Biotechnology, Santa Cruz, CA) at 4°C overnight, and then with goat anti-rabbit immunoglobulin G antibodies conjugated with horseradish peroxidase (1:2000 in blocking buffer) (Santa Cruz Biotechnology) at room temperature for 90 minutes. The antibody complexes were visualized by the ECL Plus Western Blotting Detection Reagents (Amersham Biosciences) following the manufacturer’s instructions; subsequently, densitometric analysis of each band was performed. Values were normalized to β-actin, assessed by stripping and reprobing the membranes with rabbit polyclonal anti-β-actin antibodies (1:1000) (Santa Cruz Biotechnology).

To compare values from different gels, one lane of each gel was loaded with a control sample. After transfer and blocking, the membrane was cut and the control lane incubated with rabbit polyclonal antibodies against extracellular signal-regulated kinase (ERK)1 and 2 (1:750) (Cell Signaling Technology), arbitrarily chosen as housekeeping protein, before being processed with the remaining part of the membrane as described above. Therefore, for each protein, values obtained from different experimental points were further normalized to the total ERK2 protein to offset any variations consequent to the different exposure of single membranes.

**Statistical Analysis**

For statistical evaluation, repeated-measures analysis of variance or the Student \textit{t} test was used. Cytokine concentration values were not normally distributed among young and elderly individuals. Thus, to evaluate differences between the two groups considered in the study, we performed loge transformations to normalize the data and enable parametric statistical analysis using Student’s \textit{t} test for independent samples. Values of \(p < .05\) were taken to be statistically significant.

**RESULTS**

**PHA Stimulation of PBMC Cultures Determines an Increase in IL-12 p70 and No Variations in IFN-γ Levels With Aging**

After 24-hour PHA stimulation of PBMC, IL-12 p70 production was demonstrated in 5 of 25 samples collected from young individuals versus 15 of 25 samples from elderly individuals, with the overall levels of IL-12 p70 being markedly higher in the latter group (Table 1). IL-12 p70 release could still be demonstrated after 48 and 72 hours of PBMC stimulation, featuring cytokine concentrations comparable with those detected after a short interval from cell triggering (data not shown). Despite the higher supply of IL-12 in its biologically active form, PHA-stimulated PBMC cultures from the elderly donors did not exhibit higher IFN-γ levels when compared with cultures from young donors. Table 1 shows the production of IFN-γ after 24 hours of cell stimulation. A similar trend was observed at later incubation times, even if a progressive increase in

<table>
<thead>
<tr>
<th>Cytokines</th>
<th>Young ((N = 25))</th>
<th>Elderly ((N = 25))</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-12 p70 (pg/ml)</td>
<td>3.2 ± 2.4</td>
<td>10.2 ± 9.9*</td>
</tr>
<tr>
<td>IFN-γ (ng/ml)</td>
<td>28.3 ± 11.4</td>
<td>31.2 ± 12.9</td>
</tr>
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Notes: PBMC (2 × 10\textsuperscript{5} cells/well) were stimulated for 24 hours with PHA (5 μg/ml). Afterward, supernatants were recovered and cytokine concentrations were determined by enzyme-linked immunosorbent assay. Results are expressed as mean ± standard deviation. Significance versus young controls: *\(p < .01\).

IL-12 = interleukin-12; IFN-γ = interferon-γ; PHA = phytohemagglutinin; PBMC = peripheral blood mononuclear cells.
IL-12 Fails to Enhance T-Cell IFN-γ Production in Aged Individuals

To determine whether the age-related rise in IL-12 levels is consequent to reduced cytokine activity, we mimicked conditions characterized by an abundant availability of IL-12 by supplementing PHA-activated PBMC cultures with exogenous cytokine. The effects of such an experimental approach on IFN-γ synthesis at 24 hours of stimulation are depicted in Figure 1. PBMC from aged individuals stimulated with PHA and simultaneously costimulated with exogenous IL-12 underwent only a slight increase in IFN-γ production in comparison with cells triggered with the lectin alone. This finding is very different from what was observed in PBMC from young donors, where the IL-12 costimulation gave rise to a 13.5-fold increase in IFN-γ concentrations with respect to control cultures. The age-related failure of IL-12 to enhance IFN-γ production was not due to the development of an IL-12-independent cell-stimulation pathway during aging. In fact, in parallel experiments, mAbs neutralizing the effects of IL-12 determined an inhibition of IFN-γ production in elderly persons comparable to that observed in young persons (Figure 1).

To confirm that the age-related defect in IL-12-dependent IFN-γ production was T-cell-specific and to assess whether it was restricted to a particular T-cell subset, CD4+ or CD8+ T cells were stimulated with PHA plus or minus IL-12 and then tested for their functional outcome. Results, illustrated in Table 2, show a trend similar to that observed with PBMC cultures. Therefore, whereas the two T-cell subsets released substantial and comparable levels of IFN-γ with no differences between young and elderly individuals after PHA challenge, IL-12 costimulation determined a marked increase in IFN-γ production by CD4+ or CD8+ T cells from young donors and a much less pronounced effect by similarly treated cells from elderly donors. In the light of these results, PBMC were used in the following series of experiments, and the effects of IL-12 on these cells were considered as a reflection of the cytokine effect on the T-cell compartment.

Age-Related Decline in IL-12 Biological Activity Is Not Due to an Altered Expression of IL-12R Messenger RNAs

To investigate the mechanisms responsible for the reduced IL-12 activity in elderly persons, the messenger RNA (mRNA) expression of the IL-12R chains was assessed. The results, illustrated in Figure 2, indicate that the mRNA expression time course of both IL-12R chains is similar in PHA-stimulated PBMC from elderly and young donors. In particular, the IL-12Rβ1 mRNA promptly increased, reaching a peak at 24 hours, and thereafter rapidly decreased, reaching baseline values within 7 days of stimulation, whereas the IL-12Rβ2 mRNA peaked at 72 hours and maintained substantial levels of expression after prolonged intervals of stimulation. In both groups of individuals, a higher up-regulation of the β2 chain mRNA was observed with respect to the β1 chain mRNA (Figure 2). More interestingly with regard to the scope of our study, however, PBMC from elderly donors exhibited a higher IL-12Rβ1 and especially IL-12Rβ2 mRNA expression as compared with cells from the young controls (see Figure 2).

Table 2. IL-12 Effects on IFN-γ Production by PHA-Stimulated CD4+ or CD8+ T Cells From Elderly (N = 9) and Young (N = 9) Donors

<table>
<thead>
<tr>
<th>Donors</th>
<th>CD4+ PHA</th>
<th>CD4+ PHA + IL-12</th>
<th>CD8+ PHA</th>
<th>CD8+ PHA + IL-12</th>
<th>CD4+ PHA + IL-12</th>
<th>CD8+ PHA + IL-12</th>
</tr>
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<tbody>
<tr>
<td>Young</td>
<td>56.5 ± 34.2</td>
<td>428.6 ± 125.4*</td>
<td>11.4 ± 6.6</td>
<td>60.9 ± 41.5</td>
<td>524.2 ± 224.6*</td>
<td>12.1 ± 7.2</td>
</tr>
<tr>
<td>Elderly</td>
<td>62.8 ± 39.6</td>
<td>110.8 ± 56.4*</td>
<td>2.2 ± 0.9*</td>
<td>52.1 ± 38.6</td>
<td>122.4 ± 55.2*</td>
<td>3.1 ± 1.8*</td>
</tr>
</tbody>
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Notes: CD4+ or CD8+ T cells (1.8 × 10^5 cells/well) + monocytes (2 × 10^5 cells/well) were stimulated for 24 hours with PHA (5 μg/ml) in the presence or absence of IL-12 (2 ng/ml). Afterward, supernatants were recovered and cytokine concentrations were determined by enzyme-linked immunosorbent assay. Results are expressed as ng/ml (mean ± standard deviation). Significance versus homologous cells stimulated with PHA only: *p < .001; significance versus young controls: #p < .001.

IL-12 = interleukin-12; IFN-γ = interferon-γ; PHA = phytohemagglutinin.
When the effect of exogenous IL-12 on PHA-stimulated PBMC cultures was analyzed, only the IL-12Rβ2 chain mRNA was found to be significantly up-regulated (Figure 3). However, depending on age, a different IL-12-mediated outcome was observed, with young individuals showing a 4-fold increase and elderly donors a 1.5-fold increase in IL-12Rβ2 mRNA levels with respect to PHA-induced values. As a result, there was no longer any difference in IL-12Rβ2 mRNA expression between elderly and young donors as an effect of IL-12 stimulation (see Figure 3).

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**PHA- and IL-12-Stimulated PBMC From Elderly Donors Show a Defect in STAT4 Phosphorylation**

STAT4 is critical for the biological functions of IL-12, including its ability to induce IFN-γ production (21,22). Therefore, in a subsequent series of experiments we investigated the possible presence of age-related changes in PHA- and IL-12-induced STAT4 phosphorylation. Interestingly, we found that despite the increased availability of IL-12, PHA-stimulated PBMC from elderly donors underwent a STAT4 tyrosine phosphorylation similar to that seen in analogously treated cells from young individuals (Figure 4). Furthermore, IL-12 addition to cell cultures previously stimulated with PHA gave rise to a significant increase in the levels of tyrosine-phosphorylated STAT4 in young volunteers, whereas only a mild enhancement of STAT4 phosphorylation was seen in PBMC cultures from elderly donors under the same experimental conditions (Figure 4). The defect in STAT4 activation observed with age was not due to a reduction of total protein levels of STAT4, which were comparable in elderly and young individuals (see Figure 4). Although STAT4 is crucial for IL-12-stimulated biological activities, other pathways are activated by IL-12 as well, including p38 MAPK (23). However, when PHA-stimulated PBMC from elderly donors were analyzed for p38 MAPK activation, higher levels of tyrosine-phosphorylated p38 MAPK were found in comparison with the younger counterparts (Figure 5). Moreover, after additional supplementation of cell cultures with IL-12, this difference was no longer observed, owing
both to a significant IL-12-induced increase in p38 MAPK phosphorylation in young donors and to a poor IL-12-mediated effect on p38 MAPK activation in elderly donors (see Figure 5).

**SOCS3 Levels Increase With Age**

SOCS proteins are a newly identified family of inhibitory proteins, playing a key role in the regulation of cytokine signaling through the inhibition of the Janus kinase (JAK)/STAT signal transduction pathway (24,25). In accordance with recent evidence showing that SOCS3 inhibits IL-12-induced STAT4 activation (26), it seemed to be of interest to assess whether changes in the levels of this protein might occur with age. By assaying PBMC under the same experimental conditions adopted to assess STAT4 activation, we found that basal expression of the SOCS3 protein was significantly higher in the cells of elderly individuals than in their younger counterparts (Figure 6). PHA stimulation had no effect on SOCS3 expression in PBMC of the elderly group but produced a substantial reduction of SOCS3 levels in the cells of the young controls (Figure 6). Finally, due to the short interval of stimulation, no modulatory effects of IL-12 on protein levels of SOCS3 were observed in PHA-preactivated cells in either group (see Figure 6).

**DISCUSSION**

Naive, uncommitted Th precursor cells activated in the presence of IL-12 differentiate into Th1 cells, predominantly secrete IFN-γ, and promote cell-mediated immune responses (16,17). In line with data reporting an age-related impairment of the cellular arm of the immune system (1–5), herein we show that the IL-12-driven Th1 cell differentiation program is profoundly altered with aging. We found, in fact, that PHA-stimulated T cells from elderly persons produced amounts of
IFN-γ similar to those found in young persons, but this target was achieved only because of a higher production of functionally active IL-12 by antigen-presenting cells. Furthermore, unlike young individuals, elderly donors displayed only a slight enhancement in IFN-γ production following T-cell costimulation with high amounts of exogenous IL-12. It has been recently reported that IL-12 acts primarily on naive T cells, whereas it has a poor effect on memory T cells (27). In line with these studies, preliminary experiments from our group suggest that although the contribution of naive cells to the overall PHA-induced IFN-γ production by T cells is minimal, it dramatically increases after IL-12 costimulation. When compared with naive T lymphocytes, memory T cells show a higher PHA-triggered IFN-γ release and a lower, yet substantial, IL-12-mediated enhancement in cytokine production. In both cell types, however, the IL-12 costimulatory effects are markedly reduced in elderly donors as compared to young donors (C. Tortorella, G. Piazzolla, V. Cappiello, E. Panella, S. Antonaci, unpublished observations, 2005). Taken together, these data indicate that the biological activity of IL-12 is reduced in elderly persons. This impairment results in a defect of IL-12-mediated T-cell functions that might somehow be signaled to antigen-presenting cells, which respond by increasing the production of IL-12 via a classic feedback mechanism. Although this adjustment may be effective within limits, in critical conditions where there is a high demand for T-cell functions, the defective IL-12 activity may become manifest, hampering the ability of cells to set up Th1-driven cell-mediated immune responses to antigenic stimulations. A condition of this type is to be expected when clinically using IL-12 as immune therapy against malignancies or as vaccine adjuvant, but it may occur physiologically as

**Figure 4.** Signal transducer and activator of transcription 4 (STAT4) phosphorylation in phytohemagglutinin (PHA)- and interleukin-12 (IL-12)-stimulated peripheral blood mononuclear cells (PBMC) of elderly and young individuals. PBMC were stimulated with 5 μg/ml PHA for 24 hours; afterwards, they were further incubated with or without 10 ng/ml IL-12 for 20 minutes. a, Representative immunoblots with anti-phosphorylated STAT4 antibodies (Abs) or with Abs that recognize both phosphorylated and unphosphorylated STAT4 are shown. An immunoblot performed by stripping the membrane incubated with anti-pSTAT4 Abs and reprobing it with anti-β-actin Abs is also shown. Histograms show (b) the levels of phosphorylated STAT4, normalized to β-actin, and (c) the increase in IL-12-induced STAT4 phosphorylation with respect to PHA-induced values, using data from densitometric analysis of 10 experiments. Significance versus homologous cells stimulated with PHA only: *p < .001. Significance of elderly versus young group: *p < .001.
well. In this regard, a large number of studies has provided evidence that although IFN-γ production in response to nonspecific stimuli is unaffected by aging, T-cell stimulation with trivalent influenza vaccine, live influenza virus, PPD (protein purified derivative), or tetanus toxoid is associated with a lower IFN-γ production in elderly persons (28), these differences being likely related to a higher request of IL-12 to accomplish antigen-specific T-cell responses.

It has been demonstrated that IL-12 synergizes with IL-15 (29), IL-18 (30), and the CD28 signaling pathway (31) to determine efficient IFN-γ production in human T cells. However, in our experience, PHA is unable to induce effective IL-15 synthesis (18), whereas increased levels of circulating IL-18 have been reported in healthy elderly individuals (32). At the same time, although senescence is paralleled by a decline in the absolute number of CD28 positive cells (4,5,33), the IL-12-related T-cell functional defect was found to be independent of CD28 expression, as it involved CD28+ and CD28− T cells from elderly persons alike (18).

Age-associated changes in the expression of the IL-12 receptor as well as in the activity of kinases involved in cytokine-triggered cell signaling were therefore considered to be intriguing alternatives and became the objects of further investigation. The receptor for IL-12 is composed of two noncovalently linked chains, termed IL-12Rβ1 and IL-12Rβ2 (34). These receptor subunits cooperate in IL-12 binding and signaling. In addition, both receptor chains associate with members of the JAK family of tyrosine kinases, the IL-12Rβ1 chain interacting with tyrosine kinase 2 (TYK2) and the IL-12Rβ2 chain interacting with JAK2 (35). When IL-12Rβ1 and β2 mRNA expression was evaluated, higher levels were found in PHA-stimulated PBMC of the elderly individuals with respect to the young individuals. This finding is not surprising because IL-12 has been shown to up-regulate the expression of its own receptor (36,37), and elderly persons have an increased production of IL-12. Therefore, an increase in the expression of IL-12R mRNAs is what we expected if IL-12R synthesis had not been altered, and the pathway involved in the IL-12-dependent up-regulation of IL-12R was normally functioning with aging. Similarly to the

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**Figure 5.** p38 mitogen-activated protein kinase (MAPK) phosphorylation in phytohemagglutinin (PHA)- and interleukin-12 (IL-12)-stimulated peripheral blood mononuclear cells (PBMC) of elderly and young individuals. PBMC were stimulated as described in Figure 4. a, Representative immunoblot with antibodies (Abs) that recognize the phosphorylated form of p38 MAPK is shown. Immunoblot on the same membrane stripped and reprobed with anti-β-actin Abs is also shown. Histograms show (b) the levels of phosphorylated p38 MAPK, normalized to β-actin, and (c) the increase in IL-12-induced p38 MAPK phosphorylation with respect to PHA-induced values, using data from densitometric analysis of 5 experiments. Significance versus homologous cells stimulated with PHA only: *p < .01. Significance of elderly versus young group: *p < .01.
effects on IFN-γ production, costimulation with high amounts of IL-12 determined a poor increase in IL-12R chain mRNA expression in PBMC from the elderly group, confirming that during senescence IL-12 signaling works very close to its upper limits.

After IL-12/IL-12R interaction, the IL-12Rβ2 chain is tyrosine-phosphorylated in the proximity of its cytoplasmic tail in a JAK2-dependent manner; this results in the recruitment and activation of STAT4 to a specific docking site (38). Phosphorylated STAT4 molecules then dimerize and translocate to the nucleus, where they interact directly with DNA sequences in the IFN-γ promoter to increase gene transcription (39). In this context, it is noteworthy that, despite the higher availability of IL-12, PHA-stimulated PBMC from elderly donors showed phosphorylated STAT4 levels comparable to those found in the young donors. Furthermore, unlike cells from young individuals, no increase in tyrosine STAT4 phosphorylation was observed after additional IL-12 cell challenge. These results are entirely in line with the production of IFN-γ by either PHA-stimulated or IL-12-costimulated cells, suggesting that defective activation of STAT4 may play a critical role in the impairment of IL-12-dependent T-cell responses during senescence. Furthermore, tyrosine phosphorylation of p38 MAPK, activated by IL-12 through a STAT4-independent mechanism (23), was closely correlated with the levels of IL-12 as well as with the expression of IL-12R chain mRNAs, clearly indicating that the age-related defect in IL-12 signaling is STAT4-restricted and does not represent a reflection of an overall dysfunction of the IL-12R transduction capacity. It has been reported by Lawless and colleagues (36) that IL-12Rβ1 and IL-12Rβ2 are IL-12-inducible in a STAT4-dependent manner. This finding seems to be in contrast with our findings showing an up-regulation of IL-12R mRNA expression in the presence of “normal” STAT4 activation in PHA-stimulated PBMC of elderly donors. However, Lawless and colleagues drew their conclusion on the basis of experiments carried out in STAT4-deficient cells, where they found a decreased basal expression of IL-12Rβ1 and IL-12Rβ2 in comparison with control cells. According to their data and ours, it is therefore reasonable to speculate that an alternative pathway to STAT4 is implicated in the IL-12-dependent up-regulation of IL-12R
expression and that, although necessary, STAT4 merely has a permissive function on this pathway.

Several systems which negatively regulate the JAK/STAT activation pathway have been characterized. Particular interest has recently been paid to SOCS proteins, whose members share two structural features: the Src homology (SH2) domain and a carboxyl-terminal region termed the SOCS box, which are involved in phosphotyrosine binding to activated signaling molecules and in the induction of degradation of signaling molecules through the ubiquitin-proteasome pathway, respectively (24,25). Of the eight members of this family so far identified, SOCS3 has been shown to play an inhibitory role in STAT4-mediated IL-12 signaling by binding to the STAT4 docking site in IL-12Rβ2 (26). As reported by Yu and colleagues (40), SOCS3 is constitutively expressed in naive Th cells and temporarily decreases following cell stimulation, the latter event being likely important in promoting T cell activation and proliferation. When we evaluated the expression of SOCS3 in resting or PHA-stimulated PBMC, a trend similar to the one described by Yu and colleagues was observed in young individuals. Notably, however, SOCS3 levels were markedly elevated in unstimulated cells from elderly persons and did not diminish as a result of PHA cell triggering, which may largely account for the IL-12-dependent defect of STAT4 activation observed in senescent cells. The mechanisms leading to the up-regulation of SOCS3 expression with aging are unknown, and their definition may presently be only a matter of speculation. Egwuagu and colleagues (41) found that SOCS3 expression is 23-fold higher in Th2 than in Th1 cells, whereas Th1 cells contain 5-fold higher levels of SOCS1, raising the possibility that differentiation toward the Th1 or Th2 pathway may be mediated in part by the selective repression of IL4/STAT6 or IL-12/STAT4 signaling pathways, respectively. Accordingly, it is plausible to hypothesize that the increased levels of SOCS3 found in PBMC of elderly persons is just the reflection of a preferential Th2 cell commitment during senescence. Alternatively, it is possible that SOCS3 expression is the physiological response to an age-related increase in natural inducers of the protein. These include the proinflammatory cytokines IL-1, IL-6, and TNF-α (24,25), the levels of which have been found to be significantly increased in elderly persons (42), such as to induce Franceschi and Bonafè to postulate the “inflamming” theory of senescence (43).

Taken together, our results are consistent with the conclusion that a defect of STAT4 activation, likely dependent on elevated SOCS3 levels, may affect the IL-12 signaling efficacy in aging. This may have an in vivo counterpart in clinical conditions in which a prompt and efficient T-cell function is critical. Controlling the expression of SOCS3 might be therefore an important therapeutic target for the restoration of normal T-cell responses during senescence.

**Acknowledgments**

This work was supported by grants from Ministero dell’Istruzione dell’Università e della Ricerca (MIUR), Rome, and Università di Bari, Bari, Italy.

We thank Professor Francesco Pallone and Dr. Giovanni Monteleone (Department of Internal Medicine, University of Roma Tor Vergata, Rome, Italy) for their technical assistance.

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**References**

20. Tortorella C, Schulze-Koops H, Thomas R, Splawski JB, Davis LS, Lipsky PE. Expression of CD45RB and CD27 identifies subsets of...
26. Yamamoto K, Yamaguchi M, Miyasaka N, Miura O. SOCS-3 inhibits IL-12-induced STAT4 activation by binding through its SH2 domain to the STAT4 docking site in the IL-12 receptor β2 subunit. Biochem Biophys Res Commun. 2003;310:1188–1193.

Editor Nominations

The Gerontologist

The Gerontological Society of America’s Publications Committee is seeking nominations for the position of Editor-in-Chief of The Gerontologist, the Society’s multidisciplinary journal.

The position will become effective January 1, 2007. The Editor-in-Chief makes appointments to the journal’s editorial board and develops policies in accordance with the scope statement prepared by the Publications Committee and approved by Council (see the journal’s General Information and Instructions to Authors page). The Editor-in-Chief works with reviewers and has the final responsibility for the acceptance of articles for his or her journal. The editorship is a voluntary position. Candidates must be dedicated to developing a premier scientific journal.

Nominations and applications may be made by self or others, but must be accompanied by the candidate’s curriculum vitae and a statement of willingness to accept the position. All nominations and applications must be received by March 31, 2006. Nominations and applications should be sent to the Publications Committee, Attn: Patricia Walker, The Gerontological Society of America, 1030 15th Street, NW, Suite 250, Washington, DC 20005-1503.